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Toxigenic Fungi and Mycotoxin Production in Maldive Fish (Smoked Dried Tuna Fish)

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

Food Technology

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Abstract

This is the first study on the mycological safety of “Maldive fish”, a smoked dried tuna product that is both economically and nutritionally important to the Maldives. The most obvious concern with this product is the effect of fungal contamination. The initial aim of the current study was therefore to determine if Maldive fish supports the growth of toxigenic fungi and production of mycotoxins.

The uncontrolled mycoflora on the product were characterised and related to the physiological parameters of the Maldive fish. Ninety six percent of the samples (n=25) were contaminated with one or more mycotoxigenic fungi with *Aspergillus flavus* (92%), *A. tamarii* (96%), *A. niger* (40%), *A. ochraceus* (12%) and *Penicillium citrinum* (60%) identified as the significant species. Subsequently, the potentially toxigenic isolates were screened for their corresponding mycotoxins aflatoxins, ochratoxin A (OTA), cyclopiazonic acid (CPA) and citrinin. A high proportion (72%) of isolates was able to produce toxic metabolites *in vitro* indicating possible contamination of the product with mycotoxins. Almost half (46%) of the *A. flavus* isolates were able to produce the potent carcinogen, aflatoxin B. All species on the surface were also found invading the product. The huge variability in a_w levels (0.951 to 0.720) of the samples would support growth of a wide range of species. Furthermore, the slightly acidic pH (5.65 to 6.68) and low salt content (1.48 to 4.29%) together with the high ambient temperatures of the Maldives were eminently suitable for fungal growth and mycotoxin production. Quantification of aflatoxins from the product revealed two of the 25 samples to be contaminated above the legal limits and confirms potential exposure to significant levels of this toxin from Maldive fish infected with fungi.

These results led to a new question: can fungal growth and mycotoxin production in Maldive fish be eliminated or reduced to safe levels? The most practical approach would be to reduce the a_w to sufficiently low levels that inhibit fungal growth and mycotoxin production. The limiting a_w levels for the most important species were therefore evaluated. The limiting a_w for growth of *A. tamarii* was between 0.82 and 0.85 on NaCl media and between 0.79 and 0.75, on media containing sugars at

ambient storage temperatures (25 to 35°C). The a_w of Maldive fish should be maintained below 0.75 to prevent the growth of *A. tamarii*. The physiology of *A. flavus* has been extensively studied previously but the limiting values are dependent on the food matrix. A smoked fish agar was used to simulate Maldive fish for fungal growth (*A. flavus*) and mycotoxin production (aflatoxin and CPA) under varying conditions. No growth occurred at an a_w of 0.75 while the toxin production was limited at an a_w 0.80 under all incubation conditions (25°C to 40°C). Hence, control of *A. flavus* can be achieved by rapid drying of Maldive fish to an a_w of 0.75 or below.

This study has provided scientific evidence that the mycoflora on Maldive fish produce aflatoxins and other mycotoxins that are a food safety risk. Hence, control of toxigenic fungi is imperative and can be achieved through adequate drying. This information is crucial for the Maldives as well as other developing countries that consume hot smoked dried fish while it potentially has a broader application for other food products.

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List of Abreviations

a_w	Water activity
AFPA	Aspergillus Flavus and Parasiticus Agar
CCA	Coconut Cream Agar
CPA	Cyclopiazonic Acid
CYA	Czapek Yeast Extract Agar
CY20S	Czapek Yeast Extract Agar with 20% Sucrose
DG18	Dichloran 18% Glycerol Agar
DRBC	Dichloran Rose Bengal Chloramphenicol Agar
G25N	25% Glycerol Nitrate Agar
HPLC	High Performance Liquid Chromatography
HPLC-FLD	High Performance Liquid Chromatography with Fluorescence Detection
MEA	Malt Extract Agar
MY5-12 and MY10-12	Malt Extract Yeast Extract 5% (or 10%) Salt 12% Glucose Agar
OTA	Ochratoxin A
SFA	Smoked Fish Agar
TLC	Thin Layer Chromatography
YES	Yeast Extract Sucrose Agar

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List of Peer Reviewed Publications and Conference Proceedings

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Mohamed, S., Mo, L., Flint, S. Palmer, J., Fletcher, G.C. and Pitt, J.I. (2011). Effect of water activity and temperature on the germination and growth of *Aspergillus tamarii* isolated from smoked dried fish. New Zealand Microbiological Society Annual Conference, 23-25 November, Palmerston North, New Zealand.

Mohamed, S., Flint, S. Palmer, J., Fletcher, G.C. and Pitt, J.I. (2012). Assessment of the mycotoxin producing potential of toxigenic fungi isolated from smoked dried fish. New Zealand Institute of Food Science and Technology Annual Conference, 26-28 June, Hamilton, New Zealand.

Mohamed, S., Flint, S. Palmer, J., Fletcher, G.C. and Pitt, J.I. (2013). Isolation and Identification of Mycoflora and Toxigenic Fungi from "Maldive Fish". New Zealand

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CHAPTER 1

Introduction

1.1 Background

Fish is a highly nutritious food and an excellent source of proteins, vitamins, minerals and essential fatty acids. Interest in fish consumption has increased over the years, due to its recognition as a lean alternative to meat and secondly the health benefits it imparts being a rich source of omega-3 fatty acids that reduces cholesterol levels and the incidence of heart disease and pre-term birth (He, 2009; Siscovick et al., 2000). Smoked and dried fish are very important parts of the traditionally accepted diet for many in developing countries as well as a major source of protein. They are also often enjoyed for their characteristic flavour and are commonly used as a raw material for seasoned foods such as soups and sauces.

Salting, smoking and drying are the oldest known methods used in the preservation of fish. Although improvements in the techniques have evolved over the time, traditional methods of production continue to be practiced. The processing of hot smoked fish generally involves hurdle the technology using a combination of curing/salting, smoking and drying steps to deliver a product that is stable for transport and storage. Raw fish is either dry salted, pickled or boiled in salted water followed by smoking at temperatures between 40-100°C and /or drying but the process may vary considerably depending on a number of factors including the species of fish, type of product desired and the traditional practices in different locations (Burt, 1988). For tropical countries where most production takes place, direct sun drying is often the method of choice. The process whilst enhancing the flavour and textural properties, results in a product that has reduced water activity and improved microbial stability compared with the raw material.

Figure 1.1 Maldive Fish showing typical fungal growth on the surface

"Maldive fish" or "Hikimas" (ހިކަމަސް) in Maldivian language is hot smoked, salted and dried tuna fish traditionally produced in the Maldives. It is a common ingredient used mainly for flavouring and thickening and forms the protein component of many dishes in the islands of the Maldives and neighbouring Sri Lanka. In general, It is consumed at least 3 or more times per week per person in the Maldives. The product is kept almost indefinitely without refrigeration and hence it has been one of the major exports from the country for many years. Skip jack tuna (*Katsuwonus pelamis*) is the main species in the production of Maldive fish, while other tunas such as yellow fin (*Thunnus albacares*) and frigate tuna (*Auxis thazard thazard*) are less frequently used. This product has a wood like appearance and is similar to the well-known Japanese smoked bonito stick "Katsuobushi". In local markets and bulk packs for export, it is generally available as a long fillet (or two adjoining fillets). Visible fungal growth on them is considered normal and often the surface is covered with olive green to brown growth with areas of grey at the tapering ends, the colours characteristic of the natural mycoflora on this product (Figure 1.1). The demand for convenience foods has resulted in the emergence of chipped or sliced versions of this product in the market in small plastic packs or containers.

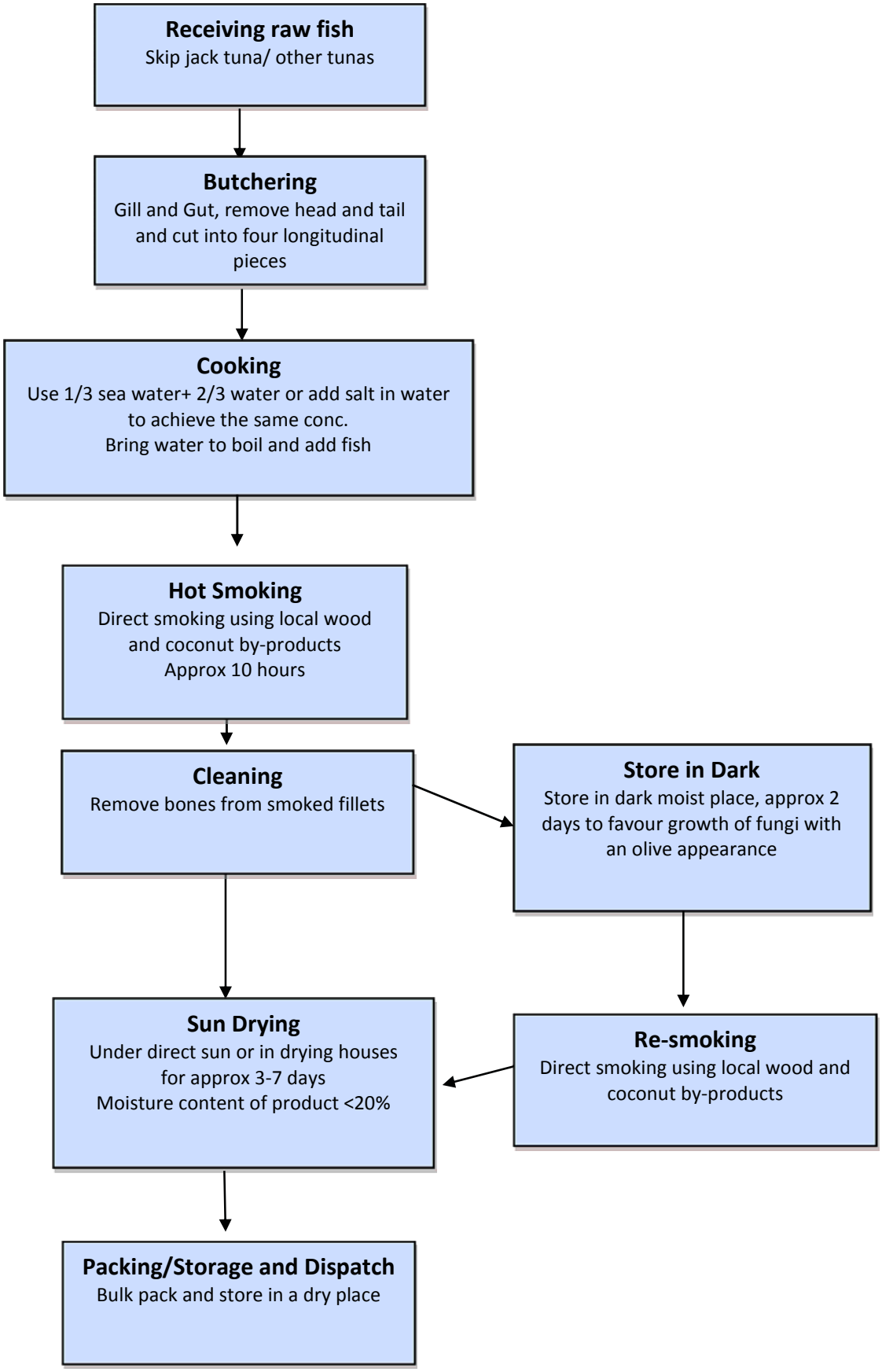


Figure 1.2 Common processing technology for Maldive fish

Figure 1.2 outlines the common processing technology for the production of Maldive fish. The fish is cut into in to four longitudinal fillets or, in case of larger fish such as yellow fin tuna, fillets are cut at cross sections in to loins. This is followed by boiling in salt water (1/3 sea water + 2/3 water or add salt in water to achieve the same concentration) until the fish is cooked. The scum formed on top of the pot is continuously removed. The fish is drained from the soup and hot smoked for approximately 8-10 hours on racks held directly above the smoke source. The temperature of the process is not controlled but can be estimated to be around 80°C. Local wood or coconut-by-products are burned to produce the smoke. After cleaning and removal of bones, it is either directly sundried or in some traditional processes stored in the dark for approximately 2 days to allow mould growth on the surface which supposedly enhances the flavour. In this case, the product is re-smoked to halt mould growth before drying in the sun. Sun drying involves direct drying in the open air on raised racks or less frequently in drying houses for approximately 3 to 7 days (or more) to achieve a final product having a wood like appearance with moisture content of 20% or below. The above recipe is produced by compiling information from different sources: five methods sourced from different areas of the country; a reference by MOFA (1995); and personal experience.

Microorganisms that can grow on smoked dried fish are limited to either spore formers that have survived the process or microorganisms introduced post production. Hot smoked fish on removal from the smoke house have low microbial counts, usually below 10^3 colony forming units (cfu) per gram (Sikorski et al., 1998). Nevertheless, several incidences of food borne diseases have been linked with such products (Bremer et al., 2003; Lindström et al., 2006; Varma et al., 2004) although it should be noted that the a_w of many of these products are quite high. The artisanal methods of production and unhygienic practices applied post production, frequently observed for Maldive fish and similar products from developing countries, are potential causes of contamination of these products. The use of unwholesome raw materials, unhygienic handling prior to production and failure to meet the production parameters can all render the product unsafe. Furthermore, inadequate storage and frequent handling involved in post-production and sale predisposes it to contamination. Most of these

products are held at ambient temperature and sold in open markets in poor packaging and sometimes with no packaging. As such post process contamination can increase the bacterial count above 10^6 cfu per gram of fish (Sikorski et al., 1998). The fact that the product is often consumed without further processing or only minimal processing imposes an additional risk to the consumer.

Potential microbial hazards associated with smoked and dried fish or similar products include the presence of pathogenic bacteria, elevated biogenic amine levels resulting from microbial action and the presence of toxigenic fungi and mycotoxins. Like any other food, smoked and dried fish can harbour food spoilage bacteria and pathogens. Inadequate processing may not sufficiently reduce the bacterial population while unhygienic post process handling can introduce a range of microorganisms. Bacterial pathogens such as histamine producing bacteria, *Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Staphylococcus aureus* and *Salmonella* serovars have been isolated from hot smoked fish (Fletcher et al., 2003; Weidmann and Gall, 2008). Although reports of food poisoning related to these products are limited and most food-borne pathogens would require significant hydration of the product before they could grow, the apparent association of organisms such as *C. botulinum* type E which produces a potent toxin is cause enough for concern (Sikorsk and Kolodziejska, 2002).

Scromboid fish such as tuna contain elevated levels of specific nitrogenous compounds in their flesh that are converted by the amino acid decarboxylation activities of selective bacteria, post mortem, into biogenic amines (Lehane and Olley, 2000). This can lead to histamine or scromboid poisoning, a common form of fish poisoning that although rarely fatal, may cause allergy type reactions in susceptible individuals. Smoked fish often contain elevated levels of histamine and other biogenic amines and have been implicated in scromboid poisoning (Fletcher et al., 1998; Hsu et al., 2009) Poor handling and temperature abuse of raw fish, high concentration of amines due to reduced moisture content, stability of the amines and synergistic effects can all contribute to histamine poisoning from Maldive fish.

The combination of salting, smoking and drying reduces the unbound water in the product limiting microbial growth to some moulds and yeasts. The association of fungal growth with smoked dried fish is well recognized and many products including Maldive fish are found to harbour visible fungi on the surface. Growth of certain types of fungi is known to enhance the acceptability of the product while others indicate spoilage (Pitt and Hocking, 2009). However, there is concern that the presence of toxigenic fungi and their toxic metabolites can lead to deleterious health outcomes in humans and animals (Pitt, 2000b; Williams et al., 2004). Mycotoxins can be hepatotoxic, nephrotoxic, carcinogenic, mutagenic and teratogenic, and in some cases may also be implicated in immunosuppresion and nutritional problems (Meggs, 2009; Wild and Gong, 2010; Williams et al., 2004) . The low water activity of the product together with the high ambient temperatures in the tropics creates an environment for potential proliferation of many toxigenic fungi in dried food commodities. Smoked dried fish from warmer climates have been reported to harbour potentially toxigenic fungi such as *Aspergillus flavus* and potent mycotoxins such as aflatoxins have been detected from some of these products (Adebayo-Tayo et al., 2008; Atapattu and Samarajeewa, 1990; Fafioye et al., 2002; Wheeler et al., 1986). Prolonged exposure of mycotoxins through continuous consumption of mouldy fish could lead to chronic sickness and potentially death.

1.2 Rationale for the study

Despite modern preservation techniques such as refrigeration, smoked and dried fish is still widely distributed in tropical countries. This remains an important commodity in many developing countries in terms of both nutritional and economic benefits. In addition, the increasing price of food commodities worldwide, including fish and the preference for seasoned foods may augment the demand for such products. The products currently available on the market are of variable quality and it is still common to find mouldy or insect infested fish on sale. Many such products fail to conform to basic food safety requirements and a large number of exports to countries with more

advanced food control systems has been rejected based on this criterion (Ward, 2003). Work to improve the safety of these products demands a detailed knowledge and understanding of the hazards associated with them. Research focusing on hot smoked and /or dried fish products is scarce and sometimes inadequate while the focus is mainly on lightly salted smoked products that are more popular in developed nations.

Although Maldive fish have been both nutritionally and economically important for centuries in Maldives, no studies have been reported on the safety aspects of this product. The safety concerns highlighted above indicate the potential for the product to be a health risk and the need for research. The study of mycotoxingenic fungi and mycotoxins associated with the product is a pressing priority for many reasons. First, the product is often naturally contaminated with fungi and the growth of fungi in products of reduced water activity overrides growth of bacteria or other microorganisms (Pitt and Hocking, 2009). Secondly, potent mycotoxins such as aflatoxins have been associated with similar products. People who have been consuming mouldy fish regularly may be exposed to detrimental levels of specific mycotoxins via this route.

Exposure to mycotoxins through contaminated foods is quite high in developing countries which frequently have somewhat lax food control systems (Wagacha and Muthomi, 2008; Williams et al., 2004). The implications are huge in terms of general health and even population mortality, although current statistics possibly underestimate the numbers resulting from mycotoxin intake. For example, the number of deaths from liver cancer induced by aflatoxin consumption has been reported to exceed a staggering 20,000 per annum in Indonesia alone (Pitt, 2000). The relationship between mycotoxigenic fungi and mycotoxins with a variety of crop and animal food products and the risk to public health has been studied widely (Barkai-Golan and Paster, 2008; Magan and Olsen, 2004). However, little work has been carried out to understand the risks in relation to traditional foods such as smoked dried fish which also forms a considerable part of the diet in many developing countries. Consumption of these foods may also contribute to the overall mycotoxin exposure levels. Hence, it is important to study mycotoxigenic fungi and mycotoxins associated with smoked

dried fish and ensure control of this hazard to achieve a safer product. This research project therefore aimed to evaluate food safety issues associated with the growth of toxigenic fungi and mycotoxin production in Maldive fish and to determine control measures that could be applied to reduce or limit their growth and/or mycotoxin production. The results of the study will contribute to a risk assessment and may be used to set criteria for the product.

1.3 Thesis Hypotheses and Objectives

The thesis seeks to test the following hypotheses:

Maldive fish supports the growth of toxigenic fungi and the production of important mycotoxins

Fungal growth and mycotoxin production in Maldive fish can be eliminated or reduced to safe levels

The individual objectives were to:

1. Isolate and identify the mycoflora associated with Maldive fish both on the surface and inside the fish and determine any potentially toxigenic species
2. Assess physiochemical properties of the product (pH, salt content and water activity) and relate these to the mycological safety of the product
3. Screen strains of potentially toxigenic species isolated for their mycotoxin producing capability
4. Determine the occurrence of major mycotoxins in the product and quantify them
5. Study and define limits for growth and mycotoxin production of important toxigenic fungi isolated from the product by inoculation on appropriate media and on product analogue under various conditions
6. Develop strategies to eliminate growth (or to reduce it to safe levels) of toxigenic fungi and mycotoxin production in Maldive fish.

1.4 Overview of the thesis

This thesis is organised in to a total of nine chapters (Figure 1.3). The current chapter (Chapter one) presents a brief introduction to the topic providing relevant background information and justification for a research hypotheses and for research objectives. It concludes with the thesis outline to clarify the direction of this thesis. Chapter two is dedicated to a review of the literature in the study area focussing on toxigenic fungi and mycotoxins in smoked and dried fish or similar products.

The subsequent chapters cover the individual objectives of the study outlining the methodology applied and presenting and discussing the experimental findings. The initial objective of this study was to isolate and identify the fungi associated with Maldive fish and determine important mycotoxin producers. The findings from this study are detailed in Chapter three. Chapter three also covers information on the physiological parameters of the product in relation to the mycoflora isolated (Objective 2). Chapter four appends Chapter five and describes developing and optimising a screening method for the mycotoxin citrinin. Chapter five is based on screening potentially toxigenic isolates for mycotoxin production (Objective 3). The occurrence of toxigenic fungi and the ability of these isolates to produce mycotoxins *in vitro* do not necessarily guarantee the production of mycotoxins in the product. Chapter six therefore reports the detection and quantification of mycotoxins found in the product (Objective 4). Chapter seven and Chapter eight support Objective five and evaluate the effects of important environmental parameters such as a_w and temperature on the growth and toxin production of the two most important species *A. flavus* and *A. tamarii* to determine the limiting conditions to control these fungi. Chapter seven defines the growth limits for *A. tamarii* on general media which provides a guide for preventing its growth on foods. There is abundant information on the physiology and limiting conditions for *A. flavus* which is the most active producer of the very potent aflatoxins. However, studies on specific food matrices are essential to yield more accurate data and Chapter eight identifies the conditions for growth and mycotoxin production of *A. flavus* isolated from the product on a smoked fish agar that

simulates Maldive fish. Chapter 9 is the general discussion based on the main findings of the experiments drawing out conclusions and recommendations for further work.

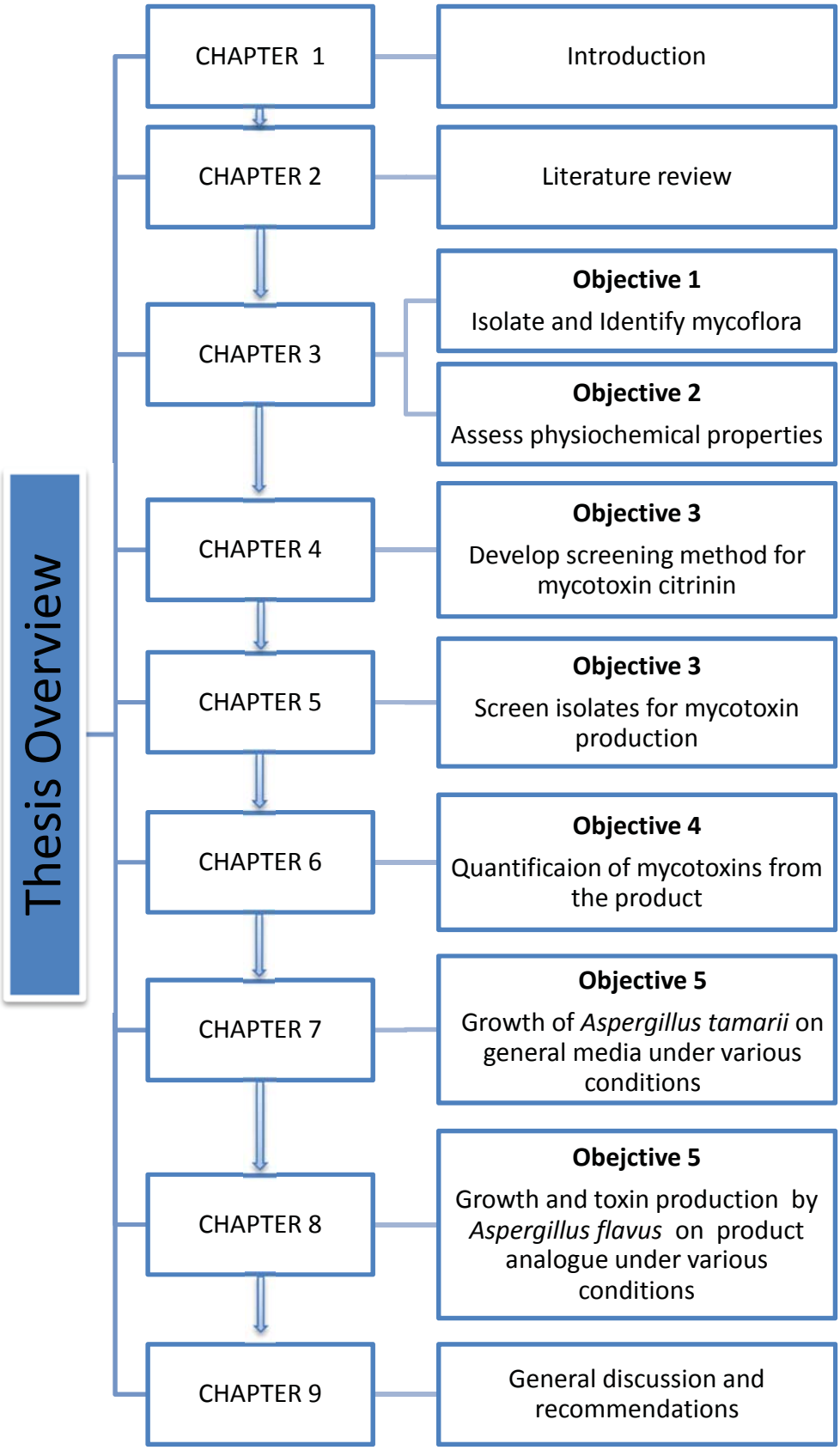


Figure 1.3 Overview of the Thesis

CHAPTER 2

A Review of Literature Relating to Toxigenic Fungi and Mycotoxins Associated with Hot Smoked and Dried Fish

2.1 Introduction

The reduced water activity of dried foods limits microbial growth to some filamentous fungi which can cause spoilage but more importantly could produce mycotoxins. An array of mycotoxins many of which are deleterious to health is produced by the different fungi that could contaminate food products. Thus, smoked and dried fish and related products could be a source of mycotoxins for populations that frequently consume such products. The microbiological safety of hot smoked and dried fish that are often consumed in developing countries has not been studied well, whereas emphasis was placed on lightly smoked fish which are popular in developed nations. In this chapter, an attempt is made to review the literature pertinent to the toxigenic fungi and mycotoxins associated with hot smoked and dried fish. A background on the types of product as well as a general role of the fungi is provided. This is followed by review of the studies conducted on smoked dried fish to elucidate the range of fungi that are found on these products. Methods for the isolation and identification of fungi and mycotoxin quantification are discussed. Information on the mycotoxigenic fungi and mycotoxins associated with smoked dried fish is critically evaluated together with factors that may affect fungal growth and toxin production. Finally, any practical control strategies that could be applied are considered.

2.2 Range of products and their characteristics

The types of salted hot smoked and dried fishery products available throughout the world are diverse. They can range from very hard products such as the Japanese bonito

stick “Katsuobushi” to mildly hot smoked products such as smoked salmon which are mainly processed for the desirable flavour rather than for preservation. Dried fish products can be broadly categorized into “fully dried” products dried to a uniform moisture content and an a_w close to or below 0.75 and “partially dried” products with higher a_w values (Burt, 1988). The former have a shelf life of several months at ambient conditions while the latter have a typical shelf life of up to one week and are usually held at refrigeration temperatures.

Dried fish can either be cured and dried or dried only, such as Stock Fish from Scandinavia. Stock fish primarily includes Cod that is slit lengthwise and rinsed in fresh water before hanging to dry for approximately one and a half to two months in a relative humidity of 70-80% (Doe, 2002). It is fully dried with a typical a_w of 0.74. Klipfish from Norway is fully dried salted Cod that is culturally and economically significant in Scandinavia and Mediterranean Europe. The fish is slit open, ripened in salt and dried on racks according to a pretermined drying program until an a_w of 0.75 is achieved (Burt, 1988). Dried anchovies or “Bilis”, a very popular side dish in Malaysia and other Southeast Asian countries, is another fully dried product. It is processed by boiling the fish in 10% brine water for one minute, before draining and cooling and eventually sun drying for a day (Burt, 1988). The a_w of this product is approximately 0.79. Partially dried products include heavily salted and dried Indonesian Mackerel, Japanese dried squid (Surume) and Malaysian Catfish conforming to an approximate a_w of 0.89, 0.84-0.88 and 0.82, respectively (Burt, 1988; Doe, 2002).

Smoked fish products can be divided into three categories: cold smoked at a temperature not exceeding 30°C, hot smoked at a temperature sufficient for thermal denaturation of the proteins, and hot smoked dried fish (Yean, 1998). During hot smoking, the temperature of the smoke ranges from 40-100°C and the centre of the product may reach a temperature of 85°C or more (Arvanitoyannis and Kotsanopoulos, 2012). Hence, hot smoked products can be classified as heat treated or pastuerised products. Examples of hot smoked and dried products include dried smoked bonito stick or “Katsuobushi” from Japan with an a_w of around 0.76 and smoked skipjack or “Maldive fish” from Sri Lanka with an a_w between 0.64 to 0.67 (Doe, 2002). Traditional

African smoked products are rarely brined, but some may be sun dried prior to smoking (Poulter, 1988).

The use of various salt concentrations that can range from 2 to over 20% and addition of other ingredients increases the range of products. Ingredients such as sugar and spices and curing salts such as nitrates form part of the formulation of some products. Burt (1988) provides a comprehensive summary of the processing methods and nutritional composition of an extensive range of dried and smoked fish products available throughout the world. Sen (2005) reviews in detail the traditional salted and dried fish products in India. Cold smoked products or products smoked for desirable flavours only rather than for preservation, will not be covered in this review as they are significantly different to hot smoked and dried products in terms of product characteristics and microbial hazards associated with them.

2.3 The role of fungi

Filamentous fungi of some species are generally considered safe and have been intentionally used in the production of medicine and foods including cheeses and commercial microbial fermentations such as miso (soybean paste) and sake (rice wine)(Abe and Gomi, 2008; Jørgensen, 2007; Yokotsuka and Sasaki, 1998). The growth of certain types of fungi in some foods is known to enhance flavour and aroma due to their lipolytic activities (Comi et al., 2004; Selgas et al., 1999). Moulds are inoculated on to the product during processing to achieve desirable sensory attributes. For example, in the production of “Katsuobushi” (dried bonito stick), cultures of *Eurotium* or other safe species are inoculated onto the product towards the end of production (Miyake et al., 2010; Sikorski et al., 1998). This process, in addition to providing a desirable flavour, also controls the moisture content, reduces the apparent fat content and makes the flesh surface smoother.

On the other hand, fungal growth on food most of the time indicates the onset of spoilage and deterioration of the product (Pitt and Hocking, 2009). This could result in negative effects such as discolouration, rotting and the production of off odours

making food unmarketable incurring huge losses to manufacturers or distributors of the food. A broad range of food and agricultural products are predisposed to fungal attack and subsequent spoilage during storage and sale if kept under conditions of temperature, moisture, a_w and relative humidity favourable for fungal growth. Dehydrated or reduced water activity products such as stored cereals, nuts and spices are especially prone. Products of animal origin that are likely to spoil from fungal growth include cured fermented meat products such as sausages and salted, dried and smoked seafood.

However, most importantly, fungi that form the natural spoilage mycoflora of foods may include species capable of producing toxic secondary metabolites (Pitt, 2000b; Williams et al., 2004). History has recorded several epidemics caused by mycotoxins (Blount, 1961) but it is only in the past couple of decades that their importance has been realized. Since then, extensive research in this area has led to the discovery of more than 100 toxigenic fungi and 300 mycotoxins from different food products across the world (Keller et al., 2005). The toxicity of these compounds can vary quite widely with a range of undesirable health effects that could either be acute or chronic. The most important of these include various types of cancers and immunosuppression. However, only a few of these compounds have been described as toxicologically significant with legal limits imposed for the following mycotoxins only: aflatoxins, ergot alkaloids, fumonisins, ochratoxin A, patulin, trichothecenes and zearalenone (Samson et al., 2010). Furthermore, one species can produce a range of mycotoxins while several species can produce a common mycotoxin. Hence, the safety of food products contaminated with more than one mycotoxin is compromised even more due to possible synergistic effects (Miller, 2008).

The relationship between mycotoxigenic fungi with particular crops and animal food products and the risk to public health has been the focus of many studies (Barkai-Golan and Paster, 2008; Magan and Olsen, 2004; Sinha and Bhatnagar, 1998). Mycotoxins are reported to occur naturally on a wide variety of agricultural products including cereal grains, oil seeds and tree nuts and major food crops such as corn, rice, wheat and their products. They have also been reported from a range of processed

and preserved food products including protein based products. The three main genera that are of concern to human health are *Aspergillus*, *Penicillium* and *Fusarium*. (Bennett and Klich, 2003). *Fusarium* species are predominantly plant pathogens that grow and produce mycotoxins before or just after harvest on a range of maize and wheat crops. *Aspergillus* and *Penicillium* species, on the other hand, are mainly introduced to food as contaminants during drying and storage although some *Aspergillus* species play a role in plant pathogenicity as well. Fungi belonging to these species can produce a wide range of secondary metabolites under favourable conditions, some of which are toxic (Pitt and Hocking, 2009; Samson et al., 2010).

2.4 Analytical methods for fungi and mycotoxins

2.4.1 Methods for isolation of fungi

Methods for isolation, enumeration and identification of foodborne fungi are based on recommendations from the International Commission on Food Mycology (ICFM)(ICFM, 2012). A brief outline of these methods is provided here - for details refer to Pitt and Hocking (2009) and Samson et al. (2010). Direct plating is the method of choice for detection of fungi from particulate foods such as grains and nuts. This involves directly placing the particles on solidified agar. In most cases, surface disinfection prior to plating is essential to remove environmental contamination and permit growth of fungi that is actually growing in the food (Hocking et al., 2006). The standard regimen includes incubation at 25°C for 5 days. Results can be expressed as the percentage of particles infected by fungi. Dilution plating is preferred for liquid foods and powders as well as in instances where a count of the total mycoflora is required. Spread plating is recommended with enumeration at 25°C for 5 days. A higher temperature of 30°C is suitable for both these techniques in foods from tropical areas.

Media for isolation and enumeration of fungi are dependent on the type of food; several media have been recommended for the purpose (Hocking et al., 2006; Pitt and Hocking, 2009; Samson et al., 1992). Dichloran rose bengal chloromphenical agar (DRBC) is a general purpose medium for high moisture foods and fresh foods. Dichloran 18% glycerol agar (DG18) is widely used for enumerating fungi from reduced

water activity foods with a_w below 0.95. Low nutrient media such as potato dextrose agar are no longer in favour due to the selectivity against some fungal species. Selective media are also available for specific fungi: DG18 for xerophilic fungi; malt extract yeast extract 50% glucose agar (MY50G) for extreme xerophiles; and malt extract yeast extract 5% salt and 12% glucose agar (MY5-12) and malt extract yeast extract 10% salt and 12% glucose agar (MY10-12) agar for halophilic xerophiles.

2.4.2 Methods for identification of fungi

Traditional morphological methods have been the primary tool for identification of fungi from foods. This includes culturing on appropriate media based on the type of fungi to observe typical growth and sporulation (Samson et al., 2010). The identification keys of Pitt and Hocking (2009) are based on growth of fungi on three standard media: Czapek yeast extract agar (CYA); malt extract agar (MEA); and 25% glycerol nitrate agar (G25N); for 7 days at 25°C as well as at 5°C and 37°C. Additional media are required for some species. For example *Eurotium* species do not develop characteristic fruiting structures on high a_w media such as CYA and MEA and need inoculation onto Czapek yeast extract agar with 20% sucrose (CY20S) or MEA with 20 or 40% sucrose (Pitt and Hocking, 2009; Samson et al., 2010). The cultures are examined for colony characteristics and morphology under the microscope followed by identification using established taxonomic monographs (Klich, 2002; Larone, 2002; Pitt, 2000a; Pitt and Hocking, 2009; Samson et al., 2010).

Molecular methods are now gaining popularity for both detection and identification of fungi with significant advances in the methods. They are often used to supplement morphological identification. Molecular methods are reproducible, unambiguous and rapid compared to morphological methods which require more expertise for accurate identification. Various techniques such as pulsed field gel electrophoresis, ribotyping and PCR based finger printing have been applied but DNA sequencing is by far the most popular (Samson et al. 2010). Identification of important genera such as *Aspergillus* is frequently based on the sequence of the ITS region together with housekeeping genes such as calmodulin, β-tubulin and elongation factor 1-α (Peterson,

2008; Varga, 2006). Full genome sequences are now available for many important foodborne fungi. Samson et al. (2010) summarises the recommended loci for sequencing and databases for identification of important genera of fungi.

Use of secondary metabolite profiling or chemotaxonomy based on the unique metabolite profiles of different species is also a reliable method for species identification especially when coupled with morphological and molecular methods (Frisvad et al., 2008). Rapid Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) are frequently applied. TLC is often used for routine screening followed by more advanced techniques. TLC methods based on direct application of agar plugs on to the plate have enabled rapid screening of a large number of isolates (Filtenborg and Frisvad, 1980; Filtenborg et al., 1983). A microscale agar plug extraction method with HPLC-diode array detection (DAD) has been developed as an effective method for screening many isolates (Smedsgaard, 1997). HPLC-DAD is the acclaimed method for detailed taxonomical studies (Nielsen and Smedsgaard, 2003; Smedsgaard and Nielsen, 2005).

2.4.3 Methods for analysis of mycotoxins from foods

Analysis of mycotoxins in foods is a challenge for many reasons, such as the heterogeneity of food matrices, diverse structures of different mycotoxins as well the frequent requirement for detection at very low levels (ng/g or ppb). Hence, it is not possible to apply a single technique for all mycotoxins. A plethora of methods have been reported in the last decade as the importance of mycotoxins has been realised. Several detailed reviews have been recently published on mycotoxin methods (Köppen et al., 2010; Rahmani et al., 2009; Shephard, 2008; Turner et al., 2009) while reviews of specific mycotoxins such as aflatoxins (Shephard, 2009), ochratoxin A (Visconti and De Girolamo, 2005) and citrinin (Xu et al., 2006) are also available.

HPLC with UV or fluorescence detection is by far the method of choice for mycotoxin analysis but other chromatographic methods such as TLC and gas chromatography (GC) have also been applied. TLC, which was often used in the past is still common, especially for screening purposes. While this technique is simple and can be used for

screening huge number of samples simultaneously, it has been largely replaced by HPLC due to disadvantages such as the low sensitivity of this method for some mycotoxins and poor precision (Pascale and Visconti, 2008). HPLC has the advantages of high sensitivity, selectivity and repeatability while short analysis time and automation are a bonus. However, use of expensive equipment and expertise for operation are drawbacks when these are not available. Immunological techniques such as ELISA (Enzyme-linked immunosorbent assay) have also found wide applications especially for rapid analysis of mycotoxins using commercially available kits (Urusov et al., 2010). Liquid chromatography linked with mass spectroscopy (LCMS) is the most promising technique for multi-toxin determination and confirmatory purposes (Songsermsakul and Razzazi-Fazeli, 2008). Pascale and Visconti (2008) lists in detail the advantages and disadvantages of both the conventional and emerging methods for mycotoxin analysis.

Mycotoxin analysis in foods generally requires extraction and clean-up of the samples prior to analysis. Several methods have been employed for extraction based on the food substrate such as liquid/ liquid extraction, supercritical fluid extraction (SPE) and solid phase extraction (SPE) (Turner et al., 2009). SPE using small pre-packaged cartridges has been applied for clean-up of the samples for many years. The analyte is adsorbed onto the surface of the package and retained while impurities are washed away before eluting it. Use of Immunoaffinity columns (IAC) based on mycotoxin specific antibodies has gained recognition as a clean-up method (Şenyuva and Gilbert, 2010). The toxin is generally extracted with methanol and the solution diluted with water or phosphate - buffered saline prior to application to the IAC. The toxin which is retained in the column is eluted using methanol which denatures the antibody and releases the toxin. The method offers excellent recovery although the disadvantage for routine analysis is the high cost and single use of the column. IAC columns are now commercially available for the major mycotoxins.

2.5 Fungi associated with smoked and dried fish

Dun spoilage, a brown surface discolouration, is the most common type of fungal spoilage in salted dried fish from the temperate climates. The condition is caused by the xerophilic fungus *Wallemia sebi* , which is able to grow in salt concentrations between 5 and 26% (Horner, 1997). It was found later that this tiny brown fungus comprises three different species, *W. sebi*, *W. muriae* and *W. ichthyofaga*, all of which are xerophiles (Zalar et al., 2005).

However, in tropical countries where most drying and smoking of fish occurs, *Aspergillus* species and its telemorphic state *Eurotium* are the dominant fungi (Pitt and Hocking, 2009). Species belonging to this genus are ubiquitous in tropical environments and dominate the spoilage mycoflora of dried foods from the tropics (Pitt and Hocking, 2009). *A. flavus, A. fumigatus*, *A. niger, A. ochraceus* and *A. sydowi* are common isolates from smoked and dried fish originating from the tropics (Adebayo-Tayo et al., 2008; Atapattu and Samarajeewa, 1990; Essein et al., 2005; Fafioye et al., 2002; Wheeler et al., 1986). *Pencillium* species were reported as the second most frequently occurring genus on such products (Ahmed et al., 2004; Essein et al., 2005) although they are more common contaminants of food from the temperate areas. Table 2.1 collates the data from several studies on smoked dried fish or similar products, comparing the most frequently encountered fungi from these products.

Smoked and or dried fish remain an important protein source in many African countries. Several studies have been conducted on smoked dried fish products from Nigeria (Adebayo-Tayo et al., 2008; Diyaolu and Adebajo, 1994; Edema and Agbon, 2010; Essein et al., 2005; Fafioye et al., 2002; Fafioye et al., 2008). As expected, species belonging to *Aspergillus* such as *A. flavus*, *A. niger*, *A. fumigatus,* were the most frequent isolates. Other species from *Aspergillus*, *Eurotium* and *Penicilium* were also commonly isolated. Dried salted fish from markets in Burundi show infection with similar species including *A. niger*, *A. flavus*, *A. fumigatus*, *A. glaucus, A. restricus, Basipetospora halophila, Cladosporium herbarum* and *Penicillium chalybeum*. Smoked

dried "Bonga" fish from local markets and homes in Sierra Leone were contaminated with *A. flavus, A. ochraceus, A. tamarii, A. niger* and *Syncephalastrum racemosum* (Jonsyn and Lahai, 1992). Despite the absence of the very commonly isolated *A. flavus*, traditional Egyptian salted fish were contaminated with *A. niger, A. nidulans, A. terreus, Eurotium* species and *Penicillium* species (Ahmed et al., 2004).

In a study of fungi associated with dried fish in Sri Lanka (n=25), 33 out of 61 cultures isolated were *Aspergillus* with *A. niger* as the most prevalent fungus (Atapattu and Samarajeewa, 1990). Other *Aspergillus* species isolated include *A. flavus*, *A. fumigatus* and *A. restricus* while *Basipetospora halophila*, a genuinely halophilic xerophile well adapted to the ecological niche of dried, salted fish was also reported (Andrews and Pitt, 1987; Pitt and Hocking, 2009). *A. niger* was also the most common species isolated from Malaysian salted dried fish while lower numbers of *A. flavus, A. fumigatus, A. ochraceus* and *Penicillium chrysogenum* were recorded (Ito and Abu, 1985). Salted and sundried fish from India were frequently contaminated with *A. flavus, A. niger, A. fumigatus, A. oryzae, A. sydowi* and *Penicillium* species (Prakash et al., 2011).

In a study of salted and unsalted dried fish from Indonesia, *Aspergillus* was the dominant mycoflora with *A. flavus, A. tamarii, A. ochraceus, A. sydowii* observed as common isolates. Other *Aspergillus* species such *A. carbonarius, A. candidus* and *Eurotium* species were also present while species belonging to *Penicillium, Absidia, Mucor* and *Fusarium* were also reported (Santoso et al., 1999). Wheeler et al. (1986), however, in their study of Indonesian salted dried fish, found that *Polypaecilum pisce* which is a salt tolerant xerophile, was the dominant fungus on this product. *P. pisce* was isolated from 42% of the fish with many samples showing conspicuous white growth on the surface, characteristic of this fungus. Other frequently isolated species were *E. rubrum, E. repens, E. amsteldami, E. chevalieri, A. niger, A. flavus, A. sydowi, A. penicilliodes* and *A. wenti*. *P. citrinum* was the most frequently encountered *Penicillium* species. The a_w of the 74 samples of Indonesian dried fish ranged from 0.65 to 0.79. Dried bonito stick or Katsoubushi from Japan was predominantly contaminated with *A. ochraceus*, *A. oryzae* (domesticated form of *A. flavus*), *A. tamarii* and

Syncephalastrum racemosum (Motohiro, 1988). In earlier studies, dried marine products from Japan and Southeast Asia were found to be dominated by *Eurotium* species (Ichinoe et al., 1977; Okafar, 1968; Wu and Salunkhe, 1978). In a study of salted dried fish originating from Pakistan, Malaysia, Thailand and Hong Kong, *E. emstelodami, E. chevalieri* and *E. rubrum* were frequently isolated (Phillips and Wallbridge, 1977).

Table 2.1 Fungi most commonly isolated from smoked dried fish or similar products

Source	Common Isolates	Isolation media	Reference
Smoked dried fishes (stock, skip jack tuna etc.) from markets in Ibom State, Nigeria	*A. flavus**, *A.terreus**, *A. fumigatus*, *A. niger* and *Absidia* sp.	Saboraud dextrose agar	(Adebayo-Tayo et al., 2008)
Traditional Egyptian salted fish from commercial sources in Ismailia city, Egypt	*A. niger**, *A. nidulans, A. terreus, E. amsteldami, E. chevalieri, P. verracosum**, *P. restrictus* and other *Penicillium* sp.	Czapek dox agar and malt extract agar with 6% NaCl	(Ahmed et al., 2004)
Dried salted fish from markets in Kandy, Sri Lanka	*A. niger**, *A. flavus* , A. *fumigatus, A. glaucus, A. restricus, Basipetospora halophila, Cladosporium herbarum* and *Penicillium chalybeum.*	Potato dextrose agar, saboraud agar, Czapek dox agar and MY5-12 agar[a]	(Atapattu and Samarajeewa, 1990)
Cured fish (salted and dried and/or smoked) from Nigeria	*A. niger**, *A. flavus* , *A. fumigatus*, *E. chevalieri*, *P. citrinum*, *C. herbarum* and *Basipetospora* sp.	Malt yeast extract agar, potato dextrose agar and Czapex-dox agar with 20% NaCl	(Diyaolu and Adebajo, 1994)
Smoked cured fish from open markets in South-western Nigeria	*A. flavus*, A. niger*, *Penicillium* sp. and *Rhizopus* sp.	Potato dextrose agar and malt extract agar	(Edema and Agbon, 2010)
Smoked shark fish from a fish processing centre and main market in Ibom state, Nigeria	*A. flavus, A.niger, A. fumigatus, P. expansum, P. viridicatum, E. repens* and *Cladosporium* sp.	Malt extract agar and potato dextrose agar	(Essein et al., 2005)

Smoked dried fresh water fish from South-western Nigeria	*A. flavus*, A. ochraceus, A. niger, A. fumigatus, Mucor racemosus, Mucor* sp. and *Fusarium* sp.	Potato dextrose agar	(Fafioye et al., 2008)
Traditionally smoked dried fresh water fish from markets in Ago-Iwoye, Nigeria	*A. flavus*, A. ochraceus, A. fumigatus, M. racemosus, Mucor* sp. and *Fusarium* sp.	Potato dextrose agar	(Fafioye et al., 2002)
Salted dried fish from local markets in Selangor, Malaysia	*A. niger*, A. flavus, A. fumigatus, A. ochraceus* and *Penicillium chrysogenum*	7.5% salt malt agar	(Ito and Abu, 1985)
Smoked dried "Bonga" fish from local markets and homes in Sierra Leone	*A. flavus*, A. ochraceus, A. tamarii, A. niger* and *Syncephalastrum racemosum*	Malt extract agar	(Jonsyn and Lahai, 1992)
Non salted Ndagala fish from markets in Burundi	*A. flavus, A. niger* and *A. sydowi*	DRBC[b] and MY5-12	(Munimbazi and Bullerman, 1996)
Salted and sundried fish including skip jack tuna from Tuticorin dry fish market, Southeast India	*A. flavus*, A. oryzae, A. niger*, A. fumigatus*, A. sydowi* and *Penicillium* sp.	Rose bengal chloromphenical agar	(Prakash et al., 2011)
Salted and unsalted dried fish from markets in Jakarta, Indonesia	*A. flavus, A. tamarii, A. ochraceus, A. sydowii, A. carbonarius, A. glaucus, A. candidus, P. citrinum, P. chrysogenum, Eurotium* sp., *Absidia* sp., *Rhizopus* sp., *Cladosporium* sp., *Mucor* sp., *Fusarium* sp., *Syncephalastrum* sp., *Chaetomium* sp., *Nigrospora/ Neurospora* sp.	Dichloran Chloramphenicol Agar	(Santoso et al., 1999)
Dried salted fish from wholesale fish markets in Jakarta, Indonesia	*Polypaecium pisce**, *Eurotium rubrum, E. repens, E. amstelodami, E. chevalieri, A. niger, A. flavus, A. sydowii, A. penicillioides* and *A. wentii.*	DRBC and MY5-12	(Wheeler et al., 1986)

* Dominant species isolated

[a] MY5-12 agar (malt extract yeast extract 5% salt 12% glucose agar)

[b] DRBC (dichloran rose bengal chloromphenical agar)

The information from some of these studies should however be taken with caution as the media and methods used are sometimes not adequate to elucidate the whole range of fungi growing on that product. For example, in the study by Jonsyn and Lahai (1992), any halophilic species on the product will not be isolated as malt extract agar was the only plating media used (Pitt and Hocking, 2009). Very limited work has been conducted to characterise the type of fungi associated with smoked dried fish products discussed in detail above. Yet, it is noteworthy that only a selected number of these studies were designed well enough to address the whole range of fungi associated with these products.

