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Ochratoxin A Production by *Aspergillus ochraceus*

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Abbreviations

A	Ampere
A.	*Aspergillus*
AFLP	Amplified Fragment Length Polymorphism
aqua (bi)dest.	(Doubly) distilled water
a_w	Water activity
bp	Base pair
cDNA	Copy desoxy ribonucleic acid
DNA	Desoxy ribonucleic acid
DNase	Desoxyribonuclease
dNTPs	2'-Desoxynucleosid-5´-triphosphate
dT	2'-Desoxytymidin
E	Extinction
e.g.	*exempli gratia*
et al.	*et alteri*
Fig.	Figure
g	Gravity constant (9.81 m/s²)
h	Hour
HPLC	High Pressure Liquid Chromatography
HPTLC	High performance thin layer chromatography
i.e.	*id est*
IEC	Ion Exchange Chromatography
kb	Kilobase
L	Liter
µ	Micro (10^{-6})
m	Milli (10^{-3})
M	Molar (mol/L)
MEBAK	Mitteleuropäische Brautechnische Analysenkommission
min	Minute
mRNA	Messenger Ribonucleic Acid
n	Nano (10^{-9})
OD	Optical density
OTα	Ochratoxin α
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PCR	Polymerase Chain Reaction
pH	Negative decadic logarithm of proton concentration
RNA	Ribonucleic Acid
Rnase	Rinonuclease
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic Acid
SDS	Sodium dodecyl sulfate
sec	Second
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid
U	Unit (enzyme activity)
V	Volt
v/v	Volume per volume
var.	*variatio*
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Contents

1 Introduction

Unprocessed agricultural commodities as well as any kind of comestible goods are susceptible to microbial contamination during growth, harvest and storage. Some of the microorganisms growing on food and feed are not only decaying those aliments but also are capable of producing toxic metabolites. Contamination of food and feed with those toxins represent a high risk for human and animal health. One of the toxins that has been considered of growing importance in the last decade, is ochratoxin A (OTA), a nephrotoxic mycotoxin produced by several species within the fungal genera *Aspergillus* and *Penicillium*.

In order to conduct early detection and identification of ochratoxigenic fungi on raw materials, feed and foodstuffs, molecular diagnostic methods are the most potent tools available. Ideally these diagnostic systems should be set up based on specific genetic features of OTA producers, i.e. their genes and enzymes involved in the biosynthetic pathway leading to OTA formation.
To elucidate those genes and enzymes connected to OTA production, it is first of all necessary to determine and understand environmental factors influencing the regulation of production of this mycotoxin.

As observed for almost all secondary metabolites, mycotoxin biosynthesis is dependent on environmental factors and elaborately regulated by several interwoven external and internal signals. Therefore it seems appropriate to take a look at the essential features of secondary metabolites and what is known about their regulation, function and evolution.

1.1 Secondary Metabolites

All molecules synthesized in the life cycle of any organism can be roughly grouped into primary and secondary metabolites. Primary metabolites are distinguished from secondary ones by several important characteristics:

Primary metabolites are a group of relatively few substances common and vitally important to all living organisms. They are synthesized via a limited number of intermediates almost through all growth phases in a highly concerted and complex network. Intermediates and enzymes involved in most primary metabolic pathways are well characterized. Primary metabolites are almost identical for all organisms and therefore often called general metabolites (Martin and Demain, 1980).

1.1.1 General Features of Secondary Metabolites

Secondary metabolites are also denoted as shunt metabolites, special metabolites and idiolites. Each term is connected to a special feature common to all substances classified as secondary metabolites (reviewed by Bennett and Ciegler, 1983). Main features of secondary metabolites are:

a) Specific Taxonomic Distribution

Synthesis of an individual secondary metabolite often is limited to a particular family, genus, species or even strain.

Generally however, prodution of secondary metabolites is more abundant in (micro-)organisms with relatively long generation times living in moderate environments with the ability to adapt to varioous growth conditions (pH, temperature, carbon sources etc.). Microorganisms with short generations times or living in extreme environments are usually adapted to rapid and complete utilization of energy sources available. Precursors and energy from the primary metabolism are not so rapidly processed in “slow growers” but might be more accessible for modifications in secondary metabolism. This implies a less stringent regulation and therefore greater flexibility of metabolism in those organisms.

b) Synthesis Dependent on Cell Differentiation Status

Production of most idiolites is associated with the end of the logarithmic growth phase of the producing organism or the idiophase, when active growth has ceased. Secondary metabolism in microorganisms generally seems connected with decreasing growth rates (Zähner *et al*., 1983). In fungi, production of secondary metabolites is often related to sporulation. In higher plants, secondary metabolite production is instead restricted to plant organs and/or a particular stage in growth and development. Therefore, production of secondary metabolites might be considered an expression of cellular differentiation (Bennett and Christensen, 1983).

In contrast to primary metabolism, production of secondary metabolites is not balanced and adjusted to what would actually be required in a certain stage of development. Apparent overproduction is known to occur frequently. Whether an organism synthesizes a special metabolite in the appropriate growth phase is also dependent on several environmental factors. Parameters possibly influencing the production of secondary metabolites are temperature, water activity, trace elements, carbon source, nitrogen source, pH, biological interactions, chemical agents etc.

c) Synthesis in Chemical Families and Synthesis from "Blocks"

Although a gigantic variety of secondary metabolites with diverse structure and biological activity have been isolated and characterized during the last century, they are synthesized from a limited number of simple precursors via only a few different pathways. Therefore Mann (1978) classified the secondary metabolites into 3 groups according to their precursors:

– Shikimic acid: precursor of aromatic compounds like aromatic amino acids, cinnamonic acid and polyphenols,
– Amino acids: precursor of alkaloids and peptide antibiotics and
– Acetate: precursor of isoprenoids (terpenes, steroids, carotinoids) and acetogenins (polyacetylene, fatty acids, polyketides).

Variation occurs from manifold combination of those pathways and/or complex modifications of common intermediates in later stages of the synthetic pathways. In most cases, only these intermediates have been isolated and characterized, not the enzymes catalyzing secondary metabolite synthesis. Furthermore, linkage between primary and secondary metabolism pathways is very complex and unidentified for most idiolites.

d) Synthesis not Essential for the Producing Organism

Secondary metabolites are dispensable in cellular development and survival. For most of them their relevance for the producing individual remains unknown. Nevertheless, complex synthetic pathways for substances seemingly not essential for the synthesizing organism have been developed in various species or strains.

1.1.2 Possible Importance and Evolution of Secondary Metabolites

There have been numerous speculations on the possible origins and functions of secondary metabolites. More specific theories about functions for the producing organisms have been drafted for some secondary metabolites, serving as competitive weapons (e. g. β-lactam antibiotics), metal transporting agents (e.g. siderophores produced by *Streptomyces* spp.), messengers in symbiosis (e. g. auxin production by *Rhizobium* spp.), sexual hormones (e. g. trisporic acids produced by *Mucorales*) or effectors in differentiation of spores (e. g. close relationship between spore formation and antibiotics production in *Bacillus* spp.) (Demain and Fang, 2000). More general hypotheses include laboratory artifacts (Woodruff, 1980); waste products to drain overflow of components of the primary metabolism and to detoxify those intermediates (Zähner *et al*., 1983); a messenger in cell differentiation and sporulation (Zähner *et al*., 1983); a selective advantage that has been

irreproducible under laboratory conditions, like an ecological role in defense or concurrence reactions (Aharonowitz and Demain, 1980) or a general increase of survival fitness (Maplestone *et al.*, 1992). Jarvis (1995) has summarized these speculations into the following hypothesis: Idiolites perhaps first arose as waste or shunt products but have been modified in the course or evolution to serve as messengers in internal or external communication: "secondary metabolites are chemicals released within a *system* by one component which are meant to convey *information* and *instructions* to another component(s) within the system."

This indicates, that random mutations and evolutionary pressure lead to the natural selection of substances because of their significance to the producer that remains yet to be determined. As only a limited percentage of the infinite variations that would be possible for secondary metabolites were reported to occur in nature, it seems reasonable that some kind of selection is at work (Zähner *et al.*; 1983). In fact, biological activity (which does not equal toxicity!) of secondary metabolites tends to increase in the course of the biosynthesis pathways (Jarvis, 1995; Bu'Lock, 1980). On the other hand, it seems plausible, that the great variety observed with secondary metabolites would only be possible if the substances remained "neutral" in relation to evolutionary benefits or detriments (Bu'Lock, 1975). As evolution is not a past process but continually in progress, both explanations might be suitable: Some of the secondary metabolites might be neutral and therefore not selected in any way. Some might become beneficial to the producing organisms with precursors and energy enough to spare in order to modify them further to present an advantage in the systemic environment they inhabit.

It is very likely that genes involved in secondary metabolism originated from duplication of genes involved in primary metabolism. Those spare gene copies were free to random mutation and in consequence developing new functions that might have been beneficial, neutral or detrimental concerning the producer's survival fitness. Enzymes catalyzing biochemical reactions in secondary metabolism often are in composition and function very similar to or a variation of those from primary metabolism.

However, there is a notable difference in gene arrangement: Genes involved in primary metabolism are usually scattered throughout the genome; genes involved in secondary metabolism are usually at least partially clustered. In filamentous fungi, this is true for the tricothecenes (Hohn *et al.*, 1993), the aflatoxins (Yu *et al.*, 1995) and the β-lactam antibiotics (Martin, 1998; Diez *et al.*, 1990). Some of the organisms possessing one of these gene clusters might have obtained it or a less evolved version of it by horizontal gene transfer (Jarvis, 1995). This might explain the dispersal of the β-lactam antibiotics, produced both in bacteria and fungi (Smith *et al.*, 1990), or ochratoxin biosynthesis in some *Aspergillus* as well as some *Penicillium* species. Another possibility for parallel development of similar pathways in secondary metabolism might be the accumulation of similar intermediates because of the gene duplication of the same primary metabolism genes (Zähner).

The effects of secondary metabolites reported so far on other organisms, especially toxicity against humans, has to be in no way identical to the actual evolutionary role of this substances for the producer (Bu'Lock, 1975). Toxicity of some microbial and plant idiolites to humans might be an accidental parallel development in evolution or more likely a question of common ancestors to receptor structure (Jarvis, 1995). Moreover, even for the best studied secondary metabolites, the antibiotics, inhibitory effects on other microorganisms have been observed in laboratory culture but very rarely in soil. In low concentrations, however, some of those antibiotics even act as growth stimulants. It simply appears impossible to survey the complex network of chemical interactions within a naturally occurring system (Bu'Lock, 1975).

1.2 Mycotoxins

Mycotoxins are a group of secondary metabolites of diverse chemical structure and biological activity synthesized by fungi, most of all by ascomycetes. There are only five agriculturally important mycotoxins: (deoxy-)nivalenol, zeareleone, ochratoxin A, fumonisin and aflatoxin (Miller, 1995). Occurrence of mycotoxins in feed and foodstuffs as well as conditions influencing mycotoxin production on agricultural products have been reviewed by Moss (1996a, 1991) and Bu'Lock (1980).

All of the mycotoxins mentioned above are considered important because of their increasing abundance and their toxicity to plants, animals or humans (Pitt, 2000). Toxic effects, tolerance levels of daily intake and molecular mechanisms of the toxic reactions have been extensively studied in the last decade and reviewed by Peraica *et al.* (1999), Hussein and Brasel (2001) and most recently by Yiannikouris and Jouany (2002). For many mycotoxins threshold values are deployed in many countries (e.g. commission regulation of the EU, Creppy, 2002). Nevertheless, occurrence of synergistic reactions between some of those mycotoxins have been shown to occur even if the concentration of each toxin is lower than the tolerable daily intake (TDI) levels. For example, ochratoxin A has been shown to boost the mutagenic effect of aflatoxin even when both substances are given to poultry in concentration well below the TDI levels (Sedmíková *et al.*, 2001; Huff *et al.*, 1998). A possible explanation for this heavy synergism might be that aflatoxin inhibits DNA transcription and ochratoxin A represses the translation reactions (Creppy *et al.*, 1995). In other experiments synergism of OTA and citrinin has been observed (Sandor *et al.*, 1991), which is especially problematic because some *Penicillium verrucosum* strains are known to produce both substances at once (Castella *et al.*, 2002).

The moulds producing agriculturally important mycotoxins can be classified into 4 groups: (1) plant pathogens like *Fusarium graminearum*, (2) opportunists like *F. moniliforme*, (3) fungi that initially colonize the soil and only induce losses and mycotoxin production after harvest like *Aspergillus flavus*, *A. ochraceus* and (4) fungi growing on dead plants like *Sordaria* or *Mucorales* species (Størmer, 1992).

For some of those mycotoxins not only their toxic effects on any consumer but also a function in phytopathology is known like deoxynivalenol (*Fusarium* spp./barley) or alternariol (*Alternaria alternata*/tomatoe). Some toxins of the genus *Aspergillus*, e. g. Aflatoxin and sterigmatocystin were identified as phytotoxic compounds (McLean *et al.*, 1992; Stoessl, 1981), but their exact role in plant fungus interaction is unknown (Desjardins & Hohn, 1997).

1.2.1 Aflatoxin

Aflatoxin is the most extensively studied *Aspergillus* mycotoxin. Apart from identification of all intermediates, enzymes and genes involved in the aflatoxin biosynthetic pathway, also peripheral parameters have been clarified: linkage between primary metabolism and aflatoxin production, conditions favorable to aflatoxin production, evolution of aflatoxin synthesis genes, and possible functions of this mycotoxin.

The effects of temperature, pH, trace elements, carbon and nitrogen source on aflatoxin have been studied by many authors. In conclusion, aflatoxin biosynthesis seems to be closely linked to depletion of nitrogen or trace elements (apart from zinc which has a stimulatory effect on aflatoxin biosynthesis) while level of carbon source remains high. Active growth has ceased but energy sources are still available. Nevertheless, these conditions do not always trigger aflatoxin production or if they do, not always to the same extent. Instead, aflatoxin production in strains of *Aspergillus flavus* has been observed to be instable within a certain species as well as in laboratory isolates from one strain (Lemke *et al.*, 1989; Lee *et al.*, 1986; Leiach and Papa, 1974; Mayne *et al.*, 1971).

When studying aflatoxin production by *Aspergillus flavus*, Detroy and Hesseltine (1968) first made the general observation of an inverse relation between fatty acid and polyketide synthesis. Later on, the linkage of aflatoxin pathways to the primary metabolism in *Aspergillus flavus* became clearer: Shift of precursors of the fatty acids to polyketide synthesis is triggered by accumulation of acetylCoA and limited NADPH availability. Under these circumstances fatty acid synthesis is repressed and polyketide synthesis is favored (reviewed by Bennett and Christensen, 1983).

The word polyketide originated from a theoretical work of Collie (1907). Experimental proof for the existence of such substances was given almost five decades later by Birch & Donovan (1953). Biosynthesis of polyketides is analogous to the fatty acid synthesis without the enzymatic dehydration and reduction steps. Synthesis starts with condensation of an enzyme bound acetylCoA with several malonylCoA units accompanied by decarboxylation. Individual compounds arise from different ways of aromatization and following modification of the ring system such as cleavage of the aromatic ring, oxidation, reduction or methylation. Fungi produce predominantly tetra-, penta-, hepta- and octaketides, much less common are tri-, hexa-, or decaketides (Turner, 1976).

After gene duplication of fatty acid synthesis genes polyketide synthases evolved via random mutations in those genes. This led to the formation of polyketides with certain biological activities. Those (toxic) substances first provided some small selective advantage in the natural environment of the producing aspergilli. This might have led to gradual development of more powerful substances. Anthraquinone, an early precursor in aflatoxin biosynthesis, is produced by species of 6 groups within the aspergilli, xanthone in 4 groups and aflatoxin in only one (Moss, 1972). The end product of this biosynthesic pathway, aflatoxin, is the most powerful, therefore it seems likely that it was selected specifically for this biological activity (Bennett and Christensen, 1983). If the toxicity towards humans and animals coincides with the "real" evolutionary functions of aflatoxins, remains to be elucidated.

However, speculations about those functions in the natural environment are all connected to toxicity. Aflatoxin might simply be a means of defense of the fungus against being eaten by insects (Bennett and Christensen, 1983). Aflatoxin might also be a weapon of the fungus to protect its nutrient source, the hosting plant, against herbivores (Janzen, 1979). Another possibility is that the plant might protect itself against those herbivores by cultivating a toxic fungus (Harborne, 1972). Neither of these hypotheses, of course, have been or will be verifiable in laboratory experiments.

1.2.2 Ochratoxin

Ochratoxins are a family of mycotoxins produced by several *Aspergillus* and *Penicillium* species. They were initially described by Scott (1965) in *Aspergillus ochraceus* but have also been found in other species of the *sectio Circumdati: A. alliaceus*, *A. melleus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *A. sulphureus* (Hesseltine *et al.*, 1972), *A. albertensis*, *A. auricomus* (Varga *et al.*, 1996); as well as in the black aspergilli of *sectio Nigri: A. niger* var. *niger*, *A. carbonarius* (Samson, 1994). OTA production has also be shown for most strains in *Penicillium nordicum* and in lower concentrations and less frequently by strains of *P. verrucosum* (Castella *et al.*, 2002).

a) Sources of Contamination

In moderate climates, *Penicillium* species seem to be the predominant OTA producers (Gock *et al.*, 2003). OTA in European products like grain from Germany or Scandinavia is mostly due to contamination with *Penicillium* species. On the other hand, grapes from France, Spain and Italy were reported to be mainly contaminated with *Aspergillus carbonarius* (Battiliani *et al.*, 2003; Cabañes *et al.*, 2003; Sage *et al.*, 2002). Primary sources of OTA in the food chain are grains like barley, wheat and maize (Scudamore and MacDonald, 1998), rye and oats (Bauer and Gareis, 1987). OTA accumulates in grain pods (Alldrick, 1996) in concentrations up to 27.5 mg/kg (Størmer, 1992). Widespread survey of grains in Germany showed that 11-13 % of the samples were contaminated

with an average concentration of 2.9 µg OTA/kg grain (Frank, 1991). Average contamination is well below the proposal of the WHO (CCCFA, 1997) of maximal residue level (MRL) of 5 µg OTA/kg grains as well as the commission regulation of the Scientific Committee for Food (European Union, 2002): 5 µg OTA/kg raw cereal grains and 5 µg/kg for all cereal products and cereals directly for human consumption.

Furthermore, OTA can be found in beans, figs, raisins, olives, nuts and spices (Abarca *et al*., 2003; Thirumala-Devi *et al*., 2001; El-Kady *et al*., 1995) as well as in coffee (Bucheli and Taniwaki, 2002; Patel *et al*., 1997; El-Kady *et al*., 1995) and medical and tea herbs (Halt, 1998). OTA is also present in products derived from the foodstuffs listed above, like beer (Jorgensen, 1998; Scott, 1996; Scott and Kanhere, 1995), wine and grape juice (Otteneder and Majerus, 2000; Burdaspal and Legarda, 1999). Whereas the WHO suggested MRLs of 8 µg OTA/kg for green coffee beans and 4 µg/kg for roasted coffee beans, 0.2 µg/kg for beer and 0.2-1 µg/kg for wine, the EU Scientific Committee for Food only defined a compulsory MRL of 10 µg OTA/kg of dried grapes. No threshold values have been given for OTA in green and roasted coffee, beans, beer, wine, grape juice or spices.

Another source of OTA intake are animal products, contaminated by carry over of OTA from contaminated feed (Scudamore *et al*., 1998a, 1998b, 1997; Abramson *et al*., 1997) to milk (Skaug *et al*., 2001a), pork and poultry (Hohler, 1998, Jorgensen, 1998) up to 70 µg/kg (Størmer, 1992). OTA has been found in human blood sera, liver samples and mother's milk in various concentrations (Valenta, 1998; Radic *et al*., 1997; Solti *et al*., 1997).

b) Toxicity of Ochratoxin A

OTA is toxic without prior metabolic activation (Moulé, 1984). OTA itself is not mutagenic in bacterial cultures (Bárta *et al*., 1997; Heller *et al*., 1975) but displays mutagenic effects only on eukaryotic cells (Castegnaro *et al*., 1991, Bendele *et al*., 1985). After formation of adducts in liver and kidney, however, mutagenicity in Ames-tests could be observed (Pfohl-Leskowicz *et al*., 1993). Toxicity of OTA is species, cell and gender specific (O'Brien *et al*., 2001). OTA is primarily a nephrotoxin in pigs, but has also genotoxic, immunosuppressive, teratogenic and carcinogenic effects on rodents (Petzinger and Ziegler, 2000; Kuiper Goodman, 1996; Størmer, 1992; Huff, 1991). OTA was classified as a potential carcinogen for humans by the International Agency of Cancer (group 2BI, IARC, 1993).

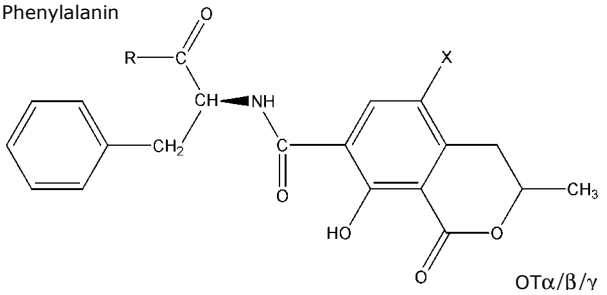
The molecular mechanism of the toxic effects of OTA so far remains unknown. Observed toxic effects of OTA include chromosomal aberrations, inhibitory effects on protein biosynthesis and coagulation, inhibition of mitochondrial ATP production and gluconeogenesis as well as induction of lipid peroxidation and apoptosis (Petzinger and Ziegler, 2000; Marquart and Fröhlich, 1992). If these toxic effects are connected to any evolutionary function of OTA for the producing fungi or if there is another ecologically valuable feature of ochratoxins, has not yet been investigated or even speculated on.

Wei *et al*. (1985) found experimental proof for one molecular toxicity mechanism: OTA non-competitively inhibits succinate dehydrogenase in mitochondria thereby decoupling the respiratory chain. Creppy *et al*. (1983) and Konrad and Röschenthaler (1977) observed experimental indications for inhibition of phenylalanine-tRNA-synthetase by OTA in a yeast model. But as McMasters and Vedani (1999) recently demonstrated, OTA cannot bind to the phenylalanine-tRNA-synthetase the same way as phenylalanine does and therefore it is not able to work as an inhibitor for this enzyme. They suggested that the toxicity of OTA was rather attributed to binding to and blocking of plasma proteins. Variable half life of OTA in blood sera is due to different binding capacity to plasma proteins and serum albumin (Hagelberg *et al*., 1989). The longest residence time of up to 840 h was observed in blood sera of pigs and humans, the most sensitive species (Studer-Rohr, 2000). Subsequent accumulation of OTA in liver and kidney is attained via active transport processes (Schwerdt *et al*., 1996). Zanic-Grubisic *et al*. (2000), however, argued that OTA displays

toxicity by binding to phenylanalnine hydroxylases and toxic effects can be reversed by high concentrations of phenylalanine.

c) Biosynthesis of Ochratoxins

Chemically, the ochratoxins are a family of mycotoxins composed of a polyketide and (modified) phenylalanine linked via a peptide bond (Fig. 1.1). Ochratoxin A, for example, is *N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)-carbonyl]-3- phenyl-L-alanin (van der Merwe *et al.*, 1965).



	-R	-X
OTA	-OH	-Cl
OTB	-OH	-H
OTC	$-OCH_2CH_3$	-Cl

Fig. 1.1: Chemical structure of ochratoxins

Integration of labeled phenylalanine into OTA has been shown by Ferreira and Pitout (1969). The polyketide residue is synthesized via the acetate malonate pathway: condensation of one acetate and 4 malonate units with subsequent cyclization and aromatization, methylation, oxidation and chlorination (Steyn and Holzapfel, 1970). The carbonyl-C(12) is derived from the C_1-pool (Steyn, 1993). Chlorine has been shown to be incorporated directly (Wei *et al.*, 1971). A possible scheme leading to the biosynthesis of OTA was published by Huff and Hamilton (1979). According to their hypothesis, 3 distinct steps occur in OTA biosynthesis: The first part is polyketide synthesis of ochratoxin α via mellein involving a polyketide synthase, followed by acyl activation: Mellein is methylated and oxidized to 7-Carboxy-Mellein (=OTβ). Chlorination by a chloroperoxidase leads to OTα. This component is then transformed to a mixed anhydride, an activation reaction using ATP. The second precursor phenylalanine is synthesized via the shikimic acid pathway, followed by ethyl ester activation so that it can participate in the subsequent acyl displacement reaction. In the third step, linkage of those activated precursors via a synthetase takes place, generating OTC, an ethyl ester of OTA: De-esterification by an esterase or transesterification is the last step in this postulated biosynthetic pathway (Fig. 1.2).

This schematic pathway has been dissented by Harris and Mantle (2001). They found no evidence for an intermediate role of (methyl-)mellein and OTC in the OTA biosynthetic pathway. Instead, they suggest a not specified pathway leading from OTβ (de-chlorinated form of OTα) to OTα to OTA.

One of the enzymes required in any of this biosynthetic schemes is a “OTA synthetase”. This enzyme is presumed to catalyze the last step of the OTA biosynthesis generating ochratoxin A from the proposed precursors (modified) ochratoxin α and (modified) phenylalanine. Existence of the OTA synthetase was originally postulated by Ferreira and Pitout (1969). They observed formation of OTA from its precursors OTα and phenylalanine in cell free extracts of *Aspergillus ochraceus* Wilh. CSIR804.

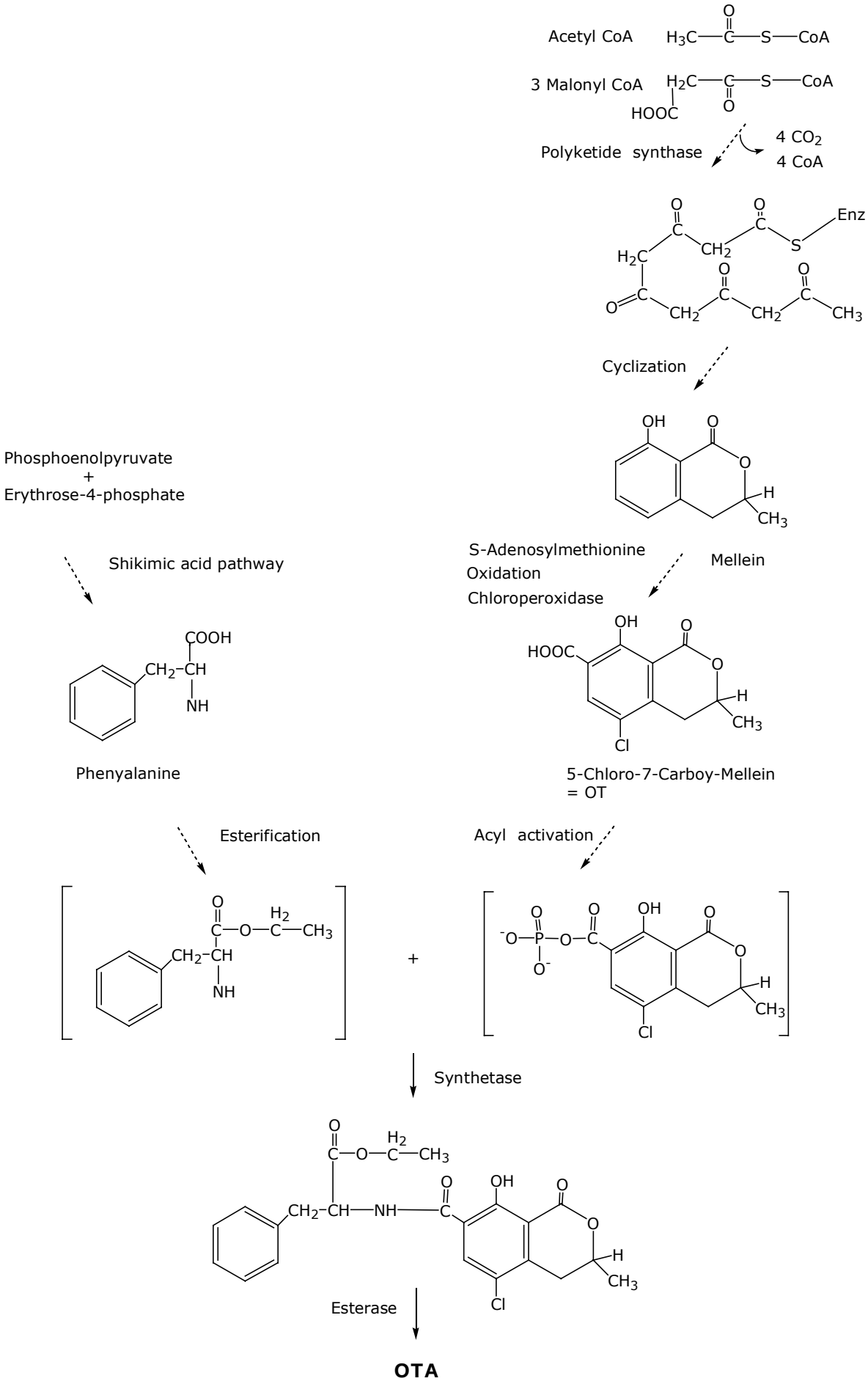


Fig. 1.2: Scheme of the hypothetic OTA biosynthetic pathway published by Huff and Hamilton (1979)

d) Regulation of OTA Production by Culture Conditions

The OTA producing organism used in this study, *Aspergillus ochraceus*, grows on several substrates between 8 and 37°C with an optimum for growth at around 24°C depending on water activity of the medium. Tolerated a_w-values range between 0.8–0.99 (Sweeney and Dobson, 1998). Temperature and water activity optimal for OTA production are different from those optimal for growth, but the values reported by several authors differ greatly: OTA production within a range of 12–37°C with an optimum at 31°C (Sweeney and Dobson 1999).

As observed for almost all secondary metabolites, ochratoxin biosynthesis is dependent on environmental growth conditions and regulated by several interwoven environmental signals. OTA production in *Penicillium* strains seems to be very abundant and relatively stable. OTA producing and non-OTA producing strains could be grouped into two distinct clusters by molecular characterization methods (Castella *et al.*, 2002; Larsen *et al.*, 2001b). On the other hand OTA producing ability in aspergilli could not be grouped so easily (Schmidt *et al.*, 2003). This might be a sign of heterogeneity occurring in OTA production by closely related strains. More likely, data concerning OTA production are not reliable because of instabilities in OTA production by *Aspergillus* strains.

Ochratoxin synthesis in aspergilli is known to be dependent on several factors and has even been reported to be sporadic or even completely lost during cultivation in the laboratory (Varga *et al.*, 1996). Percentages of OTA producing strains of *A. ochraceus* and *A. niger* isolated from various natural sources were reported to be highly variable (10–54.5% and 1.7–18.5%, resp.) (Abarca *et al.*, 2001). Several authors reported the effect of various changes in culture conditions on OTA production by *Aspergillus* strains. The results observed were as diverse as the exact conditions applied e.g. kind of medium, pH, strain, temperature and whether it was a shaken or stationary incubation. However, most authors observed no correlation between growth phase, biomass production and OTA production. Chelack *et al.* (1991) speculated on a connection between OTA production and cell differentiation dependent on the kind and phase of mycelial growth. With most *Aspergillus* strains, better OTA production on solid substrates was observed (Moss, 1991). Furthermore, differences in observations could be a result of variable production levels of OTA by different mycelia, even originating from the same spore suspension. Röschenthaler *et al.* (1984) observed varying amounts of OTA produced in single spore cultures isolated from one *Aspergillus* mycelium. This would explain divergent results on OTA producing ability and therefore difficulties in the classification of OTA positive and negative strains.

1.3 Aim of the present study

The aim of this study was to work out a suitable basis to set up a molecular diagnostic tool for early detection of ochratoxigenic fungi on raw materials, feed and foodstuffs. Development of such a molecular diagnostic method is a vital prerequisite in order to ensure safety of these agricultural products by not only being able to detect already existent contamination with ochratoxin A, but also the occurrence of organisms capable of OTA production during further storage and processing.
For optimal performance, these molecular tools need to be based on special genetic features of ochratoxigenic *Aspergillus* spp. Except for the application of species or group specific methods, the most promising approach on detection of *Aspergillus* spp. with OTA production potential would be aimed at genes and enzymes of the biosynthetic pathway leading to OTA formation.