2.6 Mycotoxigenic fungi and associated mycotoxins

Aspergillus and to a lesser degree *Penicillium,* which dominate the mycoflora of smoked and dried fish from the tropics include several important species that can produce toxic secondary metabolites. *A. flavus*, one of the most prevalent fungi isolated from such products is an important producer of aflatoxins. Aflatoxins have been extensively studied due to their ubiquitous nature and toxicity in the world's food supply (Theumer and Rubinstein, 2011). Aflatoxins occur mainly in the forms B_1, B_2, G_1 and G_2, so called because of the fluorescent colours blue or green displayed on exposure to long-wave UV light. Aflatoxin B_1, the major metabolite produced by the *A. flavus* is one of the most potent liver carcinogens and has been categorized as a Group I human carcinogen (IARC, 1993). It has been implicated in human hepatocellular carcinoma in some areas of Africa and Southeast Asia (Wang and Groopman, 1999; Wogan, 1992). Aflatoxins are mutagenic, teratogenic and carcinogenic and can cause acute liver damage including cirrhosis (Groopman et al., 2008; Wang and Tang, 2005). Long term exposure could lead to general adverse health effects such as immunosuppression, childhood stunting and interference with protein uptake (Gong et al., 2004; Khlangwiset et al., 2011; Williams et al., 2004). Although acute toxicity of aflatoxins is rare, several incidences including fatalities and deaths have been reported due to the consumption of contaminated foods (Krishnamachari et al., 1975; Lewis et al., 2005) .

The main aflatoxin producers are *A. flavus*, *A. parasiticus* and *A. nomius* belonging to the *Aspergillus* section *Flavi* (Sweeney and Dobson, 1998). Varga et al. (2011) recently presented a detailed overview of this section assigning 22 species to seven clades with several other species in the group also identified as aflatoxin producers (*A. pseudotamarii*, *A. bombycis*, *A. toxicarius*, *A. parvisclerotigenus, A. minisclerotigenes, A. arachidicola A. pseudonomius* and *A. pseudocaelatus*). These latter species are rarely encountered in foods.

A. flavus can produce only B type aflatoxins (B_1 and B_2) but it can also produce cyclopiazonic acid (CPA), a mycotoxin of lower toxicity compared with aflatoxin. CPA produces focal necrosis in most inner organs such as liver, spleen, pancreas, kidney and skeletal muscles (Frisvad et al., 2007). The toxin also acts through inhibition of the calcium pump disrupting the muscle contraction cycle (Burdock and Flamm, 2000; Chang et al., 2009a). Thus, it has been implicated in the infamous "Turkey X" disease together with aflatoxins (Cole, 1986; Smith et al., 1992). Mycotoxins CPA and/or fumuclavine A from *A. tamarii* may also have been responsible for kodua poisoning characterised by nausea, vomiting, depression, intoxication and unconsciousness after consumption of contaminated kodo millet seeds (Rao and Husain, 1985). Anthony et al. (2003) later demonstrated that consumption of kodo millet seed contaminated with CPA may lead to acute hepatotoxicity in both humans and animals. Low doses of the toxin were also reported to be immunosuppressive in test subjects (Hill et al., 1986; Nuehring et al., 1985). Some other species in the *Aspergillus* section *Flavi,* including *A. tamarii,* can also produce CPA (Varga et al., 2011). *A. tamarii* has also been isolated from smoked dried fish (Jonsyn and Lahai, 1992; Santoso et al., 1999; Wheeler et al., 1986). The incidence of both *A. flavus and A. tamarii* in smoked dried fish or similar products indicates possible contamination of such products with aflatoxins and CPA. Any other major aflatoxin producing species such *as A. parasiticus* appear to be uncommon in these products.

The frequent isolation of mycotoxin producing fungi from smoked and dried fish however does not necessarily indicate the presence of any toxins in such products. Most studies on the identification of fungi associated with these products did not

attempt to quantify the level of mycotoxins in fish. Wheeler et al. (1986) in their study of Indonesian dried fish conducted aflatoxin analysis on several fish from which *A. flavus* was isolated and found none of the samples to be positive for the toxin. The aflatoxin producing potential of *A. flavus* isolated from smoked and dried seafood has been tested by others, with negative results (Atapattu and Samarajeewa, 1990; Wu and Salunkhe, 1978). They concluded that it may be possible for the fungal spores to be present as contaminants from the environment without growing or producing toxins in the products (Wheeler et al. 1986).

However, aflatoxins have been reported from such products in other studies. Adebayo-Tayo et al. (2008) detected aflatoxins in all smoked dried fish samples ranging from 1.505-8.105 ng/g and 1.810-4.51 ng/g for aflatoxin B_1 and G_1, respectively. The 12 test samples from Nigerian markets were heavily contaminated with fungi. *A. flavus* was dominant but no aflatoxin G producers were isolated despite the presence of this toxin. The high moisture content of the samples (22.7 to 27.6% moisture, a_w not given) could have supported toxin production. In another study, TLC analysis of six mouldy fish extracts of smoked dried Bonga fish were positive for one or more of the toxins: aflatoxin B_1, G_1 or G_2 (Jonsyn and Lahai, 1992). *A. flavus* was the most prevalent fungus isolated from this product although production of aflatoxin G_1 and G_2 by them on YES medium was erroneously reported. While toxin levels in the Bonga fish samples were compared with varying amounts of the aflatoxin standards, no real quantification was achieved. In Tanzania, 16% of *A. flavus* isolates from traditionally cured fish (salted, smoked, sun dried) from markets were aflatoxigenic and contamination levels of up to 18.5 µg/kg fish were recorded (Mugula and Lyimo, 1992). In an older study surveying aflatoxin contamination of various food products from Semi- Savannah and forest areas in Nigeria, highest exposure was recorded from dried fish contaminated with exceptionally high toxin levels of 400-800 ng/g (Nwokolo and Okonkwo, 1978). Most countries have applied a regulatory limit of 2 or 4 ng/g for aflatoxin B_1 and 4 to 20 ng/g for total aflatoxin in a wide range of foods (FAO, 2004). The presence of toxin above the threshold limits in smoked dried fish indicates widespread exposure of aflatoxins through regular consumption of these foods.

Ochratoxin A (OTA) is another major mycotoxin of concern, produced by several *Penicillium* and *Aspergillus* species, that poses a risk to human health. Species belonging to *Aspergillus* section *Circumdati* and *Nigri* are responsible for the frequent contamination of tropical food commodities by this toxin (Gil-Serna et al., 2011). *Penicillium verrucosum* is the major OTA producer in cool temperate areas. OTA is a well-known nephrotoxin that can cause acute and chronic lesions of kidneys. It is also reported to exhibit carcinogenic, hepatotoxic, mutagenic, teratogenic and immunosuppressive properties in test animals (Mally and Dekant, 2009; Pfohl-Leszkowicz and Manderville, 2007). The International Agency for Research on Cancer (IARC, 1993) has classified this mycotoxin as a possible human carcinogen. OTA has also been implicated in the aetiology of Balkan Endemic Nephropathy, Chronic Interstitial Nephropathy in North Africa and the associated renal tumours (O'Brien and Dietrich, 2005; Stefanovic et al., 2006; Tatu et al., 1998)

A large number of other *Aspergillus* species, falling into four sections, can produce this toxin (Bayman and Baker, 2006). *A. ochraceus* and related species in the *Aspergillus* section *Circumdati* are producers of OTA. *A. ochraceus* was frequently encountered from some smoked dried fish products (Fafiyo et al., 2001; Ito and Abu, 1985; Santoso et al., 1999). However, other species related to *A. ochraceus* such as *A. westerdijikiae* and *A. steyni* are stronger producers of this toxin (Pitt and Hocking, 2009) but none of them were reported from smoked dried fish. This is probably because most of the reported studies pre-dated description of these relatively new species. Furthermore, A. *niger* which was a common dominant fungus in many smoked dried products are much more widespread in the tropics. Species belonging to *Aspergillus* section *Nigri* are now recognised as the major contributor to OTA levels in tropical foods. They have been found to produce OTA especially in vine fruits (Battilani et al., 2006). Spores of *A. niger* and related species are highly resistant to sunlight and can survive sun drying. Hence, it is not surprising that they have been isolated from smoked dried fish which has often been exposed to extensive drying under the sun (Essein et al., 2005; Wheeler et al., 1986). However, only a few strains of *A. niger* are capable of producing OTA (Copetti et al., 2010; Pardo et al., 2004), while *A. carbonarius* has been identified as a major

source (Frisvad et al., 2006; Romero et al., 2005). Hence, the probability of contamination of smoked dried fish products with OTA is much lower than for aflatoxins or CPA.

There have been only rare reports of the levels of OTA from smoked dried fish. Jonsyn and Lahai (1992) reported trace to moderately high levels of OTA in mouldy Bonga fish from Sierra Leone. *A. ochraceus* isolated from the fish was found to generate elevated levels of OTA on yeast extract sucrose media after three weeks of incubation. However according to Pitt (1995) the results reported in this study should be treated with caution as they were not quantified and have not been confirmed. Faraq et al. (2011) detected OTA in 20% (12) and aflatoxin B_1 in 32%(19) of 60 samples of smoked fish (*Clupea harengus*) sourced from markets in Port-Said, Egypt. Levels > 6 ng/g were reported in some samples. The moisture content of the samples was quite high with an average of 66.07%, while the mean sodium chloride content was 7.36 %. Despite only a few samples exceeding 5 ng/g, the most frequently applied regulatory limit for OTA in food stuffs (FAO, 2004), exposure of OTA via such products cannot be ignored.

Penicillium is the next most important genera of storage fungi associated with smoked dried fish. *P. citrinum*, a species of wide distribution, is however the only toxigenic species that is common in such products (Munimbazi and Bullerman, 1996; Santoso et al., 1991). It is a common contaminant of dried food commodities from Southeast Asia (Pitt and Hocking, 2009; Pitt et al., 1993, 1994). This fungus is a consistent producer of citrinin, a nephrotoxic mycotoxin that could disrupt renal functions in animals (Flajs and Peraica, 2009; Singh et al., 2011; Singh et al., 2007). Citrinin is also mutagenic, teratogenic, embryocidal, fetotoxic and immunosuppressive in animals. The toxic effects of citrinin on human T cells could increase the risk of development of allergic responses (Wichmann et al., 2002). The levels of citrinin in smoked dried fish or similar products have not been assessed. Once formed in foods, citrinin can disintegrate and the stability of the toxin in foods is thought to depend on the food substrate (Bailly et al., 2002; Bailly et al., 2005). Hence, contamination of smoked dried fish with citrinin is possible but the amount of toxin in such products is likely to depend on its stability in the products.

2.7 Factors that affect fungal growth and toxin production

Several factors can influence the range of microorganisms that can grow on hot smoked dried fish which are preserved mainly by producing an unsuitable environment in which fungi may propagate. While the conditions for toxin production are more restrictive than those required for growth (Esteban et al., 2004), the presence of toxigenic fungi in foods does not necessarily indicate mycotoxin production. Both intrinsic and extrinsic factors such as the strain, the food matrix, water activity, temperature and other environmental conditions determine the magnitude of mycotoxin production in foods by potentially toxigenic species (Klich, 2007; Sanchis and Magan, 2004). Only the main factors that could affect the growth and mycotoxin production of toxigenic fungi in smoked dried foods will be discussed in detail below.

2.7.1 Strain

The growth and mycotoxin producing ability of a particular fungus varies from strain to strain, with high variability witnessed within some species (Abbas et al., 2009; Horn and Dorner, 1999). For example, *A. flavus* exists in complex communities with different strains varying in their growth characteristics as well as the potential to produce mycotoxins (Klich, 2007). The genetic complexity of *A. flavus* enables the strains to be subdivided into several groups based on the physiological, morphological and molecular characteristics. The diversity in the colony morphology of the strains is witnessed in sclerotium production (no sclerotia to predominantly sclerotial), conidial head formation (densely sporulating to mostly mycelial), and conidial colour (bright yellow green to dark green or rarely yellow) (Horn, 2005). Some *A. flavus* strains can produce elevated levels of aflatoxins, CPA or both while other strains produce negligible levels (Abbas et al., 2009; Horn and Dorner, 1999). Based on the amount of literature on the mycotoxin production of *A. flavus*, only 30-40% of known isolates are believed to produce aflatoxins (Frisvad et al., 2007). The strain variability is also dependent on several factors such as substrate, the geographical location and the environmental factors (Horn, 2005; Klich, 2007). The incidence of aflatoxigenic *A. flavus* from various food sources in Argentina was higher in peanuts (69%) than in

wheat (13%) or soybeans (5%)(Vaamonde et al., 2003). In general, peanuts appear to be a good source for aflatoxigenic isolates compared to many other foods, regardless of the geographic location (Barros et al., 2005; Novas and Cabral, 2002; Pildain et al., 2004). Toxigenic strains of *A. flavus* isolated from peanut samples from the Northern region of Vietnam were much lower (7.7%) than from the Southern region (62.5%) (Tran-Dinh et al., 2009) most likely due to climatic differences.

Strains belonging to this species can be categorised into two morphological groups, designated as S and L strains, based on the size of the sclerotia developed in culture. The L strain forms sclerotia >400 µm in diameter and the S strain develops abundant small sclerotia <400 µm diameter. The presence of sclerotia *per se* does not seem to correlate with aflatoxin production but in general a high level of toxin formation is observed from strains with abundant small sclerotia (S strain) while strains producing large sclerotia (L strain) can be toxigenic or non-toxigenic (Cotty, 1997; Klich, 2007). S type strains atypically produce aflatoxins B and G, and some of them have been assigned as new aflatoxigenic species in the *Aspergillus* section *Flavi,* while further investigation was deemed necessary for the remaining isolates, currently assigned as *A. flavus* (Varga et al., 2011). Strains of *A. flavus* producing large sclerotia are abundant in nature while S type strains have been less commonly isolated. Vegetative compatibility groups (VCGs) are subpopulations within a fungal community that have been used as a measure of the diversity of *A. flavus* populations (Horn, 2005; Horn and Greene, 1995). The phenotypic variation in *A. flavus* populations as well as mycotoxin producing ability can be attributed to the differences among the VCGs.

Gene deletions in *A. flavus* are held responsible for the huge variability witnessed between mycotoxin producing ability of different strains of this species (Abbas et al., 2011; Yin et al., 2009). More than 25 contigous genes within the 75-kb cluster are responsible for mycotoxin production in *A. flavus* (Yu et al., 2004). Both the aflatoxin and CPA gene clusters in *A. flavus* are adjacent to each other and under the control of several of the same regulatory elements (Chang et al., 2009b). Strains with partial deletions in the aflatoxin gene cluster can still produce CPA but those strains showing complete deletion of both aflatoxin and CPA gene clusters have lost the ability to

produce either of these mycotoxins (Abbas et al., 2011). Alternatively, mutations in the pathway genes could be responsible for losing the mycotoxin producing ability (Abbas et al., 2011).

Some other species show less strain variation with regards to production of specific mycotoxins. For example, almost all strains of *A. tamarii* are capable of synthesizing CPA (Dorner, 1983; Vinokurova et al., 2007) while all *P. citrinum* strains are known as prolific producers of the mycotoxin citrinin (Heperkan et al., 2009; Malmstrøm et al., 2000; Romero et al., 2005).

2.7.2 Water activity

Water activity (a_w) relates to the availability of water in the food for use by the microorganisms and hence is the primary defining factor for the stability of dried stored products in many practical situations (Pitt and Hocking, 1991). Microbial activity leading to spoilage, and more importantly safety of the product is dependent very much on a_w. Drying and smoking processes reduce the a_w by removal of water from the system. Furthermore, addition of salt or other solutes lowers the available water as they bind with water in the food making it unavailable for microorganisms to proliferate (Doe et al., 1998). The relationship between a_w of the food and the types of microorganism that can grow is well established. Table 2.2 summarises the limiting a_w values for microorganisms found on smoked and dried fish.

Table 2.2 Limiting a_w values for microorganisms associated with smoked and dried fish (Horner 1997)

Microorganisms inhibited	Water activity
None	1.00
Gram negative rods (eg: *E.coli* and spores of Bacillaceae)	0.95
Most cocci and lactobacilli Vegetative cells of Bacillaceae	0.91
Most yeasts	0.88
Most moulds *Staphylococcus aureus*	0.80
Most halophilic bacteria	0.75
Xerophilic moulds	0.65
Osmophilic yeasts	0.60

Fungal growth often tolerates lower a_w than bacteria and the reduced water activity of smoked and dried fish predisposes these products to fungal attack and potential mycotoxin contamination. Variability in the a_w of smoked and dried fish is high due to the wide range of products available. Furthermore, even batches of the same product often show huge deviation (Fuentes et al., 2010). The types of fungi growing and dominating products, corresponds to the available water in the products. The water activity at which spore germination and growth of a fungus is inhibited can be expressed as the minimum a_w. The influence of a_w on different classes of fungi is often sharply defined, with variable minimum a_w levels. Some fungi associated with dried foods have the ability to grow at very low a_w levels that do not support the growth of many other organisms. Such fungi are termed xerophiles, literally meaning “dryness loving”. According to Pitt (1975), a xerophilic fungus is capable of growth, under at least one set of environmental conditions, at an a_w below 0.85. Pelhate (1968) defines

xerophilic fungi as those showing maximum growth rates below 0.95. The minimum a_w for important fungi according to Pitt and Hocking (2009) is listed in Table 2.3.

When the a_w of the dried food is adequately lowered to below 0.75, only a few xerophilic fungi can grow while growth of most other fungi, including the toxigenic species, will be prevented. However, products at intermediate a_w levels will support growth of a wider range of fungi including toxigenic species such as *A. flavus*. Fungal growth can be totally inhibited by rapid drying of the product to an $a_w \leq 0.65$ and maintaining this level during storage (Samson et al., 2010).

Table 2.3 Minimum water activity of some important fungi associated with foods (Pitt and Hocking, 2009)

Moulds inhibited	**Water activity***
None	1.00
Basidiomycetes Most soil fungi	0.95
Mucorales, *Fusarium*	0.90
Rhizopus *Cladosporium*	0.85
Aspergillus flavus Xerophilic *Penicillia*	0.80
Xerophilic *Aspergilli* *Wallemia* *Eurotium*	0.75
Chrysosporium *Eurotium halophilicum*	0.70
Xeromyces bisporus	0.65

*water activities are approximate minima for growth reported in the literature

2.7.3 Temperature

Temperature is a very important factor for fungal growth. Higher temperatures (> 20°C) support the growth of *Aspergillus* species, while lower levels (<20°C) tend to favour cold tolerant fungi such as *Penicillium* (Pitt and Hocking, 2009). Smoked dried foods are mostly stored under conditions that favour fungal growth. The low a_w of dried foods and the elevated ambient storage temperatures of tropical countries, provides favourable conditions for the growth of *Aspergillus* species. The optimum temperature for *A. flavus* is near 33°C, a minimum near 10-12°C and maximum near 43-48°C (Holmquist et al., 1983; Pitt and Hocking, 2009).

2.7.4 Nutrients

Most food products contain a carbon source that most common fungi are able to use. In protein rich substrates, such as smoked dried fish, amino acids may be used as a carbon source by the fungi (Pitt and Hocking, 2009). Some xerophilic fungi, such as *Chrysosporium* and *Xeromyces bisporus,* require more complex nutrients than many other fungi which can survive on almost all food products (Pitt, 1975) .

2.7.5 Smoking

Smoking imparts a preservative effect to the products due to the antimicrobial compounds of smoke such as phenolics, acetic acid and carbonyls, working together synergistically (Toledo, 2007). Vegetative bacteria including bacterial pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* are susceptible to the action of the smoke whereas bacterial spores and moulds are less affected (Asita and Campbell, 1990; Poysky et al., 1997; Toledo, 2007). The activity however depends on several factors including the type of wood or type of smoke, type of fish and the conditions of smoking (Asita and Campbell, 1990; Poysky et al., 1997; Suñen, 1998). The fungicidal activity of smoke is less effective than that for bacteria although some studies have reported positive effects. Milly et al. (2005) in a study of carbonyls and acids from smoke, reported antifungal activity against *A. niger* commonly found in dried foods. The low antifungal effects, together with the dryness of the skin on smoking and the decrease in the pH of fish to below 6.5, as well as a higher chance of

spore survival, means that smoked dried fish are increasingly prone to fungal attack and mycotoxin production (Sikorsk and Kolodziejska, 2002). Furthermore, the lengthy storage of many smoked dried products accounts for the gradual loss of any preservative effect from wood smoking, thus increasing susceptibility to the growth of fungi.

2.7.6 Salt content

Fish are often salted or brined prior to smoking and drying, adding to the preservative effect. NaCl, in addition to lowering the $a_{w,}$ also improves the antimicrobial effect of smoking. There is large variation in the amount of salt found in products ranging from 2 to over 20% NaCl. Although levels above 5% can halt growth of the majority of spoilage microorganisms associated with fish, fungi are less sensitive (Horner, 1997). In a study of different brine concentrations on the quality and safety of smoked catfish, high salt concentrations of 20 and 25% were the most effective in reducing the microbial and fungal populations but consumers preferred products containing 5% salt treatments (Omojowo et al., 2010). A sodium chloride content of 5% in a basal media was found to slightly inhibit the growth of mould species (*A. flavus, A. niger, A. ochraceus, A. candidus , E. chevalieri* , *P. citrinum* etc.) isolated from sundried and/or smoked fish with obvious inhibition when increased to 10%, except for *Basipetospora* which recorded increased growth and sporulation (Diyaolu and Adebajo, 1994). At 20% salt, growth and sporulation of all of the test species were highly or completely inhibited while 25% salt fully inhibited the growth of all fungi (Diyaolu and Adebajo, 1994). According to Uraih and Chipley (1976), aflatoxin production by *A. flavus* was inhibited at salt concentrations equivalent to or above 12% while lower concentrations of 8% and below were found to enhance toxin production in synthetic media. Hence, a high concentration of salt above 12% to 25% is necessary to prevent fungal growth and mycotoxin production, but undesirable in terms of organoleptic characteristics and health. Hence, the salt content of the product should be used in combination with other hurdles to achieve stability and safety of smoked dried fish.

2.7.7 Other factors

The role of pH in the growth of fungi becomes obvious only at high a_w. Most smoked and dried fish are of reduced water content with a_w as the overriding factor that influences fungal growth. The pH values of such products are generally >5, hence other limiting factors take over. Most fungi are unaffected by pH over a broad range between 3 to 8 (Wheeler et al., 1991). Preservatives are generally not used in stored dried products that are intended for direct consumption while fumigants or insecticides have been used to control the growth of fungi and insect infestation under some circumstances. However, the use of such chemicals is becoming undesirable and other means of ensuring safety and quality are often sought. In most products, the combined preservative factors act as a series of hurdles to prevent microbial and fungal growth (Arvanitoyannis and Kotsanopoulos, 2012). For example, the combination of low a_w, the preservative effect of smoke and salt appear to act synergistically leading to increased shelflife and improved safety of the products.

2.8 Control of fungal growth and mycotoxin production

2.8.1 Hygiene controls

As for any other food product, the establishment of hygiene standards and the adoption of Good Hygienic Practices (GHP) are essential for maintaining the safety of smoked dried fish products. Furthermore, a systematic approach to food safety, including application of Hazard Analysis Critical Control Point (HACCP), is common in the fish processing industry and often required by law. The hot smoking process is an important control point in the production of smoked dried fish and results in the pasteurisation of fish flesh (85°C or more) (Arvanitoyannis and Kotsanopoulos, 2012). The correct time and temperature is necessary to ensure inactivation of bacterial pathogens as well as fungal spores. Similarly, control over the drying process is essential, particularly in the case of fully dried shelf stable products, where adequate drying (a_w <0.68) can result in elimination of fungal growth (Pitt and Hocking, 2009). Also rapid drying of the products to low moisture content is critical as it creates less favourable conditions for fungal growth and proliferation. Stringent hygiene control is

necessary to minimise exposure to fungal spores from the environment, especially post production. The product should be protected from any environmental contamination during sun drying, the common drying technique used in the tropics.

2.8.2 Adequate drying

Drying results in the removal of water and hence the reduction in a_w of foods. During the drying process, the a_w of fish has to be brought down rapidly below 0.95 to prevent spoilage and growth of pathogenic bacteria (Sikorski et al., 1995). However, a microbiologically stable product can be obtained by decreasing the a_w to around 0.70, equivalent to 15% moisture content in lean fish (Sikorski et al., 1995). Dried foods are associated with xerophilic fungi which are able to grow rapidly at $0.77a_w$ and above. Reducing the a_w below 0.75 decreases the incidence of these fungi while a further reduction to 0.68-0.65 or below can completey arrest their growth (Pitt and Hocking, 2009; Samson et al., 2010). Control of most fungi, including major toxigenic species in smoked dried fish, can be attained by adequately drying the fish to an a_w of 0.75 or below. The most effective method for ensuring control of fungi in dried foods is to dry the product rapidly to the correct a_w and keep it dry during storage (Pitt and Hocking, 1991).

2.8.3 Packaging

Packaging is important to prevent exposure of smoked dried product to fungal contamination and reabsorption of moisture during storage and handling. Requirements for suitable packaging for dried fish include inertness, leak proof, impermeability to oxygen and moisture, low transparency and resistance to abrasion and puncture (Gopal and Shankar, 2011). Bulk packaging material commonly used in the tropics include waxed corrugated cartons, deadwood and plywood boxes, bamboo baskets, gunny bags, dried palmirah or coconut palm leaves, and multiwall paper sacks (Gopal and Shankar, 2011). The product is often bulk packed and some of these materials are very likely sources of fungal contamination, especially if hygienic standards are not maintained. Low density polyethylene (LDPE) packages and recently pouches made of polyester laminated with polythene are used for individual consumer

packages. Hot smoked dried fish that is immediately vacuum packaged after smoking displays no apparent change in the microbial levels during subsequent storage (Gram, 2009). The growth of aerobic bacteria and fungi is inhibited under vacuum due to the reduction in atmospheric oxygen. Most contamination or spoilage fungi have an absolute requirement for oxygen with growth occurring under aerobic conditions or at least when oxygen tension is appreciable. However, some fungi such as *Byssochlamys* species, can tolerate reduced oxygen or increased carbon dioxide (Pitt and Hocking, 2009). Most fungi that can tolerate such conditions are not necessary toxin producers.

2.8.4 Biological control

Many food products, such as soft ripened cheese, are inoculated with selected strains of fungi that impart a unique texture and flavour to the product due to the lipolytic and proteolytic activities of the enzymes produced by the culture. Similarly, Katsuboushi (smoked dried bonito) is inoculated with strains of flavour-inducing fungi to achieve the desirable sensory attributes (Miyake et al., 2010). This approach could have a dual role, enhancement of sensory properties and more importantly biological control by out competing any deleterious natural flora. Either non toxigenic species or non-toxigenic strains of potentially toxigenic species could be used to displace the harmful fungi growing on smoked dried fish. For example, *E. repens*, a common isolate of many smoked dried fish from tropics, has not been reported to produce any mycotoxins (Pitt and Hocking, 2009). In addition, this species is thought to have a flavour enhancing role in Katsuobushi and mould ripened ham (Comi et al., 2004; Miyake et al., 2010) and hence has the potential to be suitable to protect the product from harmful fungi. Species interactions and other environmental factors were reported to select for the dominant fungi on dried salted fish (Wheeler and Hocking, 1993). These are important factors that have to be considered when selecting a strain for inoculation in food products.

The potential for biological control of aflatoxins through competitive exclusion has been explored in many agroecosystems including peanuts and maize (Abbas et al., 2011; Probst et al., 2010) . The ability of non-toxigenic strains of *A. flavus* to effectively

compete for the same ecological niche as toxigenic strains forms the basis for this approach. Several non aflatoxigenic *A. flavus* strains have been patented and commercialized for control of aflatoxins in food crops. However, there is concern that some of these strains produce CPA and the use of strains that have lost the ability to produce both aflatoxins and CPA is encouraged (King et al., 2011). *A. flavus* is a dominant fungus in many smoked dried fish products and hence, inoculation with non-toxigenic strains of *A. flavus* may prevent the growth of toxigenic species. In fact, *A. oryzae*, often described as domesticated form of *A. flavus,* has been used in the fermentation of oriental food products for many decades. Some strains of *A. oryzae* were found to produce the mycotoxins CPA and kojic acid (Tanaka et al., 2006). Considerable care should be taken during selection of strains for biological control to ensure safety of such products.

2.9 Conclusion

Smoked dried fish or related products are frequently contaminated with important toxigenic species such as *A. flavus* and *A. niger*. Furthermore, some products are reported to contain elevated levels of aflatoxins and OTA exceeding legal limits. Hence, there is a food safety risk in the consumption of smoked dried fish infected with fungi. Exposure to mycotoxins through contaminated foods are high in many developing countries (Williams et al., 2004) and as such, mycotoxins ingested through smoked dried fish are likely to contribute to this burden. It is therefore imperative to ensure control of this hazard. Many methods can be applied to control the growth of fungi and mycotoxin production in smoked dried fish. The most practical of these would be to reduce the a_w to a sufficiently low level to inhibit toxigenic fungi. Other strategies such as modified atmospeheric packaging or biological control could also be adopted.

CHAPTER 3

Isolation and Identification of Mycoflora and Toxigenic Fungi from Maldive Fish

Abstract

The uncontrolled natural mycoflora growing on the surface of "Maldive fish" was studied. Twenty five samples with visible fungi were cultured before and after surface sanitisation and the fungi identified by morphological techniques following appropriate keys. Physiochemical parameters of the samples were also determined. *Aspergillus* and its telemorph *Eurotium* were the predominant genera isolated. More than eighteen species were isolated of which *Aspergillus flavus* (92% of samples), *A. tamarii* (96%) and *Eurotium repens* (92%) were the dominating flora. Other frequently encountered species include *A. sydowii* (64%), *A. niger* (40%), *A. candidus* (16%), *E. rubrum* (32%), *E. chevalieri* (20%), *Penicillium citrinum* (60%), *Absidia corymbifera* (16%) and *Syncephalastrum racemosum* (20%). Most isolates on the surface were also found invading the product. Water activity of the samples varied widely from 0.951 to 0.720 supporting growth of a wide range of fungi. The pH (5.65 to 6.68) and NaCl (1.48% to 4.29%) levels were unlikely to influence the mycoflora. The presence and prevalence of *A. flavus* which is a major producer of the very potent liver carcinogen aflatoxin as well as other potentially toxigenic fungi (*A. tamari*, *A. niger*, *A. ochraceus* and *P. citrinum*) implies possible contamination of the product with mycotoxins of varying potency if provided with a conducive environment. Hence, consumption of products infested with fungi could pose a health hazard.

3.1 Introduction

The reduced water activity of dried foods limits growth of competitive microorganisms to some moulds and yeasts which can cause spoilage. Many filamentous fungi associated with food products have a role in deterioration contributing to undesirable effects such as discolouration, rotting and production of off odours that could render the food unsalable. On the other hand, some species are generally considered safe and have been intentionally used in the processing of foods such as cheeses and commercial microbial fermentations (Abe and Gomi, 2008; Jørgensen, 2007; Yokotsuka and Sasaki, 1998). However, most importantly the natural spoilage mycoflora of foods may include species capable of producing toxic metabolites that could lead to adverse health effects in humans and animals (Pitt, 2000b; Williams et al., 2004). Contamination of products with potentially toxigenic fungi should therefore be avoided.

The mycoflora or the fungi associated with a particular type of food are often specifc to that food type (Samson et al., 2010). Generally only a limited number of species, in the order of one to three, dominate the spoilage mycoflora of a particular food product. This is dependent on many factors such as nutrient availability, water activity and other prevailing environmental factors (Pitt and Hocking, 2009). Detailed knowledge of the mycoflora associated with a specific food is crucial for the prevention of spoilage or mycotoxin production in the product. Physiological data and mycotoxin profiles for most of the important food related fungi are comprehensively covered in the literature (Klich, 2002; Pitt and Hocking, 2009; Samson et al., 2010). Hence, elucidation of the fungi to the species or sub-species level will assist in deciding their potential toxicity in foods.

The uncontrolled natural mycoflora growing on the surface of Maldive fish has not been characterised previously. This study aims to define the mycoflora associated with Maldive fish by isolation and identification of the fungi growing on the product using cultural and morphological methods following established taxonomic monographs. This will determine any potentially toxigenic species. Another important objective was

to assess the physiological parameters that could have an impact on the contaminating fungi and relate this to the mycological safety of the product.

3.2 Methodology

3.2.1 Sample Collection and Preparation

Samples were purchased from local markets in the capital island Malé, Maldives, over a period of three years. Twenty five smoked dried fish (1-2 fillets from the same batch depending on the size, approximately 100 g) harbouring visible fungi were collected randomly, that is one to two samples per supplier and directly placed in individual sterile polythene bags using aseptic techniques. Each sample was designated an alphabetical letter for a code X (X= A, B, C etc). They were then imported in to New Zealand (approximately 3 days at ambient temperature) and directly transferred to the testing laboratory. The samples were stored at -80°C until analysis. Details of sampling locations and times are provided in Appendix A.

Samples were removed, thawed at room temperature and half of each sample ground without any treatment (X1). The other half was surface sanitised using 70% ethanol and rinsed with sterile distilled water before grinding (X2). Surface sanitisation aimed to remove fungal spores from the surface and ensure enumeration of those fungi actually invading and contaminating the food (Samson et al., 2010).

3.2.2 Media Preparation

The following media were prepared according to the formulation of Pitt and Hocking (2009): Czapek yeast extract agar (CYA); Czapek yeast extract agar with 20% sucrose (CY20S); malt extract agar (MEA); malt extract yeast extract 5% salt 12% glucose agar (MY5-12); malt extract yeast extract 50% glucose agar (MY50G); and Aspergillus flavus and parasiticus agar (AFPA). See Appendix B for media formulations. Dichloran rose bengal chloromphenicol agar (DRBC) was procured from Merck, Germany and Dichloran 18% glycerol agar (DG18) from Fort Richard Laboratories Ltd, Auckland, New Zealand.

3.2.3 Isolation of fungi

Fungi were isolated from both untreated (X1) and surface sanitised (X2) samples by application of direct and dilution plating according to Figure 3.1. For direct plating, the ground sample was sprinkled directly onto the isolation agars in three replicates. For the dilution plating, 10 g of the ground sample was weighed into a stomacher bag with 90 ml of 0.2% peptone water and macerated for 2 minutes. Serial ten-fold dilutions were made (up to 10^{-4}) and spread plated onto the isolation agars in three replicates.

The following isolation agars were used: DRBC, a medium suitable for enumeration and isolation of fungi in high a_w foods (note that some of the products sampled had an a_w of ≥0.9); DG18 which is a selective agar for enumeration and isolation of xeropihilic fungi from low a_w and dried or semi-dried foods; and MY5-12 suitable for isolation of halophilic fungi from salted foods.

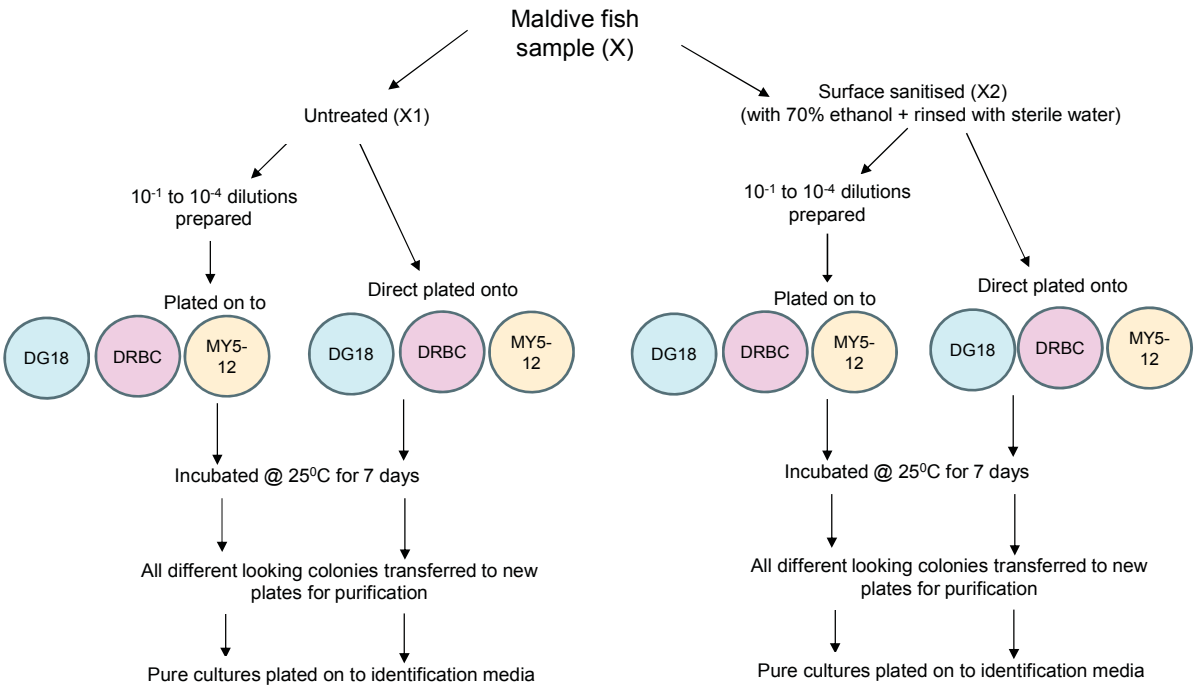


Figure 3.1 Protocol for isolation of fungi from Maldive fish

Petri dishes were incubated at 25°C for 7 days. Initially, that is for the first five samples, two sets of three replicates for each treatment was prepared, one set incubated at the standard 25°C and the other at 30°C to imitate the product storage temperature in the tropics. However, overgrowth of some species at the latter temperature obscured growth of other more slowly growing species. Hence, the standard incubation temperature (25°C) was used for all further samples. Colonies with visually distinct cultural characteristics were inoculated onto appropriate media to obtain pure cultures before transfer to the identification media.

3.3 Identification of fungi

The fungi were identified to species level using the media, methods and plating regimen of Pitt and Hocking (2009). Other keys were also consulted when required (Pitt, 2000a; Samson et al., 2010). The plating regimen is depicted in Figure 3. Each colony exhibiting different cultural characteristics was inoculated onto Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) at 25°C and Czapek yeast extract agar (CYA) at 37°C. The plates were inoculated at three points equidistant from the centre and incubated for a standard time of seven days. To avoid growth of stray spores on the plates, spores were first suspended in 0.2-0.4 ml semi solid agar in a vial consisting of 0.2% molten agar and 0.05% Polysorbitan 80 (Tween 80)(Pitt and Hocking, 2009) using a sterile needle. A sterile loop was then used to mix the vial contents and inoculate the standard plates. Plates incubated at 37°C were enclosed in polythene bags to prevent drying up of the media.

At the end of the incubation period colonies were examined for cultural and morphological characteristics as follows:

Colony diameter: measured as the diameter of the colony in millimetres from the reverse side of the plate

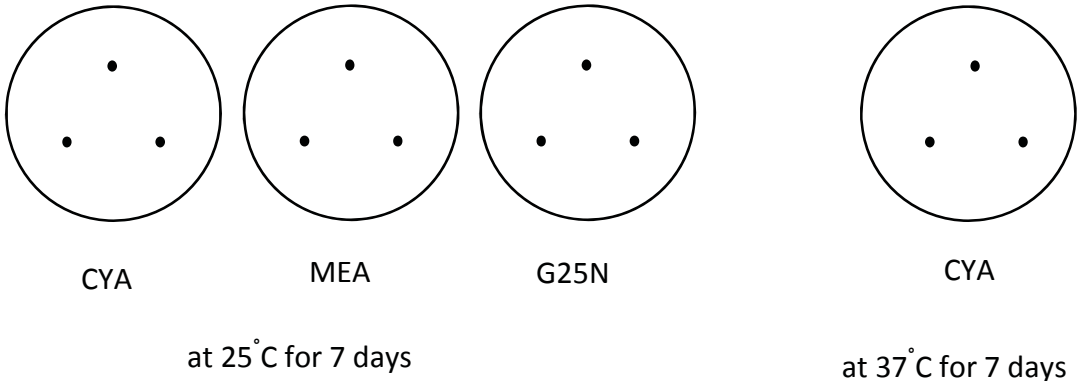


Figure 3.2 Regimen for culturing fungal isolates for identification (Pitt and Hocking, 2009)

Colony characteristics: Petri plates were examined by eye under natural daylight and under a stereomicroscope to determine the characteristics of the colony. The stereomicroscope was used to assist examination of characteristics such as type of sporing structures and their location and the extent of sporulation. Colony colours including reverse colours were checked by daylight.

Microscopy: microscopic features of the isolates were determined using a wet mount (Pitt and Hocking, 2009) and/ or by the transparent tape technique (Samson et al., 1995). A wet mount was prepared by cutting off a small portion of the colony using an inoculation needle and placing it on a slide with the aid of a drop of 70% ethanol. A drop of lactophenol cotton blue was added when required. A cover slip was placed on top and excess liquid removed by gently blotting with tissue paper before examination. For the transparent tape technique, a length of transparent adhesive tape was gently pressed down onto the surface of the colony and transferred to a glass slide containing a drop of lactophenol cotton blue. The tape was pressed at the extreme ends of the slide. The slides were viewed using a high resolution light microscope initially under 40 x objective for examining major structures and under 100 x using oil immersion for studying details of the spores and surface textures. Microscopic features of the isolates such as conidial heads, stipes, shapes of conidia, roughness of conidial walls, existence of ascospores or cleistothecia, lengths of philiades and metulae were determined. Only a single isolate from each species was recorded per sample. The frequency of isolation

of a fungus was determined based on the number of samples from which the species was isolated from the twenty five samples, expressed as a percentage. A representative number of isolates from each species per sample was transferred to storage for future experiments (refer section 5.2.1).

The above general media are not satisfactory for identification of some genera of fungi and hence special media is utilised in such cases. One such genus is *Eurotium* as *Eurotium* species do not develop characteristic fruiting structures on high water activity media such as CYA and MEA. *Eurotium* species were inoculated onto CY20S agar to assist their identification (Pitt and Hocking, 2009). The cultures were initially incubated at 25°C for seven days and colony diameter and appearance recorded. Plates were returned to the incubator and wet mounts of the cleistothecia examined at intervals until the development of mature ascospores which normally occurs within 14 days. Genuinely xerophilic fungi, such as *Polypaecilum pisce,* should be incubated for a longer period of 14 days, after transfer to the identification media. Isolates of this species were also inoculated on to MY5-12 and MY50G media and incubated at 25°C for 14 days.

3.3.1 Confirmation of Species

The major aflatoxin producing species *A. flavus* and *A. parasiticus* have some resemblance to *A. tamarii* on culture media. Aspergillus flavus and parasiticus agar (AFPA) is recommended as a useful diagnostic to distinguish *A. flavus* from *A. tamarii* (Pitt and Hocking, 2009). Isolated colonies of *A. tamarii* and *A. flavus* were inoculated on to AFPA and incubated at 30°C for 42-48 hours. At the end of the incubation period, plates were checked for reverse colouration under daylight. Colonies of *A. flavus, A. parasiticu*s and *A. nomius* are distinguished by bright orange yellow reverse colouration whereas *A. tamarii* produces a deep brown reverse colouration (Figure 3.3).

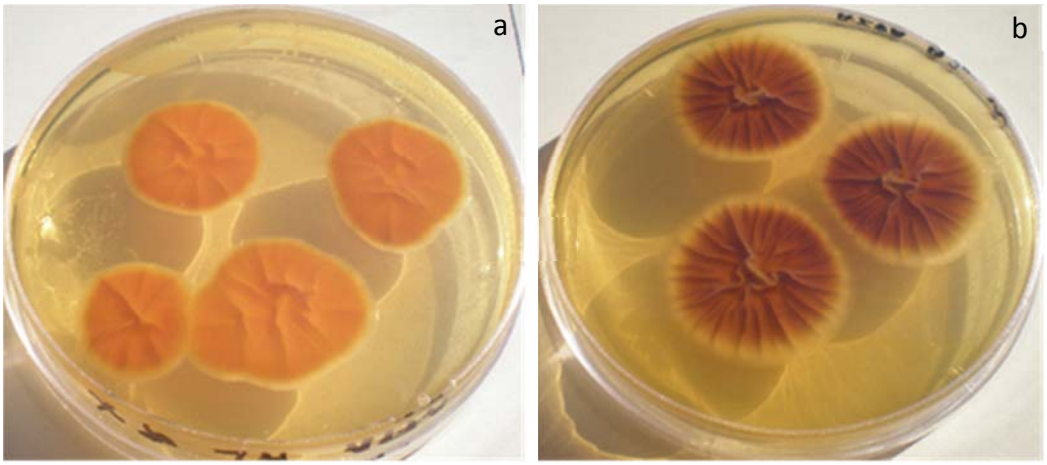


Figure 3.3 a) Orange yellow reverse colouration of *A. flavus* and b) deep brown reverse colouration of *A. tamarii*, on AFPA agar

3.3.2 Physiochemical Parameters

All of the 25 Maldive fish samples (untreated) were subjected to physiochemical analysis.

3.3.2.1 Water Activity

Water activity (a_w) of the samples was measured in duplicate using a Decagon water activity meter (model CX-2, Formula Foods Corp. Ltd).

3.3.2.2 Measurement of pH

pH of the sample was determined by mixing 1 part of sample with 9 parts of distilled water and pH of this suspension was measured, in duplicate using a pH meter (Orion Model 420A).

3.3.2.3 Sodium Chloride

Sodium chloride content of the samples was measured by the volumetric method for determination of salt (chloride as sodium chloride) in seafood, AOAC method 937.09 (AOAC, 1999).

Reagents

- 0.1 N Silver nitrate standard solution: (Dissolve 17 g silver nitrate in 1 L distilled water. This solution was stored in an amber glass stoppered bottle away from light).
- 0.1 N Ammonium thiocyanate standard solution: 7.612 g NH_4SCN in 1 L distilled water.
- Ferric indicator: Saturated solution of $FeNH_4(SO_4)_2.12H_2O$.
- Purified silver nitrate.
- Potassium chloride.
- Nitric acid (1+1): 5 ml concentrated nitric acid diluted in 5 ml distilled water.
- 5% Potassium chromate solution.

Each sample was analysed in triplicates. Sample (1 g) was weighed into an Erlenmeyer flask and 40 ml of 0.1 N silver nitrate solution (more than enough to precipitate all Cl^- as AgCl) was added. Concentrated nitric acid (20 ml) was added followed by gentle boiling on a hot plate until all the solids, except AgCl, dissolved (15 minutes). After cooling, 50 ml of water and 5 ml ferric indicator solution was added and the mixture titrated against 0.1 N NH_4SCN solution until it became a permanent light brown. A blank test was carried out using the same procedure and same reagents but omitting the test sample. The NaCl content was calculated as follows:

$$\% \text{ NaCl} = \frac{40 \text{ ml } (AgNO_3) - \text{ml } NH_4SCN \times 0.1 \text{ (N of } AgNO_3) \times 5.85}{\text{Sample weight (g)}}$$

The standard solutions were standardised as below.