First of all, constancy of OTA production in laboratory culture as well as the availability of a reliable means to induce or suppress OTA production by altering no more than one growth factor were important preconditions. In order to be able to predict and control OTA production by *Aspergillus* spp. used in this study, detailed knowledge about the external factors affecting OTA production had to be gathered prior to work on OTA biosynthesis enzymes and genes.

In this study, one of the approaches on setting up a basis for molecular diagnostic tools was characterization of the putative "OTA synthetase" on the level of enzyme activity. This key enzyme presumably catalyzes the final step of the OTA biosynthetic pathway. The approach was based on the reasonable hypothesis, that this apparently new and unknown type of enzyme should be common to all ochratoxigenic fungi and thereby presenting a suitable target for a generic molecular diagnostic tool for the relatively heterologous group of OTA producing fungi.

In parallel, elucidation of key genes of OTA biosynthesis was attempted using molecular genetic methods. By means of techniques like cDNA-AFLP, gene expression patterns under OTA submissive or non-submissive conditions in growth stages before and after onset of OTA production can be compared. Genes expressed in connection to OTA biosynthesis can be detected employing this multifactor comparison.

Finally, PCR based diagnostic assays should be set up for detection of ochratoxigenic fungi exploiting sequence information from genes or enzymes connected to OTA biosynthesis acquired in the enzymatic and genetic approaches used within this study.

2 Material and Methods

2.1 Material

2.1.1 Equipment

a) Consumables and Kits

2-K-2 Ultra Component Cell Preps Kit	Bio Basic Inc., BioCat, Heidelberg
Centrifuge vessels	15 mL and 50 mL, Sarstedt
E.Z.N.A. Gel Extraction Kit	peqlab GmbH, Erlangen
E.Z.N.A. Plasmid Miniprep Kit I	peqlab GmbH, Erlangen
Filters	fluted filter 595 1/2 Ø 110 mm, Schleicher & Schuell
	fluted filter 595 1/2 Ø 385 mm, Schleicher & Schuell
	Spartan 3/0.45 PA syringe filters, Schleicher & Schuell
Flasks	Duran, ISO 4796, Schott Glas
Conical flasks	25 and 50 mL, Witeg
Funnel	100 mL, Squibb, Witeg
LightCyclerTM capillaries	Roche Molecular Biochemicals, Mannheim
Mikrowell plates	FluoroNuncTM, Cat.-No.: 475523 F16, Nunc GmbH
MultiTM SafeSeal Tubes 1.5 mL	peqlab GmbH, Erlangen
oligo (dT)15 primer	Promega GmbH, Mannheim
Petri dishes	92 x 16 mm,Sarstedt
Qiaquick® PCR Purification Kit	(Qiagen GmbH, Hilden)
QuantiTectTM SYBR® Green PCR Kit	Qiagen GmbH, Hilden
RNase-Free	BioLogicalTM Molecular Reagents, Continental Laboratory Products
RNAfree DNase	Promega GmbH, Mannheim
RNase H minus	M-MLV Reverse Transcriptase, Promega GmbH, Mannheim
	peqlab GmbH, Erlangen
RNase free PCR reaction tubes	Qiagen GmbH, Hilden
RNeasy® Plant Mini Kit	
Safe GuardTM filter tips	peqlab GmbH, Erlangen
Sucrose/D-Glucose/D-Fructose UV-method Kit	Cat. No. 0716260, r-biopharm, Darmstadt, Germany
TLC-plates	TLC-aluminium foils 20 x 20 cm, silicagel 60 F254, Art. No. 1.05554, Merck

b) Equipment

Analysis scales	BP 210 S, Sartorius AG, Göttingen
Autoclave	2540 ELV, Systec GmbH, Wettenberg
Centrifuges	Sigma 1 K 15, Sigma Labortechnik
	Hermle Z 383 K, Hermle Labortechnik
Clean bench	Hera Safe, Heraeus, Hanau
Climatic exposure cabinet	Memmert SLP 500, Memmert GmbH & Co. KG
Electrophoresis chamber	12*14 cm, MWG Biotech AG, Ebersberg
FPLC	BioLogic HR Controller, BioLogic HR Workstation,
	Model 2128 Fraction Controller, BioRad Laboratories
Gel filtration column	SuperdexTM 200 prep grade, Amersham Biosciences, Freiburg

Ion exchange column	BioRad EconoPac Q Cartridge 5 mL, BioRad Laboratories, Richmond, CA, USA
HPLC	Merck LiChroCART 250-4 LiChrospher 100 RP 18e Fluorescence Detector LC 240, Perkin Elmer Binary LC Pump 250, Series 200 LC Pump, Advanced Sample Processor ISS 200, Perkin Elmer
Laboratory shaker	Certomat BS-1, B. Braun Biotech International REAX 2, Heidolph Instruments
Light CyclerTM	Roche Molecular Biochemicals, Mannheim
Membrane vacuum pump	ME4, Vakuubrand GmbH & Co., Wertheim
pH meter	Mikroprozessory 761 Calimatic, Knick GmbH & Co., Berlin Mettler-Toledo-Electrode InLab 412, pH 0...14
Photometer	Pharmacia Biotech Novaspec II, Amersham Biosciences, Freiburg
Rotary evaporator, Water bath	VV 2200, WB 2000, Heidolph Instruments GmbH & Co KG , Schwabach
Membrane pump	Vacuumcontroller Laboport, KNF Neuberger GmbH, Freiburg
SPECTRAFluor	TECAN SPECTRAFluor, TECAN Deutschland GmbH Filter: exitation: 380±10 nm, emission 448±5 nm
Thermal cycler	Mastercycler® Gradient, Eppendorf AG Hamburg
Ultrasonic Processor	UP 200s, Dr. Hielscher GmbH, Teltow (Berlin)
UV transilluminator	UVIS-Doustrahler, 75 W, 254 nm und 366 nm, Desaga

2.1.2 Chemicals

All chemicals not listed below were obtained in analysis purity from Merck KGaA, Darmstadt.

Agar	European Agar	Difco
Complete™ Protease-Inhibitor-Cocktail		Roche
DTT (Dithiothreitol)	high purity	Gerbu
Ethyl acetate	HPLC-grade	J.T. Baker
$Fe_2(SO_4)_3$ x 5 H_2O	97%	Sigma-Aldrich, Chemie GmbH, Schnelldorf
Lipase A "Amano" 6		Amano
Methanol	HPLC-grade	Sigma-Aldrich Chemie GmbH, Schnelldorf
$(NH_4)_6Mo_7O_{24}$ x 4 H_2O		Sigma-Aldrich Chemie GmbH, Schnelldorf
Ochratoxin A		Fluka Riedel-de-Haen, Neu Ulm
Ochratoxin B		Sigma, St. Louis
Tris Base	"Ultra Pure"	ICN
Tris-HCl		Boehringer, Mannheim
Water for molecular biologiy	certified DNase and RNase free	Sigma-Aldrich Chemie GmbH, Schnelldorf
Yeast extract		Difco, BD Biosciences, Heidelberg

2.1.3 Oligonucleotides

Bt2a primer (Glass and Donaldson, 1995)	5'GGTAACCAAATCGGTGCTGCTTTC 3'
Bt2b primer (Glass and Donaldson, 1995)	5'ACCCTCAGTGTAGTGACCCTTGGC 3'
AFLP adapter (Lan and Reeves, 2000)	5'CCTGATTGCTACAACTGAACGATGAGTCCTGAG 3'
	3'TACTCAGGACTC 5'
*Mse*I primer (Vos *et al.*, 1995)	5'GATGAGTCCTGAGTAA 3'
MAC primer (Vos *et al.*, 1995)	5'GATGAGTCCTGAGTAAAC 3'
3for	5'GGGCTGTCCTGACCGATG 3'
3rev1	5'TCACATGCCCGGCCGACA 3'
3rev2	5'ACGGGAGGCGGAGGAAGA 3'
4for	5'GACGACGGTGTTCCTGGA 3'
4rev1	5'CGGGACCCAATAGGGGTT 3'
4rev2	5'CCGGGCGAGAGTTCAATC 3'

2.1.4 Buffers

TBS (Tris Buffered Saline)

pH 7.5

2,4 g	Tris Base
8,0 g	NaCl
0,2 g	KCl

ad 1000 mL with aqua dest.

Tris Buffer

50 mM, pH 7.5

6,46 g	Tris-HCl
1,09 g	Tris-Base

ad 1000 mL with aqua dest.

Protein Extraction Buffer (Ferreira and Pitout, 1969)

20 mM $NaHCO_3$, 50 mM Tris, 0,001 M DTT

6,46 g	Tris-HCl
1,09 g	Tris Base
1,68 g	$NaHCO_3$

ad 1000 mL with aqua dest.
Add 7.7 mg DTT and 1 tablet Complete™ Protease Inhibitor to 50 mL buffer before use.

Elution Buffer A for Ion Exchange Chromatography (low salt buffer)

50 mM Tris, pH 7.5

6,46 g	Tris-HCl
1,09 g	Tris Base

ad 1000 mL with aqua dest.

Elution Buffer B for Ion Exchange Chromatography (high salt buffer)

50 mM Tris, pH 7.5, 1 M NaCl

6,46 g	Tris-HCl
1,09 g	Tris Base
58,50 g	NaCl

ad 1000 mL with aqua dest.

PCR Reaction Buffer (10x)

pH 8.5 pH 9.2

100 mM	Tris-HCl
500 mM	KCl
25 mM	$MgCl_2x6H_2O$

PCR Reaction Buffer N (10x)

100 mM	Tris-HCl
500 mM	KCl
15 mM	$MgCl_2x6H_2O$

AFLP Buffer

pH 8.3

11.26 mM	Tris-HCl
1.69 mM	$MgCl_2$
56.30 mM	KCl

Stop Buffer for Electrophoresis

0.25 %	Bromophenol blue
0 % (w/v)	Sucrose

100 bp Ladder

100 µL	Stock solution (Amersham Biosciences, Freiburg)
200 µL	Stop buffer
1700 µL	Aqua dest.

T(B)E Buffer (10x)

pH8

0.9 M	Tris Base
(0.9 M	Boric acid)
0.2 mM	EDTA

PAGE Running Buffer

pH 8.3

9.0 g	Tris-HCl
43.2 g	Glycine
(3.0 g	SDS) (+ SDS for denaturing PAGE, – SDS for native PAGE)

ad 600 mL with aqua dest.

2.1.5 Media

If not indicated otherwise, all media were sterilized by autoclaving at 121 °C, 2 bar for 21 min. Media containing agar were poured into Petri dishes. Additives were sterilized by filtration and added after autoclaving. Media and additives were either prepared freshly before use or stored at 4 °C (media, trace element stock solutions) or – 20 °C (ampicillin, X-Gal), respectively.

2.1.5.1 Media for Cultivation of Fungi

Malt Extract Agar (MEA)

pH 5.6

20.0 g	Malt extract
2.0 g	Peptone
15.0 g	Agar

ad 1000 mL with aqua dest.

Autoclave for 15 min only!

Yeast Extract Sucrose (YES) (complex medium)

pH 6.5

20.0 g	Yeast extract	(Merck GmbH, Darmstadt/Difco, BD Biosciences,
150.0 g	Sucrose	Heidelberg)
(1.0 g	$MgSO_4x7\ H_2O$)	

ad 1000 mL with aqua dest.

If necessary, adjust pH with 0.25 M NaOH.

AM-Medium (synthetic medium modified from Adye and Mateles, 1964; Lai *et al.*, 1970)

pH 6.5

5.1 g	KH_2PO_4

ad 380 mL with aqua dest.

Adjust pH to pH 6.7 (initial pH: 6.5) or pH 5.3 (initial pH: 5.0), respectively.

27.5 g	Glucose x H_2O

ad 100 mL with auqa dest.

Autoclave both solutions separately!

Combine both solutions after autoclaving and add stock solutions:

Stock Solutions (filter sterilized)

0.35 g / 50 mL	$Na_2B_4O_7$ x 10 H_2O
0.27 g	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O
0.61 g	$Fe_2(SO_4)_3$ x 5 H_2O
0.15 g	$CuSO_4$ x 5 H_2O
0.04 g	$MnSO_4$ x H_2O
0.88 g	$ZnSO_4$ x 7 H_2O

Add 50.0 µL each to 500 mL of AM medium after autoclaving.

15.0 g / 50 mL	NH_4NO_3
5.0 g	KCl
5.0 g	$MgSO_4$ x 7 H_2O

Add 5.0 mL each to 500 mL of AM medium after autoclaving.

2.1.5.2 Media for Cultivation of Bacteria

LB Medium (Sambrook *et al.*, 1989)

pH 7.2 -7.3

10.0 g	Peptone
5.0 g	Yeast extract
5.0 g	NaCl
(15.0 g	Agar)

ad 1000 mL with aqua dest.

Optional: add 100 mg / L ampicillin and 40 mg / L X-Gal.

SOB Medium (Ausubel *et al.*, 1987)

2 %	Tryptone
0.5 %	Yeast extract
10.0 mM	NaCl
2.5 mM	KCl
10.0 mM	$MgSO_4x7H_2O$
10.0 mM	$MgCl_2x6H_2O$

ad 1000 mL with aqua dest.

2.1.6 Organisms

All *Aspergillus* spp. (selected for their OTA production capabilities as specified by the supplier) used in this study are listed in Table 2.1. Fungi were maintained as spore stock cultures in 80% glycerol (v/v) at – 80°C.

Table 2.1: Strains used in this study. AM: AM liquid medium. CCA: Coconut cream agar. CZA: Czapek agar. MEA: Malt extract agar. YES: Yeast extract sucrose liquid medium

Species	Strain	Source of isolation	OTA production described by the Supplier (medium)
A. awamori	CBS 101704 [1]	unknown	+ (CZA, MEA)
A. carbonarius	M 335 [2]	coffee, Thailand	+ (CCA)
A. carbonarius	M 336 [2]	coffee, Thailand	+ (CCA)
A. fresenii	CBS 550.65 [1]	soil, India	+ (MEA, CZA)
A. ochraceus	A8 [3]	unknown	+ (YES)
A. ochraceus	CBS 588.68 [1]	Piper sp., USA	+ (MEA, CZA)
A. ochraceus	CBS 589.68 [1]	Piper sp., USA	+ (MEA, CZA)
A. ochraceus	CCT 6819 [4]	drying area, Saõ Paulo	– (MEA)
A. ochraceus	M 337 [2]	coffee, Thailand	+ (CCA)
A. ochraceus	NRRL 3174 [5]	unknown	+ (MEA)
A. ochraceus	TMW 4.1357	comm. coffee sample	– (YES, AM)
A. niger	CBS 101698 [1]	coffee, Kenya	+ (MEA, CZA)
A. niger var. *niger*	CBS 101697 [1]	coffee, Kenya	+ (MEA, CZA)
A. usamii var. *shiro-usamii*	CBS 101700 [3]	unknown	+ (MEA, CZA)
P. verrucosum var. *verrucosum*	CBS 603.74 [1]	unknown	+ (MEA)
P. verrucosum	M 222 [2]	coffee, Thailand	+ (CCA)

[1] Centraalbureau voor Schimmelcultures (CBS, Fungal Biodiversity Center, Utrecht, The Netherlands)
[2] H.M.L.J. Joosten (Nestle Research Center, Nestec Ltd., Lausanne, Switzerland)
[3] F.J. Cabañes (Universitat Autònoma de Barcelona, Spain)
[4] M.H. Taniwaki (Instituto de Tecnologia de Alimentos, Campinas-SP, Brazil)
[5] obtained as KA 103 from J. Chelkowski (Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland)
[6] R. Geisen (Federal Research Center for Nutrition, Karlsruhe, Germany)

2.2 Methods

2.2.1 Culture Conditions

2.2.1.1 Incubation Conditions

Working cultures were inoculated from the stock cultures and incubated on MEA for 7–10 d at room temperature under UV light.

Spores were harvested by suspension in 5 mL of glycerol:water mixture (1:1) with 200 µL of 25% SDS added. 500 mL of YES or AM liquid medium were inoculated with 1 mL of this suspension (approx. 10^7 spores). All *Aspergillus* spp. were grown as submerse cultures in 1000 mL Schott flasks with screw caps not tightly fastened. Incubation flasks were shaken at 120 rpm in a climatic exposure cabinet (Memmert, Schwabach) at 25°C in the dark for incubation periods up to 504 h (21 d).

2.2.1.2 Modifications of AM Liquid Medium

A recipe for standard AM medium is given in 2.1.5.1. To determine influence of certain growth factors on OTA production, several components of AM liquid medium were modified as described below.

a) Initial Medium pH

Standard AM medium was adjusted to pH 6.5 prior to inoculation. For growth experiments, media were adjusted to various pH values within the range from pH 3.5 to pH 9 with 2 M HCl or 2 M NaOH, respectively.

b) Trace Metals

Standard AM medium was supplemented with trace elements as listed in 2.1.5.1. For growth experiments, AM media were prepared without Zn, Fe, Zn and Fe, Cu and any trace metal originally supplied to AM synthetic medium. Trace metal supply was altered in AM media with 5% glucose at both pH 5.0 and pH 6.5 and in AM media with 2.5%, 0.625% glucose or 5% sucrose, respectively, at pH 6.5.

c) Carbon Source

Standard AM medium contained 5% glucose as carbon source. For growth experiments, glucose concentration of AM media was altered to 10%, 2.5%, 1.25% and 0.625%, respectively. In other setups, 5% glucose was replaced by 5% sucrose, fructose or lactose, respectively.

d) Nitrogen Source

Standard AM medium was supplemented with 3 g/L NH_4NO_3. For growth experiments, this nitrogen source was substituted by 0.5 g $(NH_4)_2SO_4$ and 0.5 g KNO_3, respectively.

2.2.2 Sampling

20 mL of *Aspergillus* spp. cultures grown in AM liquid medium were sampled at several growth stages, usually at intervals of 6, 12 and 24 h, respectively, to determine pH of the culture medium and mycelial dry weight, glucose utilization, and OTA production of the fungal culture.

2.2.2.1 Determination of Mycelial Dry Weight

20 mL of *Aspergillus* cultures at a certain growth stage were filtered through a pre-dried and pre-weighed fluted filter (Ø 110 mm, Schleicher & Schuell). Dry weight of mycelium washed two times with aqua dest. to remove residual glucose was determined after 4 h at 105 °C (MEBAK, 1993).

At the end of each incubation period, the remaining culture medium was filtered through a pre-dried and pre-weighed fluted filter (Ø 385 mm, Schleicher&Schuell). Dry weight of the whole mycelium washed two times with aqua dest. was determined after 48 h at 105 °C.

2.2.2.2 Quantification of Carbon Source Utilization

Utilization of carbon source was determined by the remaining amount of glucose, sucrose, fructose or lactose, respectively, in the culture medium. Glucose, sucrose and fructose concentrations of filtrated culture samples were measured by a photometric method according to the manufacturer's manual (Sucrose/D-Glucose/D-Fructose UV-method Kit, Cat. NO. 0716260, r-biopharm, Darmstadt, Germany).

Lactose concentration was measured with a HPLC detection method (Polyspher® CH PB column, Merck, Darmstadt; mobile phase: aqua dest. 0.4 mL/min, refractive index detector, Gynkotek, Germany).

2.2.3 Detection of OTA Production

Cultures of *Aspergillus* spp. were tested for OTA biosynthesis with different methods, depending on growth media and whether "online" or quantitative data were required.

2.2.3.1 Qualitative Analysis of Ochratoxin Production on Solid Culture Media

7–10 d old cultures of *Aspergillus* strains grown on malt extract agar were tested for OTA production by the agar plug technique as described by Filtenborg *et al.* (1983). Small plugs were cut out of the agar near the middle of the mycelium. Mycotoxins were extracted with 2 drops of cloroform:methanol (1:1). Plugs were briefly set upside down on HPTLC plates and adhering extract was subjected to TLC (see below).

2.2.3.2 Qualitative and Quantitative Analysis of Ochratoxin Production in Liquid Medium

For "online" survey of growing mycelia for OTA production, ochratoxins were verified qualitatively by a rapid detection method. Ochratoxin A in fungal culture samples collected during the entire incubation period stored at –80 °C was quantified HPLC detection method.

a) Qualitative Analysis of Ochratoxins by HPTLC (Nesheim and Trucksess, 1986)

Filtrated samples of cultures of *Aspergillus* spp. were tested for OTA by a rapid detection method: 5 mL of filtrated sample were extracted with 5 mL ethyl acetate after acidification with 1 mL HCl (1 M). Water phase was discarded and solvent was evaporated to dryness in a rotary evaporator at 60 °C. The residue was redissolved in 100 µL methanol. 10 µL of each sample were then subjected to HPTLC (HPTLC-aluminium foils, silicagel 60 F_{254}, Merck; mobile phase: toluol: ethyl acetate: formic acid 6:3:1). Ochratoxins was identified by fluorescence at 254 nm, R_f value and comparison to ochratoxin A, α and B reference substances. Amount of OTA was roughly estimated by comparison to a dilution series of OTA standard substance.

b) Quantification of Ochratoxin A by HPLC (Stettner, 2000)

2 mL of each filtrated culture medium sample were extracted once with 4 mL dichloromethane. Phases were separated by centrifugation (3,500 g, 15 min, 15 °C). Water phase was discarded and 3 mL of a the organic phase were twice extracted with 3 mL 0.13 M $NaHCO_3$. Water phases were pooled and evaporated to dryness. 1 mL of 43 % methanol was added to the dry samples. OTA was then confirmed and quantified by HPLC (isocratic elution with acetonitrile : H_2O : acetic acid 99:99:2 (v:v:v); LiChroCART 250-4 LiChrospher 100 RP 18e; fluorescence detection at 332/465 nm).

2.2.4 Harvesting of Mycelia for Enzyme and RNA Preparations

Fungal mycelia in the appropriate growth stages were separated from the culture media by filtration through a double layer of fluted filters (Ø 385 mm, Schleicher & Schuell). Mycelia were washed twice with aqua dest. and residual water was removed by squeezing the mycelia and filters using several layers of cheese cloth. Semi-dried mycelia were quickly frozen in liquid nitrogen and stored at –80 °C until further use.

2.2.5 Enzyme Purification

The following protocol was set up for purification of a putative OTA synthetase from mycelia harvested shortly before onset of OTA production or during the OTA producing phase of the submerse culture in AM liquid medium.

2.2.5.1 Crude Protein Extract

Mycelia were retrieved from storage at –80 °C, weighed and ground to fine powder in a pre-cooled mortar under constant cooling with liquid nitrogen. Mycelial powder was suspended to a adequate volume of pre-cooled protein extraction buffer. Additional cell disruption was accomplished by 1 min ultrasonic treatment while cooling the cell suspension on ice (Dr. Hielscher UP 200S, 50 % amplitude, cycle 0.5). Lysate containing all soluble proteins was separated from cell debris by centrifugation (10 min, 10,000 g, 4 °C).

2.2.5.2 Further Purification of Crude Extracts

The crude protein extract from mycelia of *A. ochraceus* NRRL 3174 was further purified by precipitation with ammonium sulfate and subsequent separation of protein fractions by gel filtration and/or ion exchange chromatography.

a) Protein Precipitation with $(NH_4)_2SO_4$

Cell lysate prepared as described above was placed on ice and slowly mixed with ammonium sulfate to a concentration of 20 % (w/v). Precipitated protein was separated by centrifugation (15 min, 5,000 g, 4 °C). More $(NH_4)_2SO_4$ was added to the remaining lysate to a concentration of 40 % (w/v). After separation of precipitated protein by centrifugation, $(NH_4)_2SO_4$ concentration of the lysate was increased to the maximum concentration of 60 % (w/v). Precipitated protein was again separated by centrifugation.

Samples of protein precipitated at different $(NH_4)_2SO_4$ concentrations (20, 40 and 60 % (w/v), respectively) were tested for *in vitro* OTA synthesis as described in 2.2.6.2a). Most *in vitro* OTA synthesis activity could be found within the "40 % (w/v) $(NH_4)_2SO_4$ protein fraction". This is in accordance to the results of Ferreira and Pitout (1969), who observed highest levels of activity in protein fractions precipitated with relative $(NH_4)_2SO_4$ saturation of 68–87%.

b) Gel Filtration

A small aliquot (10–100 mg) of a 40% (w/v) $(NH_4)_2SO_4$ protein fraction was suspended in 300 µL TBS buffer and briefly centrifuged (3 min, 10,000 g). The cleared protein suspension was applied with a syringe to the gel filtration column (Superdex™ 200 prep grade, Amersham Biosciences, Freiburg). Fractions of 1 mL volume were collected during the entire run (isocratic flow with 100% TBS, 0.45 mL/min for 40 mL) supported by an FPLC and fraction collector (BioLogic HR Controller, Model 2128 Fraction Controller, BioRad Laboratories).

c) Ion Exchange Chromatography

As ion exchange chromatography (IEC) requires low salt concentrations of the samples initially loaded onto the column, no precipitation with $(NH_4)_2SO_4$ was performed prior to application of this technique. Instead, 1 mL of cell lysate, prepared as described in 2.2.5.1, were filtered with a sterile filter and samples were subjected to a strong basic ion exchange column (EconoPac column, type UnoQ 5 mL, BioRad). Proteins not bound to the ion exchange matrix were washed away with 100% low salt buffer. Elution of bound proteins was accomplished with a linear gradient of 0–100% high salt buffer mixed with low salt buffer. Eluate was fractioned in volumes of 1 mL and collected during the entire FPLC supported run (Isocratic Flow with 100% 50 mM Tris pH 7.5 at 0.2 mL/min for 1 mL; linear gradient with 0% to 100% 50 mM Tris pH 7.5 + 1 M NaCl at 0.20 mL/min for 20.0 mL).

2.2.6 Activity Tests

To monitor the purification progress of the putative OTA synthetase, protein samples taken after continuing purification steps were tested for *in vitro* OTA production from its proposed precursors OTα and phenylalanine. To conduct activity tests it was necessary to set up a reaction for OTα formation, as this substance was not commercially available.

2.2.6.1 Formation of Ochratoxin α from OTA Standard Substance

1 mg of OTA standard substance (Fluka Riedel-de-Haen, Neu-Ulm) dissolved in methanol was evaporated to dryness in a rotary evaporator. Residue was redissolved in 5 mL TBS buffer. 10 mg of Lipase A "Amano 6" (Amano Enzyme Europe Ltd.) were added and the mixture was incubated for 24 h at 37°C. This crude lipase extract from *A. niger* includes a carboxypeptidase degrades OTA into OTα and phenylalanine (Stander *et al.*, 2000, 2001). OTα was then purified by extracting the acidified mixture twice with 10 mL ethyl acetate. Combined organic phases were evaporated and residue was redissolved in 1 mL methanol.

2.2.6.2 Activity Tests

Crude protein extracts and gel filtration fractions were tested in two different setups, both modifications of a method originally published by Ferreira and Pitout, 1969.

a) **Activity Tests with Crude Protein Extracts**

Crude protein extracts and resuspended fractions after 40% ammonium sulfate precipitation were tested for *in vitro* OTA synthesis in reaction tubes.

Setup for each test tube:

50.0 µL	Resuspended sample
30.0 µL	OTα (0.1 mg/mL)
8.0 µL	L-phenylalanine (250 µg /mL)
412.0 µL	Tris-HCl

Setups were incubate overnight at 30°C. Samples were then acidified and extracted twice with 500µL ethyl acetate. Organic phases were pooled and evaporated to dryness. Residue was redissolved in 50 µL methanol and 10 µL were subjected to TLC as described in 2.2.3.2a).

b) Activity Tests with Gel Filtration Fractions

Samples of gel filtration and ion exchange chromatography fractions were tested for *in vitro* OTA synthesis in micro well plates (NUNC PolySorp) in a fluorescence photometer (SPECTRAFluor, Tecan, Germany). Production of OTA was detected by a characteristic enhancement in fluorescence emission at 448±5 nm (excitation: 380±10 nm, gain: 100, flashes: 3, read: top, integration time: 40 ms). A spectrophotometric method for detection and quantification of OTA was first established by Hult and Gatenbeck (1976).

Setup of activity test for each micro well:

50.0 µL	Gel filtration sample
18.0 µL	OTα (0.1 mg /mL)
5.0 µL	L-phenylalanine (250 µg/ mL)
177.0 µL	Tris-HCl

Fluorescence was detected after 2 h of incubation at 30 °C, and >48 h incubation at room temperature.

c) Activity Tests in Native PAGE

Protein samples from continuing purification step (2.2.5) were separated by native PAGE according to 2.2.7.2 and overlaid with a 0.5% agar layer containing 6 ng/µL OTα. After up to 24 h of incubation at room temperature, gel was checked for fluorescence alteration on a UV transilluminator (UVIS-Doustrahler, 75 W, 254 nm und 366 nm, Desaga).

2.2.7 Separation of Proteins by One Dimensional Polyacrylamide Gel Electrophoresis (PAGE)

For estimation of protein sizes and purification progress, continuing protein purification steps were separated by PAGE.

2.2.7.1 SDS PAGE (Laemmli, 1970)

For separation of denatured proteins according to their molecular size, polyacrylamide gels were prepared according to the following protocol:

Stacking Gel (4 %)
pH 8.8

6.1 mL	Aqua dest.
2.5 mL	1.5 M Tris-HCl
40.0 µL	25 % (w/v) SDS
1.3 mL	Acrylamide/Bisacrylamide (29:1)
50.0 µL	10 % (w/v) Ammoniumpersulfate
5.0 µL	TEMED

Separating Gel (12 %)
pH 6.8

3.35 mL	Aqua dest.
2.5 mL	1.5 M Tris-HCl
40.0 µL	25 % (w/v) SDS
4.0 mL	Acrylamide/Bisacrylamide (29:1)
50.0 µL	10 % (w/v) Ammoniumpersulfate
5.0 µL	TEMED

Both solutions were degassed before adding TEMED. Stacking and separating gel were prepared in the Min Protean Gel Preparation Station (BioRad, Munich).

For electrophoresis, 4 µL of 5x sample buffer (12.5% 0.5 M Tris-HCl, 7.5% Glycerol, 2.9% SDS, 5% 2-Mercaptoethanol, 0.2% Bromophenol blue) were added to 16 µL of protein samples from continuing purification steps (2.2.5; appropriately diluted with TBS buffer). Samples were heat denatured at 95°C for 5 min. Samples were applied to gel wells along with a protein size marker (LMW–SDS Calibration Kit, Amersham Biosciences, Freiburg). Gels were developed in a Mini Protean II Electrophoresis Cell (BioRad, Munich) in SDS running buffer for 2 h at 180 V, 120 mA (Power Supply PPS 200-100, MWG Biotech AG, Ebersberg).

2.2.7.2 Native PAGE

Native PAGE was conducted similar to SDS PAGE. No SDS or 2-Mercaptoethanol was added to gels, running buffer or sample buffer. Protein samples were not heat denatured prior to native PAGE.

2.2.7.3 Silver Staining of Proteins Separated by PAGE (Blum *et al.*, 1987)

Proteins in the gel matrix were visualized by silver staining. The following solutions were prepared freshly before use and gels were incubated according to the staining protocol:

Fixing Solution

40.0 mL	Ethanol
10.0 mL	Acetic acid
ad 100 mL with aqua bidest.	

Silvering Solution

0.2 g	$AgNO_3$
20.0 µL	Formaldehyde
ad 100 mL with aqua bidest.	

Developing Solution

3 g	Na_2CO_3
250.0 µL	Thiosulfate solution (2 mg/10 mL)
50.0 µL	Formaldehyde
ad 100 mL with aqua bidest.	

Stop Solution

30.0 mL	Ethanol
12.0 mL	Glycerol
ad 100 mL with aqua bidest.	

Staining Protocol

Fixing	> 2 h
Water	3x 20 min
Thiosulfate	1 min
Water	3x20 sec
Silvering	20 min
Water	2x20 sec
Developing	> 5 min
Water	20 sec
Stopping	5 min
Water	3x10 min

2.2.8 RNA Isolation from Fungal Mycelia

In order to compare gene expression in fungal mycelia at different growth stages cultivated under (non-)submissive conditions, total RNA (a mixture of approx. 20:1 rRNA and mRNA) was extracted from the cells.

To protect RNA from degradation, it is necessary to ensure that all utilized materials are free of RNases. Mortar, pestle, glass and spatula were freed from RNases by baking at 180 °C overnight. All surfaces were treated with RNase-Free (BioLogical™ Molecular Reagents, Continental Laboratory Products) immediately before commencing the isolation procedure.