For the 0.1 N $AgNO_3$ standard solution, 0.3 ± 0.005 g of KCl was weighed and transferred to a 250 ml glass stoppered Erlenmeyer flask with 40 ml distilled water. A volume of 1 ml of 5% (50 g/l) K_2CrO_4 solution was added and titrated with the $AgNO_3$ solution until the first perceptible pale red-brown appeared. A blank determination

was conducted and the blank reading was subtracted from the sample reading, the resulting volume added (ml) was used for the normality calculation as follows:

$$\text{Normality} = \text{g KCl} \times 1000/\text{ml } AgNO_3 \times 74.555$$

The 0.1 N ammonium thiocyanate standard solution was prepared by weighing 0.7 ± 0.005 g of purified $AgNO_3$ onto a watch glass and transferring with H_2O through a glass funnel into a 250 ml glass stoppered Erlenmeyer flask. Distilled water was added to make the volume up to approximately 75 ml followed by 5 ml HNO_3 (1+1) and 2 ml Ferric indicator solution. The mixture was titrated against an ammonium thiocyanate solution until a reddish brown colour appeared and remained after vigorous shaking for 1 minute. Additional thiocyanate solution was added, if necessary to produce a permanent end point. The Normality of the solution was calculated as below.

$$\text{Normality} = \text{g } AgNO_3 \times 1000/\text{ml } NH_4SCN \times 169.87$$

3.4 Results

The species of fungi isolated from the 25 Maldive fish samples and the percentage frequency of their isolation are illustrated in Figure 3.4. A Total of nineteen species were isolated. Seven species were members of the genus *Aspergillus* while three were from *Eurotium*, an *Aspergillus* telemorph. *A. flavus* and *A. tamarii* were the dominant fungi isolated, with a frequency of 92% (23/25 samples) and 96% (24/25), respectively. They were found to co-exist and were recovered in high numbers from many samples which displayed profuse growth of *A. flavu*s and *A. tamarii* on the surface. One of the *A. flavus* isolates was yellow, an uncommon but known variant of the olive green colour characteristic of the species. The growth of *A. flavus* and *A. tamarii* on identification media together with some morphological characteristics are shown in Figures 3.5-3.6. Other frequently isolated *Aspergillus* included *A. sydowi* and *A. niger,* detected in 64% (16/24) and 40% (10/24) of the samples respectively. The remaining *Aspergillus* species (*A. ochraceus*, *A. terreus* and *A. candidus*) were encountered at

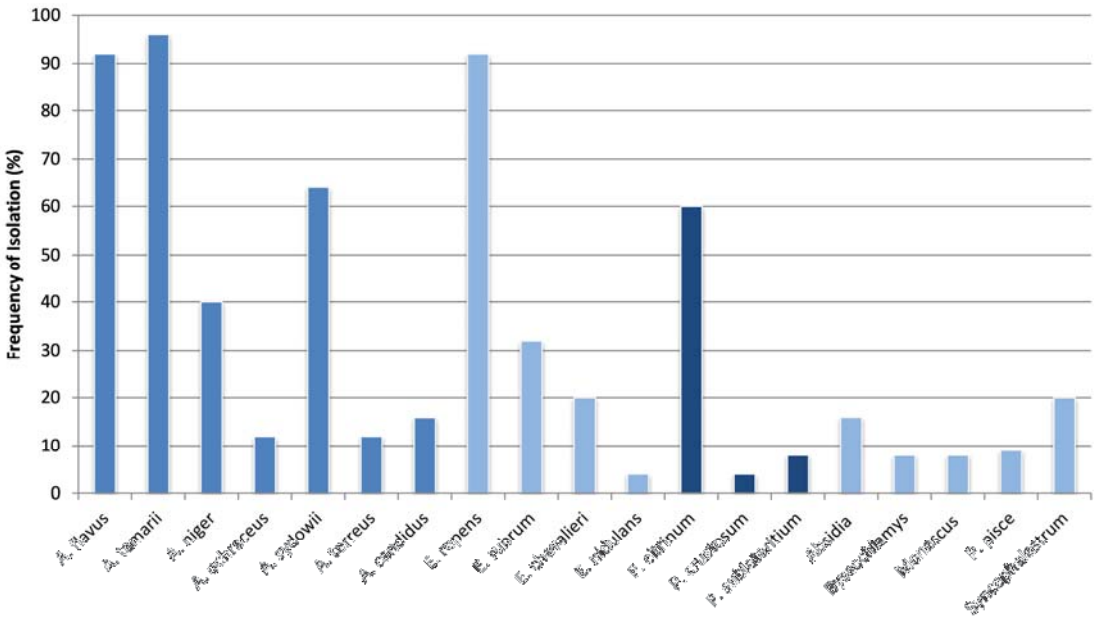


Figure 3.4 Species of fungi isolated from Maldive fish and their percentage frequency of isolation (25 samples)

lower frequencies (12-16%). *A. ochraceus* grows at 37°C but closely related species *A. westerdijikiae* and *A. steynii* fails to grow at this temperature (Pitt and Hocking, 2009). Growth at 37°C was observed for all *A. ochraceus* isolates confirming the identity of this fungus. The *A. candidus* isolates, unlike the normal isolates of this species, were not able to grow at 37°C. Pitt and Hocking (2009) also reported similar isolates, originating from tropical dried fish that did not grow at 37°C. Isolates that grow at this temperature are likely to be *A. tritici*, a closely related species in the *Aspergillus* section *Candidi* (Varga et al., 2007).

Eurotium species also occurred at high frequencies as expected due to the xerophilic nature of this genus. *Eurotium repens* was as commonly isolated as *A. flavus* and was isolated from 92% (23/25) of the samples. This species was sometimes isolated in high numbers and found to dominate the surface mycoflora of the tapering edges of many of the samples. *E. rubrum* was isolated from 32% (8/25) of the samples whereas *E. chevalieri* was present in only 20% (5/25) of the samples. *Emericella nidulans,* another telemorph of *Aspergillus* characterised by the formation of white cleistotheica

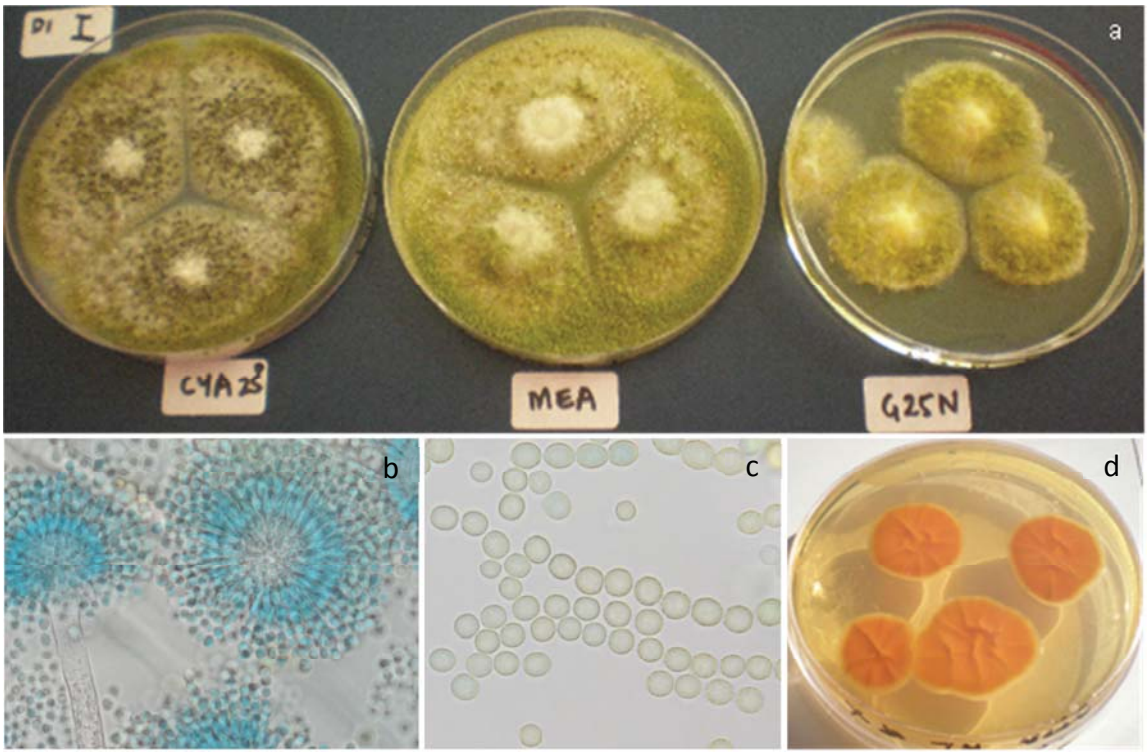


Figure 3.5 *Aspergillus flavus* a) on identification media at 25°C for 7 days, b) light microscopy of conidial heads on MEA, c) conidia showing finely roughened walls, and d) bright orange reverse colouration on AFPA agar

surrounded by Hülle cells and purple ascospores, was also isolated from one sample (4%).

The second most important genus was *Penicillum,* although the incidence was much lower than *Aspergillus*. *P. citrinum* was the only species commonly isolated from the samples, occurring at a frequency of 60% (15/25). Other *Penicillum* species isolated included *P. sublateritium* and *P. crustosum,* seen only occasionally. Fungi not belonging to these two genera occurred randomly and at a low frequency. These included *Absidia corymbifera* (16%, 4/25), *Byssochlamys fulva* (8%, 2/25), *Monascus ruber* (8%, 3/25) and *Syncephalastrum racemosum* (20%, 5/25). The halophilic xerophile *Polypaecium pisce* was also present although at a low frequency of 12% (3/25). A few nonsporing white fungi were isolated which failed to produce fruiting structures on identification media.

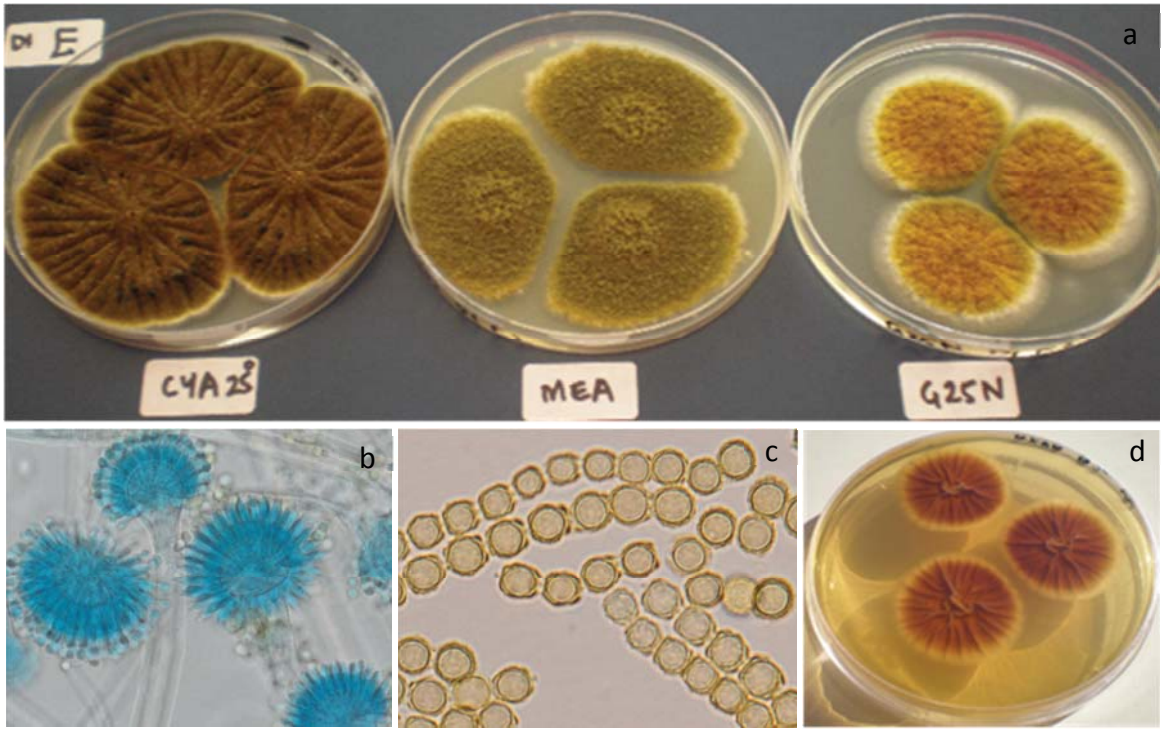


Figure 3.6 *Aspergillus tamarii* a) on identification media at 25°C for 7 days, b) light microscopy of conidial heads on MEA, c) conidia showing characteristic thick and rough, spiny walls, and d) deep brown reverse colouration on AFPA agar

The samples were plated before and after surface sanitisation to compare any differences in the mycoflora simply present on the surface and actively growing in the product. The frequency of isolation of the fungi both before and after treatment is shown in Figure 3.7. The results suggest that the sanitising treatment did not have much impact on the fungal species isolated from the product. Sanitisation of the surfaces resulted in a decline in the isolation frequency of most species as expected. However, 14 of the 19 species were still present in the treated samples. Although the frequency of *A. flavus* decreased from 92% to 88% and *A. tamarii* from 96% to 92% after the treatment, they still represent the dominant flora growing on the product. The isolation frequency of *A. sydowii* almost halved (64% to 32%) after surface treatment. Some species such as *A. ochraceus*, *P. sublateritium* and *P. crustosum* were completely eradicated following treatment and therefore were likely to be contaminants on the surface rather than growing on the product. The effect of surface

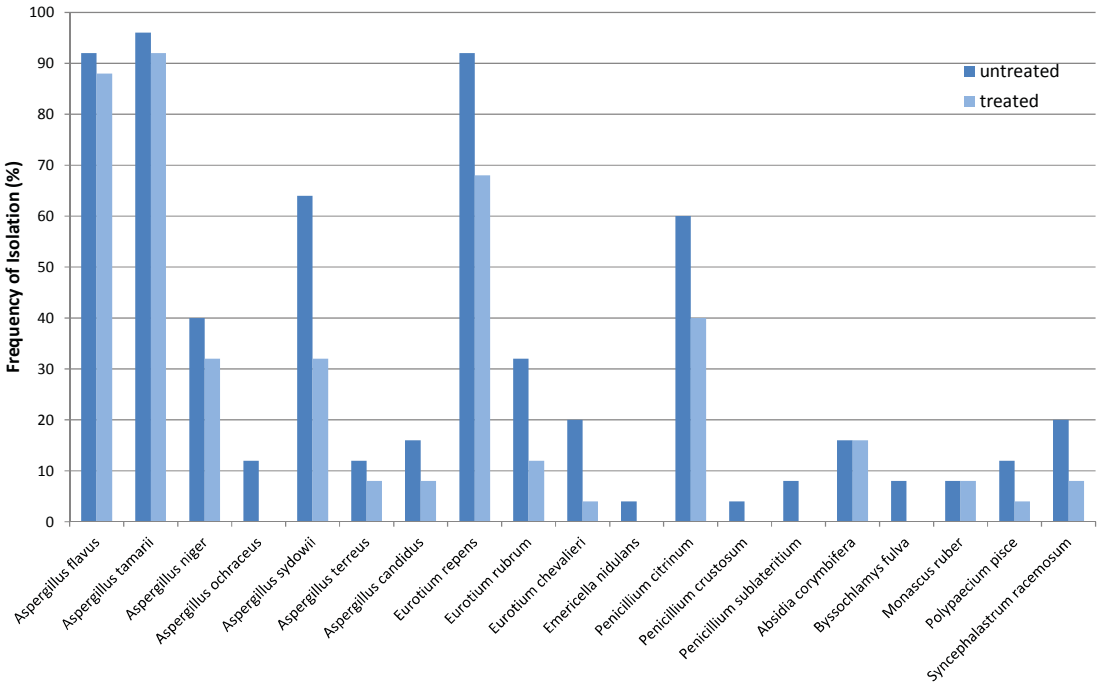


Figure 3.7 Comparison of the species of fungi isolated from Maldive fish (25 samples) before and after surface sanitisation.

sanitisation on *Eurotium* species was more apparent, although all species belonging to this genus were still isolated from the treated samples. Isolation of *E. repens* dropped from 92% to 68% whereas the other two species showed a more dramatic decrease to less than half of the original incidence. The occurrence of *Absidia corymbifera* and *Monascus ruber* remained constant after surface sanitisation while the percentages of *P. pisce* and *S. racemosum* decreased by less than half.

The a_w of the samples was quite variable, in the range 0.951 to 0.720 (Figure 3.8). Of the 25 samples, 76% (19/25) were above 0.80 a_w which is the limiting value for most fungi (Pitt and Hocking, 2009). Six samples (24%) had a_w equal to or below this level while only 3 (12%) of the samples had values below 0.75 a_w. None of the samples comply with the critical a_w below 0.68, that limits the growth of xerophilic fungi (Pitt and Hocking, 2009). The pH of the samples ranged from 5.65 to 6.68 showing little variation. The salt content of the samples was between 1.48 to 4.29% with an average of 2.08%. This level of salt would be expected to have some inhibitory effect at low a_w.

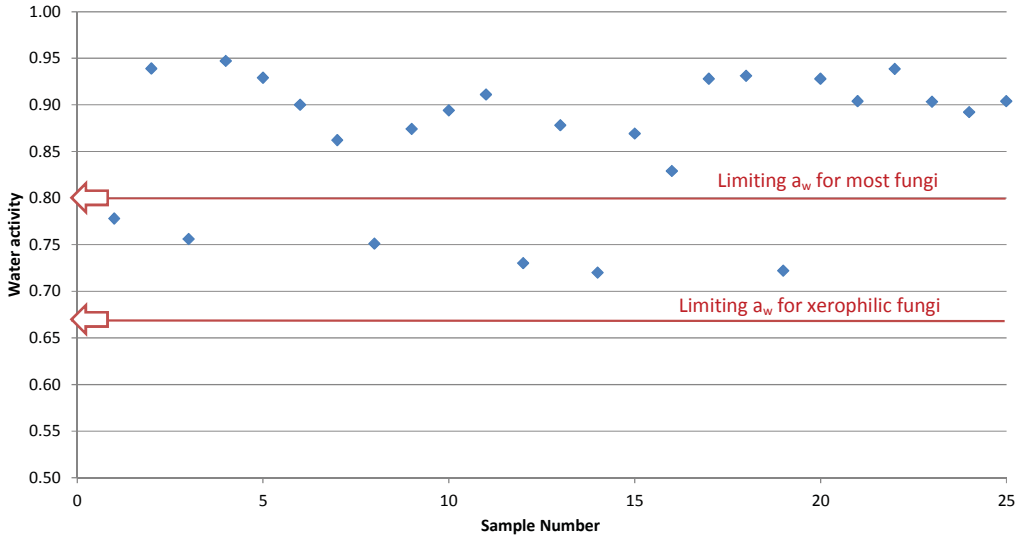


Figure 3.8 Comparison of the water activity of twenty five Maldive fish samples

3.5 Discussion

The natural surface microflora of the Maldive fish samples was dominated by fungi belonging to the genus *Aspergillus* and its telemorph, *Eurotium*. This was expected as many species are xerophiles capable of withstanding elevated temperatures and low a_w. Hence they can outcompete other mycoflora under these conditions. The warm atmospheric conditions of the Maldives provide a conducive environment for their growth on dried foods such as Maldive fish. In support of these findings, both *Aspergillus* and *Eurotium* are recognised as the dominant species associated with dried food commodities from the tropical and subtropical regions (Pitt and Hocking, 2009). Extensive studies conducted on the mycoflora of a range of dried foods from the Southeast Asia showed them to be the most common storage fungi (Pitt and Hocking, 1991). Furthermore, smoked and/ or dried fish originating from warmer regions are frequently dominated by *Aspergillus* and *Eurotium* species (Adebayo-Tayo et al., 2008; Atapattu and Samarajeewa, 1990; Essein et al., 2005; Fafioye et al., 2002; Wheeler et al., 1986).

A. flavus (92%) and *A. tamarii* (96%), two closely related species from *Aspergillus* section *Flavi* were the dominant species isolated from the samples. They were found to co-occur with the surface of some of the products covered with a conspicuous olive green to olive brown growth characteristic of these two species. *A. flavus* is ubiquitous in the air of many tropical countries (Adhikari et al., 2004; Gupta et al., 1993; Hedayati et al., 2007) and hence it is no surprise that a very high percentage of fish was contaminated with this species. Maldive fish are mainly sundried in open spaces accessible to any contamination from the environment. The product is also commonly stored in bulk and sold in open markets without any packaging, increasing the chances of airborne and contact contamination. These results are in agreement with many other studies where *A. flavus* was the dominant fungus on smoked dried fish or similar products (Adebayo-Tayo et al., 2008; Edema and Agbon, 2010; Essein et al., 2005; Fafioye et al., 2008). Studies on the mycoflora of a wide variety of smoked dried fish from Nigerian markets and processing centres revealed *A. flavus* to be the dominant species (Adebayo-Tayo et al., 2008; Edema and Agbon, 2010; Essein et al., 2005; Fafioye et al., 2002; Fafioye et al., 2008; Jonsyn and Lahai, 1992). *A. flavus* was also the dominant species growing on smoked dried fish from Sierra Leone followed by *A. ochraceus*, *A. tamarii* and *A. niger* (Josyn and Lahai, 1992). *A. flavus* was also isolated from dried fish originating from Sri Lanka and Indonesia (Atapattu and Samarajeewa, 1990; Wheeler et al., 1986).

Reports of *A. tamarii* from smoked and dried fish are not as frequent as *A. flavus* although a high incidence was observed in this study of Maldive fish. In contrast *A. tamarii* was isolated from only 6.8% of Indonesian dried fish samples compared with 27% for *A. flavus* (Wheeler et al., 1986). The incidence for smoked dried “Bonga” fish (*Ethmolosa* sp.) from Sierra Leone was more in line with our findings with 36% and 27% out of the thirteen fungal isolates testing positive for *A. flavus* and *A. tamarii*, respectively. (Jonsyn and Lahai, 1992). Interestingly, *A. tamarii* was more prevalent than *A. flavus* in dried unsalted fish from traditional markets in Jakarta, Indonesia with a frequency of 43% (13/30 samples) in contrast to only 3% (1/30) for *A. flavus* (Santoso et al., 1999). Salted dried fish sampled in the same study however showed similar

incidences for both these fungi. The low salt content of the Maldive fish could therefore be an explanation for the slightly higher incidence of *A. tamarii*.

A. sydowii with an isolation frequency of 64% was another common *Aspergillus* species isolated in this study. This is a species widely distributed across the world, especially as a marine organism (Samson et al., 2010). It is also halotolerant and therefore not surprisingly could be associated with marine products such as smoked dried fish. *A. sydowii* was a common isolate of dried fish from markets in Jakarta (Santoso et al., 1999; Wheeler et al., 1986).

A. niger was another important *Aspergillus* species which was frequently encountered in this study (40%). Species in *Aspergillus* section *Nigri* to which *A. niger* belongs, are dominant in sundried food products due to the resistance of the characteristic black spores to the sunlight (Pitt and Hocking, 2009). Hence, the presence of this species was anticipated and is supported by data from other studies. *A. niger* was the most prevalent fungus isolated from Sri Lankan dried fish products with 30% of all fungal colonies isolated from the 25 samples positive for this species (Atapattu and Samarajeeva, 1990). In the same study *A. niger* comprised 20% of the fungal colonies isolated from skipjack tuna. *A. niger* was the dominant species on a variety of dried fish originating from local markets in Malaysia (Ito and Abu, 1985). It was also a frequent contaminant of smoked dried fish or similar products from other tropical countries (Edema and Agbon, 2010; Essein et al., 2005; Jonsyn and Lahai, 1992; Munimbazi and Bullerman, 1996; Prakash et al., 2011).

Other *Aspergillus* such as *A. ochraceus*, *A. terreus* and *A. candidus* which were isolated in lower frequencies in this study have also been reported from other smoked and/ or dried fish products (Jonsyn and Lahai, 1992; Santoso et al., 1999; Wheeler et al., 1986). The popular Japanese product katsoubushi or dried bonito stick which closely resembles the Maldive fish, were found to be predominantly contaminated with *A. ochraceus*, *A. oryzae* (domesticated form of *A. flavus*), *A. tamarii* and *Syncephalastrum racemosum* (Motohiro, 1988). All four species were encountered from the Maldive fish samples analysed in this study.

Many species belonging to *Aspergillus* are important producers of toxic secondary metabolites. The dominant *A. flavus* is of major significance and has been widely studied due to its toxicity and abundance in food commodities especially those in hot climates. Aflatoxin $B_{1,}$ produced by *A. flavus,* is the most potent liver carcinogen known (IARC, 1993). *A. flavus* also produces cyclopiazonic acid (CPA), a mycotoxin of lower toxicity compared to aflatoxin nonetheless with organ damaging properties (Frisvad et al., 2007). Almost all isolates of *A. tamarii* also have the ability to produce CPA (Dorner, 1983; Vinokurova et al., 2007). *A. parasiticus,* which is also a major producer of aflatoxins, has been reported from similar products (Munimbazi and Bullerman, 1996) but was not isolated in this study. *A. parasiticus* appears to be very uncommon in Southeast Asia (Pitt et al., 1994; Tran-Dinh et al., 2009). Another important mycotoxin associated with tropical foods is ochratoxin A (OTA). *A. ochraceus* and related species in *Aspergillus* section *Circumdati* are significant producers of this toxin. However, they do not appear to be widespread in the tropics and the incidence in Maldive fish from the current study was quite low (12%). Species belonging to *Aspergillus* section *Nigri* are now recognised as the major contributor to OTA levels in tropical foods (Pitt and Hocking, 2009). However, only few strains of *A. niger* are capable of producing this toxin while *A. carbonarius* has been identified as a major source (Frisvad et al., 2006; Romero et al., 2005). *A. niger* was frequently isolated (40%) from the samples tested in this study but *A*. *carbonarius* was absent. Other *Aspergillus* isolates such as *A. sydowii*, *A. terreus* and *A. candidus* do not produce any mycotoxins of significance and hence are of less concern.

Eurotium species are able to grow under particularly dry conditions. Not surprisingly, *Eurotium* species, especially *E. repens,* were prominent species isolated from Maldive fish. *Eurotium* species are xerophiles generally capable of growing at a_w just slightly above safe levels (Pitt and Hocking, 2009). The tapering ends of the fish fillets, where the a_w is expected to be lower than in other areas, were often covered with grey areas characteristic of *Eurotium* species. This could be clearly observed when the samples were viewed under a stereomicroscope. Nevertheless, none of the species belonging to this group have the ability to produce any significant mycotoxins (Pitt and Hocking,

2009). They however are likely to have a role in enhancing the flavour and other sensory attributes of the product. *E. repens* has been commonly isolated as the dominant species from the surface of traditional mould ripened ham and other dried meat products. The typical aroma of such ham is thought to be due to this fungus which is also used as an indicator for ripening (Comi et al., 2004). Furthermore, *E. repens* is used for mould fermentation of katsuobushi (dried bonito stick), in order to acquire the desirable organoleptic attributes (Miyake et al., 2010). *Eurotium* species have been frequently isolated from dried and/ or smoked fish originating from other countries. Dried marine products from Japan and Southeast Asia were found to be dominated by this genus (Ichinoe et al., 1977; Okafar, 1968; Santoso et al., 1999; Wu and Salunkhe, 1978). In a study of salted dried fish originating from Pakistan, Malaysia, Thailand and Hong Kong, *E. amstelodami*, *E. chevalieri* and *E. rubrum* were the most frequently isolated fungi (Phillips and Wallbridge, 1977).

In addition to *Aspergillus*, the other important storage fungi that are of concern in relation to toxin production are *Penicillium*. However, species of this genus are more common in temperate zones where they contribute to contamination of foods and produce mycotoxins (Pitt and Hocking, 1991). *P. citrinum* was the only *Pencillium* species of significance isolated in this study, in terms of percent occurrence and toxigenic potential. This fungus is a consistent producer of citrinin, a nephrotoxic and teratogenic mycotoxin (Flajs and Peraica, 2009; Malmstrøm et al., 2000; Pitt and Hocking, 2009) . It has a worldwide distribution and occurs on many different kinds of foods in temperate, subtropical and especially tropical regions (Samson et al., 2010). The mesophilic nature (5-37°C, optimum 26-30°C) of this species and its ability to grow down to a_w 0.80 is responsible for its widespread occurrence in foods and the environment including the soil of tropical and subtropical areas (Domsch et al., 1980; Houbraken et al., 2010; Pitt and Hocking, 2009). Hence, *P. citrinum* is a species that has reserved a niche in Maldive fish and is not present as a mere contaminant, as proven by its isolation from both untreated and treated samples. In agreement with our findings, *Penicillium* species were less frequently encountered in dried non-salted fish from Burundi and most *Penicillium* isolates were reported as *P. citrinum*

(Munimbazi and Bullerman, 1996). This species was a common contaminant of dried food commodities from Indonesia (Pitt and Hocking, 2009) and has been reported from Indonesian dried fish (Santoso et al., 1999).

Samples were also cultured after surface sanitisation to remove any environmental contamination and allow enumeration of fungi that was actually invading the fish. Furthermore, the surface of the Maldive fish is often removed by trimming before consumption or use especially if visible fungal growth is present. Hence this could be a more accurate estimation of the risk resulting from the consumption of this product. Most species (14/ 19) growing on the surface were also found to grow inside the fish. The species which were removed after surface sanitisation were also isolated at a low frequency prior to this treatment. Hence they could be present as mere contaminants on the surface. *A. ochraceus,* isolated from three untreated samples, falls in to this category although the physiological characteristics of this species qualify its growth on the samples (Pitt and Hocking, 2009). The fungi dominating the flora of the untreated samples were still prevalent after surface sanitisation although the incidence decreased variably for different species. *A. flavus*, *A. tamarii* and *E. repens* were still the major species growing inside the food, confirming their potential role in spoilage and mycotoxin production in Maldive fish.

The natural mycoflora of foods is influenced by many factors which provide an ecological niche for the growth of specific species. In dried fish, a_w is the key factor responsible for regulating and selective growth of spoilage and toxigenic fungi (Samapundo et al., 2007; Sautour et al., 2002). The smoking and drying processes as well as the use of salt for cooking in the production of Maldive fish would contribute to the low a_w of the product. The a_w of the samples analysed in this study was much more variable than expected, ranging from 0.951 to 0.720. This would support the growth of a whole range of fungi from normal spoilage microflora to extreme xerophiles. The a_w of the majority of the samples (76%) was above the limiting a_w 0.80 for most fungal species (Pitt and Hocking, 2009) hence providing favourable conditions for their growth. Xerophilic fungi can grow below 0.80 a_w including genuinely xerophilic species at the lower end of the scale which are able to grow rapidly at 0.77 a_w and above and

more slowly below 0.75 a_w down to approximately 0.68 a_w (Pitt and Hocking, 2009). The remaining samples (24%) had water activities below 0.80 but above 0.70, including only 12 % below 0.75 a_w. None of the samples were adequately dried to limit the growth of all fungi. The limiting a_w for the most commonly isolated fungi in this study is 0.81 to 0.78 for *A. flavus* (Ayerst, 1969; Pitt and Miscamble, 1995), 0.78 for *A. tamarii* (Ayerst, 1969) and 0.72 to 0.69 for *E. repens* (Andrews and Pitt, 1987). It should however be emphasized that Maldive fish fillets are uneven with a thick centre and thin areas at the tapering ends. Hence unbound water is unevenly distributed in the product and some areas could support growth of a fungus that is limited in other areas. The values reported here are therefore estimates of the overall a_w of the samples. The large variation in the samples required the use of isolation media suitable for high a_w foods and for low a_w foods to ensure enumeration of all important species associated with this product.

Sodium chloride content can influence fungal growth in a food either by contributing to the reduced a_w of the product or due its own inhibitory effects (Jennings, 1995). However, the salt content of the Maldive fish samples was below levels (max 4.29% with an average of 2.08%) that could halt the growth of the microflora. According to Prakash et al. (2011), *A. flavus* and *A. niger* originating from dried seafood products in India showed resistance up to 18% salt. Attapattu and Samarjeeva (1990) reported concentrations of 20-30% salt to be effective in controlling the growth of fungi isolated from Sri Lankan dried fish. Yet, many dominant species isolated in this study such *A. flavus*, *A. tamarii*, *A. sydowi* and *A. niger* are halotolerant (Samson et al., 2010) and have been reported as prevalent in high salt environments such as salted dried fish (Santoso et al., 1991; Wheeler et al 1986). Wheeler et al. (1986), however, in their study of Indonesian salted dried fish, found the incidence of *Polypaecilum pisce* which was isolated at a frequency of 42%, to be higher than any other fungus. This species is a halophilic xerophile, very well adapted to the unique environment of salted dry fish (Andrews and Pitt, 1987). *A. penicilliodes*, an extreme xerophile with optimal a_w for growth at 0.91 to 0.93 was also a frequent isolate of Indonesian dried fish (Andrews

and Pitt, 1987; Wheeler et al., 1986). The low salt content is probably responsible for the decreased incidence of these species in Maldive fish.

The pH of the samples showed little variation from 5.65 to 6.68 and these values are not sufficiently low to affect fungal growth. Most fungi can tolerate a broader pH range, generally pH 3-8, compared to bacteria and other microbes (Wheeler et al., 1991). The optimal pH range for growth of *A. flavus* is a broad range between 3.4 to 10 (Gock et al., 2003; Wheeler et al., 1991) and *E. repens* is from 4.5 to 5.5 (Gock et al., 2003). Hence, the pH of the samples is optimal for the growth of these fungi.

The product is exposed to direct sun when drying and maintained at the ambient temperature during storage and sale. The Maldives is a tropical country with an average day time temperature between 28°C to 30°C (MMS, 2012). The high ambient temperature, together with the lowered a_w of Maldive fish, predisposes it to conditions suitable for the growth of *Aspergillus* and *Eurotium* species. The optimum growth temperatures for the most prevalent species isolated are close to the environmental temperatures for the Maldives. For example, the optimum temperature for *A. flavus* and *A. tamarii* is reported as 33°C (Ayerst, 1969; Pitt and Hocking, 2009).

Samples assessed in this study were collected over a period of three years. The species most frequently encountered were consistently isolated despite the time or year of sampling. *A. flavus* and *A. tamarii* as well *E. repens* remained the dominant species. Maldives is located on the equator and although it experiences a monsoonal climate, the temperature varies little throughout the year (MMS, 2012) The humidity despite being relatively high remains almost constant throughout the year. Hence no obvious seasonal variations in the isolated mycoflora were observed.

Markets and local stores in the capital island from which the samples were collected, are supplied from different areas of the country and hence represent Maldive fish originating from the whole country. However, the sample size was small and limited to fish with visible fungal growth only. As this is the first mycological study on Maldive fish, the pupose of the current study was to determine if there is a problem with

toxigenic fungi and mycotoxin production associated with this product rather than focussing on the extent of the problem.

Fungal growth on the surface of this product is considered normal, frequently witnessed and is often thought as having a flavour enhancing role (personal experience). There are many possible reasons for this contamination such as the common practice of sun drying in the open air, bulk storage under moist conditions, inadequate packaging and frequent handling post production, exposing it to fungal spores from the environment. These practices have to be improved to ensure increased quality and safety of Maldive fish.

Both direct and dilution plating was used in this study for the isolation of fungi from the product. Direct plating was more effective and resulted in enumeration of a wider range of species compared with dilution plating. Dilution plating requires more time and resources and hence is not recommended for use in any future studies on Maldive fish or similar products. Impression plating was not used in this study but according to Wheeler et al. (1986), this technique was very good for the isolation of the less common species. The incubation temperature of 30°C was initially used for isolation in addition to the standard 25°C to mimic actual storage temperature of this product. However, at 30°C species such as *A. flavus* and *A. tamarii* which although are the most important spoilage agents in this product over grew the other fungi which may also have an important role in spoilage or mycotoxin production. Hence use of the standard 25°C is recommended.

This study employed cultural and morphological methods for identification of fungi and did not use molecular methods or secondary metabolite profiling for either more accurate identification or confirmation. One disadvantage of the methods used is that some species are morphologically similar and could not be differentiated during this study. For example, accurate distinction of species belonging to *Aspergillus* section *Flavi* is not possible based on morphological features alone (Frisvad et al., 2005; Pildain et al., 2008). Varga et al. (2011) have recently presented a detailed overview of this group based on a polyphasic approach that includes morphological and molecular

methods as well as secondary metabolite profiling, assigning 22 species to seven clades. Several species (*A. flavus, A. parasiticus, A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. toxicarius*, *A. parvisclerotigenus, A. minisclerotigenes, A. arachidicola A. pseudonomius* and *A. pseudocaelatus*) are recognised as aflatoxin producers, some of which cannot be differentiated on the basis of traditional techniques alone. However, most of these species are uncommon. Houbraken et al. (2010) reviewed the taxonomy of *P. citrinum* and related species using a similar approach, and designated seven species in section *Furcatum* series *Citrina,* many of which have the ability to produce citrinin. Some species from *Aspergillus* section *Nigri,* such as *A. carbonarius,* can be easily recognised but those belonging to *A. niger* aggregate cannot be accurately identified by morphological techniques (Abarca et al., 2004). The focus of this study lies in the identification of fungi in Maldive fish that have the potential to produce important toxic metabolites. Due to the ability of these similar species to produce the mycotoxins of interest, more accurate identification was not deemed necessary. Furthermore, Pitt and Hocking (2009) claims that variations of readily identifiable common species are seldom encountered and are little more than mere variants of this species. According to them, identification to the common species level is sufficient in most cases.

In conclusion, all samples were contaminated by filamentous fungi with 96% (24/ 25) containing one or more mycotoxigenic species. The physiochemical parameters such as reduced a_w, high storage temperature and low salt content provide an ecological niche for species such as *Aspergillus* and *Eurotium* that dominated the mycoflora of Maldive fish. Most fungi growing on the surface of the product were also invading the food. The toxigenic species of significance isolated in this study together with their prevalence and the undesirable health effects they can deliver are listed in Table 3.1. *A. flavus*, *A. tamarii*, *A. niger*, *A. ochraceus* and *P. citrinum* are five species of concern based on their toxicity and the frequency of occurrence. *A. flavus* should be given some detailed attention due to its ability to produce highly potent aflatoxin B_1 and its prevalence in the product. Both *A. flavus* and *A. tamarii* have the potential to produce CPA and given their dominance in Maldive fish, their control is crucial. *A. ochraceous*

and *A. niger* are both producers of ochratoxin A while *P. citrinum* has the potential to produce citrinin in the product. The presence of these mycotoxigenic species, often at high frequencies, indicates possible contamination of the product with mycotoxins of varying potency, if provided with a favourable environment. A high probability of co-occurrence of these mycotoxins is expected to increase the food safety risk due to possible synergistic effects (Bouslimi et al., 2008; Smith et al., 1992). Hence, consumption of products infected with fungi is likely to pose a health hazard. Not all strains of these toxigenic species are mycotoxin producers and their role in the formation of these deleterious substances must be evaluated by screening the isolates for their mycotoxins.

Table 3.1 Potentially toxigenic species of fungi isolated from Maldive fish together with the mycotoxins they produce and the health risks

	Species	**Frequency (%)**	**Mycotoxins and their effects**
1	*A. flavus*	92	• Main producer of B aflatoxins • Aflatoxins cause acute liver damage and liver cancer; probably immunosuppression, childhood stunting and interference with protein uptake • Aflatoxin B_1 is the most potent liver carcinogen known • Only some strains produce mycotoxins • Some strains also produce cyclopiazonic acid, a mycotoxin of lower toxicity
2	*A. niger*	40	• Commonly used in enzyme production and fermentation of foods • few isolates capable of producing OTA • OTA is nephrotoxic, carcinogenic, teratogenic, genotoxic and immunosuppressive in animals; effects in humans remain unclear
3	*A. ochraceus*	12	• Produces OTA but only minority of the isolates are toxigenic
4	*A. tamarii*	96	• Almost all isolates produce CPA • CPA may cause kidney damage by inhibition of the calcium pump • Along with aflatoxin, CPA is suspected of involvement in "turkey X" disease and kodua poisoning • CPA may cause acute hepatotoxicity and immune-suppression • CPA co-occurs with aflatoxins
5	*Penicillium citrinum*	60	• Major producer of moderately toxic mycotoxin, citrinin • Citrinin is nephrotoxic and carcinogenic in animals • Citrinin was recently found to cause haemolysis of human erythrocytes • All strains produce citrinin • Frequently occurs and acts synergistically with the nephrotoxic OTA

CHAPTER 4

A Simple Screening Method for Detection of Citrinin using Coconut Cream Agar

Abstract

A simple and rapid screening method was developed for citrinin using Coconut Cream Agar (CCA) described earlier for detecting aflatoxins and ochratoxin A. Fifteen isolates of *Penicillium citrinum* from Maldive fish were inoculated onto CCA and incubated at 25°C and 30°C for 10 days. The isolates were also grown on CCA, Czapek yeast extract agar (CYA) and yeast extract sucrose agar (YES) and screened for citrinin semi-quantitatively by the TLC agar plug method. All isolates produced a distinct yellow green florescence on CCA when the reverse side of the plates were viewed under long wavelength UV indicating citrinin production. The measurement was optimum at 25°C after four to five days of incubation. The results from CCA direct method were comparable to those from the TLC runs.

4.1 Introduction

Citrinin is a toxic secondary metabolite that was originally isolated from *Penicillium citrinum* (Hetherington and Raistrick, 1931). Several species belonging to the genera *Aspergillus*, *Pencillium* and *Monascus* have the ability to produce this toxin (Bragulat et al., 2008; Pitt and Hocking, 2009; Wang et al., 2005). Citrinin possess nephrotoxic activity disrupting renal functions in all animal species tested (Phillips et al., 1980) while it is also mutagenic, teratogenic, embryocidal, fetotoxic and immunosuppressive in animals (Flajs and Peraica, 2009; Singh et al., 2011; Singh et al., 2007). In addition, Wichmann et al. (2002) demonstrated the toxic effects of citrinin on human T cells that could increase the risk of eliciting allergic responses.

Fungi producing citrinin can contaminate a wide range of food products, stored grains in particular and other foods such as fruits, herbs and spices (EFSA, 2012). While the fungal species used in cheese production are not citrinin producers, accidental contamination with other *Penicillium* species could result in production of this mycotoxin (Bailly et al., 2002). A *Monascus* species with the ability to produce citrinin has been used for centuries in the fermentation of many food products (Lee et al., 2010). Hence, some populations may be exposed to citrinin more frequently than generally expected (Flajs and Peraica, 2009). To ensure the safety of foods, it appears vital to screen the contaminating fungi, especially those used in fermentation, for deleterious mycotoxins such as citrinin. Screening for secondary metabolites such as mycotoxins, combined with other methods are also used for the identification of fungal species (Houbraken et al., 2010; Varga et al., 2011).

Coconut Cream Agar (CCA) was described as a simple and inexpensive screening medium for fungi producing aflatoxins (Dyer and McCammon, 1994) and later for ochratoxin A (Heenan et al., 1998). Colonies of fungi producing aflatoxins and OTA, fluoresce distinctly when the reverse side of the CCA plate is viewed under long wavelength UV light. Similarly, colonies of *P. citrinum* isolates from Maldive fish fluoresced on CCA on the reverse side upon exposure to long wavelength UV. *P. citrinum* isolated from various sources around the world are consistent producers of

citrinin (Heperkan et al., 2009; Malmstrøm et al., 2000; Romero et al., 2005) which is a naturally fluorescent compound (Xu et al., 2006). Hence, it is hypothesised that the fluorescence on CCA by this species is due to citrinin. The aim of this study was to confirm this finding and optimise CCA as a simple method for screening for the production of citrinin by fungi. This technique has not been reported previously.

4.2 Methodology

4.2.1 Fungi

Fifteen strains of *Penicillium citrinum* isolated from 15 different Maldive fish samples were studied using this method. The isolates were grown on Czapek yeast extract agar (CYA) at 25°C for seven days to obtain heavily sporulating cultures. Isolates used for negative control were *Aspergillus tamari* (K1B1 and L1B1) and *Eurotium repens* (J1E1).

4.2.2 Media

CCA was prepared from commercial coconut cream (Trident brand purchased at a local supermarket) according to Dyer and McCammon (1994), using 400 g of coconut cream, 400 mL distilled water and 12 g agar. The media was sterilised at 121°C for 15 minutes and approximately 10 ml was dispensed into 90 mm Petri dishes.

4.2.3 Inoculation and observation

P. citrinum isolates were inoculated onto CCA in duplicate, using single point inoculation at the centre of the plate. To avoid growth of stray spores on plates, mature spores were first suspended, using a sterile needle, in 0.2-0.4 ml semi solid agar in a vial consisting of 0.2% molten agar and 0.05% Polysorbitan 80 (Tween 80) (Pitt and Hocking, 2009). A sterile loop was then used to mix the vial contents and inoculate the plates. Plates were incubated the right way up in the dark at 25°C for seven days. To detect fluorescence, the reverse side of the plates was viewed under long wave length UV (366 nm) from day 4 to day 7. An uninoculated CCA plate was used as a control. Species that do not produce citrinin were also screened as negative controls.

4.2.4 Confirmation of fluorescence from citrinin

To confirm that the fluorescence produced on CCA by *P. citrinum* is due to citrinin the following tests were conducted:

Citrinin standard (1 ml, 500 ng/ml) was transferred to the centre of a 90 mm petri dish, in triplicate. CCA (10 ml) was transferred to the plate and mixed by swirling. After the agar was set, the reverse sides of the plates were examined under long wave length (366 nm) UV light for fluorescence.

The fluorescence exhibited by citrinin can be strongly enhanced in an acidic environment (Vázquez et al., 1997). The 15 isolates of *P. citrinum* on CCA plates were treated with acid by spraying with 10 M HCL (10 ml) at the end of the seven day incubation period. The obverse and reverse sides of the plates were then viewed under long wave length (366 nm) UV light to observe any enhancement of fluorescence.

Fluorescence produced on CCA by *P. citrinum* cultures was also confirmed by TLC using the agar plug method (Filtenborg et al., 1983; Samson et al., 2010). An agar plug 5 mm in diameter was removed from the centre of the colony (Filtenborg et al., 1983; Samson et al., 2010) and transferred agar side down on to the designated track on a 20 x 20 silica gel 60 TLC plate (Merck) previously impregnated with 2% oxalic acid in methanol and air dried (Xu et al., 2006). The plug was held in position until the appearance of the application spot under it, discarded and the spot allowed to dry. A second plug was removed from the same colony and superimposed on the previous spot. The spots were dried completely before the plates were developed in a tank containing toluene/ethyl acetate/formic acid (5:4:1 v/v/v) (Samson et al., 2010). The plates were then dried (in a fume hood for 20 minutes) and examined under long wavelength UV light (366 nm). Citrinin was detected as an intense yellow green band with the R_f value of 0.68, similar to the standard (100 ng/ml).

4.2.5 Optimisation of the method

To optimise conditions for the screening of citrinin formation on CCA, the isolates were tested under a range of conditions. Plates were incubated at 25°C and 30°C for a longer period of 10 days. For each temperature, two sets of plates were prepared, one set using glass petri dishes and the other using plastic Petri dishes, to see if plastic plates were adequate for this purpose. Glass Petri dishes are not commonly used having been replaced by plastic Petri dishes in most modern science laboratories. However, many plastics limit the transmission of UV light and therefore there was some concern that plastic Petri dishes may produce a false result. All plates were checked under long UV once every day starting from day 3. The fluorescence intensity was measured by a 3 point scoring system (+ for weak, ++ for medium, +++ for strong).

4.2.6 Comparison with other screening methods

P. citrinum isolates were three-point inoculated onto CCA, Czapek yeast extract agar (CYA) and yeast extract sucrose agar (YES) and incubated the right way up in the dark at 25°C for five days. Agar plugs were taken from the centre of the plates and screened by TLC using the agar plug method as described in section 4.2.4. A range of citrinin standards of different concentrations (10 µL of 100, 80, 60, 40, 20 and 10 µg/ml standard) used as references for a semi quantitative assessment of citrinin concentration, were run in parallel. The TLC plates were viewed under long wave length UV light and positive bands given a score based on the intensity of the band compared to the intensity of the corresponding standard.

4.3 Results

4.3.1 Type of fluorescence

All 15 isolates of *P. citrinum* exhibited an intense yellow green fluorescent halo around the colony on CCA when reverse side of the plates was viewed under long wavelength UV on day four of incubation at 25°C. The fluorescent ring around the colony was larger and the fluorescence more intense with the age of the colony until day seven when it started fade for some of the isolates. The uninoculated control plate as well as the negative controls did not induce any noticeable fluorescence on CCA. Figure 4.1 depicts the fluorescence produced by a test isolate after 4 and 7 days of incubation together with the control when viewed under long wavelength UV.

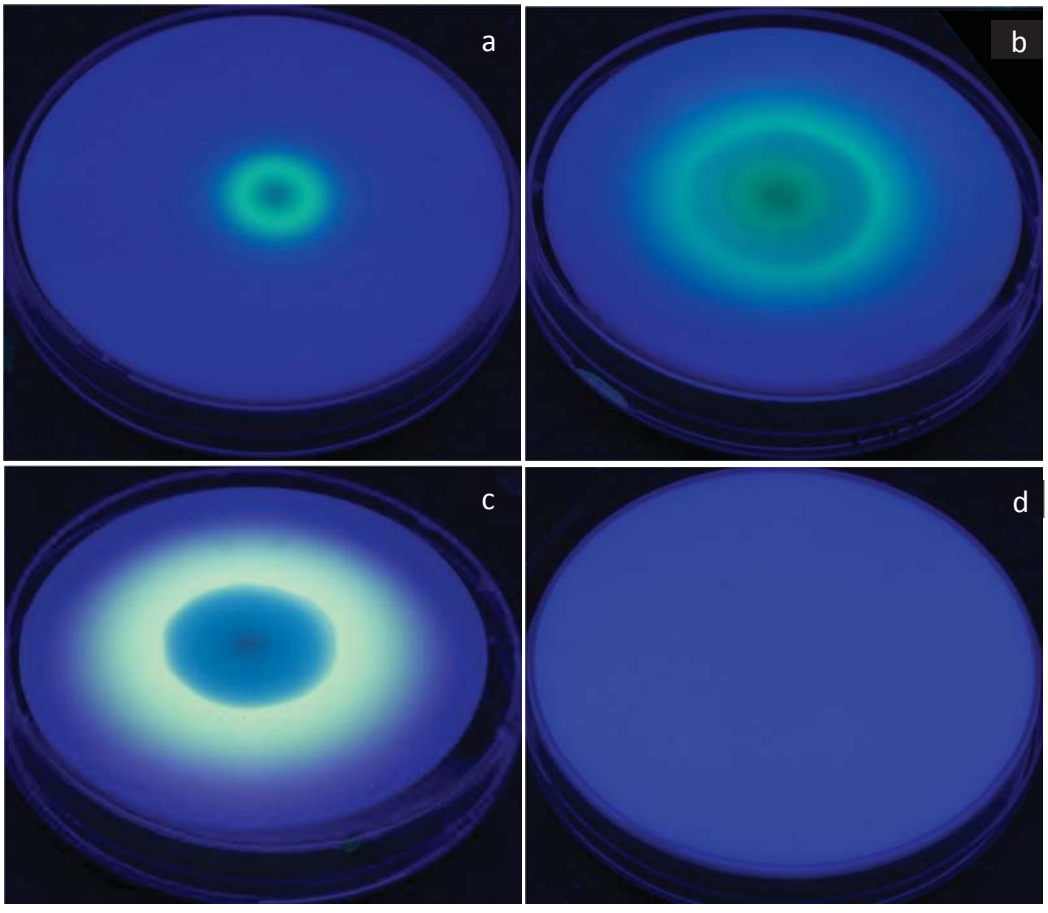


Figure 4.1 Yellow green fluorescence halo of *P. citrinum* culture H1C1 on CCA a) after 4 days at 25°C b) after 7 days at 25°C c) after HCl treatment and d) uninoculated CCA control plate, viewed under long wavelength UV (366 nm).

4.3.2 Confirmation of fluorescence

Several tests were conducted to confirm that the fluorescence produced by *P. citrinum* on CCA was due to citrinin. CCA incorporated with citrinin standards produced areas of intense yellow green fluorescence similar to the fluorescence from *P. citrinum* cultures when checked under long wavelength UV (366 nm). Furthermore, cultures of *P. citrinum* on CCA treated with 10 M HCl resulted in the change of the fluorescent ring to a very intense yellow. This reaction is expected from citrinin as exposure to an acidic environments is found to enhance the fluorescence of this compound (Vázquez et al., 1997). TLC conducted from agar plugs removed from the fluorescing area of the CCA plates also produced intense yellow green bands that correspond to the citrinin standards (Figure 4.2). No other fluorescent bands were observed.

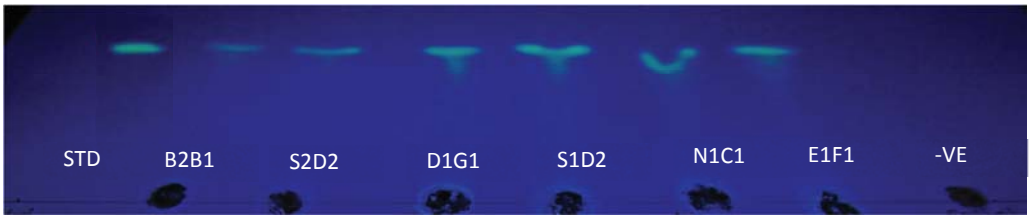


Figure 4.2 TLC of *P. citrinum* isolates after 7 days of incubation at 25°C with standard and negative control, viewed under long wavelength UV (366 nm)

4.3.3 Optimisation of the method

The fluorescence viewed on plastic Petri dishes was similar to glass Petri dishes at both temperatures. As plastic petri dishes were adequate for the purpose of screening on CCA, they were used in all subsequent assessments.

Figure 4.3 compares the fluorescence intensity of the 15 *P. citrinum* test isolates on CCA starting from day three to day ten of incubation at both 25°C (a) and 30°C (b). On day three of incubation, 13 of the 15 isolates were observed to produce noticeable

fluorescence under long wavelength UV while two isolates failed to do so at both temperatures. However, all cultures showed positive fluorescence at both temperatures after four days which improved further after 5 days. Extended incubation at 25°C resulted in enhancement of the fluorescence until after day seven when it started to decrease for some isolates. Incubation of plates for longer than seven days was undesirable as the fluorescence started to fade for some isolates while one isolate failed to register any fluorescence after 8 days. Three of the isolates exhibited intense fluorescence (+++) on day four and the number increased to eight on day seven which reduced to four on day ten. Hence at 25°C the recommended time period for the measurement of fluorescence intensity is between four to seven days with the earliest at day four to five. The fluorescence intensity of the isolates incubated at 30°C showed a steady increase from day four until day ten when a decrease in the intensity was observed.

In general, the fluorescent intensity of *P. citrinum* isolates on CCA was higher when incubated at 25°C compared with 30°C. Strong fluorescence was observed for 8 isolates at day 7 of incubation at 25°C, and for five isolates at day 9 of incubation at 30°C. Fluorescence occurred faster at 25°C compared with 30°C. For example, on day four of incubation, only four isolates showed weak fluorescence at 25°C while an increased number (10) of isolates were at this early stage at 30°C. Rest of the isolates at 25°C exhibited medium to high fluorescence. Optimum fluorescence at 25°C was observed at day seven while at 30°C fluorescence was best on day nine. One of the requirements of a screening method is the need for rapid results. Hence, for screening of citrinin using CCA, an incubation temperature of 25°C is recommended with examination after 4 days.

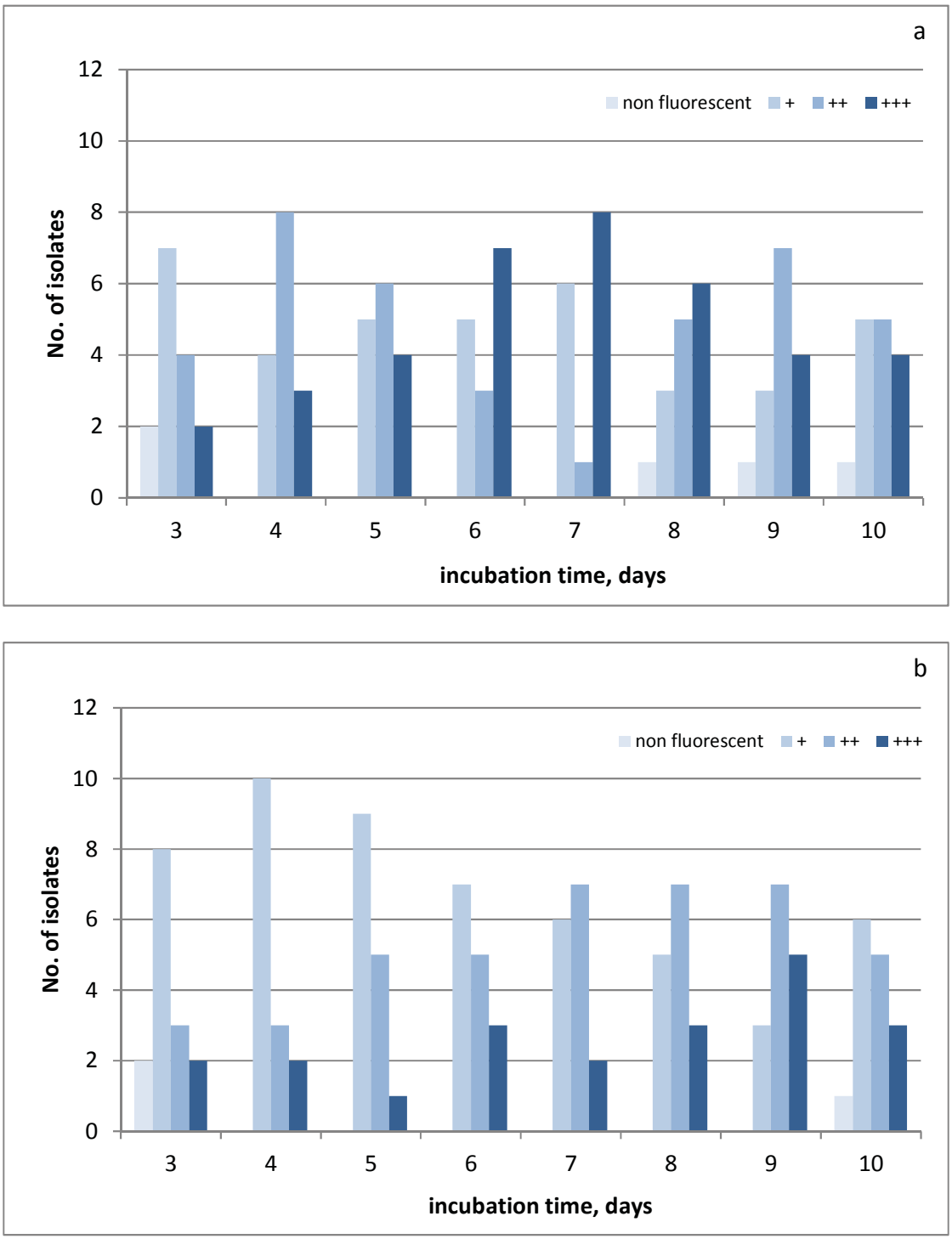


Figure 4.3 Comparison of the fluorescence produced by *P. citrinum* isolates (n=15) on CCA after three to 10 days incubation at **(a)** 25°C and **(b)** 30°C. +, ++, +++ indicates increasing colour intensity on CCA plates under long wavelength UV (366 nm).

4.3.4 Comparison to other screening methods

Fluorescence from CCA plates incubated at 25°C for five days was comparable to the results from TLC runs from the same CCA plates as well as TLC after growth of the test isolates on standard agars (YES and CYA) under the same conditions (Table 4.1). All *P. citrinum* isolates that produced positive TLC bands after growth on standard agars were also positive with the direct CCA plate as well as TLC conducted after extraction of agar plugs from CCA. The three negative control fungi did not produce any apparent fluorescence on CCA and citrinin bands by any of the methods. The fluorescence produced by direct observance of the CCA plates under the UV cannot be directly compared to the fluorescent intensity of the TLC bands. TLC of agar plugs directly removed from the CCA plates after four or five days of incubation, can be used as a confirmatory method for citrinin production. Production of this mycotoxin on CCA (TLC) appears to be better than on YES agar after 5 days of incubation although CYA induced stronger fluorescence, apparently due to increased toxin production on this media.

Table 4.1 Detection of citrinin by CCA direct method (without HCl treatment) and by the TLC agar plug method after incubation at 25°C for 5 days on CCA, YES and CYA. +, ++, +++ indicate increasing colour intensity on CCA and of TLC bands under long wavelength UV (366 nm)

Isolate	Species	CCA direct	CCA TLC	YES TLC	CYA TLC
O1C1	*P. citrinum*	+++	++++	+	++++
N1C1	*P. citrinum*	++	+++++	+++	+++++
I1B1	*P. citrinum*	+	+++	++	++++++
Q1C1	*P. citrinum*	+++	++++	+++++	++++++
S1D2	*P. citrinum*	++	+++++	++	++++++
S2D2	*P. citrinum*	+	+++	+	++++
D1G1	*P. citrinum*	++	++++	++++	++++++
J1P1	*P. citrinum*	+++	++++++	++++++	++++++
A1H1	*P. citrinum*	++	+++	+++	++++++
D1G2	*P. citrinum*	++	++	+++++	++++++
E1F1	*P. citrinum*	+++	++++	+++	++++++
N2B1	*P. citrinum*	+	++	+	++++++
B2B1	*P. citrinum*	+	+++	+	++++
H1B1	*P. citrinum*	++	++	++++	++++++
N1C2	*P. citrinum*	+	++	+	++
K1B1 (-ve control)	*A. tamarii*	-	-	-	-
L1B1 (-ve control)	*A. tamarii*	-	-	-	-
J1E1 (-ve control)	*E. repens*	-	-	-	-

4.4 Discussion

Citrinin has a conjugated planar structure which results in the natural fluorescence of this toxin (Xu et al., 2006). Citrinin appears as an intense yellow green fluorescent halo around the colony that is visibly distinct from the fluorescence produced by aflatoxins and OTA (Figure 4.1). Aflatoxins are indicated by the presence of a pastel blue fluorescent halo around the colony under UV, but they are also accompanied by a distinct yellow pigment that can be seen directly under normal light (Dyer and McCammon, 1994). OTA produces a blue green fluorescence usually covering the whole colony (Heenan et al., 1998). Citrinin can be further confirmed by exposing the plate to an acidic environment that enhances the fluorescence resulting in a clear yellow fluorescence (Vázquez et al., 1997). The fluorescence of OTA changes to purple blue when viewed under UV after exposure to ammonia vapour (Heenan et al., 1998).

Citrinin production on CCA appears to be higher at 25°C than at 30°C as the fluorescence intensity was stronger at the lower temperature. This is however in conflict with the conditions reported previously on other substrates (Domsch et al., 1980; Montani et al., 1988). *P. citrinum* is a mesophilic species which can grow at temperatures from 5°C to 37°C (Pitt, 1973) with an optimum growth temperature between 26-30°C (Domsch et al., 1980). On Czapek agar with maize extract, the optimum temperature for the growth of this species was 30°C and the maximum citrinin accumulation was also observed at this temperature (Montani et al., 1988). In concurrence with the findings of this study, observable florescence by direct visual detection of fungal culture in YES broth under acidic conditions, was detected on day four and five after incubation at 25°C (Vázquez et al., 1997). According to the authors, spots of yellow fluorescence were observed on day three which improved and was quantifiable by the direct method and HPLC after five days of incubation. Similarly, citrinin accumulation was noticed approximately four to five days with the optimum of around six to seven days of incubation at 25°C on Czapek agar with maize extract (Montani et al., 1988).

CCA medium was a suitable substrate for production of citrinin by *P. citrinum* with the toxin production comparatively better than on YES agar. YES agar is increasingly used for culturing fungi to monitor citrinin production (Franco et al., 1996; Malmstrøm et al., 2000; Vázquez et al., 1997). Agar plugs or contents of the plate can be directly removed from the CCA plate for confirmatory TLC or HPLC if required instead of growing on other general media such as YES or CYA. The TLC agar plug method of (Filtenborg et al., 1983) used in this study for confirmation is a simple and inexpensive method for qualitative confirmation of the mycotoxin. However, in cases where quantification is essential, the plugs from CCA or the contents of the plate can be extracted and run with TLC or HPLC against a range of standards.

The most commonly applied techniques for the determination of citrinin contamination in foods include TLC, HPLC and immunochemical methods with HPLC-FLD as the key method used for routine analysis (Xu et al., 2006). The same techniques have been reported for the screening of fungal cultures for citrinin production although the reports are quite infrequent compared to screening methods for other major mycotoxins. Vázquez et al. (1997) developed a method permitting direct visual detection of citrinin in a fungal culture, by enhancing the natural fluorescence of this mycotoxin in an acidic environment. Other than this, little has been published on the development of simple and cheap screening methods for detecting citrinin in culture.

In conclusion, a simple and rapid method for the screening of the mycotoxin citrinin in fungal cultures has been developed. This method provides a direct indication of the presence of citrinin as early as four to five days after incubation at 25°C. The CCA media used is a simple formulation with relatively cheap ingredients and the reading is simple using a long wavelength UV lamp. No expensive instrumentation or expertise in operation is required. The method has been tested in this study using fifteen isolates of *P. citrinum* and is very promising for the screening of a large number of cultures for citrinin production. However, further evaluation is necessary to confirm its suitability for screening other citrinin producing species and optimisation of test conditions for this purpose.

CHAPTER 5

Assessment of the Mycotoxin Producing Potential of Toxigenic Fungi Isolated From Maldive Fish

Abstract

The mycotoxin producing capability of the potentially toxigenic fungal species isolated from Maldive fish samples was determined. One hundred isolates belonging to important mycotoxin producing species *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. tamarii* and *Penicillium citrinum* were screened for their corresponding toxins using coconut cream agar (CCA) and thin layer chromatography (TLC) and confirmed by high performance liquid chromatography (HPLC). Nineteen out of the 41 isolates (46%) of *A. flavus*, one of the predominant species isolated from the product, were able to produce aflatoxin B, ranging from very low levels of 10 ng/g of agar to 78000 ng/g agar aflatoxin B_1. Ninety five per cent (39/41) of *A. flavus* isolates also produced varying amounts of the less potent cyclopiazonic acid (CPA) ranging from 7000 to 242000 ng/g agar. Those isolates capable of producing aflatoxins also produced CPA. In addition, 19 of the 21 isolates of *A. tamarii* were able to produce CPA, although at much lower concentrations (2000 to 16000 ng/g agar) than the *A. flavus* isolates. Of the 8 isolates of *A. ochraceus* screened for ochratoxin A (OTA), 6 were positive producers of this toxin at levels of 400 to 700 ng/g agar. However, only 3 of the 15 *A. niger* isolates were positive for OTA at lower levels (30 to 60 ng/g agar). All of the 15 *P. citrinum* isolates were consistent producers (115 to 596 ng/g agar) of the mycotoxin citrinin which is of moderate toxicity. The ability of a high proportion of potentially toxigenic isolates to produce toxic metabolites *in vitro* implies possible contamination of the product with mycotoxins of varying potency if provided with a favourable environment.

5.1 Introduction

Several factors influence the production of mycotoxins in dried foods and the presence of toxigenic fungi does not necessarily guarantee the formation of these toxins. Both intrinsic and extrinsic factors such as the type of strain, the food matrix , water activity, temperature and other environmental conditions determine the magnitude of mycotoxin production in foods by potentially toxigenic species (Klich, 2007; Sanchis and Magan, 2004). It is well acknowledged that the mycotoxin producing characteristic of a particular fungus is variable from strain to strain with high variability commonly witnessed within a species (Abbas et al., 2009; Horn and Dorner, 1999). Furthermore, both toxin-producing and non-producing strains of the same species often co-occur in foods. The mycotoxin producing capacity of the fungal population is therefore a defining factor for contamination of foods.

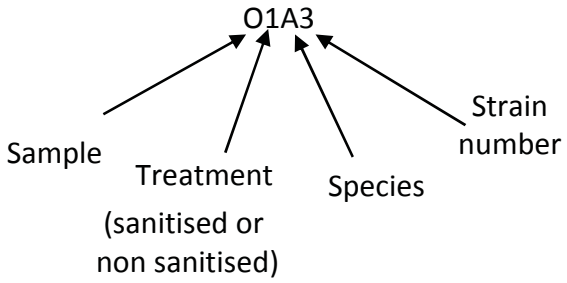
A number of toxigenic fungi were isolated from “Maldive fish” that may have the potential to produce mycotoxins in the product (Chapter 3). *A. flavus* (92%), the major producer of aflatoxins and cyclopiazonic acid (CPA), and *A. tamarii* (97%) which can also produce CPA were the predominant fungi on the product. *A. ochraceous and A. niger* which occurred at lower frequencies may synthesise Ochratoxin A (OTA), a known nephrotoxin, while *P. citrinum* a common contaminant of Maldive fish is a known producer of the mycotoxin citrinin, also a nephrotoxin. These mycotoxins can induce a range of adverse health effects from immediate toxic response, immuno-supression and acute organ damage to chronic carcinogenic, mutagenic and teratogenic effects (Jolly et al., 2008; Richard, 2007; Williams et al., 2004). The ability of the fungal isolates to produce any significant level of mycotoxin in the product may be evaluated by their capacity to produce toxins *in vitro*. This chapter therefore investigates the mycotoxin producing capacity of isolates originating from “Maldive fish” that belong to potentially toxigenic species of importance.

5.2 Methodology

5.2.1 Toxigenic Fungi

One hundred out of more than 1000 potentially toxigenic isolates from the "Maldive fish" samples that were identified to the species level were screened for their corresponding toxins. One to three isolates from the positive samples were selected for the assessment. Table 5.1 summarises the fungi screened and the methods applied in screening, confirmation and quantification of the toxins.

A sterile water system was used for the storage of fungi where agar cubes cut from the growing margin of a 7 day old culture were transferred to Bijou bottles containing approximately 2 ml of sterile water and maintained at 4°C (Pitt and Hocking, 2009; Smith and Onions, 1994). Cultures maintained by this method have been found to retain their viability and original characteristics for up to seven years (Smith and Onions, 1994). The isolates were coded for accurate identification where the first section of the code is the sample number and second is the species and strain. For example:



To obtain mature spores for subsequent analysis, an agar plug from each of the test fungi was transferred to Czapek yeast extract agar (CYA) plates and incubated at 25°C for 7 days.

Table 5.1 Summary of the methods applied in screening and confirmation of mycotoxins

Species	No. of isolates	Mycotoxin	Screening method	Confirmation method
Aspergillus flavus	41	Aflatoxin	Coconut Cream Agar	HPLC-FLD
			TLC agar plug from YES	
		Cyclopiazonic acid	TLC agar plug from CYA	HPLC-UV
Aspergillus tamarii	21	Cyclopiazonic acid	TLC agar plug from CYA	HPLC-UV
Penicillium citrinum	15	Citrinin	Coconut Cream Agar	HPLC-FLD
			TLC agar plug from CYA	
Aspergillus niger	15	Ochratoxin A	Coconut Cream Agar	HPLC-FLD
			TLC agar plug from YES	
Aspergillus ochraceus	8	Ochratoxin A	Coconut Cream Agar	HPLC-FLD
			TLC agar plug from YES	

5.2.2 Chemicals and standards

Mycotoxin standards, aflatoxins (mixed B and G, B_1 only, G_1 only and B_2 only), OTA, CPA and citrinin were supplied by Sigma-Aldrich (St. Louis, MO, USA). All HPLC grade organic solvents were sourced from Merck. All other solvents and chemicals were of analytical grade. Deionised water (Milli-Q) prepared in-house was used for HPLC.

5.2.3 Presumptive Screening of Isolates

5.2.3.1 Fluorescence on Coconut Cream Agar (CCA)

A preliminary screening for Aflatoxin, OTA and citrinin producers was performed on CCA which indicates a positive fluorescence under long wavelength UV (366 nm) after growth on this medium (Dyer and McCammon, 1994; Heenan et al., 1998). CCA has not

been previously reported for screening of citrinin and Chapter four explored this method for citrinin detection. This method cannot be employed for CPA as the compound is non-fluorescent.

CCA was prepared as described in section 4.2.2. Mature spores of the isolates were inoculated onto CCA in duplicate using single point inoculations at the centre of the plates. Plates were incubated upright in the dark at 30°C for 5-7 days for potential aflatoxin producers and 25°C for 7 days for potential OTA producers following the recommendation of Pitt and Hocking (2009). *P. citrinum* isolates were also incubated at 25°C as this temperature favoured optimum fluorescence production on CCA (refer Chapter 4).

The reverse side of the plates was periodically viewed under long wave UV (366 nm) to inspect fluorescence from day 4 to day 7. Aflatoxin production was indicated by the presence of a pastel blue fluorescent ring around the colony accompanied by a yellow reverse colouration when viewed under daylight (Dyer and McCammon, 1994). OTA was indicated by a blue green fluorescence usually covering the whole colony (Heenan et al., 1998). Citrinin was represented by a yellow green fluorescence around the positive colony (Chapter 4). An uninoculated CCA plate was used as a control and known non citrinin producers (*A. tamarii* and *E. repens*) were also screened as negative controls.

5.2.3.2 TLC agar plug method

All isolates were qualitatively screened for toxin production by a TLC agar plug method for intracellular and extracellular mycotoxins (Filtenborg et al., 1983; Samson et al., 2010). Mature spores of the isolates were single point inoculated on YES agar for potential aflatoxin and OTA producers and CYA for all other toxins (Heperkan et al., 2012). The plates were then incubated at 25°C for 7days.

For extracellular mycotoxins (aflatoxins, OTA and citrinin) an agar plug 5 mm in diameter was removed from the centre of the colony (Filtenborg et al., 1983; Samson et al., 2010) and transferred agar side down on to the designated track on a 20 x 20

silica gel 60 TLC plate (Merck). For citrinin, the TLC plate was previously impregnated with 2% oxalic acid in methanol and air dried (Xu et al., 2006). The plug was held in position until the appearance of the application spot under it, discarded and the spot allowed to dry. A second plug was removed from the same colony and superimposed on the previous spot.

For the intracellular mycotoxin CPA, the mycelium side of the plug was used instead of the agar side. The plug was treated with a few drops of chloroform: methanol (2:1 vol/vol), gently pressed onto a TLC plate and immediately removed. The spot was allowed to dry before the application of a second plug.

For weak or uncertain results, the test was repeated with superimposed application of three agar plugs. The spots were dried completely before the plates were developed in a tank containing the recommended eluent system as detailed in Table 5.2.

Table 5.2 Eluent systems for TLC of mycotoxins

Analyte	**Eluent system**	**R_f value***	**Reference**
Aflatoxins	toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v)	0.8 (B_1) 0.6 (B_2) 0.5 (G_1) 0.5 (G_2)	(Copetti et al., 2011)
Ochratoxin A	toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v)	0.55	(Copetti et al., 2010)
Cyclopiazonic acid	ethyl acetate: 2-propanol: ammonium hydroxide(40:30:20, v/v/v)	0.5	(Fernández Pinto et al., 2001)
Citrinin	toluene/ethyl acetate/formic acid (5:4:1 v/v/v)	0.68	(Samson et al., 2010)

*R_f value is the distance travelled by the metabolite from the application spot divided by the distance of the eluent front from the application spot

The plates were run until the eluent front was approximately 10 cm above the application line. They were then dried (in a fume hood for 20 minutes) and examined under long wavelength UV light (366 nm) for all mycotoxins except CPA. CPA was visualised in daylight after spraying with Ehrlich's reagent (1 g 4-dimethylamino benzaldehyde in 75 ml ethanol and 25 ml concentrated HCl). Standards (20 µl) and negative control strains were run in parallel on each plate. The aflatoxins were detected as intense blue (B_1 and B_2) and blue green (G_1 and G_2) bands whereas OTA was seen as a fluorescent blue band and citrinin as an intense yellow green band when exposed to long wavelength UV (366 nm). CPA was visualised as purple blue bands under daylight.

5.2.4 Confirmation and quantification of mycotoxin production

The mycotoxin producing capabilities of the isolates were verified and quantified by an HPLC agar plug method (Bellí et al., 2004; Bragulat et al., 2001; Riba et al., 2010; Smedsgaard, 1997). Mature spores of the isolates were inoculated on YES agar for potential aflatoxin and OTA producers and CYA for other isolates. The plates were then incubated at 25°C for 7 days. Three agar plugs were removed from the colony (inner, centre and outer area to represent variability in a colony) (Samson et al., 2010) and weighed in to a vial, in duplicates. Plugs were extracted (sonicated) with 1 ml of methanol for one hour. The contents of the vial was then centrifuged at 11000 x g for 10 minutes at 4°C and the clear solution transferred to a clean vial. For OTA and citrinin, the extract was directly injected into the HPLC system. For CPA, the extract was evaporated near dryness under a gentle stream of nitrogen and the residue reconstituted in 1 ml of the mobile phase. The solvent was filtered through a 0.2 µm hydrophilic Polyvinylidene fluoride (PVDF) filter (Millipore) and transferred to HPLC vials for injection.

Aflatoxin analysis included precolumn derivatisation performed with trifluoroacetic acid according to AOAC method 990.33 (AOAC, 2000). The extract was evaporated to near dryness under a gentle stream of nitrogen. Hexane (200 µl) and triflouroacetic

acid (50 µl) were added to the residue and vortexed for 30 s. The solutions were allowed to stand for 5 minutes before adding 1.95 ml of water-acetonitrile (9+1). The mixture was vortex mixed for 30 s and let stand for 10 minutes to facilitate separation of the layers (or centrifuged at 100 x g for 30 s). The lower layer (1 ml) was removed and filtered through a 0.2 µm PVDF filter and transferred to HPLC vials for injection.

The HPLC was a Dionex UltiMate 3000 System equipped with a UV and a fluorescence detector. A C18 column was used for separation of aflatoxins and OTA whereas an NH_2 column was applied for CPA. For citrinin, a C18 column with an extended pH operational range was necessary to withstand the highly acidic mobile phase. The injection volume was set at 25 µl. HPLC conditions varied for each mycotoxin and are summarised in Table 5.3.

Stock solutions of the mycotoxin standards were prepared using appropriate solvents (aflatoxins in benzene: acetonitrile (98:2, v/v), CPA and OTA in methanol and citrinin in acetonitrile) and stored at -20°C. Except for aflatoxins, a range of working standard solutions was made by dilution with suitable solvent or mobile phase and filtered through a 0.2 µm hydrophilic PVDF filter before HPLC injection. As with the samples, aflatoxin standards were subjected to precolumn derivatisation with trifluoroacetic acid according to AOAC method 990.33 (AOAC, 2000) before analysis. Appropriate quantities of the stock solution were transferred to 15 ml screw capped vials to achieve the desired concentration of the toxins in 2 ml of the solvent. The solutions were evaporated to dryness under a gentle stream of nitrogen and derivatised following the protocol for the samples above.

Standard curves with linearity (r^2 value) >0.99 were obtained for each mycotoxin (refer to Appendix D). Samples were considered positive if they yielded a peak at the retention time identical to that of the standard and with a concentration above the detection limits. Quantification was achieved by comparison with the standard curve and the amount of toxin produced per gram of agar was calculated. The highest of the duplicate value was reported. Negative isolates were run for each mycotoxin to confirm the peaks.

Table 5.3 HPLC conditions for mycotoxin analysis

Analyte	Column	Mobile phase	Detection	Reference
Aflatoxins	RP C18 Phenomenex Jupiter (5 µm 300A)	Acetonitrile: methanol: water (20:20:60, v/v/v) under isocratic conditions pumped at 0.75 ml/min	fluorescence detector λ_{exc} = 360 nm λ_{em} = 440 nm	(Hussain et al., 2010)
Ochratoxin A	RP C18 Phenomenex Jupiter (5 µm 300A)	Acetonitrile: water: acetic acid (57:41:2, v/v/v) under isocratic conditions pumped at 1.0 ml/min	fluorescence detector λ_{exc} = 330 nm λ_{em} = 460 nm	(Bellí et al., 2004)
Cyclopiazonic acid	NH_2 column Phenosphere, Phenomenex (5 µm 80A)	acetonitrile: 50mM ammonium acetate (3:1, v/v), pH 5 under isocratic conditions pumped at 1.0 ml/min	UV detector λ_{exc} = 285 nm	(Soares et al., 2010)
Citrinin	RP 18e Lichrospher 100, LichroCART (5 µm)	Acetonitrile: water(1:1 v/v), pH 2.5 adjusted with H_3PO_4 under isocratic conditions pumped at 1.0 ml/min	fluorescence detector λ_{exc} = 331 nm λ_{em} = 500 nm	(Zheng et al., 2009)

5.2.5 Sclerotia formation by *A. flavus* isolates

Seven to 14 day old cultures on CYA and MEA agar incubated at 25°C were examined under a stereomicroscope for the presence of sclerotia. Structures (5-10) from each

culture were transferred to slides and the dimensions of the sclerotia measured at low magnification on a light microscope.

5.3 Results

5.3.1 Aflatoxin production by *A. flavus* isolates

When screened on CCA, 14 of the 41 isolates of *A. flavus* produced the pastel blue fluorescent halo around the colony characteristic of aflatoxins when the reverse side of the plates was viewed under long wavelength UV (366 nm). In agreement with Dyer and McCammon (1994), all positive isolates were also accompanied by a yellow pigment visible against the white CCA background when viewed in daylight. Eighteen of the 41 cultures on YES agar were positive for type B aflatoxins with 10 displaying bands for both aflatoxin B_1 and B_2. Figure 5.1 depicts a positive CCA plate and typical TLC for aflatoxins when viewed under long wave UV (366 nm).

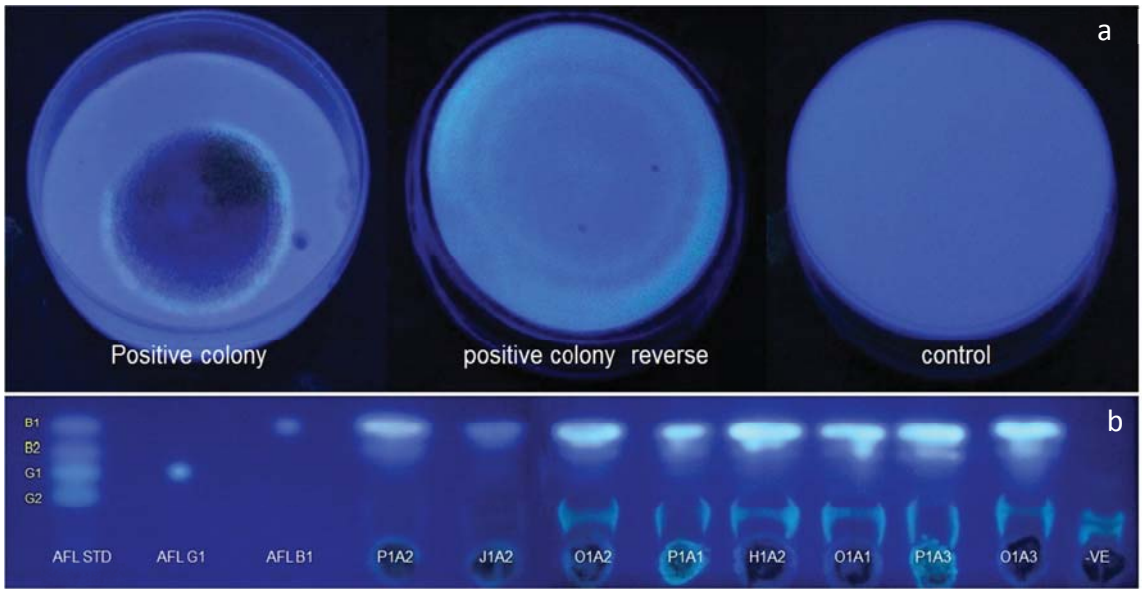


Figure 5.1 Screening of *A. flavus* isolates for aflatoxin production a) positive isolate on CCA showing the characteristic pastel blue fluorescence halo and uninoculated CCA control plate b) typical TLC for aflatoxins with the standards (3000 ng/g), test isolates and negative control, viewed under long wavelength UV.

When the HPLC agar plug method was used for confirmation, 19 of the 41 isolates were producers of aflatoxin B_1. The isolate that was negative with the TLC method produced very low levels of aflatoxin B_1 (10 ng/g agar). Thirteen of the 19 positive isolates were capable of producing both aflatoxin B_1 and B_2 on YES agar after 7 days of incubation.

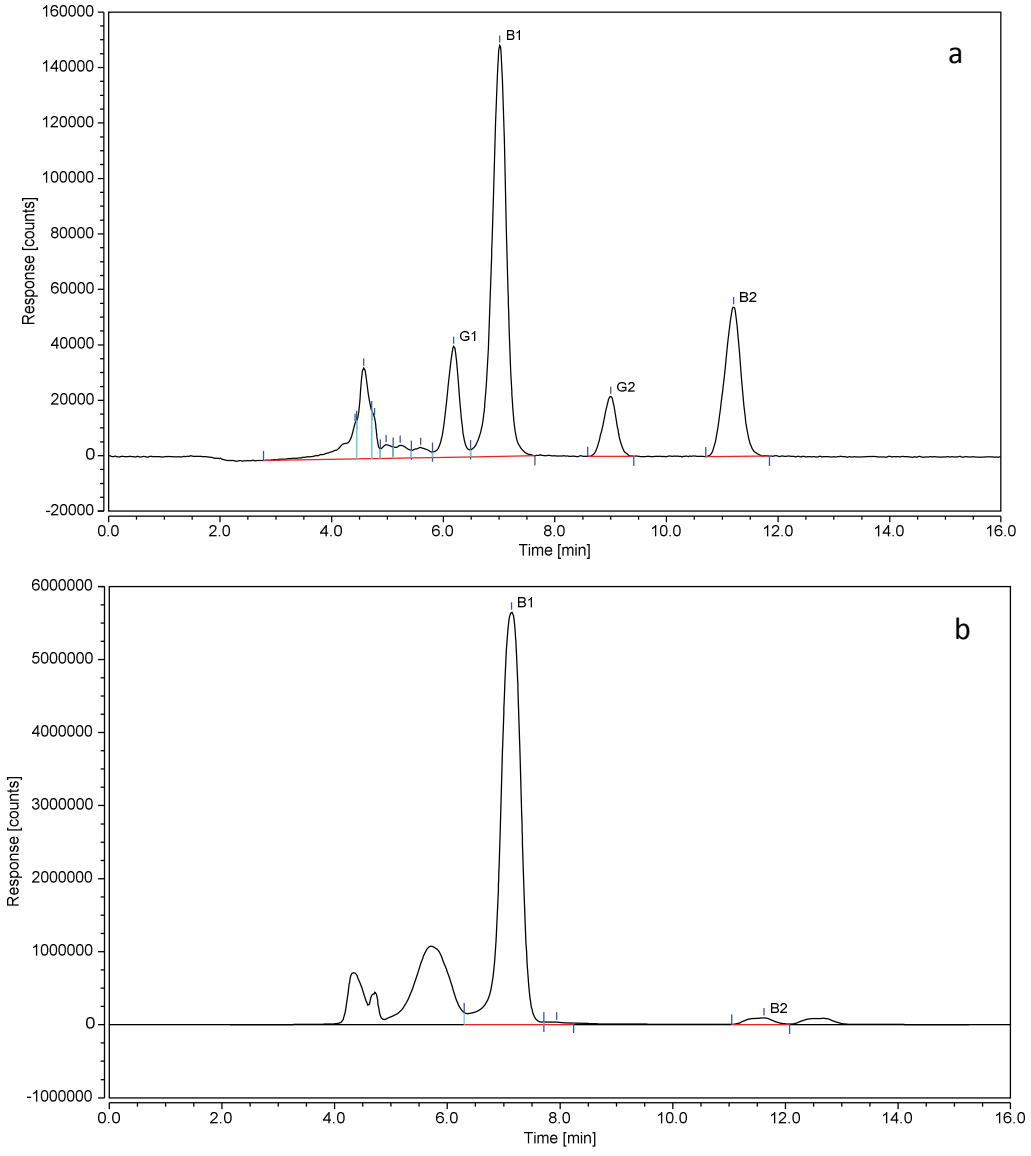


Figure 5.2 HPLC chromatographs for aflatoxins a) mixed aflatoxin standard (B_1, B_2, G_1, G_2) b) positive *A. flavus* isolate (G1E1)

The remaining 6 isolates were able to produce aflatoxin B_1 only. In addition to this three more isolates produced trace amounts of aflatoxin B_1 below 5 ng/g agar. None of the isolates produced any G aflatoxins. Figure 5.2 shows a typical HPLC chromatogram of the aflatoxin standard (mixture of B and G toxins) and a positive isolate.

HPLC also provided quantitative information on the ability of the isolates to produce aflatoxin on YES agar after 7 days of incubation at 25°C. Two replicates were tested and as differences were usually small, the higher of the two results is reported here. The concentration of aflatoxin B_1 produced ranged from very low (10 ng/g of agar) to 78,000 ng/g agar. As was to be expected, overall the isolates produced lower levels of aflatoxin B_2 (5 - 3560 ng/g of agar). The *A. flavus* isolates were categorised into 5 groups based on their capacity to produce aflatoxin B_1 in culture (Table 5.4). Seven point three percent (3) of the isolates were able to produce the aflatoxin B_1 in abundance above 50000 ng/g agar while a further 7.3% produced toxin at levels between 10000 to 50000 ng/g of agar. Intermediate levels in the range 100 to 10000 ng/g of agar were detected in 17.1% of the isolates. Minute amounts of aflatoxin B_1 (5 to 100 ng/g agar) were produced by 12% (5) of the isolates while 54% (22) can be termed as non-toxigenic as they did not have the capability to produce any toxins or produced only negligible amounts, less than 5 ng/g agar.

Table 5.4 Categorisation of *A. flavus* isolates based on the quantity of aflatoxin B_1 produced in culture

Aflatoxin B_1 (ng/g)	**No of isolates**	**Percentage %**
<5	22	53.7
5 - 100	5	12.2
100 - 10 000	7	17.1
10 000 - 50 000	3	7.3
>50 000	3	7.3

Thirty one of the 41 (76%) isolates were able to develop sclerotia under the culture conditions. All positive isolates were L-type strains, that is they produced sclerotia with a diameter greater than 400 µm. No S-strain isolates capable of producing abundant small sclerotia (diameter <400 µm) were detected.

5.3.2 CPA production by *A. flavus* isolates

All isolates screened for aflatoxins were also tested for the ability to produce CPA. The TLC agar plug method showed that 95% (39/41) of the isolates had the ability to produce this toxin, indicated by a positive purple band corresponding to the CPA standard (Figure 5.3). This was confirmed by the more sensitive HPLC agar plug method. The amount of CPA produced ranged from 7,000 to 242,000 ng/g of CYA agar. Twenty nine per cent of the isolates were able to produce levels of CPA above 50,000 ng/g agar. All isolates capable of producing aflatoxins also synthesized relatively high levels of CPA although some of the isolates producing CPA above 50,000 ng/g were not necessarily aflatoxin producers. Fifty six per cent of the isolates were able to produce CPA in the range 10,000 ng/g to 50,000 ng/g agar. All remaining positive isolates were able to produce less than 10, 000 ng/g but equal to or greater than 7,000 ng/g agar of this toxin. HPLC chromatograms of CPA standard and a positive isolate are presented in Figure 5.4.

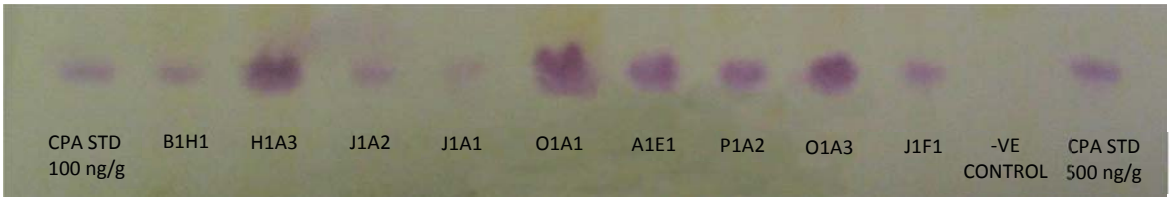


Figure 5.3 TLC for cyclopiazonic acid with the standards, test isolates (*A. flavus*) and negative control, viewed under daylight.

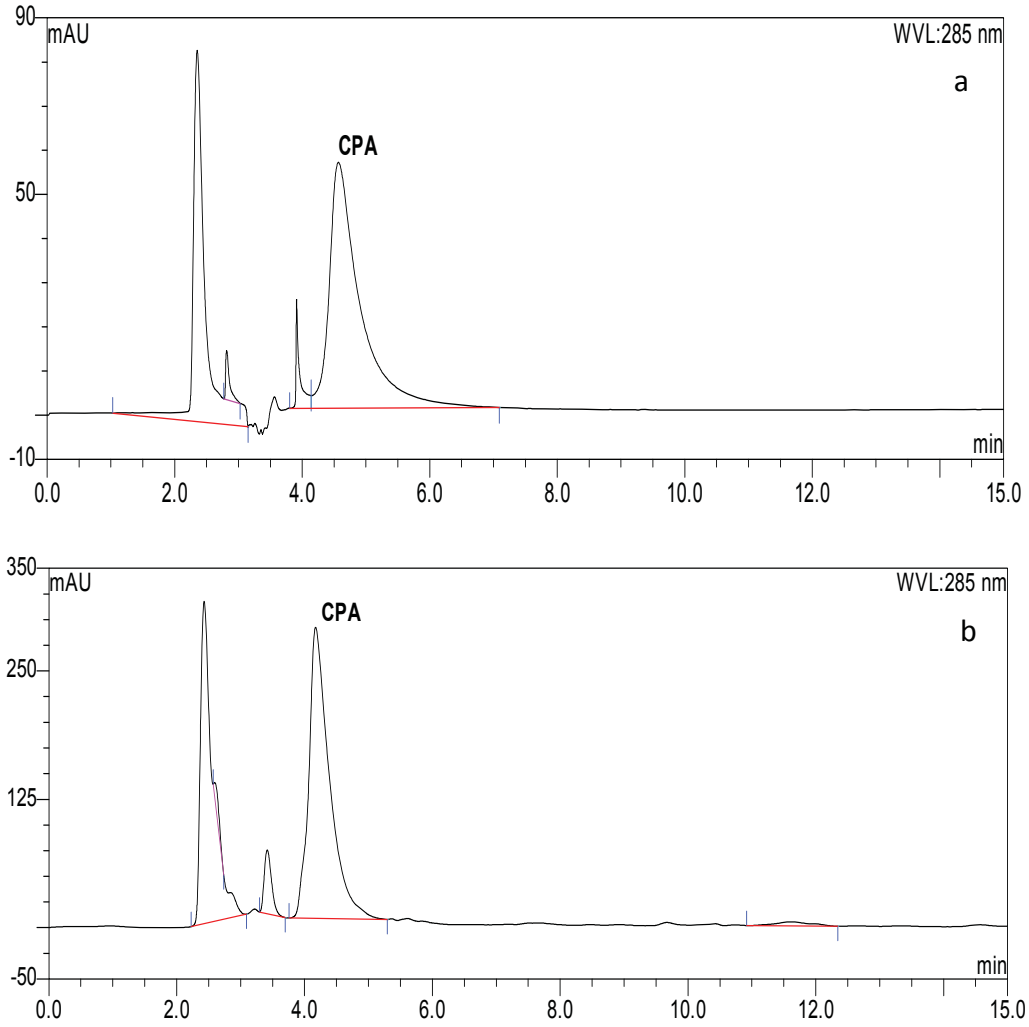


Figure 5.4 HPLC chromatographs for cyclopiazonic acid a) CPA standard (100 ng/g) b) positive *A. flavus* isolate (H1A3)

5.3.3 Identification of chemotypes in *A. flavus*

Isolates of *A. flavus* are classified into five chemotypes based on their mycotoxin producing ability according to Vaamonde *et al*. (2003) and the results from this study are shown in Table 5.5. Chemotype I was represented by the group that produces both aflatoxin B and CPA. Forty six per cent (19 out of 41) of isolates fell into this category. Forty nine per cent were able to produce CPA but no aflatoxins and belong to chemotype IV. Interestingly, only two isolates (5%) were non-toxigenic, that is lacking

the ability to produce a detectable level of either aflatoxin or CPA. Isolates that were capable of producing aflatoxin B but no CPA (chemotype III) were absent while none of the strains produced any aflatoxin G (chemotype II). Isolates under Chemotype II are now described as *A. minisclerotigenes* and *A. parvisclerotigenus* (Pildain et al., 2008).