Only certified RNase free consumables were used during the isolation procedure: RNase free PCR reaction tubes, Safe Guard™ filter tips, Multi™ SafeSeal Tubes 1.5 mL (peqlab GmbH, Erlangen), RNase free water (Sigma-Aldrich Chemie GmbH, Schnelldorf).

Mycelia harvested as described in 2.2.4 were ground to fine powder in a pre-cooled mortar. Liquid nitrogen was constantly added to prevent thawing of the mycelial cells. RNA extraction from the ground mycelia was accomplished with the RNeasy® Plant Mini Kit (Qiagen GmbH, Hilden) according to the instructions provided by the manufacturer.

After RNA extraction, residual DNA in the samples was digested with a DNase (RQ1 RNAfree DNase, Promega GmbH, Mannheim) according to the following protocol:

20.0 µL	RNA sample
2.4 µL	DNase buffer
2.0 µL	RQ1 DNase (200 U)

Incubate for 30 min at room temperature.
Stop digestion reaction with 1/10 vol. stop buffer at 65 °C for 10 min.

Short term storage of working samples (up to 2 weeks) took place at –20 °C, long term storage of RNA samples at –80 °C.

2.2.9 Reverse Transcription of RNA Isolated from Fungal Mycelia

RNA isolated from fungal mycelia was translated into double stranded cDNA in two sequential reactions.

2.2.9.1 First Strand cDNA Synthesis

Synthesis of the first cDNA strand from RNA was accomplished with M-MLV Reverse Transcriptase (RNase H minus, Promega GmbH, Mannheim). The total RNA mixture isolated from the fungal cells also includes non-coding ribosomal RNA. Therefore, selective transcription using the polyA$^+$ tails of eukaryotic messenger RNA is necessary. To ensure that only mRNA was transcribed to cDNA, an oligo(dT)$_{15}$ primer (Promega GmbH, Mannheim) was used. Setup according to the following protocol:

Add into a sterile, RNase free 0.2 mL reaction tube:

2.0 µL	RNA sample
1.0 µL	Oligo(dT)$_{15}$ primer (10 µM)
2.0 µL	H_2O (RNase free)

Incubate in a thermal cycler at 72 °C for 2 min. Immediately cool on ice

Add

1.5 µL	H_2O (RNase free)
2.0 µL	M-MLV-RT-buffer (5x)
1.0 µL	dNTPs (10 mM)
0.5 µL	M-MLV RT (100 U)

Incubate in a thermal cycler at 42 °C for 1.5 h.
Terminate reaction by incubation at 72 °C for 15 min.

2.2.9.2 Second Strand cDNA Synthesis

To synthesize a complementary cDNA strand to the one transcribed from mRNA in the first strand reaction, RNA was partially digested by RNase H. Fragments of nicked RNA were used as primers for DNA synthesis by DNA polymerase I. Gaps between the newly synthesized cDNA fragments were closed by *E. coli* DNA ligase (all enzymes: Promega GmbH, Mannheim). Second strand synthesis reaction were set up according to the following protocol:

Add the following components to the 10 µL of the first strand reaction:

48.4 µL	H_2O (RNase free)
16.0 µL	second strand buffer (10x DNA ligase buffer + 100 mM KCl)
1.6 µL	dNTPs (10 mM)
1.0 µL	RNase H (U)
1.0 µL	DNA polymerase I (400 U)
1.0 µL	*E.coli* DNA ligase (10 U)

Incubate in a thermal cycler at 16 °C for 2 h.
Terminate reaction by adding 4 µL of 0.2 M EDTA/(1 mg/mL) glycerol mix.

2.2.10 Quality Examination of Isolated RNA Samples

Quality and integrity of isolated total RNA was checked by agarose gel electrophoresis of heat denatured RNA samples. Also, presence and integrity of a PCR product derived from the β-tubulin gene was tested (see Oakley, 2000).

2.2.10.1 Electrophoresis of RNA Samples

Integrity of total RNA was checked by agarose gel electrophoresis.

For all following steps fresh buffers and reagents were used to avoid RNase contamination. 1.3% agarose was dissolved in 90 mL 0.5% TBE buffer and a gel was poured after cooling to 50 °C using MWG Biotech electrophoresis equipment (electrophoresis chamber, 12x14 cm, MWG Biotech AG, Ebersberg). 10 µL of each RNA sample (+ 2 µL 5x stop buffer) were heat denatured at 95 °C for 5 min and immediately put on ice. 10 µL of each sample were loaded into the gel wells. As DNA size marker, a 100 bp ladder (Amersham Biosciences, Freiburg) was added. The gel was developed in 0.5% TBE buffer for 1 h at 90 V, 45 mA (PP 200-10, MWG Biotech AG, Ebersberg). DNA was dyed in ethidium bromide (1 µg/mL) for 15 min and viewed on a UV transilluminator (UVIS-Doustrahler, 75 W, 254 nm und 366 nm, Desaga).

Amount of intact, not RNase-denatured total RNA is estimated by clearly visible rRNA bands at 3.8 and 2.0 kb.

2.2.10.2 Quality Examination of RNA Samples by PCR with Specific Primers

Aliquots of RNA and cDNA were tested for any product formed in PCRs conducted with primers specific for the constitutively expressed β–tubulin gene according to the following protocol:

Add the following components to each RNA or cDNA sample:		PCR Protocol		
1.0 µL	RNA/cDNA sample	95 °C	4 min	
21.4 µL	H_2O (DNase free)	95 °C	30 sec	
2.5 µL	10x PCR buffer	56 °C	40 sec	25x
0.5 µL	dNTPs (25 mM)	72 °C	1 min	
0.25 µL	Primer Btub1 (50 µM)	72 °C	5 min	
0.25 µL	Primer Btub2 (50 µM)			
0.2 µL	Go*Taq* DNA polymerase (1 U; Promega GmbH, Mannheim)			

Subject 5 µL of each PCR to gel electrophoresis as described above.

As β-tubulin is constitutively expressed, a PCR product should occur with each cDNA sample. Presence of only one product in reactions with cDNA as template and no product in PCR reactions with untranscribed RNA samples indicated no DNA contamination of the RNA samples.

2.2.11 Quantification of RNA and DNA

Concentration of RNA, DNA and cDNA molecules were determined by spectral photometric measurement (Sambrook *et al*., 1989). Accordingly, an extinction $E_{260\ nm} = 1$ corresponds to a concentration of 50 µg/mL double strand DNA and 40 µg/L single strand RNA or DNA.

2.2.12 cDNA-AFLP

In order to elucidate genes connected to OTA biosynthesis by comparison of gene expression in cultures of *Aspergillus ochraceus* NRRL 3174 in different growth stages and under (non-) submissive growth conditions, cDNA-AFLP was used (Ivashuta *et al*., 1999; Bachem *et al*., 1996). In contrast to the original AFLP method (Vos *et al*., 1995), cDNA transcribed from mRNA instead of genomic DNA was used. First, the cDNA was restricted with specific enzymes. Resulting fragments were ligated to AFLP-adaptors. General and specific amplification of cDNA fragments was then accomplished using primers complementary to those adaptors with and without selective bases at the 3' end. Amplification patterns of the different samples applied were then compared on silver-stained polyacrylamide gels. DNA sequence of differentially expressed cDNA fragments was determined.

2.2.12.1 Restriction of cDNA

cDNA was cleaned up from second strand reactions with the Qiaquick® PCR Purification Kit (Qiagen GmbH, Hilden) and was restricted to fragments using *Mse* I (New England Biolabs, Frankfurt) according to the following protocol:

11.5 µL	H_2O
2.0 µL	Restriction buffer
1.0 µL	cDNA (200 ng)
0.5 µL	*Mse* I (3 U)

Incubate at 37 °C for > 3h.

2.2.12.2 Ligation of AFLP-Adaptors

Ligation of AFLP-adapters to restricted cDNA fragments was performed with T4 DNA ligase (New England Biolabs, Frankfurt) according to the following protocol:

1.0 µL	H_2O
1.0 µL	*Mse* I adaptor (20 pMol)
2.0 µL	10x ligation buffer with 1mM ATP
1.0 µL	T4 DNA ligase (400 U)

Add 5 µl to each restriction reaction.
Incubate overnight at 16 °C.

2.2.12.3 Preamplification Reaction

For non-selective preamplification of all cDNA fragments with an *Mse*I adapter at both ends, a *Mse*I primer fully complementary to the *Mse*I adapter was used. Restriction/ligation mixture was diluted 1:10 in TE buffer and the following components were added:

5.0 µL	restriction/ligation mix (1:10)
13.3 µL	AFLP buffer
0.4 µL	dNTPs (25 mM)
1.2 µL	*Mse* I Primer (10 µM)
0.1 µL	Go*Taq* DNA polymerase

PCR Protocol

95 °C	4 min	
95 °C	30 sec	
56 °C	40 sec	20x
72 °C	1 min	
72 °C	5 min	

2.2.12.4 Selective Amplification Reaction

A selective amplification step was performed to reduce the number of bands in cDNA fragment patterns on silver stained PPA gels. Selectivity was ensured by using MAC primer with two selective bases at the 3' end in a touch-down PCR. Preamplification mixture was diluted 1:10 in aqua dest. and the following components were added:

10.0 µL	preamplification mix (1:10)
26.6 µL	AFLP buffer
0.8 µL	dNTPs (25 mM)
2.4 µL	MAC Primer (10 µM)
0.2 µL	Go*Taq* DNA polymerase

PCR Protocol

95 °C	4 min	
95 °C	30 sec	
65 (-0.7) °C	40 sec	12x
72 °C	1 min	
95 °C	30 sec	
56 °C	40 sec	22x
72 °C	1 min	
72 °C	5 min	

2.2.12.5 Separation of Amplified cDNA Fragments on Polyacrylamide Gels

The amplification mixture was freeze-dried and resuspended in 5 µL TE buffer. 1 µLsample buffer (25 mL Rehydration buffer, 60 µL Bromophenol blue solution (1%), 40 µL Xylencyanl solution (1%), 250 µL 0.2 mol/L EDTA) was added. 6 µL of each sample and of a base pair ladder (10 µL of 100 bp ladder (2.1.3), 1290 µL sample buffer) were applied to sample wells on rehydrated ready gels. Either Clean Gel DNA-HP 15% with the Delect Buffer System or Long Distance Clean Gel Large-10 with DNA LongRun Buffer System (ETC, Kirchtellinsfurt) were used in a Multiphor electrophoresis chamber (Amersham Biosciences, Freiburg) according to the manufacturers protocol.

2.2.12.6 Silver Staining of PAA Gels (ETC, 2002)

The following solutions were prepared freshly before use and gels were incubated according to the staining protocol:

Fixing Solution

1.2 g	Benzene sulfonic acid
60.0 mL	Ethanol
ad 200 mL with aqua bidest.	

Staining Protocol

Fixing	40 min
Washing	3x 10 min
Silvering	40 min
Water	2 min
Developing	> 7 min
Stopping	3x 10 min

Washing Solution

0.35 g	Benzene sulfonic acid
ad 600 mL with aqua bidest.	

Silvering Solution

0.4 g	$AgNO_3$
0.14 g	Benzene sulfonic acid
260.0 µL	Formaldehyde
ad 200 mL with aqua bidest.	

Developing Solution

5.0 g	Na_2CO_3
200.0 µL	Thiosulfate solution (0,5 g ad 25 mL with aqua bidest.)
260.0 µL	Formaldehyde
ad 200 mL with aqua bidest.	

Stop Solution

25.0 mL	Acetic acid
25.0 mL	Glycerol
200.0 mL	Aqua bidest.

2.2.13 Characterization of Differentially Expressed cDNA Fragments

Several of those cDNA fragments only appearing in certain lanes on silver stained cDNA-AFLP PAA gels were cut out. cDNA was resuspended by overnight incubation in TE buffer at 37°C. Resuspended DNA fragments were amplified by PCR as described in 2.2.12.4. 10 µL of each PCR sample were by analyzed by agarose gel electrophoresis as described in 2.2.10.1. Resulting DNA fragments of appropriate size were isolated from the gel by use of the E.Z.N.A. Gel Extraction Kit (peqlab GmbH, Erlangen).

2.2.13.1 Cloning

For further handling and storage, reamplified cDNA fragments were ligated into pSTBlue-1 and transformed to *E. coli* Dh5α.

a) Ligation

Reamplified cDNA fragments were ligated into pSTBlue-1 AccepTor™ Vector (Novagen, Madison, USA) with T4 DNA ligase (New England Biolabs GmbH, Frankfurt) according to the following protocol:

15.0 µL	H_2O
2.0 µL	10x ligation buffer with 1mM ATP
1.0 µL	insert DNA (100 ng/µL)
1.0 µL	linear vector DNA (200 ng/µL)
1.0 µL	T4 DNA ligase (400 U)

Incubate overnight at 16 °C.

b) Transformation

250 mL SOB medium were inoculated with 250 µL of an overnight LB culture of *E. coli* Dh5α. At OD_{578nm}=0.4, cells were harvested by centrifugation (5,000 g, 10 min, 4 °C) and processed with the 2-K-2 Ultra Component Cell Preps Kit (Bio Basic Inc., BioCat GmbH, Heidelberg). 100 µL aliquots of competent cells were stored at –80 °C.

For transformation, 100µL *E. coli* Dh5α competent cells were carefully thawed on ice. 10 µL of ligation mixture were added. After an incubation on ice for 30 min, mixture was heated to 42 °C for 1 min. 1 mL pre-cooled LB medium was added and the mixture was incubated on ice for 5 min and at 37 °C for 1 h. 100 µL of the cell suspension was plated on selective LB agar (+Amp, +X-Gal) and incubated overnight at 37 °C.

2.2.13.2 Determination of DNA Sequences of Cloned cDNA Fragments

E. coli Dh5α clones containing plasmids with cDNA inserts were selected by their resistance to ampicillin coded on the pSTBlue-1 vector and their white color, due to the disruption of β-galactosidase gene in the pSTBlue-1 vector. Plasmid DNA was extracted and subjected to sequencing.

a) Extraction of Plasmid DNA from *E.coli* DH5α

White colonies grown on selective LB agar were cultivated overnight at 37°C in 100 mL LB liquid medium with 100 mg/L ampicillin. Plasmid DNA was isolated using the E.Z.N.A. Plasmid Miniprep Kit I (peqlab GmbH, Erlangen).

b) Sequencing of cDNA Fragments

Sequencing of cDNA fragments with the MAC primer was accomplished by Dr. W. Metzger (Sequiserve, Vaterstetten) using the ABI 373 Stretch Sequencer and ABI Prism Dye Terminator-cycle Sequencing Ready Reaction Kit with Ampli*Taq* polymerase FS (Perkin Elmer, Weiterstadt).

2.2.13.3 Quantitative PCR

Quantification of cDNA was accomplished by real time PCR using a LightCycler™. Reactions were setup in LightCycler™ capillaries (Roche Molecular Biochemicals, Mannheim) with QuantiTect™ SYBR® Green PCR Kit (Qiagen GmbH, Hilden).
For verification of differential expression of cDNA fragments obtained from cDNA-AFLP, two primer pairs derived from those fragments were used (3for/3rev1; 4for/4rev1). Primers Bt2a and Bt2b were used as an external standard for cDNA quantification. Calibration of DNA concentrations was achieved by a dilution series of genomic DNA of *A. ochraceus* NRRL 3174 using primers 4for/4rev1. Genomic DNA was prepared acooriding to the protocol published by Al-Samarrai and Schmid (2000).

Reactions were set up according to the following protocol:

10.0 µL	QuantiTect™ SYBR® Green PCR Master Mix
1.0 µL	cDNA/genomic DNA sample
7.0 µL	H_2O (Dnase free)
1.0 µL	Primer 1 (10 µM)
1.0 µL	Primer 2 (10 µM)

Results of real time PCR were analyzed with LightCycler™ Software Version 5.32 (fluorescence channel F1, Second Derivative Maximum with Arithmetic Baseline Adjustment, Polynomial Evaluation of Melting Curve).