Table 5.5 Chemotypes of *A. flavus* isolates based on aflatoxin and cyclopiazonic acid production

chemotype	Aflatoxin B	Aflatoxin G	Cyclopiazonic acid	No of isolates	Percentage (%)
I	+	-	+	19	46
II	+	+	+	0	0
III	+	-	-	0	0
IV	-	-	+	20	49
V	-	-	-	2	5

5.3.4 CPA production by *A. tamarii* isolates

Nineteen of the 21 *A. tamarii* isolates produced positive purple bands by TLC with the R_f value corresponding to the CPA standard. The two isolates that did not produce any bands were also negative with the more sensitive HPLC agar plug method suggesting that they are non-producers or CPA production is negligible. The 19 isolates that were positive were confirmed as positive producers of CPA by HPLC, although production of

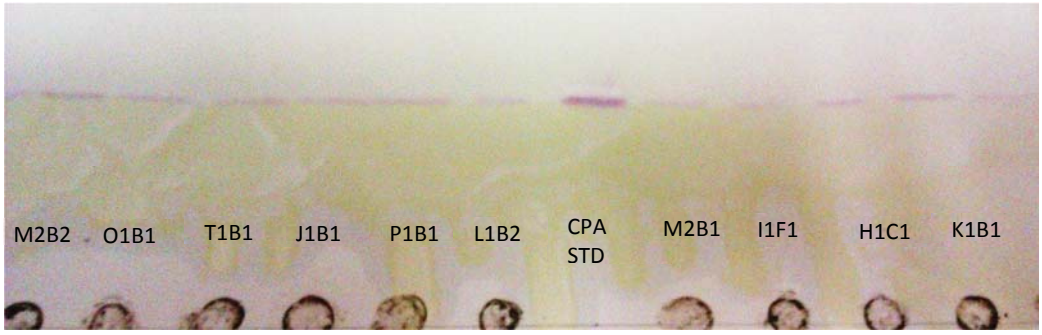


Figure 5.5 TLC for cyclopiazonic acid screening, *A. tamarii* isolates and CPA standard (1000 ng/g) viewed under daylight.

CPA was at a much lower level (2000 to 16000 ng/g of agar) than the *A. flavus* isolates (7000 to 242000 ng/g of CYA agar).

5.3.5 OTA production by *A. ochraceus* and *A. niger*

A. ochraceus and *A. niger* are potential producers of OTA. Six of the eight isolates of *A. ochraceus* screened on CCA produced blue green fluorescence covering the whole colony indicating positive toxin production (Figure 5.6a). After assay by TLC, the positive isolates also produced clear fluorescent bands when viewed under long wavelength UV (Figure 5.5c). OTA production was confirmed by HPLC. Toxin production on YES agar after 7 days of incubation at 25°C ranged from 400 to 700 ng/g of agar. The remaining two isolates of *A. ochraceus* were negative for OTA by the methods applied in this study. None of the 15 *A. niger* isolates produced any apparent fluorescence on CCA and TLC but three were positive for OTA by HPLC. However, the positive isolates produced between 30 to 60 ng/g of agar of the toxin, levels much lower than those produced by the *A. ochraceus* isolates examined in this study. Figure 5.7 represents HPLC chromatographs of the standard and a positive *A. ochraceus* isolate.

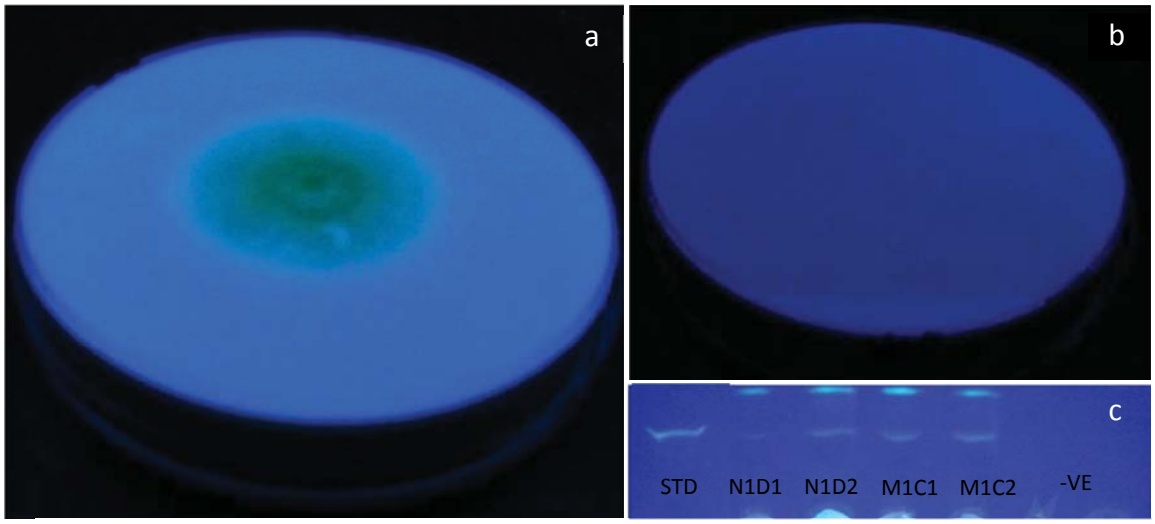


Figure 5.6 Screening of isolates for OTA production a) positive isolate on CCA exhibiting green blue fluorescence on colony reverse b) uninoculated CCA control plate c) typical TLC plate for OTA with the standards, test isolates and negative control, viewed under long wavelength UV (366 nm).

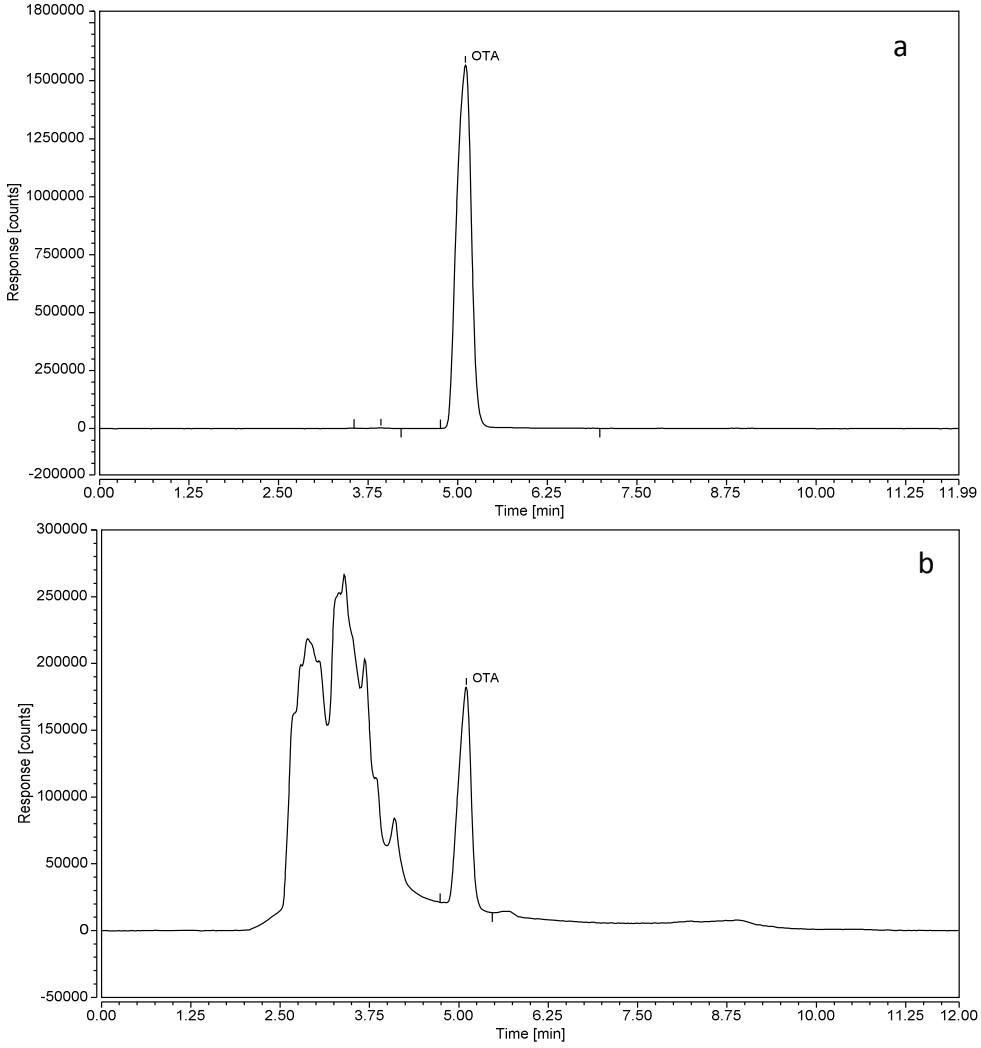
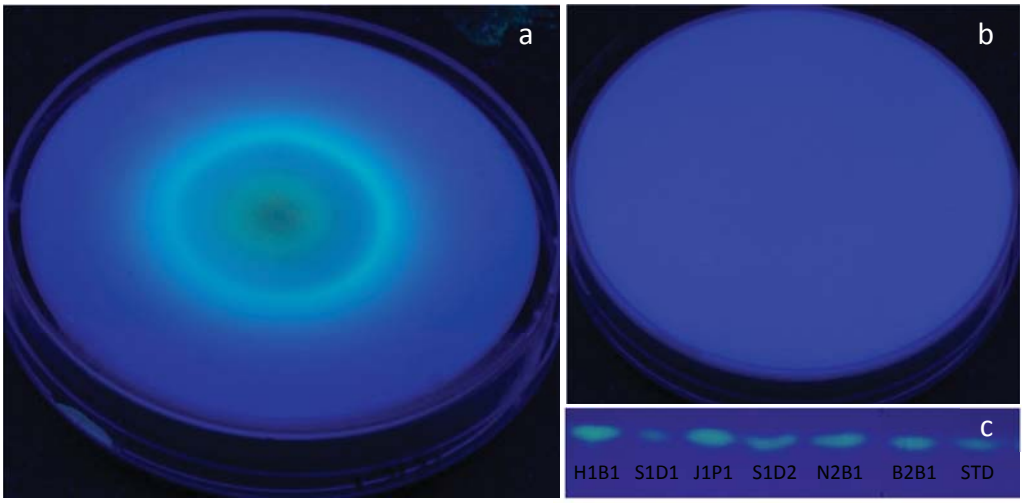


Figure 5.7 HPLC chromatographs for ochratoxin A a) OTA standard 1000 ng/g b) positive *A. ochraceus* isolate (N1D2)

5.3.6 Citrinin production by *P. citrinum*

All of the 15 *P. citrinum* isolates studied were prolific producers of citrinin exhibiting strong positive fluorescence on CCA and TLC plates (Figure 5.8). This was confirmed by HPLC and all isolates produced abundant citrinin, ranging from 115 to 596 ng/g agar. Figure 5.9 illustrates HPLC chromatographs of the standard and a positive isolate.



Figue 5.8 Screening of *P. citrinum* isolates for citrinin production a) positive isolate on CCA showing the characteristic yellowish green fluorescence on reverse b) uninoculated CCA control plate c) typical TLC plate for citrinin with the standards (100 ng/g), test isolates and negative control, viewed under long wavelength UV (366 nm).

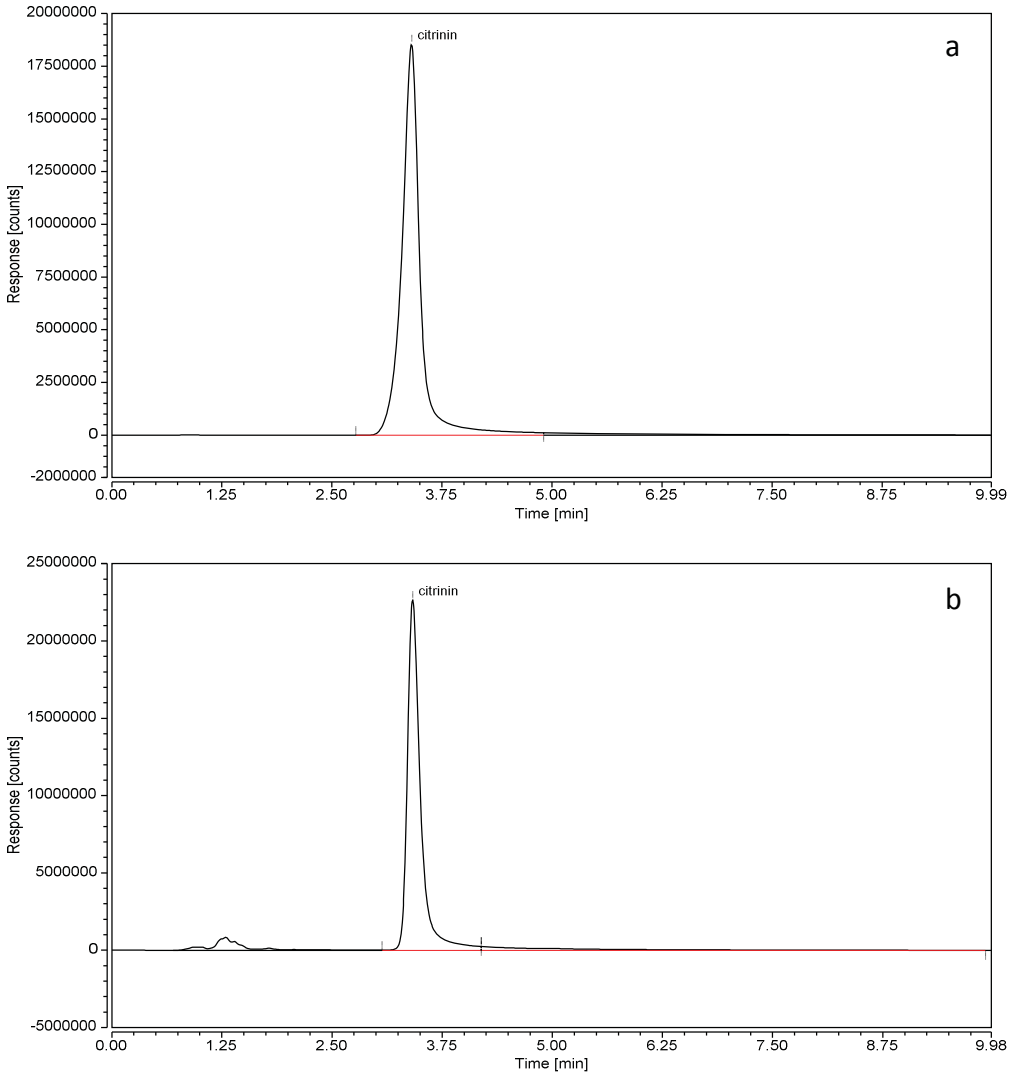


Figure 5.9 HPLC chromatographs for citrinin a) citrinin standard 3000 ng/g b) positive *P. citrinum* isolate (H1B1)

5.4 Discussion

The screening results for toxigenic fungi isolated from smoked Maldive fish are presented in Table 5.6. This study confirms that approximately half (46%) of the *A. flavus* isolates from the 25 Maldive fish samples are aflatoxin producers and 95% of the isolates are producers of CPA although the production varied greatly from isolate to isolate. Numerous studies have been conducted on the toxicity of *A. flavus* from different sources and only 30-40% of known isolates are estimated to produce aflatoxins (Frisvad et al., 2007). However, there is great variation on the reported incidences of aflatoxin and CPA producing ability of *A. flavus* and their prevalence appears to vary widely depending on the nature of the substrate and other environmental factors (Horn, 2005; Klich, 2007). Aflatoxin production by *A. flavus* isolates from smoked and dried foods or similar products have been rarely reported. In a study of the traditional market cured fish from Tanzania, 16% of *A. flavus* strains isolated were aflatoxigenic (Mugula and Lyimo, 1992) while none of the nine isolates from Sri Lankan dried fish produced any positive fluorescence on coconut agar (Atapattu and Samarajeewa, 1990). The aflatoxin and CPA producing ability of *A. flavus* from different sources is collated in Table 5.7, clearly showing the huge variability between the values. The current study on Maldive fish falls in the mid range of the different studies.

Table 5.6 Summary of the screening results

Species (Frequency %)	Mycotoxin screened	No screened	No positive (%)	Range ng/g agar
A. flavus (92)	Aflatoxin	41	19 (46%)	10 - 78000
	Cyclopiazonic acid	41	39 (95%)	7000 - 242000
A. tamarii (96)	Cyclopiazonic acid	21	19 (91%)	2000 - 16000
A. niger (40)	Ochratoxin A	15	3 (20%)	30 - 60
A. ochraceus (12)	Ochratoxin A	08	06 (75%)	400 - 700
P. citrinum (60)	Citrinin	15	15 (100%)	115 - 596

Table 5.7 Aflatoxin and cyclopiazonic acid producing ability of *Aspergillus flavus* from different sources

Source	% Aflatoxigenic strains (no.)	% CPA positive strains (no.)	Reference
Various food substrates, air and soil from India	48% (826/1706)	-	(Bilgrami and Choudhary, 1993)
Stored grains and smoked dried meat products from Croatia	9% (9/96)	5% (5/96)	(Cvetnić and Pepeljnjak, 1998)
Croatian dried hams	0	-	(Comi et al., 2004)
Maize from six regions in Northern Italy	70% (49)	61% (43)	(Giorni et al., 2007)
Traditional market cured fish from Tanzania	16% (16/100)	-	(Mugula and Lyimo, 1992)
Foods from markets in Burundi	39% (37/95)	71% (67/95)	(Munimbazi and Bullerman, 1996)
Pods from a peanut field in Formosa Province, Argentina	L strain 89.3 (25/28)	L strain 100 % (28/28)	(Pildain et al., 2004)
Argentinian corn	15% (5/34)	97% (33/34)	(Resnik et al., 1996)
Algerian wheat and derived products	72% (108/150)	10% (15/ 150)	(Riba et al., 2010)
Spanish dry cured hams	56% (9/16)	-	(Rojas et al., 1991)
Different food grains from China	28% (23/82)	-	(Wang et al., 1993)
Peanuts, wheat and soybean from Argentina	Peanuts 69% (27/37) Wheat 13% (2/15) Soybeans 5% (2/41)	Peanuts 94% (35/37) Wheat 93% (14/15) Soybeans 73% (30/41)	(Vaamonde et al., 2003)
Maldive fish	*46% (19/41)*	*95% (39/41)*	*Current study*

Despite the low potency of CPA compared to aflatoxins, it is still a food safety concern hence contamination of foods with this toxin should not be overlooked. Further, both aflatoxins and CPA often co-occur in food matrices as they can originate from the same source thereby magnifying the issues associated with *A. flavus* contamination of foods. A very high proportion of isolates from this study can produce CPA and all isolates producing aflatoxins were also capable of producing CPA simultaneously. It appears that *A. flavus* from "Maldive fish" are more likely to produce CPA than aflatoxins and this concurs with some other reports where a relatively higher percentage of isolates were able to produce CPA than aflatoxins (Munimbazi and Bullerman, 1996; Resnik et al., 1996; Vaamonde et al., 2003). Contrary to this, only 10% of 150 *A. flavus* isolates from Algerian wheat and derived products screened for CPA were positive whereas 72% of them were aflatoxin B producers (Riba et al., 2010). Giorni et al. (2007) also found 70% of isolates from Italian maize to be aflatoxigenic whilst 61% of them were CPA producers. A good number of isolates (19%) from the latter studies comply with chemotype III (produce aflatoxin B but no CPA) and this chemotype has been increasingly reported in other studies (Novas and Cabral, 2002; Vaamonde et al., 2003). However, none of the isolates from the current study were able to produce aflatoxins in the absence of CPA while all of the aflatoxin positive strains produced high amounts of CPA. The majority of the isolates (85.4%) of *A. flavus* from grains and smoked dried meat from Croatia failed to produce any aflatoxins or CPA (Chemotype V) (Cvetnić and Pepeljnjak, 1998). High numbers of such non-toxigenic isolates have been reported from other *A. flavus* populations (Pildain et al., 2004). However, non-toxigenic isolates in our study were rare.

Some *A. flavus* strains can produce elevated levels of aflatoxins, CPA or both while other strains produce negligible levels (Abbas et al., 2009; Horn and Dorner, 1999). *A. flavus* isolates in this study displayed extreme variability in their aflatoxin producing ability with values ranging from as low as 10 ng/g to 78000 ng/g of agar on YES for Aflatoxin B_1. The results are accordance with other studies reporting similar or even higher distribution of toxin production by *A. flavus*. Isolates of *A. flavus* from Algerian wheat and derived products were capable of producing 20 ng/g to very high levels of

234,000 ng/g of aflatoxin B_1 on CYA medium after 7 days of incubation at 28°C (Riba et al., 2010). *A. flavus* strains from smoke-dried meat products from Croatia mainly produced aflatoxin B_1, and displayed various levels of toxigenicity ranging from 1400 to 3120 ng/g of agar (Cvetnić and Pepeljnjak, 1995). Thirty three per cent of *A. flavus* isolates from different food substrates produced less than 100 ng total aflatoxins per ml of YES medium and none produced more than 8000 ng/ml (Munimbazi and Bullerman, 1996). Similarly, CPA production by *A. flavus* in the current study also shows similar strain dependent variability with values ranging from 7000 -242,000 ng/g of agar. In agreement, *A. flavus* strains from stored grain and smoke dried meat produced CPA in the range 50 to 30,000 ng/g of agar (Cvetnić and Pepeljnjak, 1998). L-strain isolates of *A. flavus* (774) from soil populations of some major peanut-growing regions of the United States show CPA production ranging from 1000 to 300,000 ng/ml of yeast extract sucrose liquid media with majority of production observed at levels between 10,000 to 99,000 ng/ml (Horn and Dorner, 1999).

A. flavus produces aflatoxin B_1 and B_2 and CPA but no G aflatoxins (Pitt and Hocking, 2009). However there have been rare reports where atypical strains of *A. flavus* produced aflatoxin G (Frisvad et al., 2005; Vaamonde et al., 2003) and some of them have now been described as new aflatoxigenic species namely *A. parvisclerotigenus* and *A. minisclerotigenes* (Pildain et al., 2008). Many other closely related species belonging to *Aspergillus* section *Flavi* such *A. parasiticus*, *A. bombycis*, *A. toxicarius* and *A. arachidicola,* have the ability to produce both B and G aflatoxins (Varga et al., 2011). Failure to detect aflatoxin G production by the isolates screened in this study suggests scarcity or absence of any such species in the Maldive fish.

A. flavus can be categorised into two morphological groups designated as S (sclerotia <400 µm diameter) and L (sclerotia > 400 µm in diameter) strains based on the size of the sclerotia developed in culture. The presence of sclerotia *per se* does not correlate with aflatoxin production but in general a high level of toxin formation is observed from S strains whereas L strains can be toxigenic or non-toxigenic (Cotty, 1997; Klich, 2007). Only L type strains were isolated from Maldive fish and the rest failed to produce any sclerotia under the experimental conditions.

A high percentage of *A. tamarii* isolates was also observed to produce CPA although at much lower concentrations (2000 - 16000 ng/g) than the *A. flavus* isolates. This agrees with other studies where almost all *A. tamarii* strains were capable of synthesizing this toxin (Dorner, 1983; Vinokurova et al., 2007). Dorner (1983) reported CPA production from *A. tamarii* for the first time and examined 23 isolates of which 22 were producers. The CPA producing ability of *A. tamarii* have not been thoroughly studied although it appears from the current findings that this species may be an important contributor to contamination of foods by this toxin.

A. flavus (92%) and *A. tamarii* (96%) were the dominant fungi isolated from Maldive fish. In this study 7.3% and 29% of the *A. flavus* isolates were capable of producing > 50,000 ng/g agar of aflatoxins and CPA, respectively. Some isolates therefore have the potential to accumulate elevated levels of these toxins in the product when provided with favourable conditions but both the intermediate and low producers can also contribute to overall levels of toxin. Both *A. flavus* and *A. tamarii* were common isolates from many of the fish samples in this study, and the surface of some of the samples was covered with the olive green to olive brown colour characteristic of their growth. Furthermore 95% of the *A. flavus* isolates and 91% of the *A. tamarii* isolates were producers of either aflatoxin and/or CPA and hence there is a high probability of co-occurrence of these toxins in the product. The high frequency of occurrence of the two species coupled with the ability of most of the isolates to produce aflatoxin and CPA, increases the likelihood of the product being contaminated with these mycotoxins.

A. ochraceus and *A. niger* are the potential ochratoxigenic species isolated from Maldive fish. The latter is often dominant in sundried products from warmer regions (Atapattu and Samarajeewa, 1990; Pitt and Hocking, 2009). Although 6 out of the 8 strains of *A. ochraceus* were able to produce OTA at levels between 400-700 ng/g when grown on YES agar, the low isolation frequency suggests this is not likely to pose any serious risk in the product. Other species related to *A. ochraceus* such as *A. westerdijikiae* and *A. steyni* are stronger producers of this toxin (Pitt and Hocking, 2009) but none of them were isolated from Maldive fish. *A. niger* was encountered

more frequently but only three isolates (20%) were able to produce OTA at levels much lower than for *A. ochraceus*. Many other studies also report a lower incidence of toxigenic *A. niger*: 5.2% of 191 isolates from Brazilian cocoa beans (Copetti et al., 2010); 6 % from green coffee beans (Pardo et al., 2004); 3% from Tunisian grapes (Lasram et al., 2007); and 1% (3 of 293 isolates) from Argentinian dried wine fruits (Romero et al., 2005). However, higher incidences of up to 47% have been reported (Chulze et al., 2006; Heperkan et al., 2011; Rosa et al., 2002). In agreement with most other reports, only minute levels of OTA ranging 30-60 ng/g CYA were produced by *A. niger*. In wine grapes from Argentina, OTA production by *A. niger* ranged from only 2 to 24.5 ng/ml of liquid media (Magnoli et al., 2003) while similar levels (2 to 61.15 ng/ml) were observed in another study on Argentinian dried vine fruits (Magnoli et al., 2004). Likewise, strains of OTA positive *A. niger* failed to produce levels above 100 ng/g of CYA after 7 days of incubation (Romero, 2005). Higher incidences and increased levels of OTA have been reported from *A. carbonarius*, another species belonging to *Aspergillus* section *Nigri* that co-occurs with *A. niger* in many foods (Heperkan et al., 2011; Magnoli et al., 2004; Rosa et al., 2002). However, *A. carbonarius* was not isolated from Maldive fish in this study. The low incidence of *A. ochraceus* from the product together with the decreased ability of the more dominant *A. niger* isolates to produce toxins shows that there is no real threat of contamination of the product with OTA.

P. citrinum is widely distributed in nature and is the major producer of the mycotoxin citrinin (Pitt and Hocking, 2009). All 15 isolates examined in this study were capable of producing citrinin on CYA agar with levels ranging from 115 - 596 ng/g of agar. This is in agreement with other studies where this species was frequently reported as a consistent producer of citrinin on different media or substrates. In a study of the mycoflora of dried figs in Turkey, 100% of the isolates investigated were positive for citrinin (Heperkan et al., 2012). Similarly, all 19 *P. citrinum* strains from Argentinian dried vine fruits were strong citrinin producers (Romero et al., 2005). Twenty five soil isolates were steady producers of citrinin on CYA and YES agar after incubation at 25°C for two weeks (Malmstrøm et al., 2000). However, more than 50% of pure citrinin

added to dry cured ham was lost after 6 hours and decreased to only 15% after 192 hours during incubation at 20°C (Bailly et al., 2005). The stability of citrinin in foods depends on the substrate (Bailly et al., 2002) and hence its presence in Maldive fish may change during storage. Citrinin undergoes photodegradation when exposed to light (Schmidt-Heydt et al., 2012). Furthermore, previous studies demonstrates that citrinin can bind to albumin and plasma proteins while the disappearance of citrinin in cheese is thought to be due to its reaction with the casein component (Bailly et al., 2002). Since Maldive fish is a protein rich substrate, a similar reaction with citrinin is likely to occur. The presence of *P. citrinum* suggests the likelihood of contamination of the product with citrinin although the concentrations could depend on the production and stability of this compound in the product.

Three analytical methods were employed in this study for detection of mycotoxins from fungal cultures. CCA was a cheap and simple screening method for aflatoxins, OTA and citrinin that can be used to screen a large number of isolates. The drawback of the method however was the low sensitivity compared to the other two methods. For example, only 34% (14 out 41) of the *A. flavus* isolates in the current study were positive by CCA compared to 46% (19 out of 41) by the HPLC method. Isolates producing lower amounts (< 20 ng/g) of aflatoxins B_1 on YES agar failed to exhibit any fluorescence on CCA. Riba *et al*. (2010) also reported a deviation of 30% to 72% between coconut agar medium and the HPLC agar plug method for detection of aflatoxigenic fungi. In addition, the CCA method can only be used for screening mycotoxins that fluoresce.

The TLC agar plug method was also a relatively simple method that involves direct application of agar plugs without any additional extraction step that normally precedes chromatography. The method was more sensitive than the CCA method with the ability to detect aflatoxin B_1 levels down to 10 ng/g of YES agar and hence a higher percentage of positives (44%) were identified. The method although qualitative provided additional information on the types of mycotoxins (separate bands for aflatoxin B_1, B_2, G_1 and G_2). The HPLC agar plug method was the most sensitive of the three methods and provided confirmatory as well as quantitative data. The extraction

step was very simple and fast compared to many HPLC extractions and requires use of few chemicals. The HPLC method for aflatoxins can be further simplified by applying post-column derivatisation. The biggest disadvantage of the method is that it requires expensive instrumentation and hence necessitates use of alternative methods such as TLC in the absence of such equipment. CCA can be used for fast screening of many isolates where the sensitivity is not an issue and low mycotoxin producers are not of concern. The TLC agar plug method is a more preferred screening method where qualitative information is adequate for the purpose. However, HPLC must be the method of choice when sensitivity and quantification is vital such as in research. For instance, OTA production by *A. niger* isolates was very low and could only be detected by the HPLC method. Furthermore, the sensitivity of the TLC and HPLC can be enhanced by increasing the number of agar plugs. Both the TLC and HPLC agar plug methods have increasingly been used for screening many different mycotoxins (Bragulat et al., 2008; Copetti et al., 2011; Mogensen et al., 2009; Pildain et al., 2004; Vaamonde et al., 2003).

In conclusion, the ability of a high proportion of potentially toxigenic isolates (72%) to produce toxic metabolites *in vitro* implies possible contamination of Maldive fish with mycotoxins of varying potency if provided with a favourable environment. The frequent association of *A. flavus* with the product and the fact that approximately half of the isolates were able to accumulate aflatoxins in culture while a higher percentage can produce CPA is cause for concern. The co-occurrence of aflatoxins and CPA can augment the risk due to their synergistic action (Smith et al., 1992). Hence, consumption of contaminated products could pose a health hazard. Measures must be taken to control the growth of these fungi and subsequent mycotoxin production to guarantee the safety of the product. The magnitude of the contamination however will depend on the stability of these compounds in the product and this has to be confirmed by quantification of mycotoxins from the product.

CHAPTER 6

Quantification of Aflatoxins and Ochratoxin A in Maldive fish

Abstract

Maldive fish samples were assessed for contamination with mycotoxins. Mycotoxins were detected and quantified by HPLC-FLD following extraction and clean-up using commercial immunoaffinity columns. Aflatoxins were detected at concentrations of 0.38 ng/g to 51.4 ng/g for aflatoxin B_1 and 0.38 ng/g to 53.6 ng/g for total aflatoxins from four of the five composite samples (n= 25 samples) analysed. Two of these samples were contaminated at levels of 20.4 and 51.4 ng/g aflatoxin B_1, far exceeding the regulatory limits of 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins. Individual samples from these two batches were tested with two samples surpassing the stated limits. This study confirms possible exposure to deleterious levels of aflatoxins, which are potent carcinogens, through regular consumption of Maldive fish infected with fungi. High aflatoxin levels could be ascribed to the frequent association of toxigenic *A. flavus* with the product and a conducive environment for their growth and toxin production. Hence, growth of *A. flavus* on this product should not be taken lightly and hurdles are needed to ensure control of their growth and subsequent mycotoxin production. Any significant exposure to ochratoxin A via this product is unlikely due to the low prevailing levels found in the samples (n=10).

6.1 Introduction

Aflatoxins, a group of toxic metabolites produced by some species in *Aspergillus* section *Flavi* are the most significant mycotoxin in foods and feeds in terms of distribution and toxicity. They occur naturally as four compounds, named B_1, B_2, G_1 and G_2, for the fluorescent colours blue or green displayed on exposure to long-wave UV light after TLC. *A. flavus* and *A. parasiticus* are the most important producers of these toxins. The consequence of exposure to these substances through contaminated food is quite variable leading to both acute and chronic effects (Krishnamachari et al., 1975; Lewis et al., 2005). Aflatoxins are mutagenic, teratogenic and carcinogenic and can cause acute and chronic liver damage including cirrhosis (Groopman et al., 2008; Wang and Tang, 2005). Aflatoxin B_1, the major metabolite produced by toxigenic strains of *A. flavus* is the most potent liver carcinogen known and has been categorized as a Group I human carcinogen (IARC, 1993). Chronic exposure can also cause general adverse health effects such as impaired growth in children and immunosuppression (Khlangwiset et al., 2011; Williams et al., 2004).

Ochratoxin A (OTA) is another major mycotoxin of concern produced by several *Penicillium* and *Aspergillus* species that pose a risk to human health. Species belonging to *Aspergillus* sections *Circumdati* and *Niger* are responsible for the frequent contamination of tropical food commodities by this toxin (Gil-Serna et al., 2011). OTA is a well-known nephrotoxin that can cause acute and chronic lesions of kidneys. It is also reported to exhibit carcinogenic, hepatotoxic, mutagenic, teratogenic and immunosuppressive properties in test animals (Mally and Dekant, 2009; Pfohl-Leszkowicz and Manderville, 2007). The International Agency for Research on Cancer (IARC, 1993) has classified this mycotoxin as a possible human carcinogen. OTA was once considered to be the cause of Balkan Endemic Nephropathy and Chronic Interstitial Nephropathy in North Africa, but that now appears to be unlikely.

Foods associated with *Aspergillus* species can be contaminated by a range of mycotoxins of which aflatoxins and OTA are of great concern. *A. flavus* was one of the dominant species isolated from Maldive fish with almost half of the isolates of this

species demonstrating aflatoxin production in YES medium by HPLC. Some isolates of *A. niger* and *A.ochraceus* isolated from this product were also able to produce OTA in culture. However, mycotoxin production in a food is governed by many influencing factors (Klich, 2007; Sanchis and Magan, 2004) and the presence of toxigenic fungi that have the ability to produce mycotoxins *in vitro* does not guarantee their presence *in vivo*. The objective of this study therefore was to assess if Maldive fish is contaminated with these major mycotoxins and determine the magnitude of contamination by quantification of toxins in the product.

6.2 Methodology

6.2.1 Chemicals and standards

Mixed aflatoxin standard (B and G aflatoxins) as a powder and individual standards of aflatoxin B_1, B_2, G_1 and G_2 in benzene: acetonitrile were supplied by Sigma-Aldrich (St. Louis, MO, USA). OTA standard in the powder state was also procured from the same supplier. All HPLC grade organic solvents were sourced from Merck and all other solvents and chemicals were of analytical grade. Deionised water (Milli-Q) prepared in-house was used for HPLC. AflaTest Immunoaffinity columns were obtained from Vicam (Watertown, MA, USA) and Ochraprep® immunoaffinity columns from R-Biopharm (Darmstadt, Germany).

6.2.2 Quantification of Aflatoxins

Aflatoxins in the samples were quantified following the method of Chiavaro et al. (2005) as modified by Hussain et al. (2010). The method involves clean-up of the sample extract by immunoaffinity columns (IAC) selective for aflatoxins followed by pre-column derivatisation by triflouroacetic acid (TFA) to enhance the fluorescence of aflatoxin B_1 and G_1 by converting them to B_2a and G_2a, respectively. An HPLC system equipped with a C18 column was used for separation and subsequent detection was via a fluorescence detector.

6.2.2.1 Sample Preparation

Finely ground Maldive fish samples stored at -20°C were thawed and composite samples prepared by combining five samples with similar water activities, 5 g of each sample, to obtain a total of 25 g per sample. Five composite samples (total 25 samples), were analysed in triplicate. Individual samples within the composite sample were analysed if the batch contained aflatoxin B_1 at a concentration greater than the limit of 2 ng/g and total aflatoxins at levels above 4 ng/g.

6.2.2.2 Sample Extraction and clean up

Each sample (25 g) was extracted (shaken) with NaCl (5 g) in methanol: water (80:20, v/v; 100 ml) for 30 minutes at high speed and the mixture filtered through a paper filter. A volume of the filtrate (10 ml) was diluted with wash buffer (40 ml) containing 0.1 % Tween 20. This solution was passed through an AflaTest immunoaffinity column (Vicam, Watertown, MA) at a flow rate of 1-2 drops per second. The column was washed with 20 ml, (2 x 10 ml portions), of deionised water at a flow rate of approximately 5 ml/minute. All liquids were removed from the column by applying downward pressure from the syringe. The toxin was eluted from the IAC with methanol (1 ml) at a rate of 1-2 drops per second into 15 ml screw capped vials. The elute was dried near dryness under a gentle stream of nitrogen and derivatised with trifluoroacetic acid according to the AOAC method 990.33 (AOAC, 2000). The residue remaining in the tube was treated with hexane (200 µl) and TFA (100 µl) and mixed by vortex for 30s. The solutions were allowed to stand for 5 minutes before adding water: acetonitrile (9:1, v/v; 1.90 ml). The mixture was mixed by vortex for 30 s and let stand for 10 minutes to facilitate separation of the layers (or was centrifuged at 100 x g for 30 s). 1 ml of the lower layer was removed and filtered through a 0.2 µm PVDF filter in to HPLC vials for injection.

6.2.2.3 Standard preparation and derivatisation

The mixed solid standard (Sigma-Aldrich) was reconstituted in benzene: acetonitrile (98:2, v/v) following manufacturer's instructions to obtain a stock solution containing

aflatoxin B_1 and G_1 (3 µg/ml) and aflatoxin B_2 and G_2 (0.9 µg/ml). The solution was stored at -20°C until further use. An intermediate stock solution of aflatoxin B_1 and G_1 (100 ng/ml) was prepared in benzene: acetonitrile (98:2, v/v). Appropriate quantities of the intermediate stock solution were transferred to 15 ml screw capped vials to achieve the desired concentrations of the toxins per 2 ml of solvent. The solutions were evaporated to dryness under a gentle stream of nitrogen and derivatised according to AOAC 990.33 as for the samples above. Individual standards of aflatoxin B_1, B_2, G_1 and G_2 were used to confirm the peak after treatment as above. Samples were considered positive if they yielded a peak at the retention time identical to that of the standard. A standard curve was developed from the standard runs with points ranging from 0.3 ng/ml to 50 ng/ml for aflatoxin B_1 and G_1 and 0.09 ng/ml to 15 ng/ml for aflatoxin B_2 and G_2. Quantification of the samples was achieved by comparison with this curve.

6.2.2.4 Recovery experiments

Spiked samples were prepared by adding appropriate levels of working solutions of the mixed aflatoxins B and G to 25 g of the ground fish samples with no detectable aflatoxins. They were spiked at levels of 2.5 ng/g and 5 ng/g, in triplicate. Spiked samples were then left at ambient temperature for at least two hours for equilibrium before extraction as in 6.2.2.2. Blank samples were extracted, in triplicate and analysed.

6.2.2.5 HPLC conditions

The HPLC system was a Dionex UltiMate 3000 System fitted with a C18 column (RP C18 Phenomenex Jupiter, 5 µm 300A) and a fluorescence detector set at emission and excitation wavelengths of 360 nm and 440 nm, respectively. The mobile phase was composed of acetonitrile: methanol: water (20:20:60, v/v/v) pumped under isocratic conditions at 0.75 ml/min. The injection volume was set at 25 µl.

6.2.3 Quantification of Ochratoxin A

The method applied for quantification of OTA was a slight modification of the method developed by Monaci et al. (2005). The method involves liquid-liquid extraction followed by clean-up using immunoaffinity columns (IAC) selective for OTA. Separation and detection was achieved using an HPLC system equipped with a C18 column and a fluorescence detector.

6.2.3.1 Sample Preparation

Ten Maldive fish samples that were positive for the potentially ochratoxigenic fungi *A. ochraceus* and *A. niger* were selected for OTA analysis. The samples stored at -20°C in finely ground form were thawed and each sample analysed in triplicate.

6.2.3.2 Sample Extraction and clean up

Each sample (10 g), in triplicate was extracted (homogenised) with 1 M H_3PO_4 (24 ml) for 15 minutes. An aliquot (3 g) was withdrawn from the homogenate and extracted twice with ethyl acetate (5 ml) containing NaCl (0.5 mol/l). The organic phases were combined and reduced to approximately 5 ml and back extracted with 0.5 M $NaHCO_3$ (pH 8.0; 5 ml). The mixture was centrifuged for 5 minutes at 1000 x g and the organic phase removed. The aqueous extract was loaded onto a Ochraprep IAC column and passed through at a flow rate of 2-3 ml/minute. The column was washed with 20 ml of deionised water (2 x 10 ml portions) at a flow rate of approximately 5 ml/minute. All liquid was removed from the column by applying downward pressure from the syringe. The toxin was eluted from the IAC with methanol: acetic acid (98:2, v/v; 2.5 ml) using slow downward motion followed by back flushing to remove all liquid. The eluted sample was evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted with mobile phase (500 µl) before injection into the HPLC.

6.2.3.3 Standard preparation

The solid standard (Sigma-Aldrich) was reconstituted in methanol to obtain a stock solution containing 1 µg/ml of OTA. This stock solution was stored at -20°C. Working

solutions were prepared by diluting appropriate amounts of stock solutions with mobile phase and directly injected in to the HPLC. A calibration curve was developed with concentrations ranging from 2.5 ng/ml to 15 ng/ml of OTA and the samples quantified against this curve.

6.2.3.4 Recovery experiments

Samples with no detectable OTA were spiked at a level of 5 ng/g by adding appropriate level of methanolic solution of the standard to the ground fish, in triplicate. The samples were maintained at ambient temperature for at least two hours and extracted as in 6.2.3.2. Blank samples were extracted, in triplicate and analysed.

6.2.3.5 HPLC conditions

The HPLC was a Dionex UltiMate 3000 System equipped with a C18 column (RP C18 Lichrospher® 100, 5 µm 300A) and a fluorescence detector with excitation and emissions wavelengths set at 334 and 460 nm, respectively. A mobile phase composed of water: acetonitrile: acetic acid (99: 99: 2, v/v/v) was pumped under isocratic conditions at 0.5 ml/minutes. Injection volume was set at 20 µl.

6.3 Results

6.3.1 Aflatoxins

The calibration curve developed using mixed aflatoxins standards was linear (correlation coefficient, r^2, was >0.995) over the calibration range 0.3 ng/ml to 50 ng/ml for aflatoxin B_1 and 0.09 ng/ml to 15 ng/ml for aflatoxin B_2 (Figure 6.1). Retention times were approximately 7 min for aflatoxin B_1 and 11.2 min for aflatoxin B_2 (Figure 6.2 a). Similar retention times were obtained using individual standards of aflatoxin B_1 and B_2 confirming the identification of the peaks. Recoveries for samples spiked at 2.5 ng/g and 5 ng/g for aflatoxin B_1 were 97.19% and 80.31%, respectively. Aflatoxin B_2 spiked at lower levels of 0.75 ng/g and 1.5 ng/g were recovered at 103.80% and 88.22%, respectively. Application of aflatoxin specific IAC columns for

sample clean-up after extraction resulted in elimination of interfering substances to elute very clean peaks of B and G aflatoxins as shown in Figure 6.2 b and c.

A total of five composite samples (five samples per batch) of Maldive fish were analysed for aflatoxins. Aflatoxin B_1 was detected in all batches except one with concentrations ranging from 0.38 ng/g to 51.36 ng/g (Table 6.1). Total aflatoxins were detected at levels 0.38 ng/g to 53.62 ng/g in the four batches that were positive.

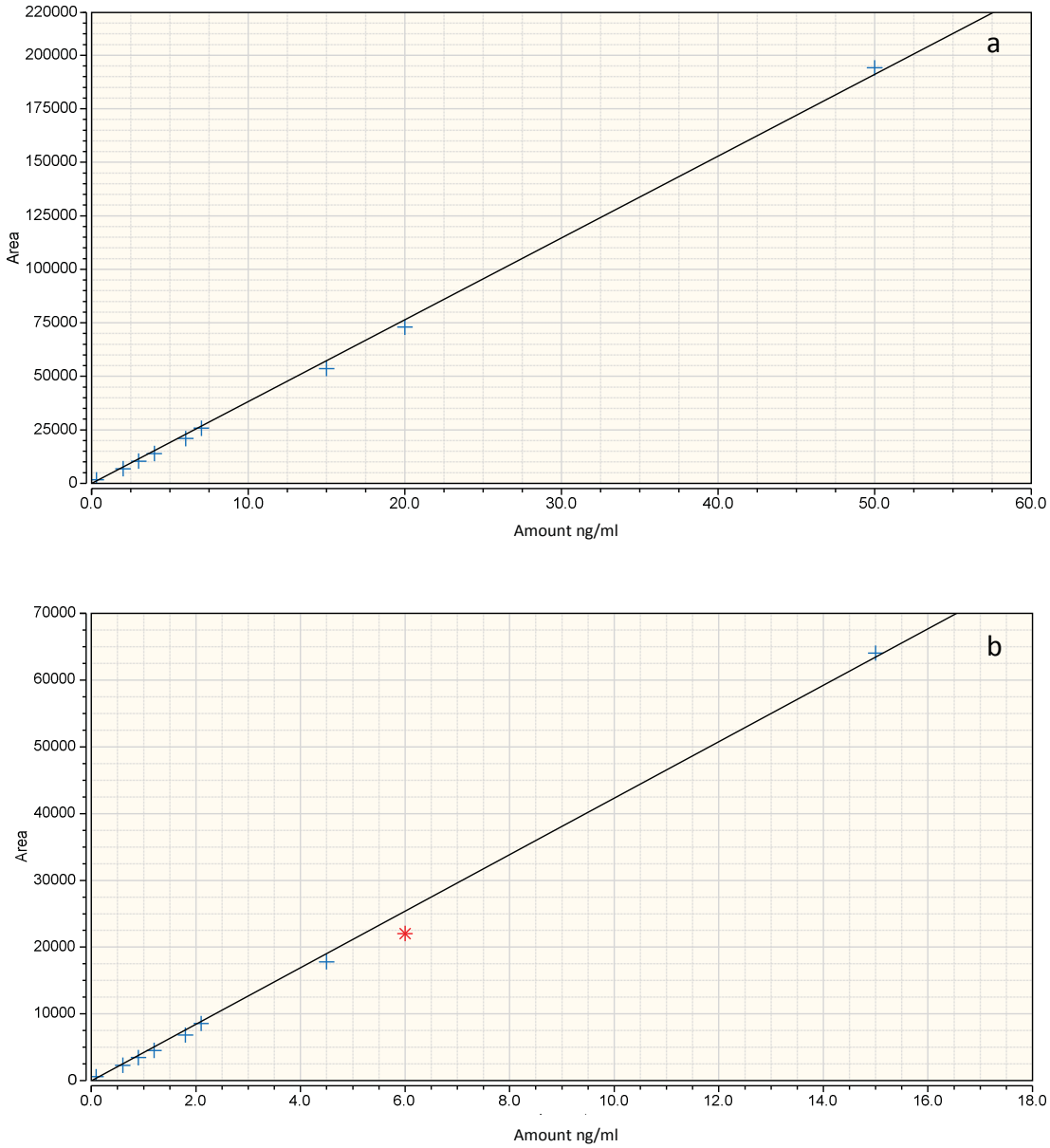
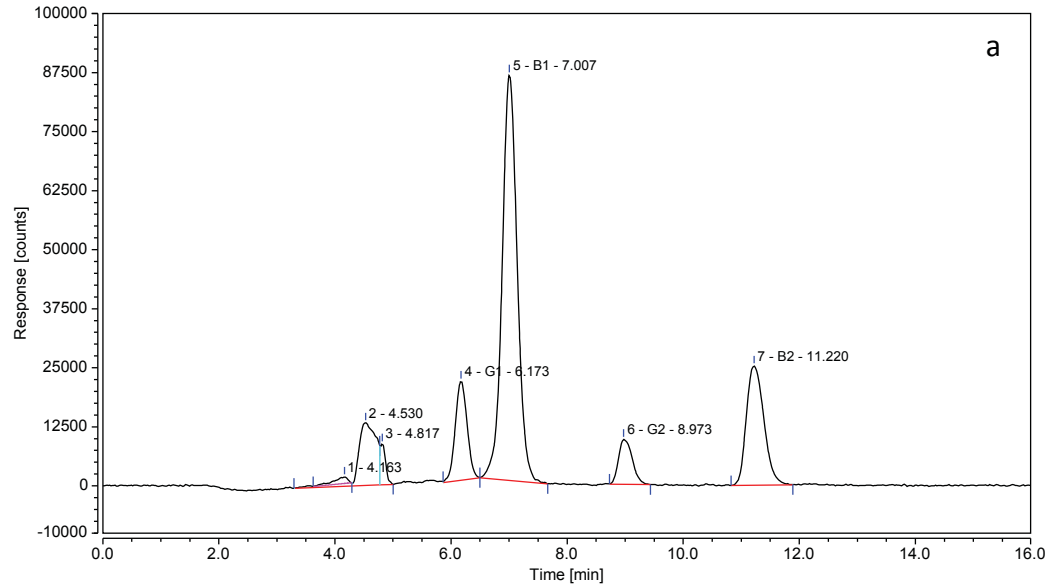
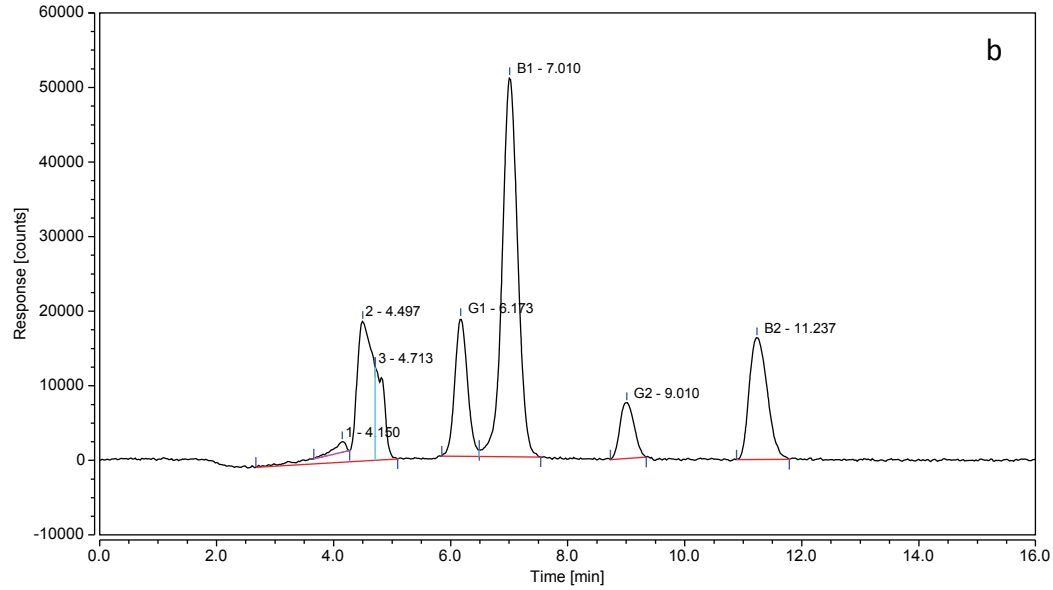
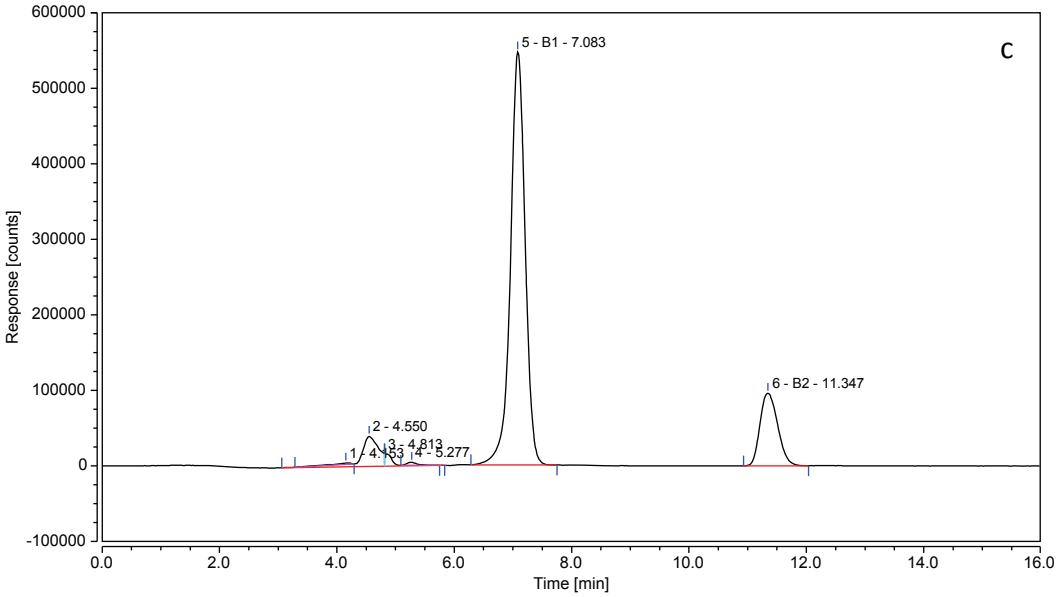


Figure 6.1 Calibration curves for a) aflatoxin B_1 and b) aflatoxin B_2


a
5 - B1 - 7.007
4 - G1 - 6.173
7 - B2 - 11.220
2 - 4.530
3 - 4.817
6 - G2 - 8.973
1 - 4.163
Response [counts]
Time [min]


b
B1 - 7.010
2 - 4.497
G1 - 6.173
B2 - 11.237
3 - 4.713
G2 - 9.010
1 - 4.150
Response [counts]
Time [min]


c
5 - B1 - 7.083
6 - B2 - 11.347
2 - 4.550
3 - 4.813
1 - 4.153
4 - 5.277
Response [counts]
Time [min]

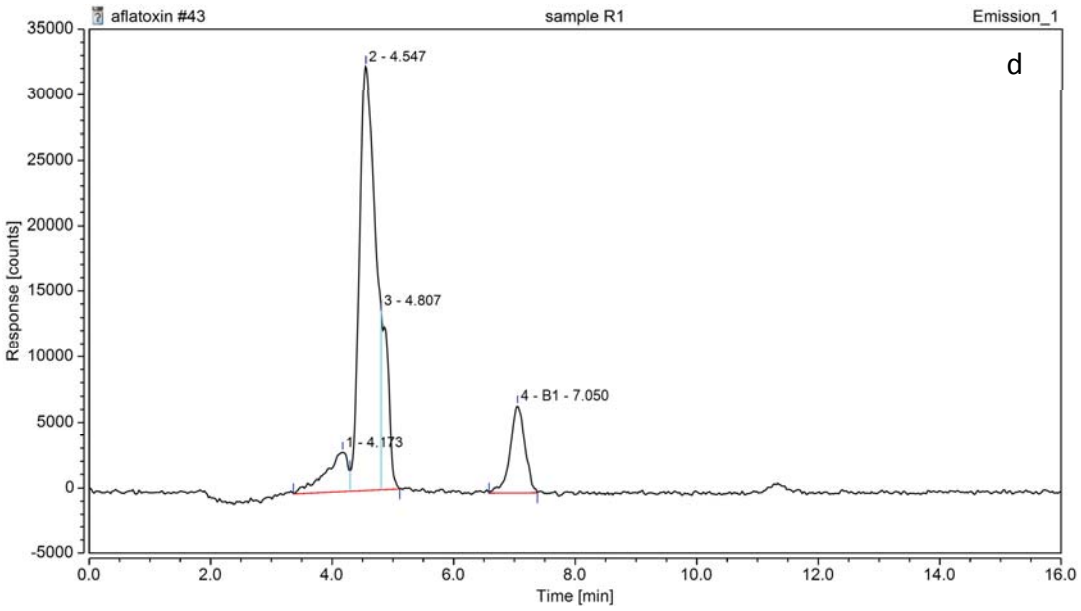


Figure 6.2 HPLC-FLD chromatograms of a) mixed aflatoxin standard 7 ng/ml (B_1, G_1) and 2.1 ng/ml (B_2, G_2), b) blank sample spiked at 5 ng/ml (B_1, G_1) and 1.5 ng/ml (B_2, G_2), c) naturally contaminated Maldive fish sample A1 with positive aflatoxin B_1 and B_2 peaks and **d**) naturally contaminated Maldive fish sample R1 with positive aflatoxin B_1 peak.

A regulatory limit of 2 ng/g for Aflatoxin B_1 and 4 ng/g for total aflatoxins are the most frequently applied limits for aflatoxins in foods worldwide (FAO, 2004). Batches IV and V were found to contain aflatoxin B_1 (20.4 and 51.4 ng/g) and total aflatoxins (24.0 and 53.6 ng/g). Other positive samples were contaminated at levels below this regulatory limit. However, Batch I could have contained only one sample that was contaminated in which case the aflatoxin level for this sample (2.6 ng/g) would exceed the regulatory limit of 2 ng/g for aflatoxin B_1. Batches IV and V with elevated aflatoxin B_1 levels were also positive for aflatoxin B_2 at lower concentrations of 3.6 ng/g and 2.3 ng/g. None of the composite samples were positive for type G aflatoxins.

Samples comprising the two batches with aflatoxin B_1 levels above 2 ng/g were subjected to individual analysis and the results are provided in Table 6.2. Three samples (samples A1, D1 and R1) from batch IV were positive for aflatoxin B_1 at concentrations of 41.2 ng/g , 1.6 ng/g and 0.49 ng/g, respectively. Similarly from batch V, aflatoxin B_1 was detected in sample E1 at 45.35 ng/g and sample T1 at 1.8 ng/g. The

Table 6.1 Mean concentrations of aflatoxin B_1, B_2 and total aflatoxins detected from Maldive fish. Each batch is a composite sample comprising five individual samples.

Batch	Aflatoxin B_1 ng/g	Aflatoxin B_2 ng/g	Total Aflatoxins ng/g
I	0.52	ND	0.52
II	ND	ND	ND
III	0.38	ND	0.38
IV	20.4	3.6	24.0
V	51.4	2.3	53.6

ND= not detected

Table 6.2 Mean concentrations of aflatoxin B_1, B_2 and total aflatoxins detected from individual Maldive fish samples in batch IV and V.

Sample	Batch	a_w	Aflatoxin B_1 ng/g	Aflatoxin B_2 ng/g	Total Aflatoxins ng/g
A1	IV	0.778	41.2	8.0	49.2
B1	V	0.939	ND	ND	ND
D1	IV	0.756	1.6	0.19	1.8
E1	V	0.947	45.4	3.5	48.8
F1	IV	0.890	ND	ND	ND
T1	V	0.931	1.8	0.47	2.3
R1	IV	0.826	0.49	ND	0.49
AC1	IV	0.892	ND	ND	ND
AD1	V	0.905	ND	ND	ND
AE1	V	0.904	ND	ND	ND

ND= not detected

aflatoxin contamination of sample A1 and E1 well exceed the previously stated limits. Total aflatoxins for these samples with a_w values 0.78 and 0.95 were 49.2 ng/g and 48.0 ng/g, respectively.

6.3.2 Ochratoxin A

The calibration curve for OTA was linear with a correlation coefficient (r^2) > 0.995 over the calibration range 2.5 ng/ml to 15 ng/ml of the standard (Figure 6.3). Interfering substances from the matrix was removed by use of mycotoxin specific IAC for clean up of the samples resulting in a clean peak for OTA with a retention time of approximately 8.16 min (Figure 6.4). Recovery for samples spiked at 5 ng/ g was 80.28%. OTA was not detected from eight of the ten samples analysed for this mycotoxin. The two positive samples contain insignificant levels of OTA at concentrations of 1.1 ng/g and 0.22 ng/g, both values below the most commonly applied legal limit of 5 ng/g of OTA in foods (FAO, 2004).

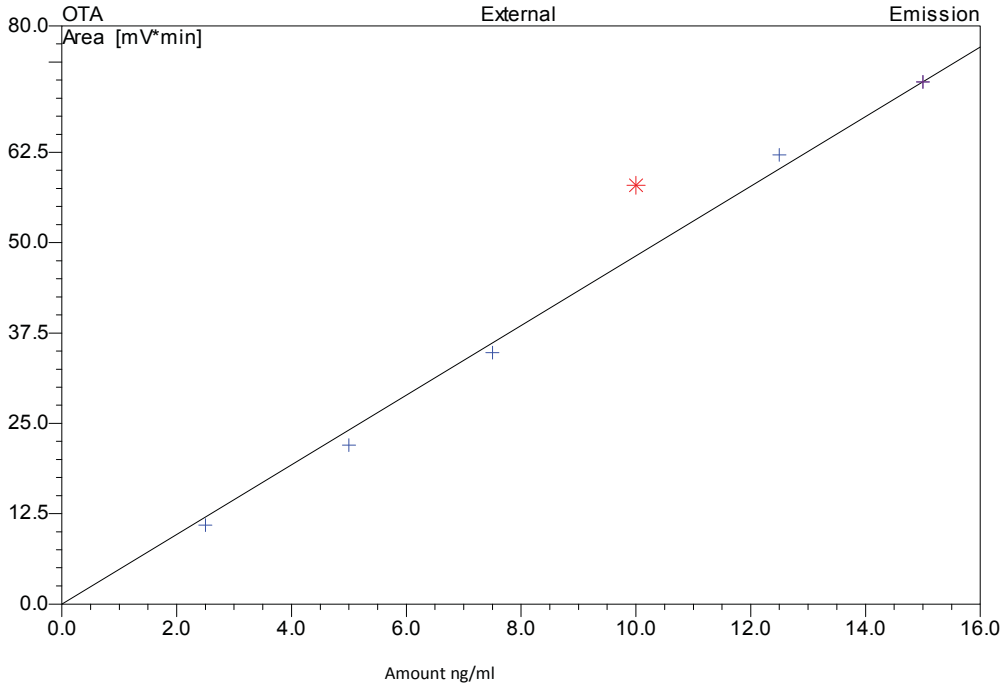


Figure 6.3 Calibration curve for ochratoxin A

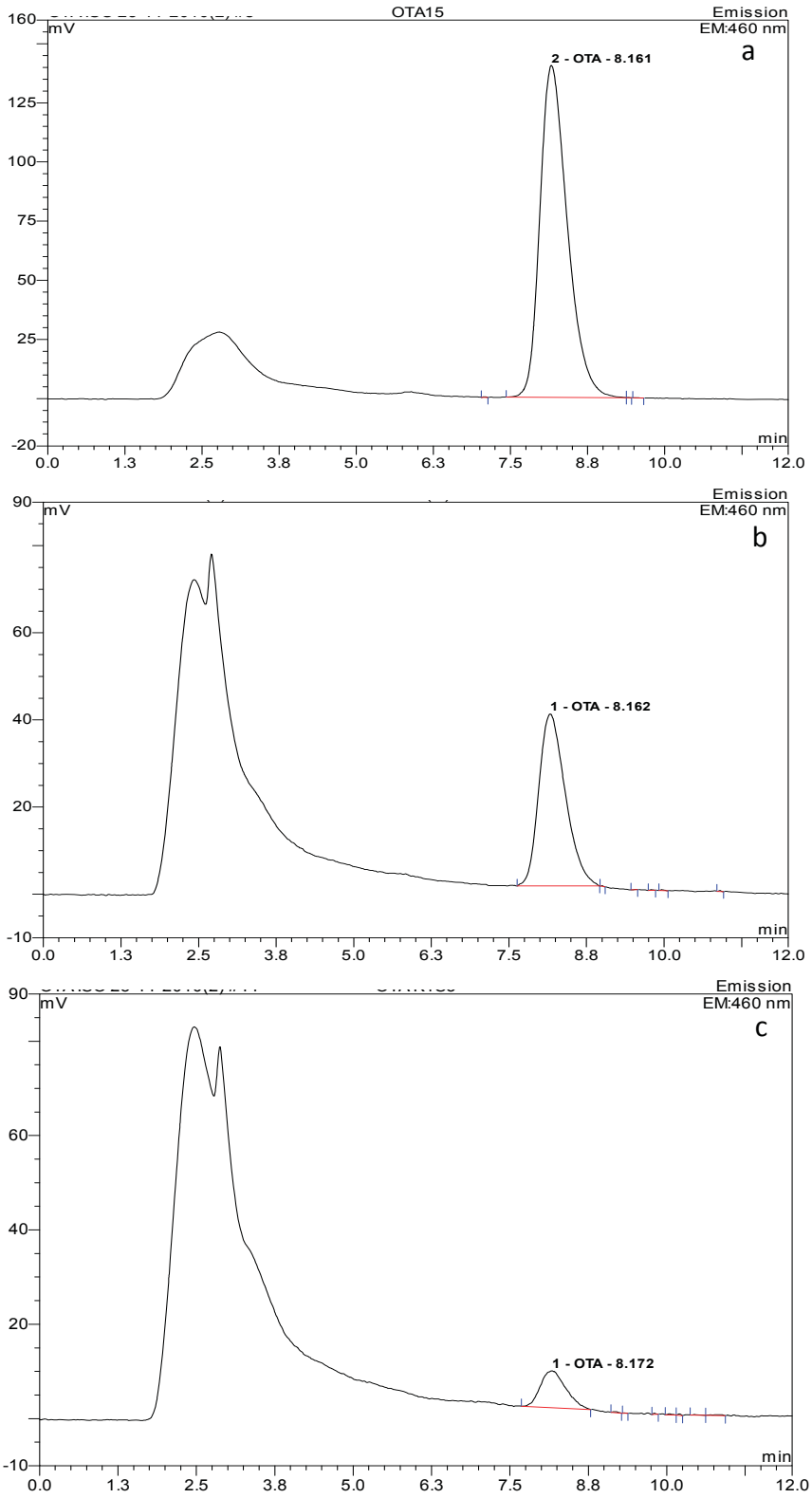


Figure 6.4 HPLC-FLD chromatograms of a) OTA standard 15 ng/ml b) blank sample spiked at 5 ng/ml, and c) naturally contaminated Maldive fish sample K1 with positive OTA peak (1.2 ng/g)

6.4 Discussion

Measurable concentrations of aflatoxins were detected from four of the five composite samples of Maldive fish tested with two samples contaminated at levels far exceeding the most commonly applied legal limits for aflatoxins. The regulatory limits for aflatoxins in foods and feeds are the most frequently imposed mycotoxin limits worldwide represented either as aflatoxin B_1 or total aflatoxins (sum of aflatoxins B_1, B_2, G_1 and G_2) (Van Egmond et al., 2007). The European community and many other countries have established a legal limit of 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins for a range of foods intended for human consumption (FAO, 2004). Other frequently established limits include 5 ng/g for aflatoxin B_1 and 10 n/g total aflatoxin set as a general level for food stuffs or for specific foods across countries in Asia, Africa and Oceania (FAO, 2004). Australia and New Zealand have set limits of 5 ng/g for foods other than peanuts (ANZFS, 2000). The US, Canada and many other countries enforce slightly higher maximum limits of 20 ng/g of total aflatoxins for foods in general (FAO, 2004). The concentrations of aflatoxin B_1 (20.4 and 51.4 ng/g) and total aflatoxins (24.0 and 53.6 ng/g) detected in two of the composite samples assessed in this study are in excess of the above regulatory limits. Furthermore, two of the individual samples showed similar aflatoxin levels above these maximum limits. It can therefore be presumed that two of twenty five samples (8%) of Maldive fish were contaminated with levels of aflatoxins that exceed the legal limits set by most of the countries across the world. No type G aflatoxins were detected from the samples supporting the previous finding on the absence of fungi such as *A. parasiticus* capable of producing this type of toxin (Pitt and Hocking, 2009).

Aflatoxin contamination of Maldive fish is not unexpected for many reasons. Firstly, *A. flavus,* which is the major producer of B aflatoxins, was one of the most dominant fungi isolated from Maldive fish with a frequency of 92% (23/25) (refer to Chapter 3). Moreover, 46% (19/41) of *A. flavus* originating from these samples were able to produce variable amounts of aflatoxins *in vitro*, while 15% (6/41) of these isolates were relatively good producers (refer to Chapter 5). As stated previously, the growth of toxigenic fungi on a food is not a reliable indicator of toxin production due to other

factors – in the case of *A. flavus*, one being that only around 50 % of isolates are toxigenic. Wheeler et al. (1986) in their study of Indonesian dried fish conducted aflatoxin analysis on several fish from which *A. flavus* was isolated and found none of the samples to be positive for the toxin. The low a_w of the samples (0.79 to 0.65) together with possibly high salt content (direct salting before drying) may have been responsible for hindering toxin production in Indonesian dried fish. It appears from the current results the hurdles applied were not adequate in many Maldive fish samples thus providing a favourable environment for aflatoxin production by toxigenic *A. flavus*.

A greater understanding is needed of the intrinsic and extrinsic factors that interact together to control aflatoxin production in Maldive fish in order to assist the development of control strategies. Aflatoxin production by *A. flavus* is influenced by many intrinsic and extrinsic factors. In general, the factors for mycotoxin production are more restrictive than those for growth of fungi (Klich, 2007). Water activity, which is an obvious overriding factor in dried foods, was quite high and hugely variable in the Maldive fish samples tested with values between 0.951 to 0.720 a_w. Aflatoxin production by A. *flavus* is greatest at relatively high a_w of 0.99 to 0.95 (Gqaleni et al., 1997; Klich, 2007) with a limiting value of around 0.82 a_w (Pitt and Hocking 2009). The a_w of most Maldive fish samples (76%) was above this critical value of 0.82, providing adequate free water for *A. flavus* to produce aflatoxin. Samples A1 and E1, which had elevated levels of aflatoxin had a_w values of 0.778 and 0.947, respectively. The high a_w of sample E1 would undoubtedly permit optimum aflatoxin production. However, sample A1 has a lower a_w value which is the average for this sample but as previously pointed out moisture content may be uneven, and aflatoxin is likely to have been produced during the slow drying process normal for these fish. Aflatoxin production in Maldive fish could therefore be controlled by a rapid drying process that is adequate to achieve an a_w below 0.82 in all parts of the product. Aflatoxin production by *A. flavus* is reported to be optimal between 16 and 31°C (Klich, 2007; Pitt and Hocking, 2009) and hence the ambient temperature conditions in Maldives (average day time temperature between 28°C to 30°C; MMS, 2012) are ideal for accumulation of

aflatoxins in the product. Furthermore, the low salt content (1.5 to 4.3%) and the slightly acidic pH (5.6 to 6.7) may even stimulate toxin production (Joffe and Lisker, 1969; Uraih and Chipley, 1976) .

Few studies on toxigenic fungi and mycotoxin production have been reported on smoked dried fish or similar products, and fewer still that agree with the findings of this study. According to Adebayo-Tayo et al. (2008), smoked dried fish including skip jack tuna from markets in Ibom State, Nigeria were contaminated with aflatoxins in the range 1.5 to 8.1 ng/g of aflatoxin B_1 and 1.8 to 4.5 ng/g of aflatoxin G_1. *A. flavus* was one of the dominant fungi isolated from these products. However *A. parasiticus* or one of the other aflatoxin G producing species was not present although aflatoxin G_1 was detected in some products. Perhaps the two species were not differentiated. It should be noted that the moisture content of these dried fish samples was high (22.7 - 27.6%) which would support fungal growth and mycotoxin production. The a_w values for the samples were not reported. In another study, TLC analyses of six mouldy fish extracts from smoked dried Bonga fish from Sierra Leone markets were positive for aflatoxins B and G (Jonsyn and Lahai, 1992), but they were not quantified. *A. flavus* was the most prevalent species isolated from this product too. In an earlier study surveying aflatoxin contamination of various food products from Semi-Savannah and forest areas in Nigeria, the highest levels were recorded from dried fish, with exceptionally high levels of 400-800 ng/g toxin (Nwokolo and Okonkwo, 1978).

None of the Maldive fish samples evaluated in this study showed any unacceptable level of contamination with OTA. Although two samples were positive, levels were below 5 ng/g, which is the most frequently applied regulatory limit for OTA in food stuffs (FAO, 2004). *A. niger* but no *A.ochraceus* was isolated from these two samples. The low levels of OTA in Maldive fish could be attributed to the limited incidence of toxigenic *A. niger* (3 out of 15) and the low isolation frequency of *A. ochraceus*. Furthermore, toxigenic *A. niger* isolates were capable of producing only small amounts of OTA (30 to 60 ng/g agar) in culture when compared to *A. ochraceus*. OTA contamination was reported in three of six smoked dried Bonga fish samples from

Sierra Leone (Jonsyn and Lahai, 1992), but quantitative assays were not used in their study.

The toxigenic potential of fungi is very much dependent on the type of food substrate. Maldive fish is high in proteins with an approximate protein content of 83% (Maldives Food and Drug Authority). Protein rich substrates such as dried meat are poor media for some mycotoxins. For example, the mycotoxin patulin is degraded in dried meat due to a reaction with sulfydryl groups in the food (Bailly et al., 2005). Similary, a strain of A. ochraceus that was able to produce 151 mg/kg of OTA on rice was only able to produce 0.4 to 6 mg/kg of the toxin on ham after 21 days of incubation (Escher et al., 1973). Production of secondary metabolites by Penicillium on ham was much lower when compared with levels produced on carbohydrate rich substrates (Núñez et al., 2007). The half life of OTA on dried cured ham is 120 hours (Bailly et al., 2005). Relatively high concentrations of citrinin (73 mg/kg after 16 days incubation) were produced on dry cured meat but citrinin was only partially stable on this substrate with a half life of about 6 hours (Bailly et al., 2005). These results indicate the low toxigenic potential of Aspergillus and Penicillium species on protein rich substrates or the rapid degradation of toxins on such substrates or both. They could also be an explanation for the low prevailing levels of OTA in Maldive fish in the current study. By contrast, the production and stability of aflatoxin in Maldive fish appears to be relatively good from the high concentrations of toxin obtained in some samples.

This study confirms possible exposure to unacceptable aflatoxin levels above common regulatory limits through regular consumption of Maldive fish infected with fungi. The presence of aflatoxins in the product could be ascribed to the frequency of toxigenic *A. flavus* found in the product together with the absence of any inhibitory factors, providing a favourable environment for fungal growth and toxin production. Hence, the growth of *A. flavus* on this product should not be taken lightly and hurdles should be created to ensure control of fungal growth and mycotoxin production. The most practical approach is to adequately dry the fish to achieve an a_w of 0.82 or below in all parts of the product. The drying process should be rapid to avoid extended periods at high a_w that would encourage toxin production. The limiting a_w for aflatoxin

production by *A. flavus* quoted above is a guideline value. Further research on the physiological behaviour and conditions of toxin production of this species on Maldive fish or a simulating environment is deemed necessary for accurate estimation of the limiting values. Any significant exposure of OTA via this product may not be expected due to the low prevailing levels found in the samples.

CHAPTER 7

Effect of Water Activity and Temperature on the Germination and Growth of *Aspergillus tamarii* Isolated from Maldive Fish

Abstract

Germination times and radial growth rates of cyclopiazonic acid producing strains of *Aspergillus tamarii* isolated from Maldive fish were studied over water activities (a_w) ranging from 0.99 to 0.79 at 25°C, 30°C, 35°C and 40°C on two laboratory media. The a_w of the media was controlled by either NaCl or a mixture of glucose and fructose. The optimum germination and growth were observed at temperatures between 30°C and 35°C. Germination was favoured at the highest a_w of 0.99 under all conditions. Growth however was dependent on the media and temperature with a lower optimum a_w of 0.95 for NaCl media and 0.95 to 0.92 a_w on media containing glucose/fructose. The minimum a_w for growth was often higher than for germination while both parameters were influenced by temperature and media type. Germination on NaCl media was prevented at a_w values below 0.82 at 25°C and 30°C, 0.85 at 35°C and 40°C. However, growth did not occur at a_w <0.85 at 25-35°C. At those temperatures on glucose/fructose media, growth was observed at the lowest a_w tested (0.79). On both media, the restrictive effect of lowered water activity was more pronounced at 40°C than at 25-35°C. Delays in germination increased and growth rates decreased with marginal a_w and temperature conditions. The fungi displayed better tolerance on glucose/fructose media than on NaCl media on which it was partly inhibited by the NaCl. The information obtained here could be used to develop strategies for the control of this xerophilic fungus on smoked dried fish and other tropical foods on which it predominates.

7.1 Introduction

Aspergillus tamarii belongs to the *Aspergillus* section *Flavi* and occurs widely in tropical and subtropical foods. It is closely related to *Aspergillus flavus* which produces toxic metabolites aflatoxins, potent carcinogens (IARC, 1993), as well as cyclopiazonic acid (CPA). *A. tamarii* only produces CPA and almost all strains display the ability to produce this toxin (Dorner, 1983; Vinokurova et al., 2007).

Cyclopiazonic acid (CPA) produces focal necrosis in most inner organs such as liver, spleen, pancreas, kidney and skeletal muscles (Frisvad et al., 2007). The toxin also acts through inhibition of the calcium pump disrupting the muscle contraction cycle (Burdock and Flamm, 2000; Chang et al., 2009a). Thus, it has been implicated in the infamous "Turkey X" disease together with aflatoxins (Cole, 1986; Smith et al., 1992). Mycotoxins CPA and/or fumuclavine A from *A. tamarii* may also have been responsible for kodua poisoning characterised by nausea, vomiting, depression, intoxication and unconsciousness after consumption of contaminated kodo millet seeds (Rao and Husain, 1985). Anthony et al. (2003) later demonstrated that consumption of kodo millet seed contaminated with CPA may lead to acute hepatotoxicity in both humans and animals. Low doses of the toxin were also reported to be immunosuppressive in test subjects (Hill et al., 1986; Nuehring et al., 1985).

Prolonged exposure to mycotoxins such as aflatoxins and CPA through the consumption of contaminated products poses a serious health risk (Milićević et al., 2010; Wild and Gong, 2010). Hence, measures must be taken to reduce unwanted fungal growth and subsequent mycotoxin production for the supply of safe and stable foods. Water activity and temperature are defining factors for the growth of fungi on dried foods and it is therefore important that these parameters be studied in order to design strategies for the control of potentially toxigenic fungi. There is abundant information on the physiology and growth limits of *A. flavus* but less has been published on *A. tamarii*. This study therefore attempts to determine the effect of water activity and temperature on the germination and growth of *A. tamarii* isolated from Maldive fish. *A. flavus* and *A. tamarii* were the dominant species on this product.

7.2 Methodology

7.2.1 Fungi

Three isolates of *A. tamarii* (J1B1, S2B2 and K1B1), with the ability to produce CPA were randomly selected from a group of isolates obtained from Maldive fish. The identity of the fungi was further confirmed by screening the isolates for aflatoxins and CPA. Recent work by Varga et al. (2011) designated 4 species within the *A. tamarii* clade of which *A. tamarii* can be differentiated on the basis of its ability to produce CPA but not aflatoxins. All isolates were positive for CPA and negative for aflatoxin production by the TLC agar plug method (described in section 4.2.3.2) and the HPLC agar plug method (described in section 4.2.3.3) after growth on CYA and YES. The isolates were grown on CYA agar at 25°C for 7 days to obtain heavily sporulating cultures to use as inocula in the following trials.

7.2.2 Media

Media with a range of a_w (0.99, 0.95, 0.92, 0.88, 0.85, 0.82 and 0.79) were prepared by adding NaCl or a mixture of equal amounts of glucose and fructose as controlling solutes to a basal medium containing 1% malt extract, 1% yeast extract, 0.1% dipotassium hydrogen phosphate (K_2HPO_4) and 2% agar (pH 7.5). The concentrations of the solute were calculated using reference data of Chirife and Resnik (1984) for NaCl and from the formula of Norrish (1966) for sugar (glucose and fructose). All chemicals used were of analytical grade. Media with a_w values above 0.92 were sterilized by autoclaving at 120°C for 15 minutes whereas the remaining media were steamed at 100°C for 15 minutes to avoid excessive browning that may result in the production of growth inhibiting compounds. Actual a_w values of the media were measured with an Aqualab CX-2 Water Activity Meter (Decagon Devices Inc., USA). The recorded deviation from the expected value was always below 0.005. Media (2ml) of all formulations were poured into Petrislides (Millipore, Bedford, MA, USA), immediately covered and allowed to set.

7.2.3 Inoculation and examination

Methods used for inoculation and examination were in accordance with Wheeler et al., (1988). Each Petrislide was inoculated at the centre with a single needlepoint of mature spores of a test strain and transferred to polyethylene food storage containers with appropriate saturated salt solutions to minimise any transfer of water from or to the media. The containers were incubated at 25°C, 30°C, 35°C and 40°C for a maximum of 100 days.

Petrislides were examined regularly to determine the germination times; thrice a day for high a_w media (>0.90) and less frequently as required for low a_w media. They were viewed under a transmitted light microscope at 100 X magnification. Germination was determined by the appearance of germ tubes of a length equal to the diameter of the spores as described by Pitt and Hocking (1977).

Colony diameters for all isolates were measured daily or less frequently for low a_w media using a light microscope with an eyepiece micrometer at the initial stages of growth and later with the help of microscope stage verniers. Measurements continued until colonies reached the edge of the Petrislide or the 100 day limit set for this experiment had passed. Radial growth rates, expressed as mm/day, were calculated from the data for the period of linear growth (initial exponential growth and late sub-optimal growth excluded).

The mean germination times (in log reciprocal) and growth rate for the three isolates were calculated and the line of best fit plotted against a_w. In addition, contour plots indicating the interactive effect of a_w and temperature on the radial growth rates were obtained using SigmaPlot 11 (Systat Software Inc.).

7.3 Results

7.3.1 Germination

Figure 7.1 shows the effect of a_w on the mean germination times (in log reciprocals) for *A. tamarii* on NaCl and glucose/fructose media. The small standard deviations indicate that any isolate to isolate variation was minimal. A general trend was observed for all treatments with the germination time increasing as the a_w of the media decreased. The fungi germinated most rapidly at the highest a_w tested (0.99) under all conditions.

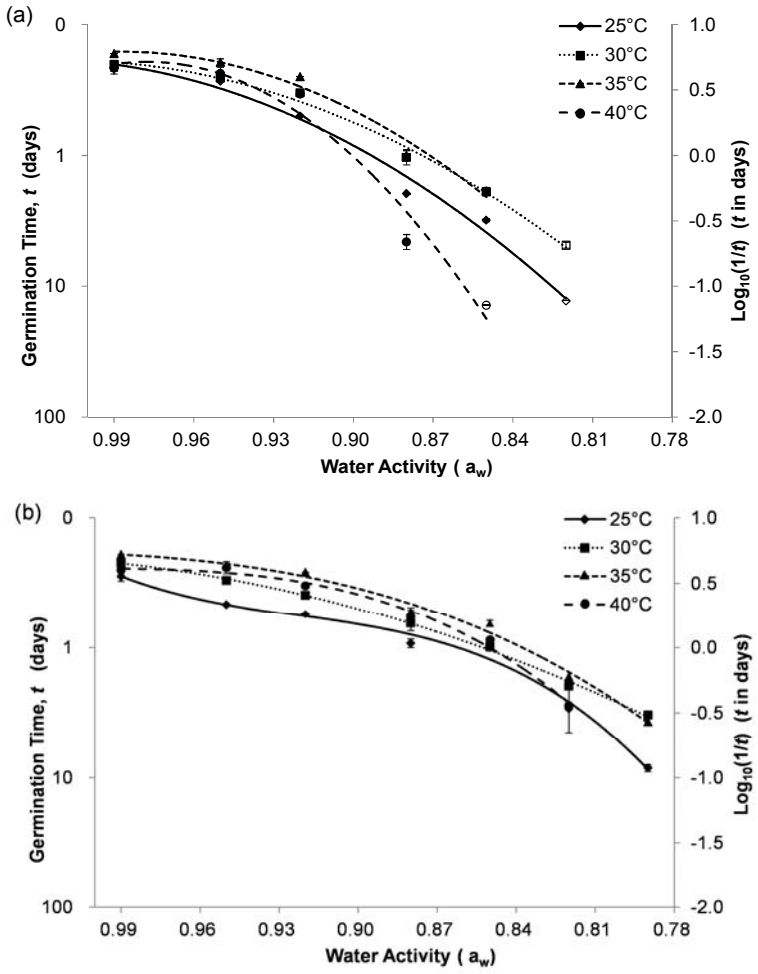


Figure 7.1 Effect of a_w and temperature on germination time (days) of *A. tamarii* on (a) NaCl and (b) glucose/ fructose media at 25°C, 30°C, 35°C and 40°C. The error bars represent the standard deviation of the three isolates. No fill indicates germination with no subsequent growth. Where no symbol is shown for a particular a_w, no germination had occurred by 100 days' incubation at that temperature.

The optimum temperature for germination was 35°C irrespective of the media type. Temperature had little effect on germination except at 40°C on NaCl media where the lag time was 14 days compared to around 2 days at 35°C in media with the minimum a_w. In addition, germination time was delayed by approximately 50% with a decrease in incubation temperature from 35°C to 25°C.

The solute type had minimal influence on spore germination at high a_w but showed a marked effect with the decrease in a_w at all temperatures. *A. tamarii* appeared to be inhibited by NaCl compared with glucose and fructose. This was most apparent at 40°C when germination at 0.85 a_w was observed after 14 days on NaCl media while it took less than a day on glucose/fructose media and at 0.82 a_W, germination did not occur on NaCl media and took approximately 3 days with glucose/fructose media. The combination of low a_w and high temperature together with the inhibitory effect of the solute on NaCl media was particularly restrictive on the germination of the spores.

The effect of incubation temperatures and solute type on the minimum a_w values for germination was quite evident (Table 7.1). On NaCl media, spore germination was prevented below a_w 0.82 at 25°C and 30°C whereas it was restricted to 0.85 a_w at higher incubation temperatures of 35°C and 40°C. On glucose/fructose media, germination was observed at the lowest a_w tested (0.79) for all temperatures except for 40°C when it was limited at an a_w of 0.82. Germination at 25°C on both media took

Table 7.1 Minimum a_w for germination of *A. tamarii* at various incubation temperatures on NaCl media and glucose/fructose media

Media type	Germination	25°C	30°C	35°C	40°C
NaCl media	Minimum a_w	0.82	0.82	0.85	0.85
	Time (days)	13.0	4.9	1.9	14.0
Glucose/Fructose Media	Minimum a_w	<0.79	<0.79	<0.79	0.82
	Time (days)	8.4	3.3	3.7	2.8

longer than at the optimum temperature of 35°C, although it should be noted that 25°C on NaCl media supported germination at a lower a_w than 35°C.

Under some of the more limiting conditions on NaCl media, growth after germination was not observed: spores were able to germinate but failed to develop any further at the minimum germination a_w at 25°C, 30°C and 40°C. Spores that failed to germinate under marginal conditions of 0.82 a_w at 35°C and 40°C on NaCl media were transferred to general media (Malt Extract Agar) and incubated at 25°C to confirm their viability. Growth was observed for spores previously incubated at 35°C but not at 40°C indicating that high temperatures combined with other limiting factors are destructive to *A. tamarii* spores.

7.3.2 Growth

Figure 7.2 depicts the effect of a_w and temperature on the mean radial growth rate (mm/day) of the three *A. tamarii* isolates on both NaCl and sugar media. Optimum and minimum a_w for growth under varying experimental conditions are summarised in Table 7.2. Contour plots (Figure 7.3) shows the interaction between a_w and temperature on radial growth of *A. tamarii* on NaCl and glucose/fructose media. A general observation for all treatments was an increase in the growth rate until an optimum level was reached followed by a gradual decline in growth as the a_w decreased. The optimum a_w for growth was lower than for germination and was also dependent on temperature and solute type. Maximum growth was observed at 0.95 a_w on NaCl across all temperatures and on sugar media at 25°C and 30°C (Table 7.2). At the higher incubation temperatures of 35°C and 40°C on sugar media, the fungi displayed optimum growth at a lower a_w of 0.92. In general, temperatures between 30°C and 35°C were favoured on both media. At the optimum a_w values, maximum growth rates of 10.6 mm/day and 11.2 mm/day on NaCl media and 17 mm/day and 14.8 mm/day on glucose/fructose media were recorded at 30°C and 35°C, respectively. Of the temperatures studied, 40°C was the least preferred by the fungi with growth almost twice as slow at this temperature then at 35°C when the a_w was optimum.

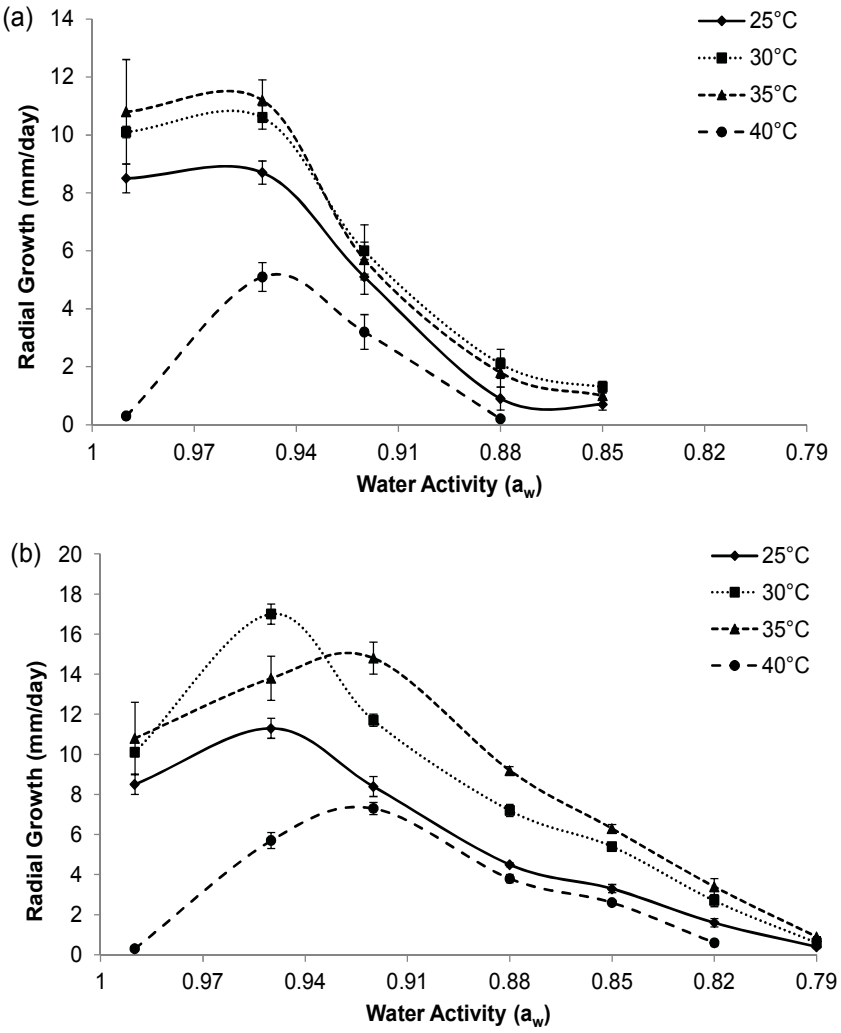


Figure 7.2 Effect of a_w and temperature on radial growth rate (mm/day) of *A. tamarii* on a) NaCl and b) glucose/ fructose media. The error bars represent the standard deviation of the three isolates. Where no symbol is shown for a particular a_W, no growth had occurred by 100 days' incubation at that temperature

The effect of the solute on the growth of *A. tamarii* was marked. Growth rates were faster and the minimum a_w levels lower on media containing glucose/fructose as the controlling solute. The maximum growth rates on glucose/fructose media and NaCl media were 17 mm/day and 11.2 mm/day, respectively. The fungi displayed better tolerance at low a_w on glucose/fructose media with growth observed over a wider range. Hence the minimum a_w on this media was below 0.79 at all temperatures except 40°C where growth was prevented at lower a_W levels, 0.88 on NaCl media and a_w 0.82 on glucose/fructose media. Similar to germination, the fungi did not grow well under restricted conditions of low a_w and high temperatures, especially on NaCl media which adds to the inhibitory effect. Growth at the minimum a_w conditions were slow at 0.2-0.6 mm/day on both media.

Table 7.2 Optimum and minimum a_w for growth of *A. tamarii* at various incubation temperatures on NaCl media and glucose/fructose media

Media type	**Radial growth**	**25°C**	**30°C**	**35°C**	**40°C**
NaCl media	Minimum a_w	0.85	0.85	0.85	0.88
	Optimum a_w	0.95	0.95	0.95	0.95
Glucose/Fructose Media	Minimum a_w	<0.79	<0.79	<0.79	0.82
	Optimum a_w	0.95	0.95	0.92	0.92

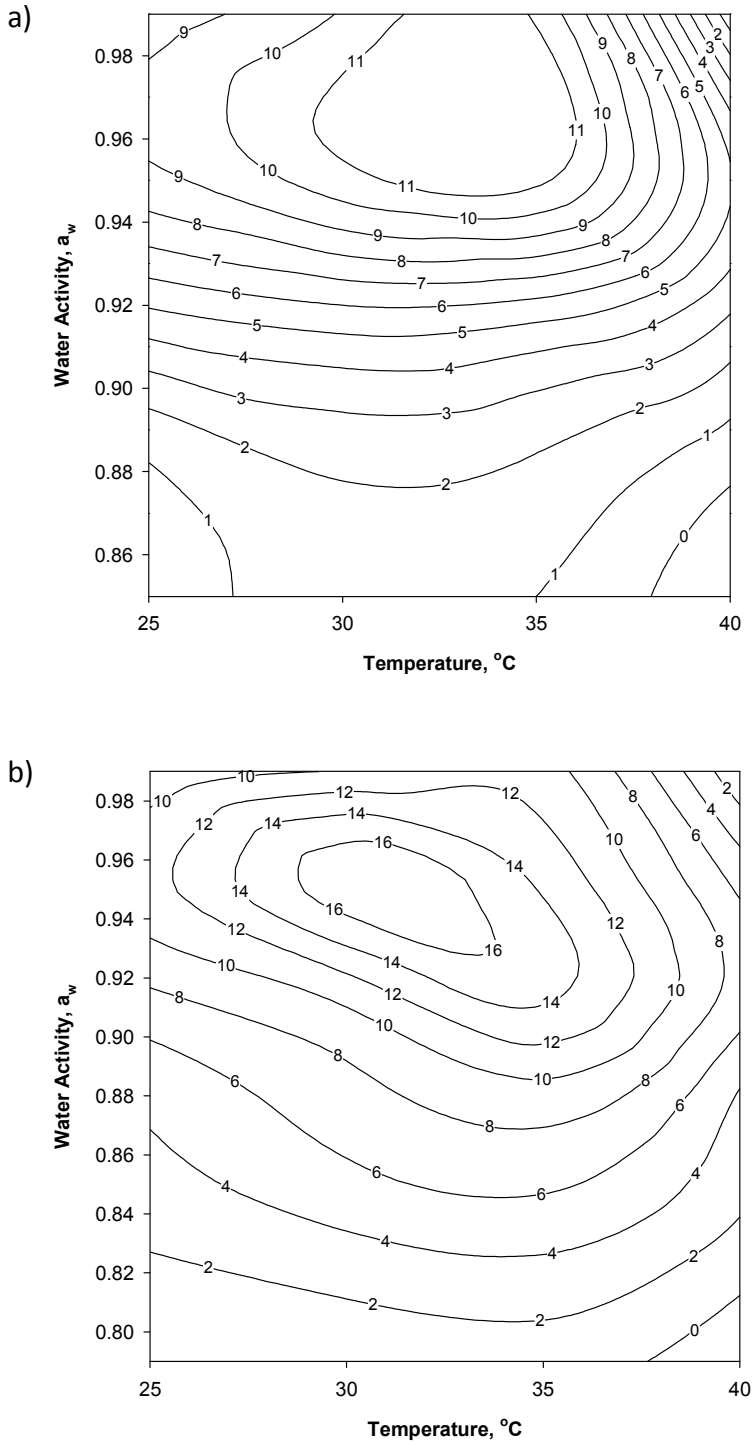


Figure 7.3 Interaction between a_w and temperature on the radial growth rate (mm/day) of *A. tamarii* on a) NaCl and b) glucose/fructose media

7.4 Discussion

The data presented in this study can provide an estimation of the optimum and limiting conditions for the growth of *A. tamarii* on food, although the behavior of the fungi may vary depending on the food matrix and other interacting factors. Water activity and temperature had a marked effect on the germination and growth of this fungus on both NaCl and glucose/fructose media. The optimum conditions for growth on both media were in agreement: 0.95 to 0.92 a_w at 30-35°C. The minimum a_w for growth is considered the limiting value as this is the point at which the fungi can proliferate in foods under the most severe conditions. On NaCl media, *A. tamarii* was able to grow at 0.85 a_w but did not grow at the next level (0.82 a_w). When the controlling solute was glucose/fructose, *A. tamarii* was able to grow at the lowest a_w tested while the literature reports the inability of the fungi to germinate and grow at 0.75 a_w (Ayerst, 1969). Hence, the minimum a_w for *A. tamarii* at 25-35°C is between 0.82 and 0.85 on NaCl media and between 0.79 and 0.75 on media containing sugars. *A. tamarii* spores did not survive under these limiting a_w conditions on both NaCl and sugar media when incubated at 40°C, demonstrating the lethal effect of two or more restrictive hurdles.