LightCycler™ program protocol:

Program	Segment	Temperature	Time	Slope	Acquisition mode	Cycles
Start	1	95 °C	10 min	20 °C/s	none	1x
PCR	1	94 °C	15 sec	20 °C/s	none	
	2	54 °C	5 sec	20 °C/s	none	50x
	3	72 °C	25 sec	20 °C/s	none	
	4	80 °C	2 sec	20 °C/s	single	
Melting curve	1	95 °C	0 sec	20 °C/s	none	1x
	2	65 °C	10 sec	20 °C/s	none	
	3	95 °C	0 sec	0.1 °C/s	continuous	
End	1	40 °C	10 sec	20 °C/s	none	1x

2.2.13.4 PCR

Genomic DNA of several OTA producing and non-producing fungi were tested for any product formed in PCRs conducted with primers 3for/3rev2 and 4for/4rev2 derived from differentially expressed fragments obtained by cDNA-AFLP. A list of all tested organisms is given in Table 2.1. 5.0 µL of each PCR were analyzed by agarose gel electrophoresis as described above. PCRs using each primer pair were conducted according to the following protocol:

Add the following components to each DNA sample:

3for/3rev2	4for/4rev2	
1.0 µL	1.0 µL	genomic fungal DNA
2.0 µL		10x PCR buffer N
	2.0 µL	10x PCR buffer
0.5 µL	0.2 µL	Form amide
	0.5 µL	Gelatine (1 %)
0.4 µL	0.4 µL	dNTPs (25 mM)
0.6 µL	0.6 µL	Primer 1 (10 µM)
0.6 µL	0.6 µL	Primer 2 (10 µM)
0.1 µL	0.1 µL	Go*Taq* DNA polymerase (0.5 U; Promega GmbH, Mannheim)

ad 20 µL with H_2O (DNase free)

PCR Protocol 3for/3rev2:			**PCR Protocol 4for/4rev2:**		
94 °C	4 min		94 °C	4 min	
94 °C	40 sec		94 °C	40 sec	
56 °C	40 sec	35x	51°C	40 sec	40x
72 °C	1 min		72 °C	1 min	
72 °C	5 min		72 °C	5 min	

3 Results

Within this study requirements and conditions were accomplished for the setup of a molecular diagnostic method for early detection of ochratoxigenic fungi on feed and foodstuffs based on special genetic features of OTA producers, i. e. their genes and enzymes of the biosynthetic pathway leading to OTA formation.

Elucidation of key genes of OTA biosynthesis was attempted via purification of enzymes involved in the OTA biosynthetic pathway, particularly the OTA synthetase catalyzing the final step of this pathway. As an alternative genetic method to elucidate genes connected to OTA biosynthesis, gene expression in cultures grown under OTA submissive or non-submissive conditions, respectively, was compared.

For both approaches on OTA biosynthesis, stability of OTA production in laboratory culture was a vital requirement. Considerable instability of OTA production patterns by ochratoxigenic aspergilli as well as total loss of OTA production capability in laboratory culture has been reported by several authors. The same phenomenon was observed in this study while cultivating *A. ochraceus* A8 as submerse culture in YES medium at room temperature.

Especially for the genetic setup, another precondition was to have a reliable means to induce or suppress OTA production in culture by altering no more than one growth factor. The results given in literature as well as early observations from the present study suggested that it is necessary to determine specific conditions determining OTA production anew for each *Aspergillus* spp. strain under any given laboratory setting.

Therefore, to be able to predict and control OTA production by *Aspergillus* spp. used in this study, detailed knowledge about the external factors affecting OTA production had to be gathered prior to further work on OTA biosynthesis enzymes and genes.

3.1 Growth Experiments

For the reasons mentioned above, stability of OTA production was a major prerequisite for the work planned in the current study. As severe instabilities in OTA production were observed while routinely cultivating *A. ochraceus* A8 as submerse culture in YES medium at room temperature, it became imperative to elucidate possible external causes influencing OTA biosynthesis.

For this purpose, correlation of biomass and OTA production patterns of several *Aspergillus* spp. depending on chosen growth parameters in complex and synthetic culture media was studied.

3.1.1 Growth and OTA Production of Several *Aspergillus* spp. in YES Complex Liquid Medium

First, influence of incubation temperature on OTA production of *A. ochraceus* A8, *A. ochraceus* NRRL 3174, *A. ochraceus* CBS 588.68, *A. ochraceus* CBS 589.68 and *A. niger* var. *niger* CBS 101697 in YES liquid media was studied. In media supplemented with 2% yeast extract provided by Merck (Darmstadt) and 15% sucrose, *A. niger* var. *niger* CBS 101697 produced OTA after 3–4 d of incubation at 25°C or 30°C, respectively. OTA production by *A. ochraceus* NRRL 3174 and *A. ochraceus* A8, on the other hand, did only occur after 10–11 d of incubation at 25°C. OTA production could only be detected in less than 50% of the cultures of these two *A. ochraceus* strains tested. *A. ochraceus* CBS 588.68 and CBS 589.68 did not produce any OTA when grown as submerse cultures in YES medium.

In order to stabilize the sporadic OTA production patterns of *A. ochraceus* A8 and NRRL 3174, medium composition was changed according to observations reported by other authors. Adding $MgSO_4$ to YES medium prepared with yeast extract provided by Merck (Darmstadt) or alternatively

using yeast extract provided by Difco (BD Biosciences, Heidelberg) as described by Filtenborg *et al.* (1990) had no effect on either biomass or OTA production. Supplying only 4% sucrose instead of 15% as described by Davis (1969) decreased biomass production but had no influence on OTA production of any *Aspergillus* spp. tested.

In summary, *A. niger* var. *niger* CBS 101697 produced detectable amounts of OTA in all setups with YES medium after 3–4 days of incubation. OTA production pattern of *A. ochraceus* A8 and *A. ochraceus* NRRL 3174 seemed to be random and therefore independent from other medium components tested.

3.1.2 Growth and OTA Production of Several *Aspergillus* spp. in AM Synthetic Liquid Medium

After experiencing those instabilities described above of OTA production in a complex medium by *A. ochraceus* A8 and NRRL 3174, several growth experiments were set up with a synthetic medium to test the influence of selected parameters on OTA production in laboratory culture. The synthetic medium was based on a medium described by Adye and Mateles (1964) and was modified as reported by Lai *et al.* (1970) for cultivation of OTA producing *Aspergillus* spp. In the course of growth experiments conducted in this study, it was further modified to AM medium (chapter 2.1.5.1).

3.1.2.1 OTA Production by Several *Aspergillus* spp. in AM Liquid Medium

For this purpose, several *Aspergillus* spp. were tested for OTA production in AM synthetic liquid medium with an initial pH adjusted to pH 6.5 at 25 °C shaking at 120 rpm in the dark for up to 15 d. Pattern of OTA production by *Aspergillus* species and strains tested are shown in Fig. 3.1. Of 11 strains, 9 described as OTA producing by the suppliers, only 3 showed significant and reproducible OTA production in AM liquid medium: *A. ochraceus* NRRL 3174, *A. ochraceus* CCT 6819 and *A. niger* var. *niger* CBS 101697.

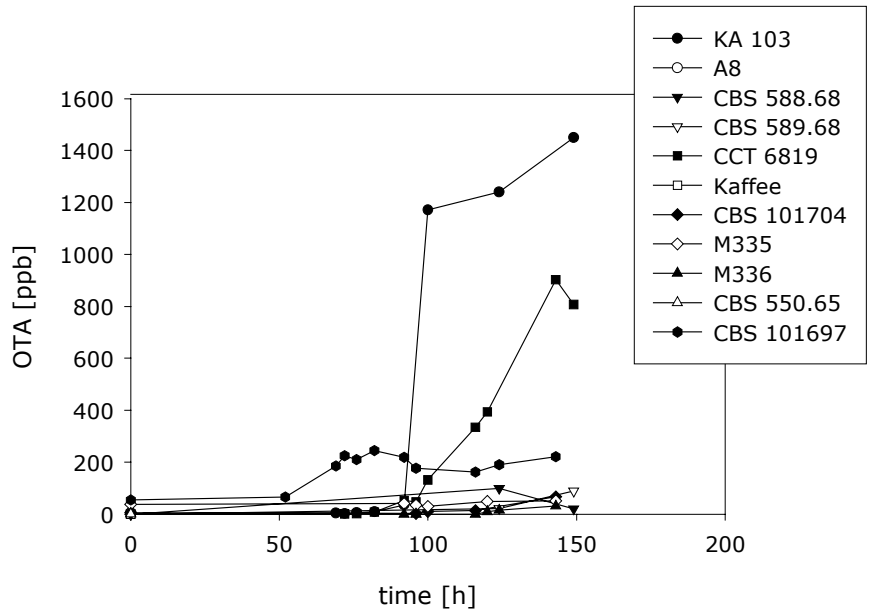


Fig. 3.1: OTA production by several aspergilli in AM liquid medium adjusted to initial pH 6.5:
A. ochraceus NRRL 3174, CBS 588.68, 589.68, CCT 6819, TMW 4.1357; *A. awamorii* CBS 101704;
A. carbonarius M335, M336; *A. fresenii* CBS 550.65; *A. niger* var. *niger* CBS 101697

While *A. ochraceu*s NRRL 3174 and *A. niger var. niger* CBS 101697 were known to have OTA production capability, *A. ochraceus* CCT 6819 was originally described as a non-OTA producing strain (Schmidt *et al.*, 2003). In our experiments, it did not produce any OTA when cultivated on malt extract agar but up to 300 µg/g mdw when grown in AM liquid medium. On the other hand, some strains described as OTA producing by the suppliers did not produce OTA when grown either on solid MEA or submerse in AM liquid medium or both (Table 3.1).

Table 3.1: OTA production capabilities of the strains used in this study. MEA: malt extract agar. CZA: Czapek agar. CCA: coconut cream agar. YES: yeast extract sucrose. AM: AM liquid medium

Species	Strain	OTA Production described by Supplier	OTA Production Detrected in this Study		
			on MEA	in YES	in AM
A. awamori	CBS 101704	+ (CZA, MEA)	+	n.d.	–
A. carbonarius	M 335	+ (CCA)	++	n.d.	–
A. carbonarius	M 336	+ (CCA)	–	n.d.	–
A. fresenii	CBS 550.65	+ (MEA, CZA)	++	n.d.	+
A. ochraceus	A8	+ (YES)	+	±	±
A. ochraceus	CCT 6819	– (MEA)	–	n.d.	++
A. ochraceus	CBS 588.68	+ (MEA, CZA)	–	–	–
A. ochraceus	CBS 589.68	+ (MEA, CZA)	–	–	–
A. ochraceus	NRRL 3174	+ (YES)	++	±	++
A. ochraceus	TMW 4.1357	– (MEA)	–	n.d.	–
A. niger var. *niger*	CBS 101697	+ (MEA, CZA)	++	+	+
A. usamii var. *shiro-usamii*	CBS 101700	+ (MEA, CZA)	–	n.d.	–

3.1.2.2 Growth and OTA Production of *A. niger* var. *niger* CBS 101697 and *A. ochraceus* NRRL 3174 in AM Liquid Medium

Two of the *Aspergillus* spp. producing OTA in the experiments described above, were studied more closely for their biomass production and OTA/biomass production when grown as submerse culture in AM liquid medium (starting pH 6.5) at 25 °C during an incubation period of 120 h.

a) Growth and OTA Production of *A. niger* var. *niger* CBS 101697 in AM Liquid Medium

Biomass and OTA/biomass production of a culture of *A. niger* var. *niger* CBS 101697 during 120 h of incubation are shown in Fig. 3.2. Initial OTA concentration of the culture medium was already 60 ppb, most likely due to high OTA concentration in the inoculating spore suspension. Also, *A. niger* var. *niger* CBS 101697 displayed very early onset of OTA production. Almost from the beginning of the cultivation period, OTA was produced accompanied by a steady drop in medium pH. Concentration of OTA in the culture medium did not rise steadily but was connected with a kind of sigmoid course instead. This phenomenon could be observed in several growth experiments conducted with this strain (see Fig. 3.1) and was not due to measurement fluctuations (about 5%).

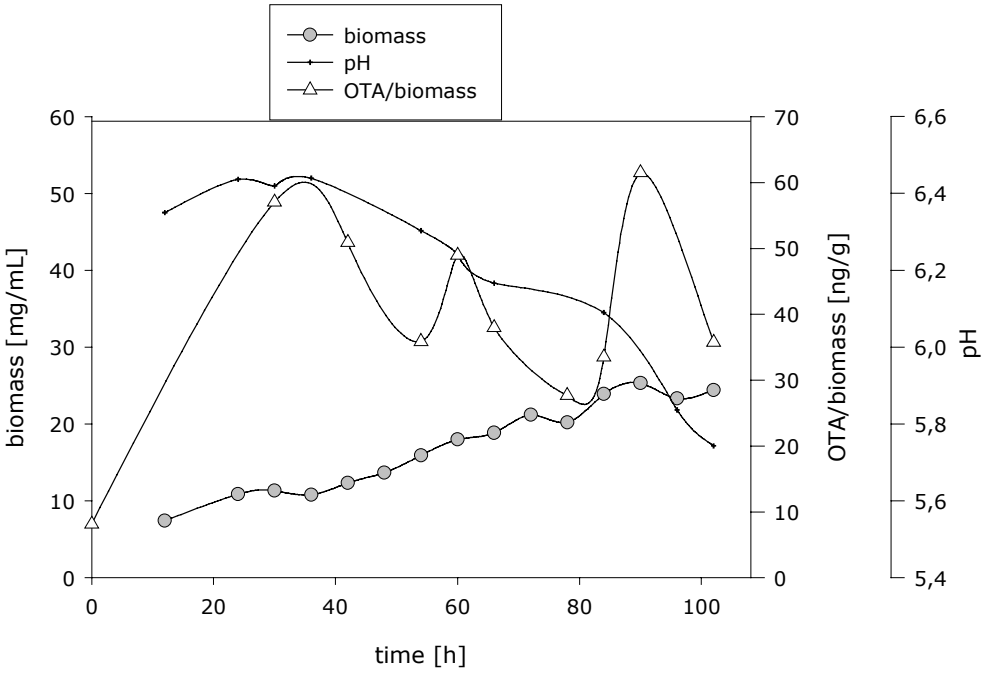


Fig. 3.2: Biomass, OTA production and acidification of culture medium by *Aspergillus niger* var. *niger* CBS 101697 in AM liquid medium (initial pH 6.5)

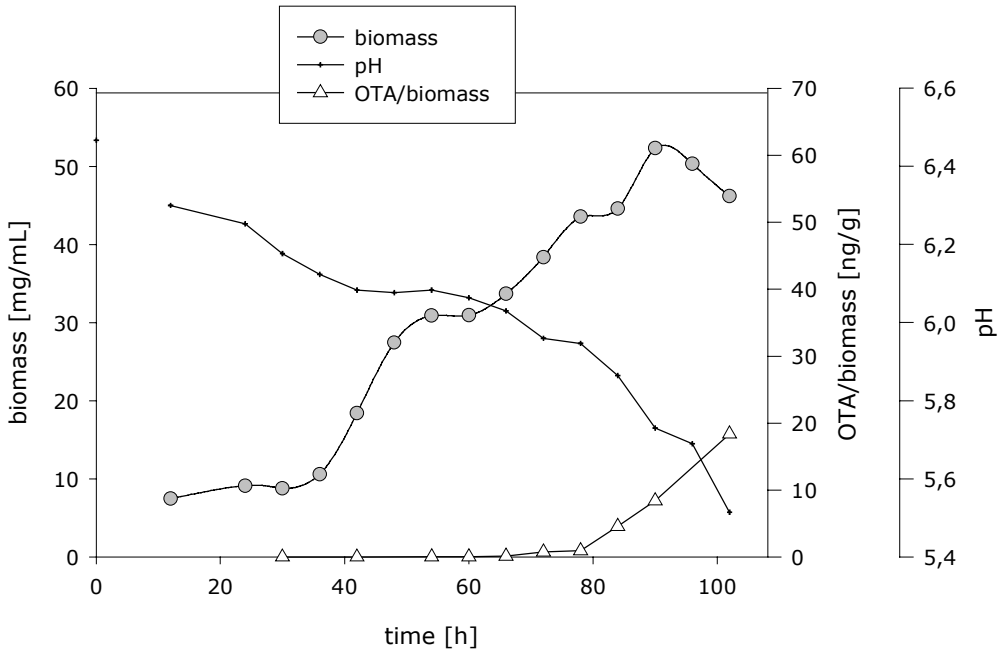


Fig. 3.3: Biomass, OTA production and acidification of culture medium by *Aspergillusochraceus* NRRL 3174 in AM liquid medium (initial pH 6.5)

b) Growth and OTA Production of *A. ochraceus* NRRL 3174 in AM Liquid Medium

In contrast to observations with *A. niger* var. *niger* CBS 101697, two distinct phases during growth concerning OTA production could be observed cultivating *A. ochraceus* NRRL 3174 in AM liquid medium: before and after onset of OTA synthesis (Fig. 3.3). OTA production started after about 72 h of incubation, within the exponential growth phase of the fungal culture. OTA concentration in the medium rose steadily during the further incubation period. After 10 d of incubation up to 2 mg OTA/L AM medium were produced. During cultivation, pH continuously dropped to pH 4.2 at the end of the incubation phase after 360 h (see Fig. 3.6b).

Growth and OTA production of *A. ochraceus* CCT6819 in AM liquid medium were similar to growth and OTA production of *A. ochraceus* NRRL 3174: steady increase of OTA concentration in the medium after onset of OTA production at 72 h of incubation accompanied by a drop in pH.

3.1.3 Dependence of OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium on Several Growth Parameters

With finding a suitable strain, *A. ochraceus* NRRL 3174, for stable and reliable OTA production under specified culture conditions, growing as submerse culture in AM medium adjusted to initial pH 6.5 at 25°C for >72 h, the first prerequisite for studies on key genes and enzymes of OTA biosynthesis was met. In order to find factors determining OTA production, further studies on the influence of selected growth parameters like medium pH, trace metals, carbon source and nitrogen source on OTA production by *A. ochraceus* NRRL 3174 were carried out.

3.1.3.1 pH Dependence of OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium

OTA production of *A. ochraceus* NRRL 3174 was determined by initial pH of the culture media. In AM liquid media adjusted to a pH value between pH 5.5 and pH 8.5 prior to inoculation, *Aspergillus ochraceus* NRRL 3174 produced up to 2 mg/L OTA after 360 h of incubation at 25°C. There was no significant difference in the amount of OTA produced in cultures adjusted to any pH value within this range. No OTA production could be observed outside the range pH 5.5–8.5, whereas growth and biomass production of *A. ochraceus* NRRL 3174 remained unaffected from pH 3.5 to pH 9. In all cultures buffered to an initial pH 5.0–9.0, pH slowly dropped to pH 4.2 within 360 h of incubation. Acidification of the culture medium was due to organic acids like citrate and pyruvate produced by the growing fungal mycelium (data not shown, see Kubicek *et al.* 1994, Jayaraman *et al.*, 1971).

OTA synthesis by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium initially adjusted to a pH outside the pH range favorable to OTA production, could be triggered by implementing a pH shift to the culture medium during the exponential growth phase (Fig. 3.4).

Initial pH adjustment of culture medium to pH 6.5 resulted in quantifiable OTA production after 72 h, when the culture was within its exponential growth phase. Initial pH adjustment of culture medium to pH 5.0 resulted in no quantifiable OTA production within an incubation time of 500 h. A shift in pH from 5.0 to 6.5 after 60 h of incubation triggered onset of measurable OTA production by *A. ochraceus* NRRL 3174 after 40 h of further incubation. However, the amount of OTA produced was notedly lower in cultures with shifted pH compared to cultures with initial pH 6.5.

Therefore, initial pH adjustment of the culture medium provides OTA permissive and nonpermissive conditions altering only one growth factor. In the following experiments, other important growth factors were tested for their influence on OTA production, most of all for further characterization of the "pH effect" described above.

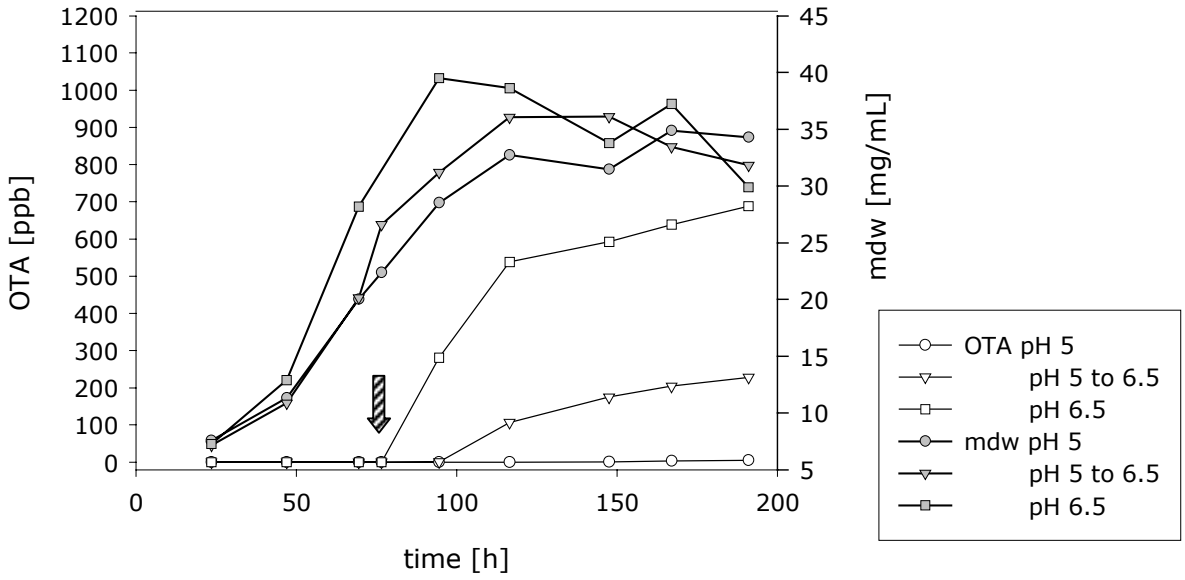


Fig. 3.4: OTA production by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium adjusted to different initial pH values. The arrow indicates pH shift in one of the setups (▽), mdw: mycelial dry weight

3.1.3.2 Influence of Trace Metals on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium

Availability of trace metals in AM medium varies with pH. At pH 6.5 considerable precipitation of zinc and iron phosphates takes place, but not at pH 5.0. Therefore at pH 6.5 the concentration of accessible Zn^{2+} and Fe^{3+} in the medium must be reduced in comparison to pH 5.0. In order to determine whether the "pH effect" described above was due to different availability of those trace metals at different pH values, Zn^{2+} and Fe^{3+} or both, respectively, were omitted from the synthetic media at pH 5.0, and pH 6.5 media for control.

The effect of not supplying particular trace metals to AM culture media on growth rates and biomass yields as well as connected parameters like medium acidification and glucose utilization, was the same at both initial pH values. At both pH values, mycelial yields, medium acidification and glucose utilization remained unaffected by omission of Fe^{3+}. When Zn^{2+}, Zn^{2+} and Fe^{3+} or all trace metal ions were left out of AM media at both initial pH 6.5 and 5.0, biomass of the fungal culture was reduced to 25% of the biomass obtained in fully supplemented media (Fig. 3.5a and Fig. 3.6a).

In cultures with normal growth rates final pH was balanced to 4.2. In cultures adjusted to initial pH 6.5, this was achieved by a steady drop in pH during the incubation period. In cultures adjusted to initial pH 5.0, pH dropped to pH 3.7 after 200 h of incubation and slowly settled down to final pH 4.2. Medium acidification was disordered in cultures not supplemented with Zn^{2+}, Zn^{2+} and Fe^{3+} or any trace metals at both initial pH values. At initial pH 6.5, final pH of these cultures only dropped to pH 6.0 after 360 h of incubation. At initial pH 5.0, on the other hand, final pH of these cultures dropped to < pH 3 after 360 h of incubation (Fig. 3.5b and Fig. 3.6b).

Glucose utilization was significantly reduced in media not supplemented with Zn^{2+}, Zn^{2+} and Fe^{3+} or any trace metals at both pH 5.0 and pH 6.5 (Fig. 3.5c and Fig. 3.6c).

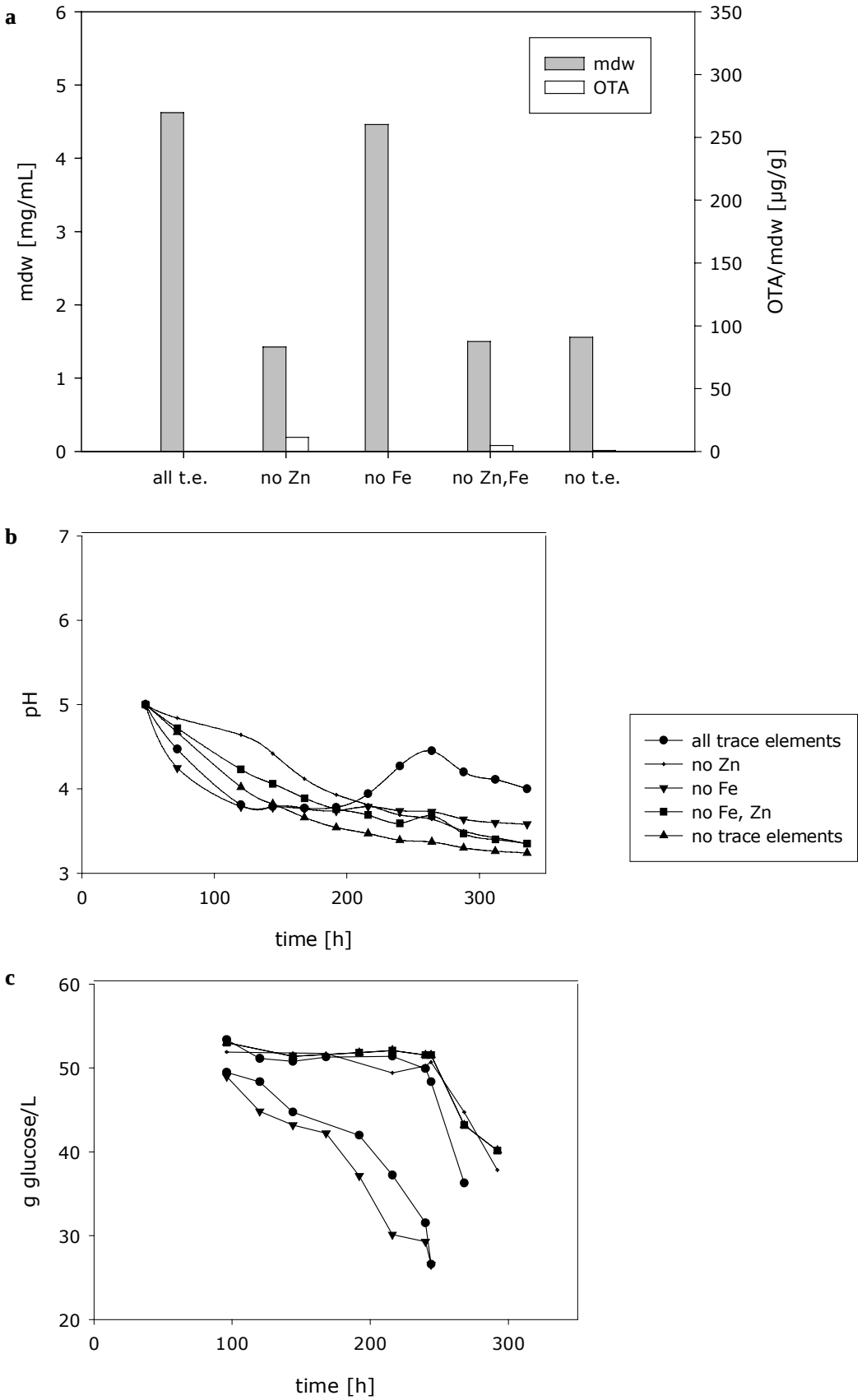


Fig. 3.5: Biomass and OTA production after 120 h of incubation (**a**) pH development (**b**) and glucose utilization (**c**) by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium at initial pH 5. Mdw: mycelial dry weight, t.e.: trace elements

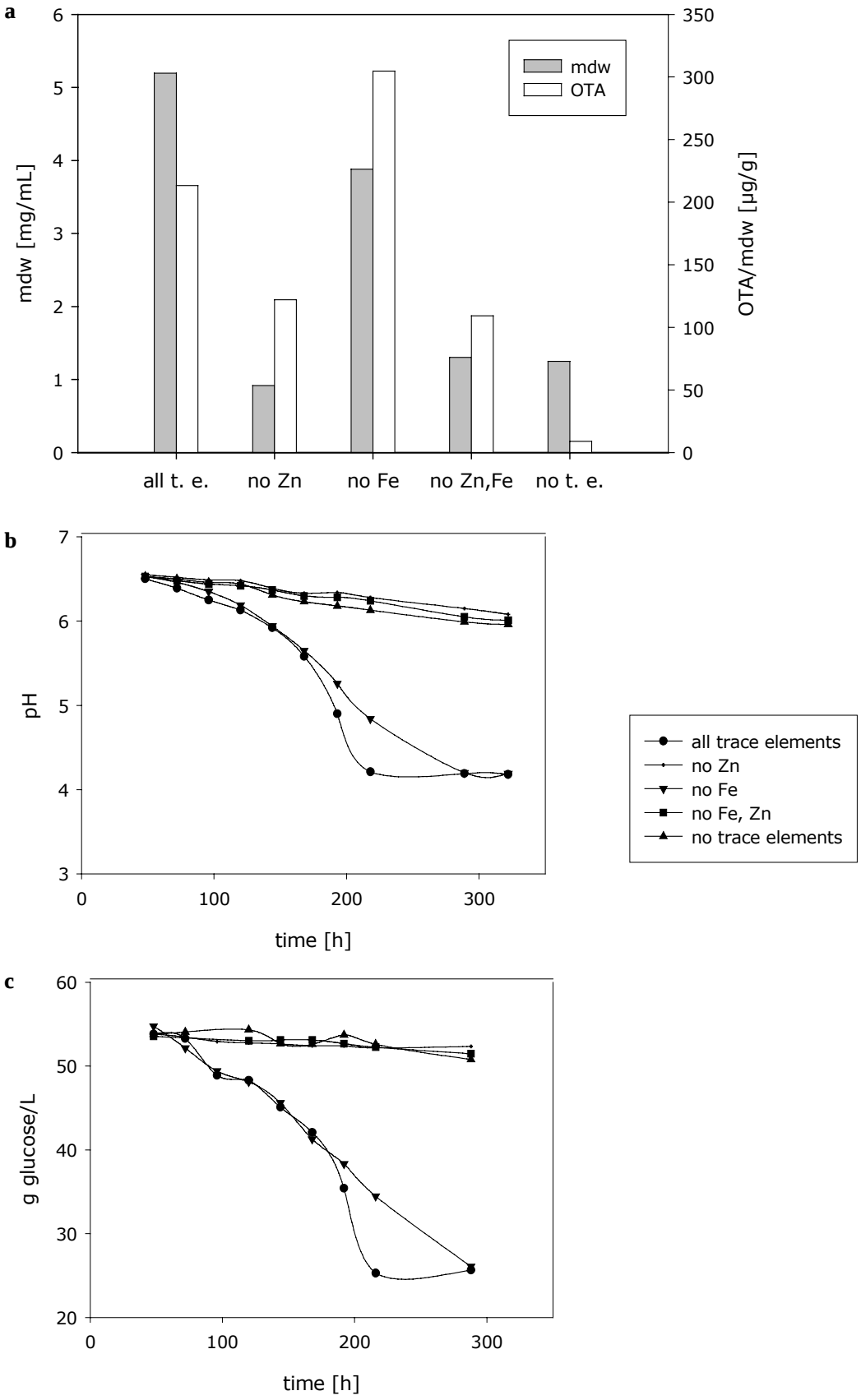


Fig. 3.6: Biomass and OTA production after 120 h of incubation (**a**) pH development (**b**) and glucose utilization (**c**) by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium at initial pH 6.5. Mdw: mycelial dry weight, t.e.: trace elements

a) Influence of Trace Metals on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 5.0

At pH 5.0, OTA production was not affected by omission of any trace metal from culture media (Fig. 3.5a). No significant amount of OTA could be detected in any setup after 120 h of incubation. A maximal OTA concentration of 8 µg/g mdw was detected after 120 h of incubation in cultures of *A. ochraceus* NRRL 3174 adjusted to initial pH 5.0 not supplemented with Zn. Amount of OTA produced did not increase with further incubation time.

b) Influence of Trace Metals on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 6.5

In contrast, in the control experiments with media initially adjusted to pH 6.5, OTA production was sensitive to variations in trace metals supply (Fig. 3.6a).