The solute type had a noticeable effect on growth and germination of fungi at lower a_w values. The minimum a_w for both germination and growth on media containing NaCl was high compared to the glucose/fructose media implicating the inhibitory role of NaCl under marginal conditions. There is no published information on the effects of a_w on the growth of *A. tamarii* on media controlled by NaCl and hence the influence of solute type has not been previously evaluated. However, this is in agreement with the effect of solutes reported for other species belonging to the *Aspergillus* isolated from dried fish or other food sources (Pitt and Hocking, 1977; Wheeler et al., 1988). Moderate solute influences were indicated for these species where they displayed improved growth on media controlled by solutes such as sugars and glycerol but was suppressed to a certain degree on NaCl media.

Studies on the physiology of *A. tamarii* are limited and have not been published as widely as some other fungi in the *Aspergillus* section *Flavi*. Ayerst (1969) reported on the effects of a_w and temperature on germination and growth of a range of fungi including *A. tamarii* and *A. flavus*. The fungi were grown on agar strips of an ordinary agar medium (2% malt extract, 2% agar) at various a_w levels under temperatures varying from 10 to 50°C. The a_w of the agar strips were adjusted by exposing them to controlling solutions (potassium hydroxide) of known water activities for 3 to 4 days to allow moisture equilibrium to occur. The optimum conditions for germination and growth of *A. tamarii* reported were 33°C and a_w of >0.98. Data from our study are comparable to this although interestingly improved growth was observed at a lower a_w of 0.95-0.92. The limiting a_w value of 0.78 observed by Ayerst (1969) was also in agreement with our data on the glucose/fructose medium where germination and growth were witnessed on the lowest a_w of 0.79 tested. However, *A. tamarii* displayed shorter germination times and faster growth on glucose/fructose as well as NaCl media compared to the figures obtained in the earlier study. A possible explanation for this deviation is the variability in the preparations and method of measurement. In our study the plates were viewed directly under the microscope which enabled us to note changes at a very early stage. Our results demonstrate the ability of the fungi to grow between 25°C and 40°C and this agrees with the limiting temperature range of 12°C and 43°C, reported previously.

A. tamarii often co-occurs with *A. flavus* in products and this was the case in "Maldive fish" where the two fungi were the dominant species isolated from the surface of the product (see Chapter 3). The physiological behavior of both fungi appears to be similar (Pitt and Hocking, 2009). Ayerst (1969) reported the same optimum and limiting conditions for both these fungi. The optimum growth of *A. flavus* occurs at the highest a_w levels of 0.99 to 0.98 (Gibson et al., 1994; Pitt and Miscamble, 1995; Wheeler et al., 1988) compared with slightly lower levels of 0.95-0.92 observed in our study for *A. tamarii*. The reported optimum temperature for *A. flavus* was variable between 33°C and 37°C whereas *A. tamarii* in our study favoured temperatures around 35°C which falls within this range.

The minimum a_w values for the growth of *A. flavus* have also been reported by many authors although the values are somewhat inconsistent. Pitt and Miscamble (1995) studied the temperature and a_w relationships for *A. flavus* and found the minimum a_w values to be dependent on temperature as follows: 0.82 at 25°C, 0.81 at 30°C and 0.80 at 37°C on glucose/fructose media. Some earlier studies have reported the inability of *A. flavus* to grow below 0.85 and 0.87 on the same media (Pitt and Hocking, 1977; Wheeler et al., 1988). We were able to witness growth at a lower a_w (0.79) for *A. tamarii* on the same media under similar temperatures. This is comparable to the value (0.78) obtained by Ayerst (1969) for both *A. flavus* and *A. tamarii* . The minimum a_w values for germination of *A. tamarii* on NaCl media observed in the current work were lower than those of *A. flavus* from previous studies. *A. tamari* was able to germinate at a_w 0.82 at temperatures between 25°C and 35°C, whereas the limiting a_w for germination of *A. flavus* on this media was 0.85 to 0.86 when incubated between 25°C and 37°C (Wheeler et al., 1988). Nevertheless, the minimum a_w values for growth of these fungi on NaCl media are in better agreement. Overall it appears that *A. tamarii* was slightly more tolerant of the low a_w than *A. flavus* especially when the a_w of the environment is controlled by sugars.

Fungi mostly associated with low a_w dried foods are xerophiles. According to Pitt (1975), a xerophilic fungus is capable of growth, under at least one set of environmental conditions, at an a_w below 0.85 while Pelhate (1968) defines xerophilic fungi as those showing maximum growth rates below 0.95. *A. tamarii* fulfills both these criteria according to the experimental results of our study and hence can be defined as xerophilic. The optimum a_w for growth and limiting a_w values for *A. tamarii* were lower than for *A. flavus* suggesting that it may be more xerophilic than *A. flavus*, and thus competitive in environments where they are both isolated. The ability to grow at low a_w and high temperatures explains the widespread occurrence of this fungus in stored dried commodities from tropical and subtropical regions. *A. tamarii* has been frequently associated with seeds and nuts, cocoa, corn, spices and other tropical raw materials and dried foods (Pitt and Hocking, 2009; Samson et al., 2010). It is also a common contaminant of food commodities originating from Southeast Asia

(Pitt and Hocking, 1997). The species has been isolated from salted dried fish and smoked fish products. *A. tamarii* comprised 14% of all fungi isolated from smoked dried fish originating from homes and markets in Sierra Leone (Jonsyn and Lahai, 1992). Samples of salted fish (6.8%) from Indonesia were also contaminated with this fungus (Wheeler et al., 1986).

This study has implications in providing a better understanding of the behavior of *A. tamarii* in foods under varying environmental conditions and the data accumulated could be used to develop predictive models to describe growth under any combination of these conditions. The optimum and limiting levels are characterized and could act as a guide to optimize processing and storage conditions for preventing growth of this fungus as well as any subsequent mycotoxin production. The data from this study therefore could be used to develop strategies such as the use of reduced a_W and salt for the control of *A. tamarii* on smoked dried fish and other tropical foods on which it predominates. However, further studies on specific food matrices are essential to yield more accurate data applicable to that particular food.

CHAPTER 8

Effect of Water Activity and Temperature on Growth and Mycotoxin Production by *Aspergillus flavus* on a Smoked Fish Agar

Abstract

Growth rate and mycotoxin production of three toxigenic strains of *Aspergillus flavus* were studied over a range of water activities (0.95, 0.90, 0.85, 0.80, 0.75) at 25°C, 30°C, 35°C and 40°C on a smoked fish agar (SFA) that simulates Maldive fish. Aflatoxin (B_1) and CPA analyses were performed after the 7th, 14th, 21st and 60th days of incubation. Water activity and temperature had a marked effect on the growth and toxin production. Optimal growth occurred at the highest a_w (0.95) at 35°C whereas toxin production was optimum at the same a_w but at lower temperatures of 25°C and 30°C. Growth did not occur at an a_w of 0.80 at 25, 35 and 40°C. At 30°C, growth was observed down to 0.80 a_w after incubation for 21 days but no growth occurred at the next level tested (0.75 a_w.) Hence, a value between 0.80 and 0.75 can be considered as the limiting a_w for growth of *A. fla*vus on SFA. Production of aflatoxin and CPA by *A. flavus* was limited at an a_w of 0.80 on SFA under all conditions. It is recommended to control the drying process in the production of Maldive fish to rapidly achieve an a_w of 0.75 or below and maintain at this level to limit the growth of toxigenic *A. flavus* and prevent mycotoxin production.

8.1 Introduction

Water activity and temperature are the most critical determining factors for fungal growth and mycotoxin production (Holmquist et al., 1983; Plaza et al., 2003). Mycotoxin production occurs under more restrictive conditions than growth of the fungi. Information on the conditions that limit the growth and toxin production of toxigenic fungi are important for the control of these fungi in foods.

The physiology of *A. flavus* including the interaction between $a_{w,}$ temperature and other environmental parameters on growth and toxin production has been well documented (Gibson et al., 1994). *A. flavus* produces both aflatoxins and cyclopiazonic acid (CPA) and their additive effect can increase the toxigenic potential of this fungus. Studies on the effects of environmental parameters on mycotoxin production by *A. flavus* often focus on aflatoxin only while very few studies address both aflatoxins and CPA (Gqaleni et al., 1996a; Gqaleni et al., 1997; Vaamonde et al., 2006). Furthermore, production of secondary metabolites by toxigenic fungi is very much dependent on the food matrix (Astoreca et al., 2012; Cuero et al., 1987; Park and Bullerman, 1983). Hence, studies on specific food matrices are essential to yield more accurate data.

This study evaluated the effects of a_w and temperature on the growth and production of both aflatoxin and CPA by toxigenic *A. flavus* isolates on a smoked fish agar. This agar simulates Maldive fish and enabled the determination of the limiting conditions for growth and mycotoxin production.

8.2 Methodology

8.2.1 Fungi

Three strains of *A. flavus* (P1A2, O1A2 and A1E1), isolated from Maldive fish, were used for this study. These strains originated from different samples and were selected out of 41 isolates (Chapter 5), based on their ability to produce CPA (CYA medium) and high concentrations of aflatoxin in culture (YES medium). The strains were single point inoculated on CYA (Pitt and Hocking, 2009) and incubated at 25°C for 7 days to obtain heavily sporulating cultures to use as inocula in the following trials.

8.2.2 Preparation of smoked dried fish (Maldive fish)

Maldive fish was prepared as the base for smoked fish agar (SFA). Yellow fin tuna fillets (6 kg) purchased from a local fish shop was transferred to the pilot plant (Massey University) on ice and stored at -20°C until further use. Figure 8.1 illustrates step by step method applied in the processing of Maldive fish. The method is based on the information compiled from different sources: 5 methods sourced from different areas of the country; reference by MOFA (1995); and personal experience. The fish was thawed (overnight at 4°C) and cut into loins for cooking. They were then transferred to a pot of boiling water (1:1, fish: water) and cooked for approximately 45 minutes. Scum formed during this process was continuously removed by scooping from the surface. The cooked loins were removed from the soup, drained and transferred to a smoking chamber (Whitlock Speedy Smoke 'N' Cooker). They were smoked for approximately eight hours at a temperature of 80°C (fan speed set at 4) using wood chips followed by drying (in the same chamber) at a temperature of 40°C for over 12 hours until the final product had a wood-like texture. The average water activity of three random samples of this product, measured using an Aqualab CX-2 Water Activity Meter (Decagon Devices Inc., USA), was 0.94.

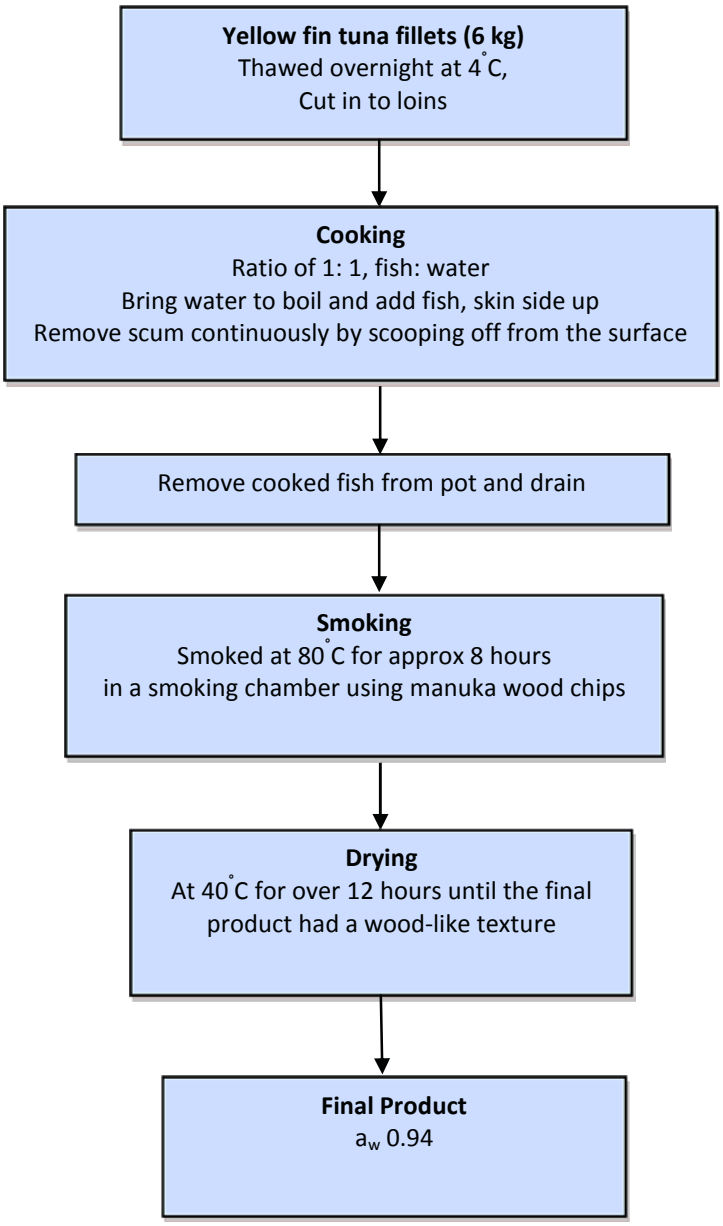


Figure 8.1 Process flow for the production of Maldive fish for use in the smoked fish agar formulation

8.2.3 Smoked fish agar

A smoked fish agar (SFA) was prepared to simulate Maldives Fish. The basal medium was comprised of the formulatio below. Smoked dried fish (Figure 8.1) were ground into a fine powder and stored at -20°C until further use.

- 50g smoked dried fish, finely ground
- 6 g of agar powder
- 300 ml of distilled water
- 6 g salt

A salt content of approximately 2% in this formulation was used to conform to the average salt content of the twenty five Maldive fish samples evaluated (Chapter 3).

SFA with a range of a_w (0.95, 0.90, 0.85, 0.80, 0.75) was prepared using glycerol to achieve the desired water activities. The a_w range used in this study reflects the range of a_w of the Maldive fish samples tested previously (Chapter three) which falls between 0.951 to 0.720 a_w. Actual a_w values of the media were measured with an Aqualab CX-2 Water Activity Meter (Decagon Devices Inc., Pullman, W.A., USA). The a_w was measured repeatedly after adding glycerol until the target a_w was achieved. The media were then sterilised at 120°C for 15 minutes. A small amount of medium was transferred to a measuring cup and allowed to set. Water activity of the set media was verified and adjusted accordingly with sterile glycerol if required; the error limit from the expected value was always maintained at ±0.005. Media of all a_w levels (approx. 20 ml) were poured into 90 mm Petri dishes and allowed to set before inoculation.

8.2.4 Inoculation and Incubation

Spores of the three isolates harvested from seven day old cultures on CYA were suspended in sterile distilled water containing 0.005% of a wetting agent (Tween 20) used to prevent spore clumping. A range of spore suspensions that matched the a_w of the media (0.95, 0.90, 0.85, 0.80, 0.75 a_w), were prepared by adding appropriate quantities of sterile glycerol. The final concentration of spores in each treatment was

determined with a Neubauer Chamber and adjusted if required to achieve 10^7 spores/ml.

Each plate was inoculated at the centre with the corresponding spore suspension using a graduated loop. The plates were then transferred to polyethylene food storage containers with salt solutions of matching a_w levels, to minimise any transfer of water from or to the media. The containers were incubated at 25°C, 30°C, 35°C and 40°C for a maximum of 60 days.

8.2.5 Growth rate

A full factorial design was used for this experiment with parameters: isolates (n=3), a_w levels (n=5) and temperature (n=4), as summarised in Figure 8.2. Three replicate plates were assayed for each treatment. Colony diameters were measured regularly: daily for high a_w media and less frequently as required for low a_w media. For each colony, two diameters at right angles were measured, initially under a stereomicroscope and later by eye with use of a ruler. Measurements continued until the colony reached the edge of the plate or the 60 day limit set for the experiment had passed. Linear regressions of radial growth against time (days) were used to obtain the growth rates, expressed as mm/day, for each treatment. The mean growth rate for each isolate was plotted against a_w.

8.2.6 Mycotoxin production

A full factorial design was used for this experiment with parameters: isolates (n=3), a_w levels (n=5) and temperature (n=4), as summarised in Figure 8.2. Three replicate plates were assayed for each treatment. Aflatoxin (B_1) and CPA analyses were performed after the 7^{th}, 14^{th}, 21^{st} and 60^{th} days of incubation. *A. flavus* can produce both aflatoxins B_1 and B_2, but the former is more prevalent and more potent, so aflatoxin B_1 was used as an indicator of aflatoxin production. Mycotoxin production was assessed by the HPLC agar plug method (Bragulat et al., 2001; Riba et al., 2010; Soares et al., 2010). Three agar plugs were removed from the centre, inner and the outer area of the colony transferred to a vial and weighed. Methanol (2 ml) was added to the vial and plugs extracted. After centrifugation, the filtrate was split into two vials in equal

portions and analysed for aflatoxins and CPA (section 5.2.4). The HPLC system and the conditions were the same as in section 5.2.4 for aflatoxins and CPA analysis. Quantification was achieved by comparison against previously developed standard curves (see section 5.2.4).

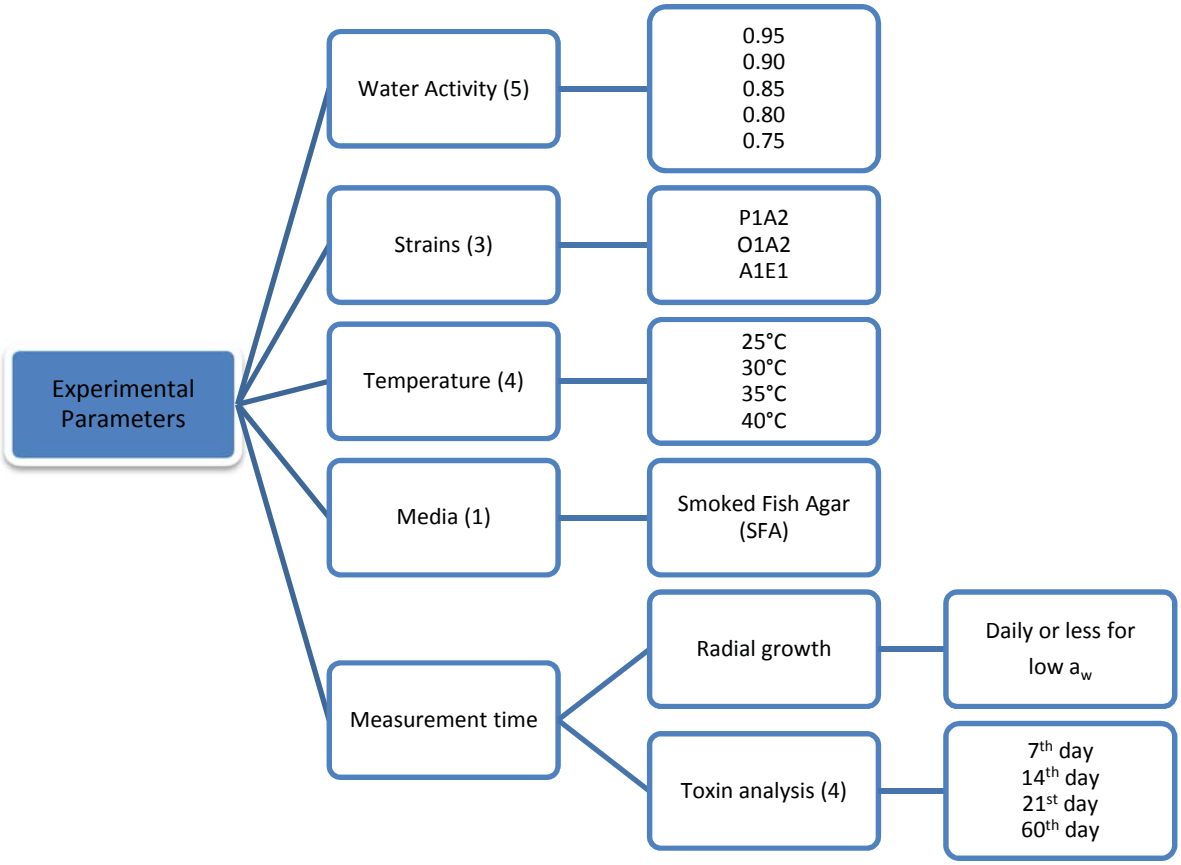


Figure 8.2 Experimental design to determine the factors affecting growth and toxin production of *Aspergillus flavus* isolates P1A2, O1A2 and A1E1 on smoked fish agar

8.2.7 Statistical analysis

The effect of different factors and their interactions on growth rate and toxin production was statistically analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM) using Minitab, version 15.

8.3 Results

8.3.1 Growth rate

Figure 8.3 illustrates the effect of a_w and temperature on the mean radial growth rate of three *A. flavus* strains O1A1, P1A2 and A1E1 on SFA. The growth data are provided in Appendix H. A general observation for all strains was that there was a gradual decline in growth as the a_w decreased from 0.95 until 0.80 when they failed to grow. The optimum a_w for growth was at the highest a_w tested (0.95), independent of temperature or strain. Growth of all the strains almost halved when the a_w of the media was reduced from 0.95 to 0.90 at 25-30°C. At these temperatures, a further reduction in the a_w of the media to 0.85 resulted in approximately a tenfold decrease in the growth rate of all test strains. At 40°C, this downward trend in the growth was more pronounced as the a_w decreased. In general, 35°C was the most favoured temperature for growth by all strains followed closely by 30°C. Maximum growth rates of 15-16 mm/day were recorded at 35°C when the a_w was optimum. Of the temperatures studied, 40°C produced the slowest growth, little more than half the rate at 35°C, the optimum a_w. Growth was observed for all strains at 0.85, irrespective of the incubation temperature, but was prevented at the next a_w level of 0.80 for all treatments, except at 30°C after 20 days of incubation. A small colony, with a diameter of 5 mm, was observed at these conditions for all strains which, however, failed to develop any further. Furthermore, no apparent growth was observed at an a_w 0.80 at the end of the 60 day period when incubated at 25, 35 or 40°C. At the end of the observation period, all strains failed to produce any colonies at the next a_w level (0.75) studied. Both a_w and temperature and their interactions were found to have a highly significant effect on the growth of *A. flavus* ($p<0.01$). Strain did not have a significant impact on growth rate.

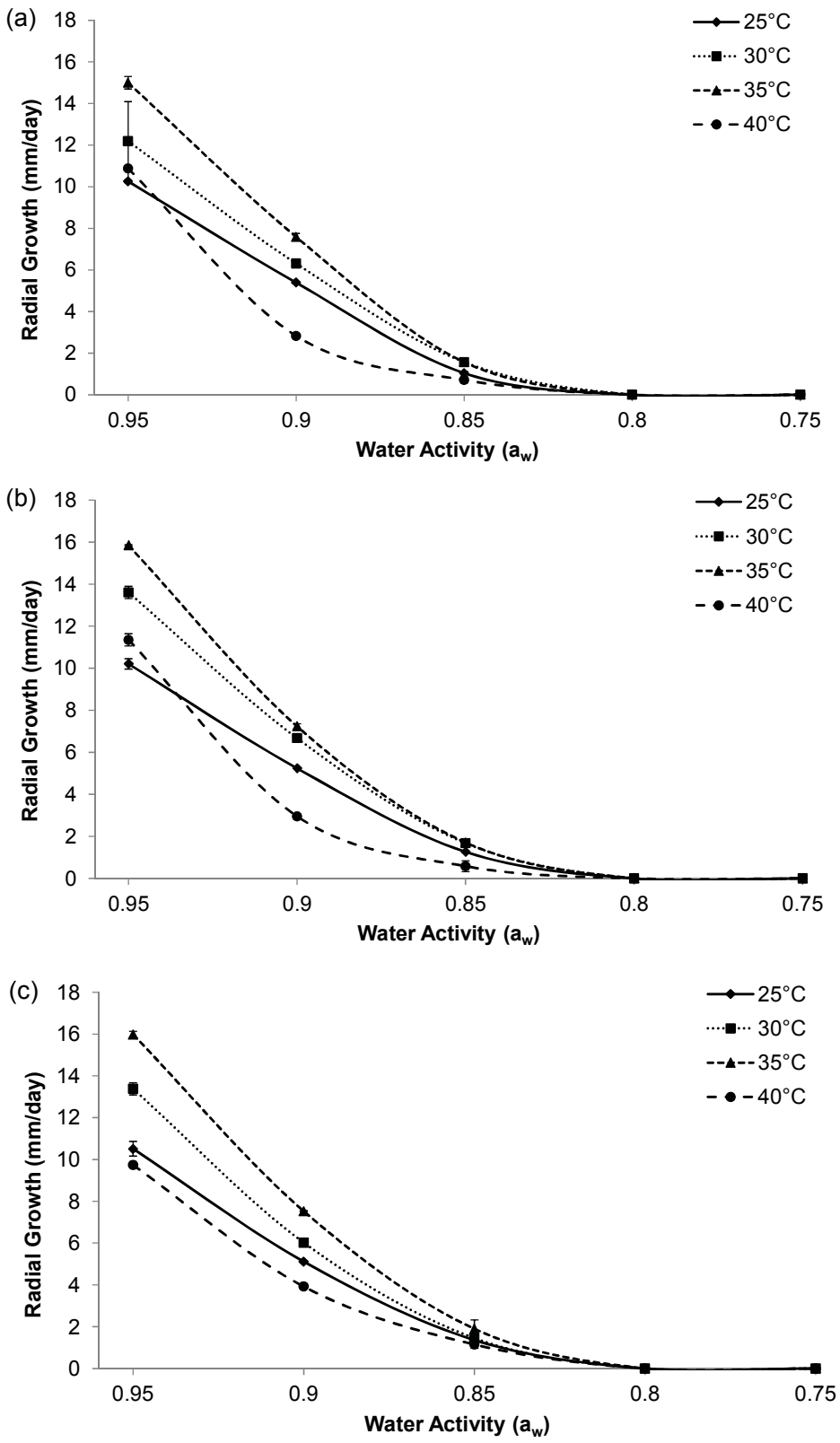


Figure 8.3 Effect of a_w and temperature on radial growth rate (mm/day) of three *A. flavus* strains a) P1A2, b) O1A2 and c) A1E1. The error bars represent the standard deviation of three replicate measurements.

8.3.2 Aflatoxin production

The influence of a_w and temperature on aflatoxin B_1 production by *A. flavus* strains P1A2, O1A2 and A1E1 over a 60 day period is presented in Figure 8.4. Some general trends were observed. The a_w of the medium had a significant effect ($p<0.01$) on aflatoxin production by *A. flavus* on SFA (Table 8.1). Synthesis of aflatoxin on SFA was greatest at the highest a_w of 0.95 for all strains regardless of the incubation temperature and the time of sampling. Aflatoxin production showed a reduction with the decrease in a_w for all strains. Aflatoxin production declined quite evidently with a decrease in a_w from 0.95 to 0.90 while at 0.85 a_w only low concentrations of the toxin were formed, generally below 10 ng/g medium. Although some aflatoxin was detected at 0.85 a_w, none was detected at the next a_w level tested (0.80). Hence, the limit for aflatoxin production on SFA by strains of *A. flavus* lies between 0.85 and 0.80 a_w.

Temperature alone, and in combination with a_w, significantly ($p<0.01$) influenced aflatoxin production by *A. flavus* isolates. Toxin production was optimal between 25°C and 30°C for all strains. The highest concentrations of the toxin were detected at 30°C which reached maximum levels after just 7 days of incubation for P1A2 (31,800 ng/g) and O1A2 (37,200 ng/g). Small quantities of toxin were detected at the elevated temperature of 40°C at high a_w (0.95). However, toxin production was generally inhibited at 40°C when the a_w of the media was reduced.

Sampling time did not have a significant impact on aflatoxin production by any of the isolates. Toxin production was detected as early as seven days of incubation and little change to these values was observed at the 14th , 21st and 60th day of sampling. After 60 days, high levels of toxin were detected under favourable conditions. For example, aflatoxin concentrations for strain P1A2 on 0.95 a_w media after 60 days were 16,300 ng/g and 19,600 ng/g SFA at 25°C and 30°C, respectively.

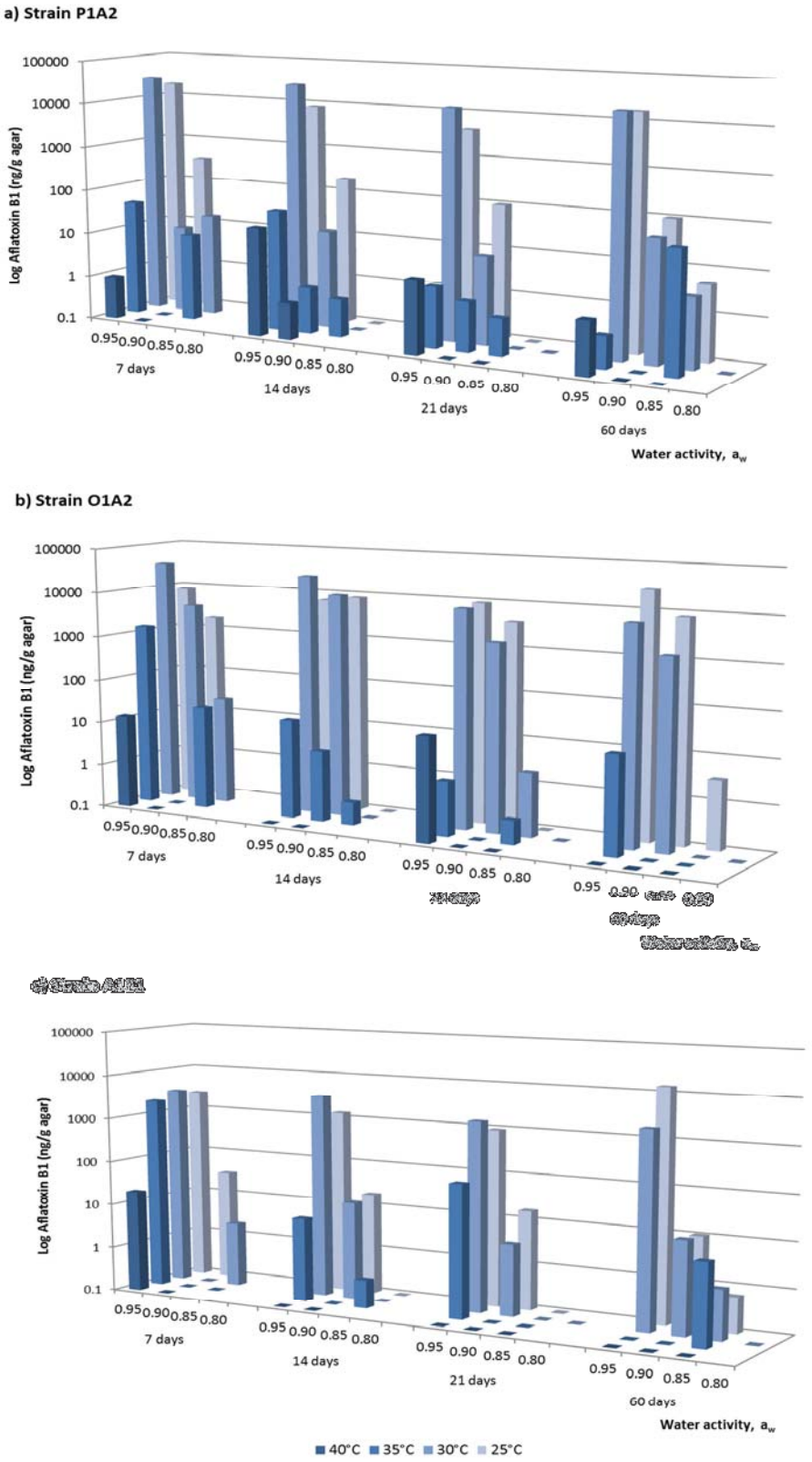


Figure 8.4 Effect of water activity and temperature on production of aflatoxin B_1 by three *Aspergillus flavus* strains (P1A1, O1A1 and A1E1) on a smoked fish agar after sampling at 7, 14, 21 and 60 days of incubation.

The three strains used in this experiment differed significantly ($p<0.01$) in their ability to produce aflatoxins. Strain A1E1 produced much lower levels of aflatoxins compared with the other two strains under similar conditions. For example, the highest concentration of aflatoxin produced by A1E1 under any of the conditions was 13,800 ng/g while strains P1A2 and O1A1 were able to produce up to 31,847 ng/g and 37,276 ng/g of aflatoxin, respectively.

Table 8.1 Analysis of variance of the effects of water activity (a_w), temperature (t), strain (s) and day of sampling (d) and their interactions on aflatoxin production on smoked fish agar

Factor	df	Mean square	F	P value
a_w	3	1268390847	82.61	<0.01
t	3	632151950	41.17	<0.01
d	3	51938645	3.38	0.018 NS
s	2	252449885	16.44	<0.01
a_w x t	9	476156500	31.01	<0.01
a_w x d	9	47470266	3.09	<0.01
a_w x s	6	187299682	12.20	<0.01
t x d	9	64327441	4.19	<0.01
t x s	6	109270466	7.12	<0.01
d x s	6	13501416	0.88	0.51 NS
a_w x t x d	27	48156232	3.14	<0.01
a_w x t x s	18	90619797	5.90	<0.01
a_w x d x s	18	12898501	0.84	0.65 NS
t x d x s	18	19851434	1.29	0.19 NS
a_w x t x d x s	54	13371361	0.87	0.73 NS

df, degrees of freedom
F, variance ratio
NS, non-significant ($p>0.01$)

8.3.3 Production of Cyclopiazonic acid

The influence of a_w and temperature on the production of CPA by *A. flavus* strains P1A2, O1A2 and A1E1 over a 60 day period is presented in Figure 8.5. The a_w of the media had a highly significant effect ($p<0.01$) on production of CPA by *A. flavus* on SFA (Table 8.2). Maximum CPA production was observed at the highest a_w of 0.95 for all strains independent of the temperature and time of sampling. A reduction in toxin production was apparent with the decrease in a_w from 0.95 to 0.90. A further decrease in a_w to 0.85 inhibited toxin production except after 60 days of sampling when low levels of CPA were detected at 25°C and 35°C for all strains. No toxin production occurred at the next a_w level tested. Hence, the limit for production of CPA by *A. flavus* on SFA is similar to that for aflatoxin production that is between 0.85 and 0.80 a_w.

Temperature, alone and in combination with a_w significantly influenced ($p<0.01$) production of CPA by *A. flavus* isolates. Toxin production was optimal between 25°C and 30°C for all strains. CPA was detected over the temperature range tested (25-40°C) when the a_w was high (0.95). However, toxin product was low at 40°C when the a_w of the medium was reduced.

The time of sampling also had a significant effect ($p<0.01$) on the production of CPA by *A. flavus* strains on SFA. Toxin was detected as early as seven days of incubation and in general increased for all strains with extended storage. For example, for strain A1E1, a gradual increase in concentrations of CPA from 70 ng/g to 284 ng/g followed 7 to 60 days of incubation at 25°C.

The *A. flavus* strains used in this study differed significantly ($p<0.01$) in their ability to produce CPA. Production of CPA occured in the following order A1E1> O1A2> P1A2. CPA production was not proportional to aflatoxin production and strain A1E1, which produced the lowest levels of aflatoxin, produced more CPA than the other strains. Higher concentrations of aflatoxin were observed for all isolates compared to the concentrations of CPA. For example, for strain P1A2 on 0.95 a_w media after 7 days of incubation, aflatoxin levels were 7,960 ng/g at 25°C and 37, 200 ng/g at 30°C. Under these conditions, CPA production was lower at 72 ng/g and 86 ng/g, respectively.

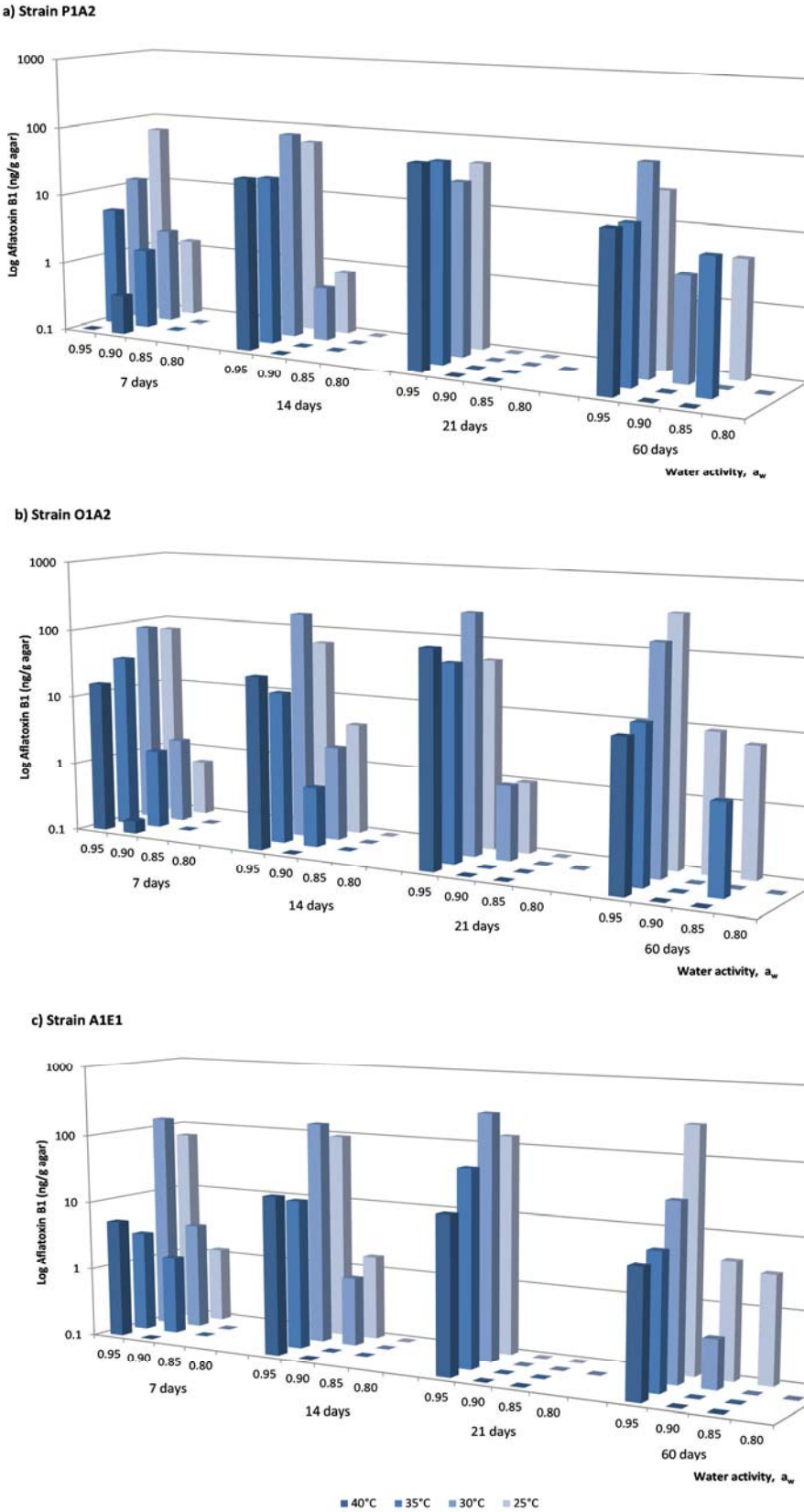


Figure 8.5 Effect of water activity and temperature on production of cyclopiazonic acid by three *Aspergillus flavus* strains (P1A1, O1A1 and A1E1) on a smoked fish agar after sampling at 7, 14, 21 and 60 days of incubation.

Table 8.2 Analysis of variance of the effects of water activity (a_w), temperature (t), strain (s) and day of sampling (d) and their interactions on production of cyclopiazonic acid on smoked fish agar.

Factor	df	MS	F	P
a_w	3	204763	295.37	<0.01
t	3	24510	35.36	<0.01
d	3	6433	9.28	<0.01
s	2	8849	12.76	<0.01
a_w x t	9	24016	34.64	<0.01
a_w x d	9	6439	9.29	<0.01
a_w x s	6	9186	13.25	<0.01
t x d	9	5824	8.40	<0.01
t x s	6	4878	7.04	<0.01
d x s	6	1768	2.55	0.020 NS
a_w x t x d	27	5369	7.74	<0.01
a_w x t x s	18	4625	6.67	<0.01
a_w x d x s	18	1830	2.64	<0.01
t x d x s	18	2769	3.99	<0.01
a_w x t x d x s	54	2610	3.76	<0.01

df, degrees of freedom
F, variance ratio
NS, non-significant (p>0.01)

8.4 Discussion

The results from this study enable the estimation of the optimum and limiting conditions for the growth and toxin production by *A. flavus* on Maldive fish and similar products. Water activity and temperature had a marked effect on the growth of this fungus on SFA. Optimal growth occurred at the highest a_w (0.95) at 35°C. This is in agreement with the most frequently reported conditions for *A. flavus* from the literature. Maximum growth for *A. flavus* is often described at the highest a_w levels of 0.99 to 0.98 on a range of different matrices (Cuero et al., 1987; Gibson et al., 1994; Pitt and Miscamble, 1995; Wheeler et al., 1988). Growth was indicated as optimal near 33°C, a minimum near 10-12°C and a maximum near 43-48°C (Holmquist et al., 1983; Pitt and Hocking, 2009). The limiting a_w for the growth of *A. flavus* on SFA was dependent on the incubation temperature. Growth was limited at an a_w of 0.80 at 25, 35 and 40°C. At 30°C, some growth was observed down to 0.80 a_w after incubation for 21 days but no growth occurred at 0.75 a_w. Hence, a value between 0.80 and 0.75 can be considered as the limiting a_w for growth of *A. fla*vus on SFA. The minimum a_w permitting growth of *A. flavus* is reported for a variety of substrates, although the values are rather variable in the literature (Table 8.3). Pitt and Miscamble (1995) found the limiting a_w for *A. flavus* to be dependent on temperature as follows: 0.82 at 25°C, 0.81 at 30°C and 0.80 at 37°C on synthetic media with glucose/fructose used to control a_w. The lowest minimum a_w recorded is 0.78, reported from both synthetic media and natural food substrates (Ayerst, 1969; Marín et al., 2012). This value approximates the estimated limiting value for SFA. As growth and hence the limiting a_w of a fungus is dependent on the type of food substrate, Maldive fish can be considered as a good medium for growth of *A. flavus*. The limiting a_w for germination of *A. flavus* on media controlled by NaCl is 0.85 to 0.86 when incubated between 25°C and 37°C (Wheeler et al., 1988). Due to the low salt content of SFA and Maldive fish, these values are not applicable.

Table 8.3 Optimum and Limiting a_w values for the growth of *Aspergillus flavus* on a range of substrates

Substrate/ controlling solute	**Temper-ature °C**	**Optimum a_w**	**Limiting a_w**	**Reference**
Corn extract medium	25-35	0.98	0.77	(Astoreca et al., 2012)
Malt Extract agar strips/ in controlled atmosphere adjusted using KOH	33	>0.98	0.78	(Ayerst, 1969)
Maize based media/ glycerol	25	0.99	<0.83	(Giorni et al., 2007)
glycerol	22-33	0.99	0.85	(Holmquist et al., 1983)
Maize extract	25	0.98	0.90	(Cuero et al., 1987)
	16	0.98	0.95	
Rice extract	25	0.98	0.95	
	16	0.98	0.95	
Pistachio nuts	20, 25, 30, 37, 42	0.98-0.93	0.78	(Marín et al., 2012)
3% Chili powder extract agar	25	0.99-0.97	0.82-0.88	(Marín et al., 2009)
Glucose and fructose	25		0.82	(Pitt and Miscamble, 1995)
	30		0.81	
	37		0.80	
Corn	25, 30		0.801	(Samapundo et al., 2007)
Glucose and fructose	20	0.99	0.85	(Wheeler et al., 1988)
	25	0.98	0.84	
	30	0.99	0.85	
NaCl	25		0.85	(Wheeler et al., 1988)
	37		0.86	
Maldive fish	*25, 35, 40* *30*	*0.95* *0.95*	*0.80* *0.75*	*Current study*

Fungal growth is not synonymous with mycotoxin production and the conditions for the latter are more restrictive than for growth (Klich, 2007; Sanchis and Magan, 2004). The optimum conditions for both aflatoxin and CPA production by *A. flavus* on SFA were also observed at the highest a_w (0.95) although the optimal temperatures were 25°C and 30°C, lower than the optimum growth temperature of 35°C. This supports published values for *A. flavus* on some other substrates where maximum toxin production was reported at high a_w values and similar temperatures. The highest concentrations of aflatoxins and CPA were produced at an a_w of 0.996 after 15 days on YES and CYA, with the optimum temperature for aflatoxin production at 30°C and CPA at 25°C (Gqaleni et al., 1997). On rice paddy, aflatoxin was detected in the range 0.86 to 0.99a_w with the optimal of 0.98 while toxin production was favoured at temperatures between 25 to 30°C (Mousa et al., 2011). Temperatures of 25°C and 30°C are also reported as favourable for aflatoxin production by *A. flavus* on peanuts (Vaamonde et al., 2006). However, CPA production was optimal between 20 and 25°C on this food (Mousa et al., 2011). This was in agreement with Gqaleni et al. (1996b), who reported higher levels of CPA at 20°C than at 30°C on maize grains held at constant a_w (0.98).

Small quantities of mycotoxins were detected at 0.85 a_w but production of both aflatoxin and CPA was prevented at an a_w of 0.80 by *A. flavus* on SFA under all conditions. Similar limiting values were obtained by other authors. On paddy, aflatoxin production by two isolates of *A. flavus* was observed in the a_w range of 0.86-0.99 with no obvious production in the next a_w level (0.82) (Mousa et al., 2011). This is in agreement with figures from ICMSF (ICMSF, 1996) describing aflatoxin production above an a_w of 0.80 by the same species. Vaamonde et al. (2006) report aflatoxin and CPA production by *A. flavus* on peanuts at 0.86 a_w, the lowest level studied. Ribeiro et al. (2006) reported aflatoxin production by the same species at 0.80 a_w on barley rootlets, but other studies indicate higher limiting conditions for aflatoxin production. For example, Gqaleni et al. (1997) reported germination but no further development of *A. flavus* strains used in their study, suggesting that neither aflatoxin nor CPA was produced at 0.85 a_w on YES and CYA.

All *A. flavus* strains used in this study displayed similar growth patterns but differed significantly ($p>0.01$) in their ability to produce mycotoxins. It is well known that the ability to produce mycotoxins by *A. flavus* is highly variable from strain to strain, ranging from high producers of aflatoxin, CPA or both to non toxigenic strains (Abbas et al., 2009; Horn and Dorner, 1999; Vaamonde et al., 2003). Furthermore, the toxigenic potential of the strains is dependent on the food substrate. The strains selected for this study were able to produce high to moderate concentrations of aflatoxin B_1 on YES after 7 days of incubation at 25°C : 77,700 ng/g by P1A2; 69,900 ng/g by O1A2; and 15,972 ng/g by A1E1 (Chapter 5). They however produced much lower concentrations of aflatoxins under these conditions on protein rich SFA (0.95 a_w), that is 18,909 ng/g, 7959 ng/g and 2487 ng/g, respectively. Similarly, they also produced high concentrations of CPA on CYA under the same conditions with 40,600 ng/g, 95,000 ng/g and 105,000 ng/g, respectively (Chapter 5). Yet again, only minute levels of CPA (65 ng/g by P1A2; 72 ng/g by O1A2; and 70 ng/g by A1E1) were detected on SFA (0.95 a_w) after 7 days of incubation at 25°C. SFA appears to be a less favourable substrate for mycotoxin production by *A. flavus* when compared to CYA and YES. This can be explained by the low toxigenic potential of *Aspergillus* on protein rich substrates in comparison to high carbohydrate or hydrocarbon rich substrates as demonstrated by other studies (Bailly et al., 2005; Escher et al., 1973).

Simultaneous production of two or more mycotoxins by a fungus is a complex relationship involving strain, substrate and the environment. Substrate has been reported to be a highly influential factor and aflatoxin production by *A. flavus* on SFA appears to be favoured over production of CPA. Gqalani et al. (1997) observed relatively high aflatoxin production in comparison with CPA production on YES agar but CPA production was relatively higher when the medium was CYA. Similarly, when *A. flavus* strains were grown on maize grains, the ratios of aflatoxin to CPA were 16.6:1, 5.7:1 and 1.6:1 after incubation times of 7, 15 and 21 days, respectively. However, the ratios were shifted in favour of CPA when YES or liquid malt extract yeast extract glucose medium was used, with only low concentrations of aflatoxins produced in 21 days (Gqaleni et al., 1996a). Vaamonde et al. (2006) observed optimal production of

CPA at 20°C when a cocktail of *A. flavus* strains, capable of producing both aflatoxins and CPA, were grown on peanuts. A similar effect was seen by Gqaleni et al. (1996a) who reported higher levels of CPA at 20°C than at 30°C by *A. flavus* on maize grains held at 0.98 a_w. The lowest temperature used in the current study was 25°C. An increase in toxin production may have been observed if lower temperatures had been studied.

The a_w of Maldive fish samples (n=25) analysed ranged from 0.951 to 0.720 with 44% (11/25) of the samples at or above 0.90 a_w. As considerable levels of both aflatoxin and CPA were detected on SFA at 0.95 and 0.90, conditions at these a_w levels are conducive to mycotoxin contamination. Although only small amounts of toxin were detected at 0.85 a_w on SFA, long term storage of Maldive fish will provide adequate time for accumulation of significant amounts of aflatoxin. The safest option is to reduce the a_w of fish to 0.75 or below, to eliminate the growth of *A. flavus*. As the average ambient day time temperature of Maldives (MMS, 2012) is 28°C to 30°C, and this corresponds to the optimum temperature observed for aflatoxin and CPA production on SFA, Maldive fish is susceptible to *A. flavus* growth and hence toxin production.

This study provides a better understanding of the behavior of *A. flavus* in Maldive fish or similar products under varying environmental conditions, and identifies the limiting conditions that could prevent fungal growth and mycotoxin production. Predictive models have been increasingly applied to describe the growth of *A. flavus* on several food substrates (Gibson et al., 1994; Marín et al., 2009; Yue et al., 2011). The data accumulated in this study could be used to develop such a model to predict growth of *A. flavus* on Maldive fish or similar substrates under different combinations of conditions. The results of this study indicate that no mycotoxin production will occur when the a_w of Maldive fish is lowered to 0.80, while the a_w has to be reduced further to approximately 0.75 to inhibit growth of *A. flavus* on this product at corresponding storage temperatures. Hence, to limit the growth of toxigenic *A. flavus* and prevent mycotoxin accumulation, it is recommended to control the drying process in the production of Maldive fish to rapidly achieve an a_w of 0.75 or below and maintain a_w at this level during storage and sale. Regulatory limits of 0.75 a_w (or equal value

translated in to moisture content) should be imposed to ensure the safety of Maldive fish.

CHAPTER 9

General Discussion and Conclusions

Maldive fish is a food product that is both economically and nutritionally important to the Maldives. The artisanal methods of production and unhygienic practices applied post production provide ample opportunity for this product to be contaminated with microorganisms. This is the first study on the safety of Maldive fish. Such a study has been needed to ensure a better quality product both in terms of revenue for the producer and health of the consumer. The most obvious immediate concern with Maldive fish is the effect of fungal contamination. The product commonly shows signs of fungal growth: the justification for the present study is that some of these fungi may be capable of producing toxic secondary metabolites.

9.1 Major Contributions and Findings

The first hypothesis of this study was that "Maldive fish supports the growth of toxigenic fungi and the production of mycotoxins". The natural mycoflora on the surface of Maldive fish has not been characterised previously. The first objective under this hypothesis was therefore to isolate and identify the mycoflora associated with the product. All of the twenty five samples assessed in this study were contaminated by filamentous fungi with 96% (24/25) containing one or more mycotoxigenic species. *Aspergillus flavus* (92%), *A. tamarii* (96%) and *Eurotium repens* (92%) were the dominant fungi contaminating Maldive fish. These results are in agreement with previous reports on the mycoflora of other smoked dried fish from the tropics (Adebayo-Tayo et al., 2008; Edema and Agbon, 2010; Essein et al., 2005; Fafioye et al., 2008; Prakash et al., 2011; Santoso et al., 1999). Although some species were isolated only from the fish surface, most were isolated after surface disinfection and so were also growing in the product. *A. flavus*, *A. tamarii*, *A. niger*, *A. ochraceus* and *Penicillium*

citrinum are the five species that were considered further due their potential to produce important mycotoxins.

Mycotoxin production by potentially toxigenic species is strain dependent (Abbas et al., 2009; Horn and Dorner, 1999) and hence, the next step of the study was to screen isolates of these fungi for their corresponding toxins. This included developing and optimising a new screening method for citrinin based on coconut cream agar. This simple and rapid method was used to screen *P. citrinum* isolates from Maldive fish for citrinin production but could also have a broader application of screening other potential citrinin producers. It is noteworthy that all isolates of *P. citrinum* were citrinin producers. Almost half (46%) of the *A. flavus* isolates were able to produce aflatoxins B_1 and B_2 in culture, of importance because of the frequent association of *A. flavus* with the product. Most *A. flavus* (95%) and *A. tamarii* (91%) isolates were producers of cyclopiazonic acid. Most isolates of *A. ochraceus* (75%) and some isolates of *A. niger* (20%) were able to produce ochratoxin A. Despite the low frequency of occurrence of *A. ochraceus* and the rarity of production of ochratoxin by *A. niger*, the highly toxic nature of OTA necessitates further assessment. The ability of a high proportion (72%) of potentially toxigenic isolates to produce toxic metabolites *in vitro* indicates potential contamination of the product with mycotoxins.

Growth of toxigenic fungi on a product does not necessarily indicate an ability to produce mycotoxins unless a favourable environment is provided. The next objective of this study was to determine if the physiochemical parameters of the product were suitable to support the production of toxin by the fungal isolates. Although the product is often considered to be very dry, a guideline limit had not been established. The lack of control of the drying process was reflected in the huge variability in water activity (a_w) levels (0.951 to 0.720) of the samples tested. None of the samples was found to be of a sufficiently low a_w level to halt the growth of the toxigenic fungi isolated. Furthermore, the only slightly acidic pH (5.65 to 6.68) and low salt content (1.48 to 4.29%) of the fish together with the high ambient temperatures (average 28°C to 30°C) of the Maldives were eminently suitable for fungal growth and mycotoxin production.

At this stage in the study, it had been shown that the environmental conditions were ideal for mycotoxin production in Maldive fish. As someone who has enjoyed this product since childhood, this was a concern. Tests on the product confirmed that two of the 25 samples were contaminated with aflatoxin levels (aflatoxin B_1 and total aflatoxins) greater than the commonly applied legal limits. This is one of the most significant findings of the current study and proves the first hypothesis that Maldive fish supports the growth of toxigenic fungi and the production of mycotoxins. Maldive fish poses a risk to food safety and therefore this information needs to be communicated to consumers of the product. This will provide incentive to draw up and enforce the draft regulations covering the manufacture of Maldive fish. Although ochratoxin A is an important mycotoxin, the low prevailing levels detected in this study suggest that significant exposure to this toxin from Maldive fish is unlikely.

These results led to the second question as to whether the mycotoxin hazard can be controlled leading to the second hypothesis that "fungal growth and mycotoxin production in Maldive fish can be eliminated or reduced to safe levels". Possible approaches include the use of improved packaging or biological control by inoculation of fish with nontoxigenic strains. These approaches would be expensive or require technical expertise which would add a burden to the small scale or cottage industries that are generally involved in the production of this product. The more practical approach would be to ensure that the a_w of the product was reduced to sufficiently low levels to control growth and toxin production of *A. flavus* and other toxigenic fungi (Pitt and Hocking, 1991). The remaining objective of this study was to evaluate the effects of the important environmental parameters such as a_w and temperature on the growth and toxin production of the two most important species *A. flavus* and *A. tamarii,* in order to determine the limiting conditions for their growth and mycotoxin production.

Studies on the physiology of *A. tamarii* are limited compared to the wide body of literature on *A. flavus* (Gibson et al., 1994; Marín et al., 2009; Marín et al., 2012; Pitt and Miscamble, 1995; Wheeler et al., 1988; Yue et al., 2011). Hence, the effect of environmental parameters on the germination and growth of *A. tamarii* on general

media was studied. The limiting a_w for *A. tamarii* was between 0.82 and 0.85 on NaCl media and between 0.79 and 0.75 on media containing sugars at ambient storage temperatures (25 to 35°C). The latter values are more applicable to this product due to its low salt content. Hence, the a_w of Maldive fish should be maintained below 0.75 to prevent the growth of *A.tamarii* and avoid CPA production. However, for a more accurate estimate, trials were needed on media that more closely simulated the product.

A smoked fish agar was used to simulate Maldive fish for fungal growth (*A. flavus*) and mycotoxin production (aflatoxin and CPA) under varying conditions. The growth of *A. flavus* was observed down to 0.80 a_w at 30°C after incubation for 21 days but no growth occurred at 0.75 a_w. The production of both aflatoxin and CPA was limited at 0.80 a_w under all incubation conditions (25°C to 40°C). Hence, the main recommendation from this study would be that Maldive fish should be dried rapidly to achieve 0.75 a_w or below. A guideline limit of 0.75 a_w (translated to moisture content) should become part of the regulations for this product to achieve better control of fungal growth and mycotoxin production in Maldive fish. These results provide enough evidence to suggest that the second hypothesis is probably correct. However, the practicality of consistently preparing such low a_w product under artisanal conditons needs to be pursued. Also, the smoked fish media results should be validated in the product e.g. by inoculating product prepared to low a_w with mycotoxigenic fungi and confirming that growth and toxin production were prevented.

In addition, exposure of the product to fungal spores should be minimised at all stages of production and storage. Hygiene standards should be in place to address microbial contamination of the product during processing and storage. Measures must be taken to avoid reabsorption of moisture from the environment by controlling the humidity during storage. These requirements should also be part of the regulations. Based on the findings of this study regulatory limits should be established for aflatoxins levels in Maldive fish.

9.2 Future Directions

The current study was limited to identification of fungi and mycotoxin quantification from 25 fish samples sourced from markets on the capital island of the Maldives. These markets are supplied from different parts of the country but are mostly intended for local consumption. A larger number of samples from different areas of the country as well as product intended for export would increase confidence in the findings of this study.

It would be worthwhile to sample for fungal growth and toxin production at different stages of processing, including smoking, sun drying and storage, to determine the major source of contamination. This is specifically important in view that one of the sample with high aflatoxin levels had an a_w lower than 0.75. Mycotoxin production must have occurred during processing before the final a_w of the sample was achieved.

An alternative explanation for the low a_w of a sample with an elevated aflatoxin level was that the measure was an average for the whole sample and some areas of the sample may contain adequate water to support mycotoxin production. The uneven shape of the fish fillet supports this opinion. Hence, a future study could assess the variation in a_w in different areas of the same sample.

CPA and citrinin from the Maldive fish samples were not quantified in this study due to limitations in time and technical difficulties (Xu et al., 2006). However, contamination of Maldive fish samples with CPA is highly probable due to the large proportion of *A. flavus* and *A. tamarii* isolates that produced this toxin *in vitro*. Similarly, *P. citrinum* isolated at a high frequency was a consistent producer of citrinin in culture. On the other hand, citrinin is also reported to breakdown over time (Bailly et al., 2005) and the stability of this compound is dependent on the substrate (Bailly et al., 2002). The occurrence of multiple mycotoxins in the product might amplify the risks due to possible synergistic action. Therefore further experiments to estimate the levels of these mycotoxins in the product are needed.

Some fungi growing on Maldive fish are thought to have a role in enhancing the flavour of this product (Comi et al., 2004) and consumers may have an acquired preference for this flavour. The Japanese product, Katsuboushi, which is analogous to Maldive fish, is inoculated with safe strains of flavour inducing fungi to achieve the desirable sensory attributes (Miyake et al., 2010). A similar approach could be used for Maldive fish which would have a dual role; flavour enhancement and, more importantly, biological control by outcompeting the deleterious natural flora of Maldive fish. One recommendation from this thesis is to select suitable candidates for this purpose and optimise conditions for application supplemented by sensory studies. Such a product has the potential to be a premium product for the local, export and tourist consumers.

It would be interesting to evaluate the extent of migration of the aflatoxins and other mycotoxins in to the product by analysing both the outer and inner layers. This is important information as the outer layer of fungi infested fish is often removed before consumption. Evaluation of outer and inner layers of dry cured and smoked hams indicates a higher occurrence of OTA in the outer layers than in the inner samples (Toscani et al., 2007). In citrinin contaminated cheese, 66% of the toxin was present in the first mm, 33% of the toxin was retained in the cheese after slight trimming while, 96% of it was lost after removal of 0.5 cm of the outer layer (Bailly et al., 2002).

A number of possible future studies using the same design (factorial) and experimental set-up as for growth and toxin production of *A. flavus* on smoked fish agar (SFA) under varying environmental conditions could provide useful data. The limiting conditions for other important toxigenic species such as *A. tamarii* and *P. citrinum* could be deduced and applied for the control of these fungi.

Studies of aflatoxin production on protein rich substrates are rare. It would be interesting to asses the protein content of the SFA medium, and compare toxin production on a high carbohydrate or high hydrocarbon versus elevated protein substrate.

While SFA is considered a suitable media for simulation of Maldive fish, the resulting information is still an estimate of the conditions needed for the control of mycotoxins. As aflatoxin production by *A. flavus* is dependent on the nature of the substrate (Horn, 2005; Klich, 2007), the results should be validated in the actual product to improve confidence in the limiting conditions recommended from this study.

Predictive models have been increasingly used to describe the growth of toxigenic fungi and mycotoxin production on several food substrates (Marín et al., 2009; Marín et al., 2012; Samapundo et al., 2007; Yue et al., 2011). Results from the study of *A. flavus* on smoked fish agar could be used to develop such a model to predict growth and toxin production on Maldive fish or similar substrates under any combinations of conditions and arrive with more accurate limiting values.

In conclusion, this study has provided some scientific evidence that the mycoflora on Maldive fish produce aflatoxins and other mycotoxins that are a food safety risk. The favourable environmental conditions often present in this product, together with the toxin producing ability of a high proportion of strains isolated, can result in the contamination of the product with levels of aflatoxins above the recommended limits. Hence, control of the growth of *A. flavus* and other toxigenic strains is believed to be necessary and could be achieved through adequate drying. This information is crucial for the Maldives as well as other developing countries that consume hot smoked dried fish or similar products. It is hoped that the results from this study can be used to help enforce standards and regulations for the manufacture of Maldive fish and produce guidelines that can be practically implemented to ensure adequate reduction in the a_w of the product. The results from this study potentially have a broader application for other food products.

Appendix A Location and Time of Sampling

Samples were purchased from local markets in the capital island Malé, Maldives, over a period of three years. Details are given below:

Date of Sampling	Sample	Location
26/03/09	Sample A, B, D, E, G	Valhomas market block no 3 Valhomas market block no 10 Local market block no 4
12-13/04/2010	Sample H, I, J, K, L, M, N	Valhomas market block no 16 Valhomas market block no 2 Local market block no 20 Gnaviyani Atolhu Fihaara
05/10/2010	Sample O, P, Q, R, S, T	Amira Fihaara Valhomas market block no 4 Valhomas market block no 10
04/07/2011	Sample U, V, X, Z	Gnaviyani Atolhu Fihaara Local market block no 22 Valhomas market block no 6
07/12/2011	Sample AA1, AC1, AF1	Valhomas market block no 14 Valhomas market block no 12

Appendix B Media Formulations

The following isolation and identification media were prepared according to the formulation of Pitt and Hocking (2009).

Aspergillus flavus and parasiticus agar (AFPA)

Peptone, bacteriological	10 g
Yeast extract	20 g
Ferric ammonium citrate	0.5 g
Chloramphenicol	100 mg
Agar	15 g
Dichloran	2 mg
(0.2% in ethanol, 1.0 ml)	
Water, distilled	1 L

After addition of all ingredients, sterilise by autoclaving at 121°C for 15 min. The final pH of this medium is 6.0–6.5.

Czapek concentrate

$NaNO_3$	30 g
KCl	5 g
$MgSO_4\_7H_2O$	5 g
$FeSO_4\_7H_2O$	0.1 g
Water, distilled	100 ml

Czapek concentrate will keep indefinitely without sterilisation. The precipitate of $Fe(OH)_3$ which forms in time can be resuspended by shaking before use.

Czapek yeast extract agar (CYA)

K_2HPO_4	1 g
Czapek concentrate	10 ml
Trace metal solution	1 ml
Yeast extract, powdered	5 g
Sucrose	30 g
Agar	15 g
Water, distilled	1 L

Refined table grade sucrose is satisfactory for use in CYA provided it is free from sulphur dioxide. Sterilise by autoclaving at 121°C for 15 min. The final pH is 6.7.

Czapek yeast extract agar with 20% sucrose (CY20S)

K_2HPO_4	1 g
Czapek concentrate	10 ml
Yeast extract	5 g
Sucrose	200 g
Agar	15 g
Water, distilled	1 L

Sterilise by autoclaving at 121°C for 15 min. The final pH is 5.2.

25% Glycerol nitrate agar (G25N)

K_2HPO_4	0.75 g
Czapek concentrate	7.5 ml
Yeast extract	3.7 g
Glycerol, analytical grade	250 g
Agar	12 g
Water, distilled	750 ml

Sterilised by autoclaving at 121°C for 15 min. The final pH is 7.0.

Malt extract agar (MEA)

Malt extract, powdered	20 g
Peptone	1 g
Glucose	20 g
Agar	20 g
Water, distilled	l L

Sterilise by autoclaving at 121°C for 15 min. Do not sterilise for longer, as thismedium will become soft on prolonged or repeated heating. The final pH is 5.6.

Malt extract yeast extract 50% glucose agar (MY50G)

Malt extract	10 g
Yeast extract	2.5 g
Agar	10 g
Water, distilled to 500 g	
Glucose, A.R.	500 g

Add the minor constituents and agar to ca. 450 ml distilled water and steam to dissolve the agar. Immediately make up to 500 g with distilled water. While the solution is still hot, add the glucose all at once and stir rapidly to prevent the formation of hard lumps of glucose monohydrate. If lumps do form, dissolve them by steaming for a few

minutes. Sterilise by steaming for 30 min; note that this medium is of a sufficiently low a_w not to require autoclaving. The final aw of this medium is 0.89. The final pH is 5.3.

Malt extract yeast extract 5% (or 10%) salt 12% glucose agar (MY5-12 and MY10-12)

Malt extract	20 g
Yeast extract	5 g
NaCl	50 g
	(100 g for MY10-12)
Glucose	120 g
Agar	20 g

Sterilise MY5-12 by autoclaving at 121°C for 10 min, and MY10-12 by steaming for 30 min. Overheating of these media will cause softening. The final a_w of MY5-12 is 0.93 and of MY10-12 is 0.88.

Appendix C Fungal Isolates

Details of the species isolated from 25 Maldive fish samples before surface sanitisation

#	Sample	Water activity	*A. flavus*	*A. flavus (yellow)*	*A. tamarii*	*A. niger*	*A. ochraceus*	*A. sydowi*	*A. terreus*	*A. candidus*	*E. chevalieri*	*E. rubrum*	*E. repens*	*Emericella nidulans*	*P. citrinum*	*P. crustosum*	*P. sublateritium*	*Absidia corymbifera*	*Byssochlamys fulva*	*Monascus ruber*	*Ppolypaecium pisce*	*Syncephalastrum recemosum*	*white non sporing fungi*
1	A1	0.778	x		x	x	x		x		x		x	x	x		x			x			
2	B1	0.939	x		x	x							x									x	
3	D1	0.756	x		x						x		x		x	x	x			x			
4	E1	0.947	x		x	x		x					x		x							x	
5	G1	0.722	x		x			x					x								x		x
6	H1	0.900	x		x	x		x				x	x		x				x				
7	I1	0.862	x		x	x		x		x	x		x		x								
8	J1	0.751	x	x	x								x		x							x	
9	K1	0.874	x		x	x						x	x									x	
10	L1	0.894	x		x			x		x			x		x								
11	M1	0.911	x		x		x	x					x		x								
12	N1	0.730	x		x	x	x	x		x	x		x		x								
13	O1	0.878	x		x			x				x	x		x								
14	P1	0.720	x		x	x		x			x	x	x										
15	Q1	0.869	x		x			x				x	x		x				x				x
16	R1	0.829	x		x	x			x			x	x		x								
17	S1	0.928	x		x	x		x					x		x			x			x		
18	T1	0.931	x		x			x					x					x					
19	U1	0.929	x		x			x	x				x					x					
20	V1	0.928	x		x			x				x	x					x					
21	X1	0.904			x										x								x
22	Z1	0.939	x		x								x									x	
23	AA1	0.903	x		x			x					x							x			
24	AC1	0.892	x		x			x		x		x	x		x								
25	AF1	0.888																			x		
Total Incidence			23		24	10	3	16	3	4	5	8	23	1	15	1	2	4	2	3	3	5	3
%Frequency			**92**		**96**	**40**	**12**	**64**	**12**	**16**	**20**	**32**	**92**	**4**	**60**	**4**	**8**	**16**	**8**	**12**	**12**	**20**	

X denotes that the sample was positive for the species

Details of the species isolated from 25 Maldive fish samples after surface sanitisation

#	Sample	Water activity	*A. flavus*	*A. flavus (yellow)*	*A. tamarii*	*A. niger*	*A. ochraceus*	*A. sydowi*	*A. terreus*	*A. candidus*	*E. chevalieri*	*E. rubrum*	*E. repens*	*Emericella nidulans*	*P. citrinum*	*P. crustosum*	*P. sublateritium*	*Absidia corymbifera*	*Byssochlamys fulva*	*Monascus ruber*	*Ppolypaecium pisce*	*Syncephalastrum recemosum*	*white non sporing fungi*
1	A2	0.778	x		x								x							x			
2	B2	0.939	x		x	x							x		x								
3	D2	0.756	x		x	x							x							x			
4	E2	0.947	x		x	x							x		x							x	
5	G2	0.722	x									x	x										
6	H2	0.900	x		x	x			x				x		x								
7	I2	0.862	x		x			x			x		x		x								
8	J2	0.751	x		x								x		x								
9	K2	0.874	x		x	x																	
10	L2	0.894	x		x																		
11	M2	0.911	x		x			x					x		x								
12	N2	0.730	x		x	x																	
13	O2	0.878	x		x																		
14	P2	0.720	x		x	x																	
15	Q2	0.869	x		x														x				
16	R2	0.829	x		x								x		x								
17	S2	0.928	x		x	x		x					x		x			x					
18	T2	0.931	x		x			x		x			x					x					
19	U2	0.929	x		x			x	x			x	x					x					
20	V2	0.928			x			x					x					x					
21	X2	0.904			x										x								
22	Z2	0.939	x		x								x									x	
23	AA2	0.903	x		x			x					x							x			
24	AC2	0.892	x		x			x		x		x	x		x								
25	AF2	0.888																			x		
Total Incidence			22		23	8	0	8	2	2	1	3	17		10	0	0	4	0	3	1	2	
%Frequency			**88**		**92**	**32**	**0**	**32**	**8**	**8**	**4**	**12**	**68**		**40**	**0**	**0**	**16**	**0**	**12**	**4**	**8**	

X denotes that the sample was positive for the species

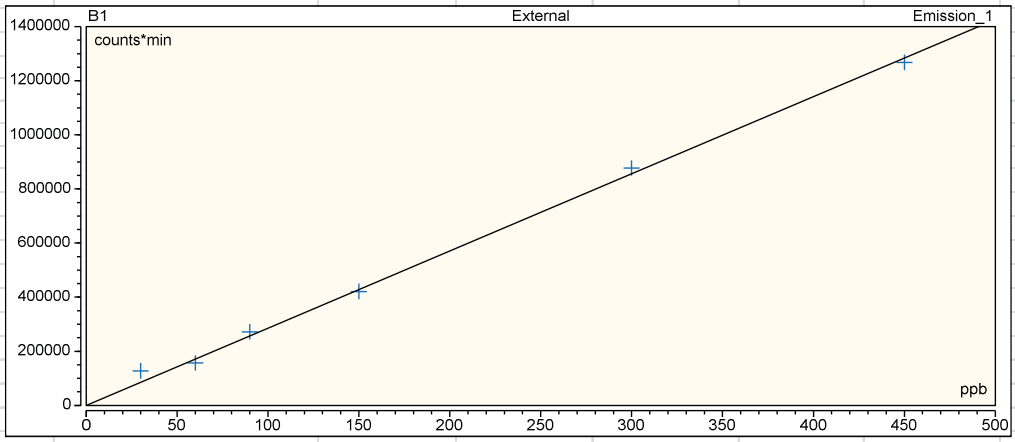
Appendix D HPLC Standard Curves

HPLC standard curves with r^2 >0.99 for screening mycotoxins

Aflatoxin B_1

Calibration Details	B1				
Calibration Type	Lin		*Offset (C0)*		0.0000
Evaluation Type	Area		*Slope (C1)*		2853.2526
Number of Calibration Points	6		*Curve (C2)*		0.0000
Number of disabled Calibration Points	0		*R-Square*		0.9972

Calibration Plot B1

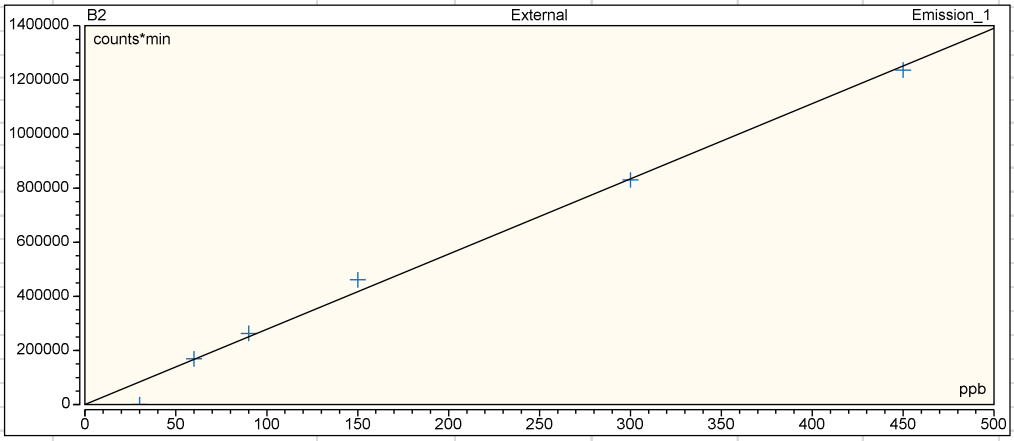


Calibration Results	B1						
No.	Injection Name	Calibration Level	X Value B1 Emission_1	Y Value B1 Emission_1	Y Value B1 Emission_1	Area counts*min B1 Emission_1	Height counts B1 Emission_1
2	30ppb	01	30.0000	127829.5233	127829.5233	127829.523	418060.594
3	60ppb	02	60.0000	156754.4286	156754.4286	156754.429	512964.028
4	90ppb	03	90.0000	271952.3742	271952.3742	271952.374	889350.900
5	150ppb	04	150.0000	421110.7040	421110.7040	421110.704	1379595.103
6	300ppb	05	300.0000	877216.7956	877216.7956	877216.796	2889371.296
7	450ppb	06	450.0000	1268173.4202	1268173.4202	1268173.420	4218362.895

Aflatoxin B_2

Calibration Details	B2		
Calibration Type	Lin	*Offset (C0)*	0.0000
Evaluation Type	Area	*Slope (C1)*	2781.3439
Number of Calibration Points	5	*Curve (C2)*	0.0000
Number of disabled Calibration Points	0	*R-Square*	0.9970

Calibration Plot **B2**



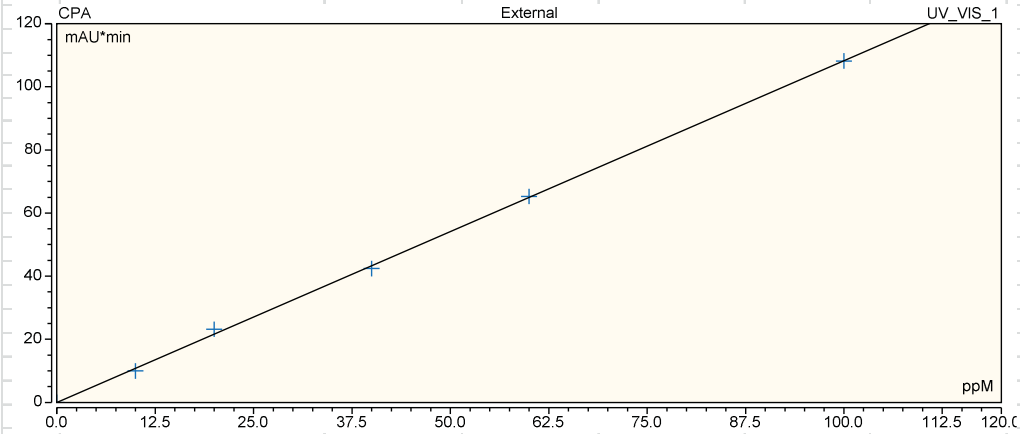
Calibration Results **B2**

No.	Injection Name	Calibration Level	X Value B2 Emission_1	Y Value B2 Emission_1	Y Value B2 Emission_1	Area counts*min B2 Emission_1	Height counts B2 Emission_1
2	30ppb	01	30.0000	n.a.	n.a.	n.a.	n.a.
3	60ppb	02	60.0000	169096.5579	169096.5579	169096.558	475512.055
4	90ppb	03	90.0000	263219.9542	263219.9542	263219.954	750994.178
5	150ppb	04	150.0000	461170.8155	461170.8155	461170.816	1326383.855
6	300ppb	05	300.0000	830683.7914	830683.7914	830683.791	2411465.442
7	450ppb	06	450.0000	1236552.6610	1236552.6610	1236552.661	3642216.389

Cyclopiazonic acid

Calibration Details	CPA		
Calibration Type	Lin	*Offset (C0)*	0.0000
Evaluation Type	Area	*Slope (C1)*	1.0824
Number of Calibration Points	5	*Curve (C2)*	0.0000
Number of disabled Calibration Points	0	*R-Square*	0.9993

Calibration Plot **CPA**



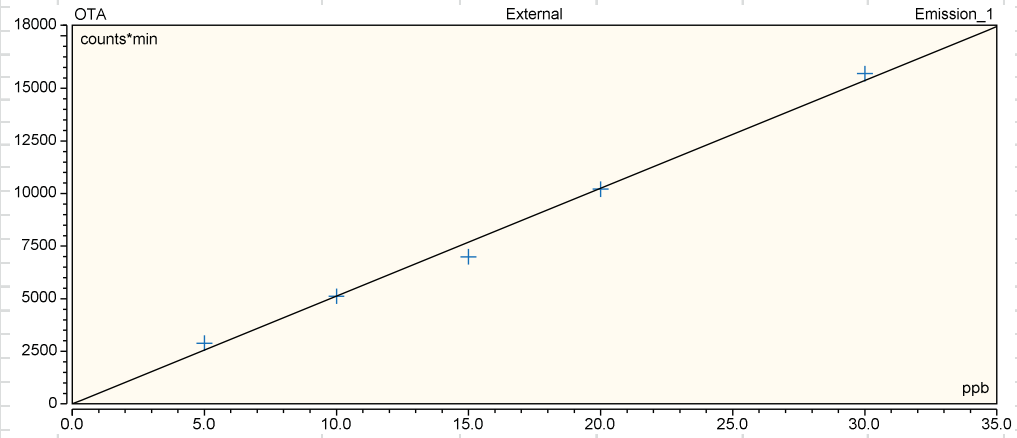
Calibration Results **CPA**

No.	Injection Name	Calibration Level	X Value CPA	Y Value CPA	Y Value CPA	Area mAU*min CPA	Height mAU CPA
12	10ppm	01	10.0000	9.9388	9.9388	9.939	19.096
13	20ppm	02	20.0000	23.1741	23.1741	23.174	38.602
15	60ppm	04	60.0000	65.2637	65.2637	65.264	98.629
17	100ppm(1)	06	100.0000	108.1971	108.1971	108.197	156.981
19	40ppm	03	40.0000	42.3997	42.3997	42.400	72.106
20	80ppm	05	0.0000	0.0000	0.0000	n.a.	n.a.

Ochratoxin A

Calibration Details	OTA		
Calibration Type	Lin	*Offset (C0)*	0.0000
Evaluation Type	Area	*Slope (C1)*	512.6006
Number of Calibration Points	5	*Curve (C2)*	0.0000
Number of disabled Calibration Points	0	*R-Square*	0.9930

Calibration Plot **OTA**

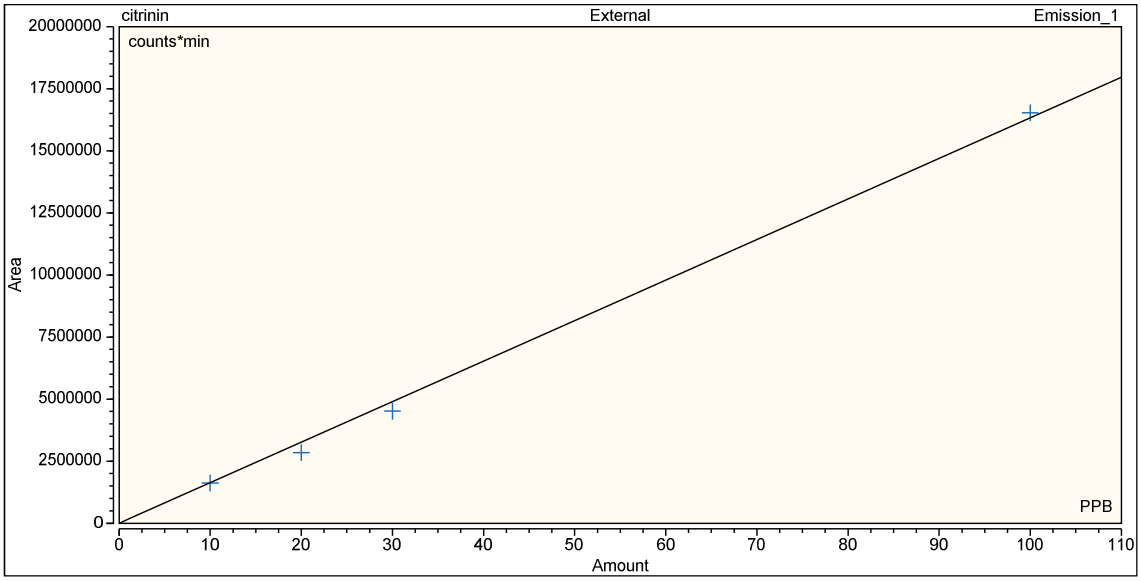


Calibration Results **OTA**

No.	Injection Name	Calibration Level	X Value OTA	Y Value OTA	Y Value OTA	Area counts*min OTA	Height counts OTA
39	std 5ppb	01	5.0000	2885.0367	2885.0367	2885.037	10655.764
40	std 10ppb	02	10.0000	5117.0950	5117.0950	5117.095	18539.045
41	std 15ppb	03	15.0000	6995.0777	6995.0777	6995.078	23866.242
42	std 20ppb	04	20.0000	10210.7067	10210.7067	10210.707	35814.889
43	std 30ppb	05	30.0000	15701.8208	15701.8208	15701.821	55212.368

Citrinin

Calibration Details	citrinin					
Calibration Type	Lin			*Offset (C0)*		0.0000
Evaluation Type	Area			*Slope (C1)*		163235.9909
Number of Calibration Points	4			*Curve (C2)*		0.0000
Number of disabled Calibration Points	0			*R-Square*		0.9974



Calibration Results	citrinin						
No.	Injection Name	Calibration Level	X Value citrinin Emission_1	Y Value citrinin Emission_1	Y Value citrinin Emission_1	Area counts*min citrinin Emission_1	Height counts citrinin Emission_1
62	STD 100	04	100.0000	16524549.4821	16524549.4821	16524549.482	72094181.655
64	STD10*	01	10.0000	1621831.4233	1621831.4233	1621831.423	5675640.614
65	STD 20	02	20.0000	2841296.9125	2841296.9125	2841296.913	11552044.983
66	STD 30	03	30.0000	4513036.5326	4513036.5326	4513036.533	18566036.659

Appendix E Mycotoxin Screening Results

Isolates of *Aspergillus flavus* for aflatoxins

#	Isolate	AFPA reverse	sclerotia size	CCA	TLC YES	HPLC Aflatoxin ng/g	
						B1	B2
1	J1F1*	orange	no sclerotia	P	P (Afl B1&B2)	4176	66
2	B1H1	orange	L strain	P	P (Afl B1&B2)	8432	215
3	A1E1	orange	L strain	P	P (Afl B1&B2)	15972	985
4	A1E2	light orange	no sclerotia	N	N	N	N
5	T1A1	orange	L strain	N	N	N	N
6	T1A2	orange	L strain	N	N	N	N
7	G1E1	orange	no sclerotia	P	strong B1 only	1815	42
8	G1E2	orange	no sclerotia	P	weak B1 only	43	5
9	Q1A1	light orange	no sclerotia	N	N	N	N
10	S1A1	orange	L strain	N	N	N	N
11	D1S1	orange	L strain	N	weak B1 only	15	N
12	P1A1	orange	L strain	P	P (Afl B1&B2)	11061	562
13	P1A2	orange	L strain	P	P (Afl B1&B2)	77776	3560
14	P1A3	orange	L strain	P	P (Afl B1&B2)	69412	3118
15	L1A1	orange	L strain	N	N	N	N
16	L1A2	orange	L strain	N	N	N	N
17	L1A3	orange	L strain	N	N	N	N
18	H1A1	orange	no sclerotia	N	N	<5	N
19	H1A2	orange	L strain	N	N	N	N
20	H1A3	orange	v. few, L strain	P	P (Afl B1&B2)	8974	234
21	H1A4	orange	L strain	N	v weak B1	12	
22	M2A1	orange	L strain	N	N	N	N
23	M2A2	orange	no sclerotia	N	N	N	N
24	M2A3	orange	L strain	N	N	<5	N
25	N1A1	orange	L strain	N	weak B1	16	N
26	N1A2	orange	no sclerotia	N	N	15	N
27	N1A3	orange	L strain	N	N	N	N
28	J1A1	light orange	L strain	N	N	N	N
29	J1A2	orange	L strain	P	P (Afl B1only)	4797	86
30	J1A3	orange	no sclerotia	N	N	N	N
31	O1A1	orange	L strain	P	P (Afl B1&B2)	23417	929
32	O1A2	orange	L strain	P	P (Afl B1&B2)	69926	2302
33	O1A3	orange	L strain	P	P (Afl B1&B2)	9736	238
34	M1A1	orange	L strain	N	N	N	N
35	M1A5	orange	L strain	N	N	<5	N
36	K1A1	light orange	L strain	N	N	N	N
37	K1A2	light orange	L strain	N	N	N	N
38	I1E2	orange	v. few, L strain	N	N	N	N
39	I1E3	orange	v. few, L strain	N	v weak B1	18	N
40	I1E4	orange	no sclerotia	N	N	N	N
41	AD1A1	orange	L strain	P	strong B1 only	318	N
	* yellow isolate		P= positive N= negative				

Isolates of *Aspergillus flavus* for cyclopiazonic acid

#	Isolate	AFPA reverse	sclerotia size	TLC CYA	HPLC CPA ng/g
1	J1F1*	orange	no sclerotia	P	35000
2	B1H1	orange	L strain	P	56000
3	A1E1	orange	L strain	P	47200
4	A1E2	light orange	no sclerotia	N	N
5	T1A1	orange	L strain	P	170203
6	T1A2	orange	L strain	P	208148
7	G1E1	orange	no sclerotia	P	40949
8	G1E2	orange	no sclerotia	P	242327
9	Q1A1	light orange	no sclerotia	P	24698
10	S1A1	orange	L strain	P	12963
11	D1S1	orange	L strain	P	14287
12	P1A1	orange	L strain	P	29850
13	P1A2	orange	L strain	P	40600
14	P1A3	orange	L strain	P	33600
15	L1A1	orange	L strain	P	6950
16	L1A2	orange	L strain	P	9150
17	L1A3	orange	L strain	P	21050
18	H1A1	orange	no sclerotia	P	29300
19	H1A2	orange	L strain	P	34400
20	H1A3	orange	v. few, L strain	P	171000
21	H1A4	orange	L strain	P	10900
22	M2A1	orange	L strain	P	11500
23	M2A2	orange	no sclerotia	P	62600
24	M2A3	orange	L strain	P	9450
25	N1A1	orange	L strain	P	8900
26	N1A2	orange	no sclerotia	P	42300
27	N1A3	orange	L strain	N	N
28	J1A1	light orange	L strain	P	18900
29	J1A2	orange	L strain	P	58600
30	J1A3	orange	no sclerotia	P	50400
31	O1A1	orange	L strain	P	80300
32	O1A2	orange	L strain	P	95050
33	O1A3	orange	L strain	P	107150
34	M1A1	orange	L strain	P	23800
35	M1A5	orange	L strain	P	10600
36	K1A1	light orange	L strain	P	11700
37	K1A2	light orange	L strain	P	18600
38	I1E2	orange	v. few, L strain	P	28350
39	I1E3	orange	v. few, L strain	P	36800
40	I1E4	orange	no sclerotia	P	16700
41	AD1A1	orange	L strain	P	48274
	P= positive N= negative				

Isolates of *Aspergillus tamarii* for cyclopiazonic acid

Isolate	AFPA reverse	TLC CYA	HPLC CPA ng/g CYA
K1B1	dark brown	P	13300
K2B2	dark brown	P	3900
L1B1	dark brown	P(weak)	2600
L1B2	dark brown	P	9900
H1C1	dark brown	P	9500
H1C2	dark brown	P	11250
M2B1	dark brown	P	3500
M2B2	dark brown	P(weak)	4900
I1F1	dark brown	P(weak)	4250
I2F1	dark brown	P	6600
S1B1	v dark brown	P	10800
S1B2	dark brown	P	15750
S2B1	brown	N	0
S2B2	dark brown	P	15450
O1B1	dark brown	P	2250
N1B1	toffee	P	13100
T1B1	toffee	P	9550
P1B1	light brown	P	2800
J1B1	dark brown	P	10850
V1B1	dark brown	N	0
V1B2	dark brown	P	10650
P= positive	N= negative		

Isolates of *Aspergillus ochraceus* and *Aspergillus niger* for Ocratoxin A

Isolate	CCA	TLC	HPLC OTA ng/g
A. ochraceus			
M1C1	P	P (weak)	435
M1C2	P	P (weak)	375
N1D1	P	P (weak)	556
N1D2	P	P (weak)	717
N1D3	P	P (weak)	380
N1D4	P	P (weak)	388
A1T1	N	N	0
A1U1	N	N	0
A. niger			
P1C1	N	N	N
P1C2	N	N	N
E1A1	N	N	N
E1A5	N	N	N
B1F1	N	N	N
R1C1	N	N	N
H2E1	N	N	60
A1S1	N	N	N
N1H1	N	N	N
K1C1	N	N	36
I1G1	N	N	N
H1E1	N	N	32
S2E1	N	N	N
S1E1	N	N	N
R1C2	N	N	N
P= positive N= negative			

Isolates of *Penicillium cintinum* for citrinin

Isolate	CCA	TLC	HPLC OTA ng/g
J1P1	P	P	446
O1C2	P	P	524
S1D2	P	P	391
H1B1	P	P	255
O1C1	P	P	410
E1F1	P	P	596
N2B1	P	P	552
B2B2	P	P	416
B2B1	P	P	483
D1G1	P	P	456
Q1C1	P	P	430
Q1C2	P	P	509
N1C1	P	P	374
J1P1	P	P	451
I1B1	P	P	139
S1D1	P	P	464
S2D1	P	P	500
A1R1	P	P	391
D1G2	P	P	402
Q1C2	P	P	465
P= positive N= negative			

Appendix F Reference Data for Adjusting Water Activity

Reference data for adjusting the water activity of culture media and controlling solutes

Water activity of NaCl solutions: values proposed to be used as standards in the range 15-50°C according to Chirife and Resnik (1984)

Conc (% w/w)	a_w	Conc (% w/w)	a_w	Conc (% w/w)	a_w	Conc (% w/w)	a_w
0.5	0.997	7.0	0.957	13.5	0.906	20.0	0.839
1 0	0.994	7.5	0.954	14.0	0.902	20.5	0.833
1.5	0.991	8.0	0.950	14.5	0.897	21.0	0.827
2.0	0.989	8.5	0.946	15.0	0.892	21.5	0.821
2.5	0.986	9.0	0.943	15.5	0.888	22.0	0.815
3.0	0.983	9.5	0.939	16.0	0.883	22.5	0.808
3.5	0.980	10.0	0.935	16.5	0.878	23.0	0.802
4.0	0.977	10.5	0.931	17.0	0.873	23.5	0.795
4.5	0.913	11 0	0.927	17.5	0.867	24.0	0.788
5.0	0.970	11.5	0.923	18.0	0.862	24.5	0.781
5.5	0.967	12.0	0.919	18.5	0.857	25.0	0.774
6.0	0.964	12.5	0.915	19.0	0.851	25.5	0.766
6.5	0.960	13.0	0.911	19.5	0.845	26.0	0.759

For adjusting the water activity of the media with glucose/ fructose, the formula of Norrish's (1966) was used:

$a_w = (1 - x_w) \cdot e^{-k \cdot {x_s}^2}$ (Equation 1), where,

a_w : water activity,

x_w: solute molar fraction of water

x_s: solute molar fraction of solute

K: correlating constant

From Equation 1, $\frac{a_w}{x_s} = e^{-k \cdot {x_s}^2}$(Equation 2), where,

K values were suggested by Baeza et al.(2010)

$K_{fructose}$	2.25	(most acceptable)
$K_{glucrose}$	2.25	(most acceptable)
Average	2.25	

Also, $X_s = 1 - X_w$

To solve Equation 2, left hand side must be equal (or as close as possible) to right hand side. This was achieved by substituting presumed X_s values into both sides of the equation. The amounts of sugar added to the media were then calculated and summarized in Table below. The actual amounts added, however, were adjusted based on the a_w values obtained using a Decagon CX-2 water activity meter.

a_w	Left	Right	Xs	Xw	Solute (sugar) (g)	Water (g)	Total sugar per 100 g water	Fructose/ Glucose (g)
0.95	0.9953	0.9954	0.0455	0.9545	8.2	17.2	47.7	23.9
0.92	0.9890	0.9891	0.0698	0.9302	12.6	16.7	75.1	37.6
0.88	0.9778	0.9778	0.1000	0.9000	18.0	16.2	111.2	55.6
0.85	0.9674	0.9674	0.1214	0.8786	21.9	15.8	138.3	69.1
0.82	0.9557	0.9556	0.1420	0.8580	25.6	15.4	165.6	82.8
0.79	0.9427	0.9427	0.1620	0.8380	29.2	15.1	193.5	96.7

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Shazla Mohamed

Name/Title of Principal Supervisor: Prof. Steve Flint

Name of Published Research Output and full reference: Mohamed et al. 2012. Effect of aw and temp. on the germination and growth of A-tamarii isolated from "Maldive fish". Int. J of Food Microbiology, 160: 119-23.

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- **The percentage of the Published Work that was contributed by the candidate:** _____
  and / or
- **Describe the contribution that the candidate has made to the Published Work:**
  This manuscript was an integral part of a PhD study and conducted entirely by the student under the guidance of the supervisory team.

Candidate's Signature — 21/12/2012 Date

Principal Supervisor's signature — 21/12/12 Date

Appendix H Supplementary Data Chapter 8

Radial growth rates (mm/day) of three *A. flavus* strains (P1A2, O1A2 and A1E1) under varying conditions of water activity and temperature

Water activity	Temp (°C)	Growth rate (mm/day)		
		P1A2	O1A2	A1E
0.95	25	10.3	10.2	10.5
0.95	30	13.3	13.6	13.4
0.95	35	15.0	15.9	16.0
0.95	40	10.9	11.7	9.7
0.9	25	5.4	5.6	5.1
0.9	30	6.3	6.9	6.0
0.9	35	7.6	7.6	7.5
0.9	40	2.8	3.1	3.9
0.85	25	1.0	1.3	1.3
0.85	30	1.6	1.7	1.6
0.85	35	1.8	1.7	1.7
0.85	40	0.7	0.6	0.9
0.8	25	0	0	0
0.8	30	0	0	0
0.8	35	0	0	0
0.8	40	0	0	0
0.75	25	0	0	0
0.75	30	0	0	0
0.75	35	0	0	0
0.75	40	0	0	0

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