In fully supplemented AM liquid media, 220 µg OTA/g mycelial dry weight (mdw) were produced by cultures of *A. ochraceus* NRRL 3174 after 120 h of incubation at 25 °C. Omitting Fe^{3+} from media at pH 6.5 resulted in slightly lower biomass production but higher OTA/biomass production (310 µg/g mdw). Not supplying Zn^{2+} or both Zn^{2+} and Fe^{3+}, respectively, to AM synthetic medium resulted in significantly reduced OTA/ biomass production (approx. 120 µg/g mdw). Omitting all trace metals resulted in only minimal OTA production (20 µg/g mdw). Onset of OTA production was delayed for about 20 h in the latter three setups compared to fully supplemented media at pH 6.5.

c) Influence of Trace Metals on OTA Production by Some *Aspergillus* spp. in AM Liquid Medium at Initial pH 6.5

In order to determine whether the gradual dependence of OTA production by *A. ochraceus* NRRL 3174 was reproducible with other aspergilli, influence of trace metal supply on three other OTA producing A*spergillus* spp. was studied (see Fig. 3.1). As an example, only the two setups “no zinc” and “no iron” were compared to fully supplemented media. No difference between the setups “no zinc” and “no zinc and iron” could be observed in the experiments with *A. ochraceus* NRRL 3174, indicating the regulatory importance of Zn^{2+} for OTA production.

The effect of not supplying particular trace metals to AM culture media on growth rates and biomass yields was the same with *A. ochraceus* CCT 6819, *A. fresenii* CBS 550.65 and *A. niger* var. *niger* CBS 101697. Upon omission of Fe^{3+}, biomass production was reduced to approx. 80% compared to fully supplemented media in cultures of all three organisms, comparable to the effect of Fe^{3+} omission on biomass production by *A. ochraceus* NRRL 3174.

However, omission of Zn^{2+} reduced biomass production by *A. ochraceus* CCT 6819, *A. fresenii* CBS 550.65 and *A. niger* var. *niger* CBS 101697 only to approx. 50–60% compared to fully supplemented media, whereas *A. ochraceus* NRRL 3174 biomass production was reduced to 20% under these conditions.

As observed with cultures of *A. ochraceus* NRRL 3174, pH in fully supplemented cultures of *A. ochraceus* CCT 6819 dropped to pH 5.3 after 170 h and to pH 4.2 after 360 h of incubation. Whereas omission of Fe^{3+} had no effect on rapid acidification of culture media, pH in cultures not supplemented with Zn^{2+} only dropped to pH 6.3 after 170 h and pH 6.0 after 360 h of incubation. *A. fresenii* CBS 550.65 showed the same tendency of a slower drop in pH in culture media not supplemented with Zn^{2+}, although differences were not as distinct as in cultures of both *A. ochraceus* strains. In contrast, pH drop in cultures of *A. niger* var. *niger* CBS 101697 was strongest in cultures not supplemented with Zn^{2+} and only a weak drop in pH was induced by the omission of Fe^{3+} (Table 3.2).

Tendency of effects of altered trace metal supply on OTA production by *A. ochraceus* CCT 6819, *A. fresenii* CBS 550. 65 and *A. niger* var. *niger* CBS 101697 was comparable to effects observed with

A. ochraceus NRRL 3174. Reduction of OTA/biomass production upon omission of Zn^{2+} from AM liquid media at initial pH 6.5 after 120 h of incubation was observed in all four *Aspergillus* spp. tested. Increase in OTA/biomass upon omission of Fe^{3+} was observed in *A. ochraceus* CCT 6819 and *A. niger* var. *niger* CBS 101697 as well as in *A. ochraceus* NRRL 3174.

Table 3.2: Drop in culture medium pH induced by several *Aspergillus* spp. after 170 h of incubation in AM media adjusted to an initial pH 6.5. T.e.:trace elements

Organism		pH after 170 h of Incubation
A. ochraceus NRRL 3174	all t.e.	pH 5.4
	no Zn^{2+}	pH 6.3
	no Fe^{3+}	pH 5.4
A. ochraceus CCT 6819	all t.e.	pH 5.5
	no Zn^{2+}	pH 6.3
	no Fe^{3+}	pH 5.3
A. niger var. *niger* CBS 110697	all t.e.	pH 4.6
	no Zn^{2+}	pH 4.2
	no Fe^{3+}	pH 5.6
A. fresenii CBS 550.65	all t.e.	pH 6.0
	no Zn^{2+}	pH 6.4
	no Fe^{3+}	pH 6.1

1. Influence of Trace Metals on OTA Production by *A. ochraceus* CCT 6819 in AM Liquid Medium at Initial pH 6.5

Cultivating *A. ochraceus* CCT 6819 in AM liquid medium with initial pH 6.5, OTA production was slightly lowered compared to *A. ochraceus* NRRL 3174.

Strong decrease in OTA production when Zn^{2+} was omitted from the culture media could be observed (Fig. 3.7). Amount of OTA/biomass was reduced to 20% compared to fully supplemented media. Obviously *A. ochraceus* CCT 6819 reacted more strongly to omission of Zn^{2+} than *A. ochraceus* NRRL 3174 (reduction to 50%).

Slight stimulation of OTA production when Fe^{3+} was omitted from the culture media could be measured (10%), but not as prominent as observed with *A. ochraceus* NRRL 3174 (>40%).

2. Influence of Trace Metals on OTA Production by *A. fresenii* CBS 550.65 in AM Liquid Medium at Initial pH 6.5

With *A. fresenii* CBS 550.65, biomass and OTA production in AM liquid medium were much lower compared to *A. ochraceus* NRRL 3174 (Fig. 3.8). OTA production was reduced upon omission of Zn^{2+} by 30%, comparable to decrease in OTA production by *A. ochraceus* NRRL 3174 due to absence of Zn^{2+}. In contrast to all other three *Aspergillus* spp. tested, omission of Fe^{3+} reduced amount of OTA/biomass produced by *A. fresenii* CBS 550.65 to 65% compared to fully supplemented media.

3. Influence of Trace Metals on OTA Production *A. niger* var. *niger* CBS 101697 in AM Liquid Medium at Initial pH 6.5

Cultivating *A. niger* var. *niger* CBS 101697 in AM liquid medium at initial pH 6.5, biomass and OTA production were much lower compared to *A. ochraceus* NRRL 3174. Absence of Fe^{3+} resulted in stimulation of OTA production nearly as strong as observed with *A. ochraceus* NRRL 3174. OTA production was increased by >40% compared to fully supplemented media. However, in contrast to *A. ochraceus* NRRL 3174, only a slight reduction of OTA/biomass could be observed when Zn^{2+} was omitted from the culture media (Fig. 3.9).

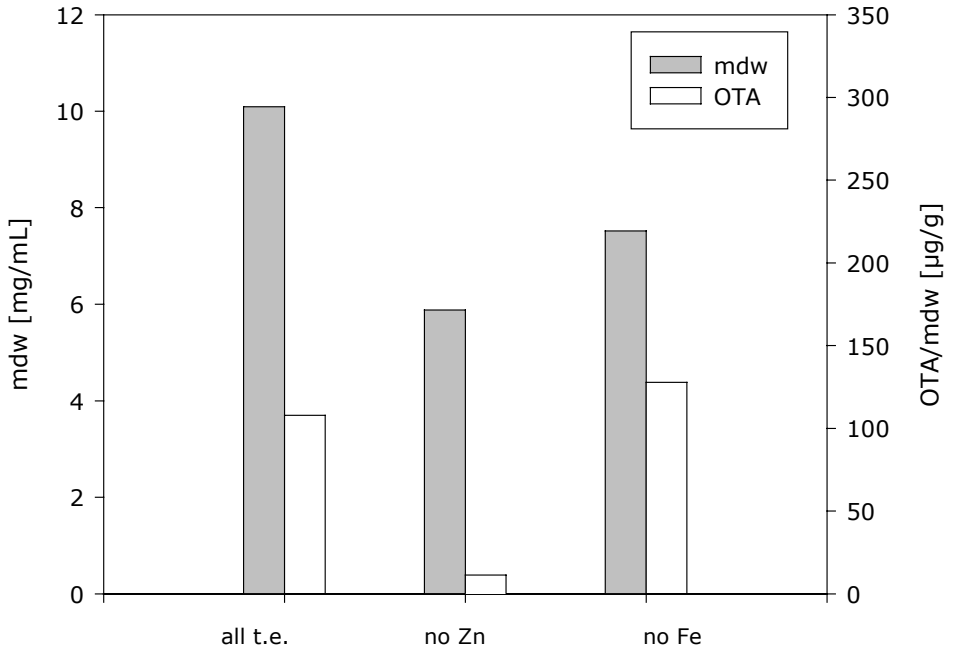


Fig. 3.7: Biomass and OTA production by *Aspergillus ochraceus* CCT 6819 in AM liquid medium at initial pH 6.5 after 120 h of incubation. Mdw: mycelial dry weight, t.e.: trace elements

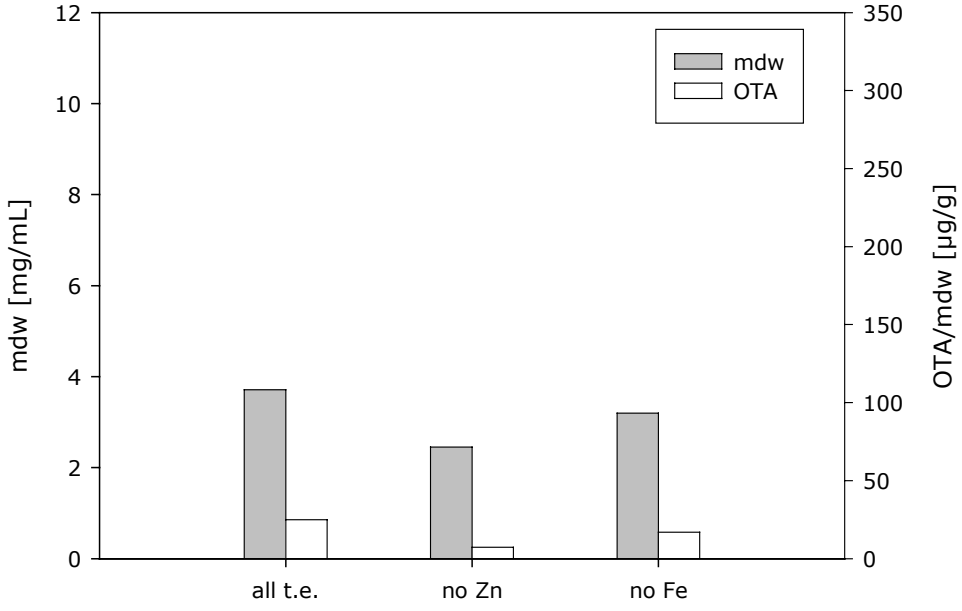


Fig. 3.8: Biomass and OTA production *Aspergillus fresenii* CBS 550.65 in AM liquid medium at initial pH 6.5 after 120 h of incubation. Mdw: mycelial dry weight, t.e.: trace elements.

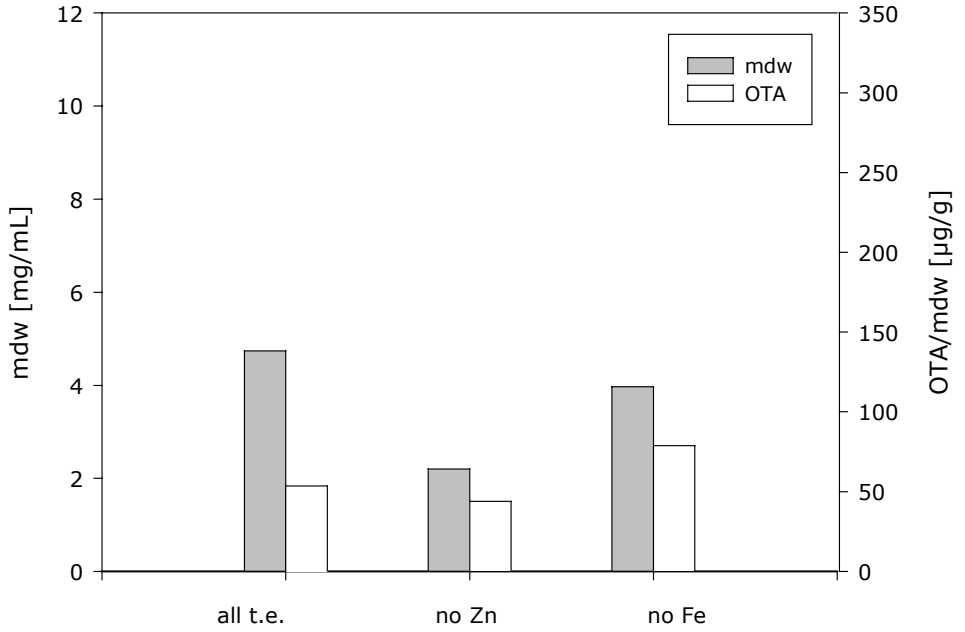


Fig. 3.9: Biomass and OTA production Aspergillus *niger* var *niger* CBS 101697 in AM liquid medium at initial pH 6.5 after 120 h of incubation, mdw: mycelial dry weight, t.e.: trace elements

3.1.3.3 Influence of Carbon and Nitrogen Source on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 6.5

As reported above, OTA production by *A. ochraceus* NRRL 3174 in AM liquid medium was determined in absolute terms by initial pH of the culture media and was gradually dependent on concentration of accessible trace metals in the culture media. Results obtained in this study implied considerable discrepancy to literature data on OTA production by *A. ochraceus* NRRL 3174 (4.1.1). Therefore, further characterization of some other growth parameters reported to determine OTA production by *A. ochraceus* NRRL 3174 was accomplished.

Experiments were set up to test influence of carbon and nitrogen source on OTA synthesis by *A. ochraceus* NRRL 3174 in AM media. Different amounts of glucose as well as a range of carbon sources (glucose, sucrose, lactose and fructose) and nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, KNO_3), respectively, were added to AM liquid medium set to initial pH 6.5.

a) Influence of Glucose Concentration on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 6.5

To test the influence of carbon source concentration on OTA biosynthesis by *A. ochraceus* NRRL 3174, 10%, 5% (standard), 2.5%, 1.25% or 0.625% glucose, respectively, were added to AM media fully supplemented with trace elements and adjusted to initial pH 6.5. The amount of OTA/biomass was dependent on concentration of glucose in culture media.

Biomass production decreased with amount of sugar available for growth. At 1/8 of standard glucose concentration, mycelial dry weight was reduced to 60% compared to standard AM media. However, fungal biomass production was also reduced to 60% when 10% glucose was added to culture media.

Mycotoxin production, on the other hand, increased with decreasing supply of glucose. This effect was not apparent when amounts of OTA produced in the different setups were expressed as

OTA/mL medium. All cultures produced similar total amounts of OTA and OTA production started after 72 h in all 5 setups (Fig. 3.10).

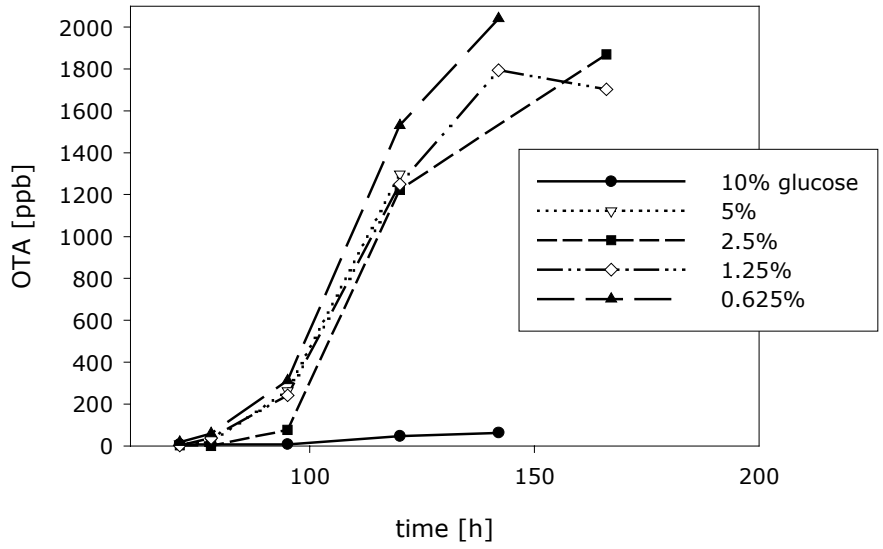


Fig. 3.10: OTA production by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium at initial pH 6.5 with different glucose concentrations supplied

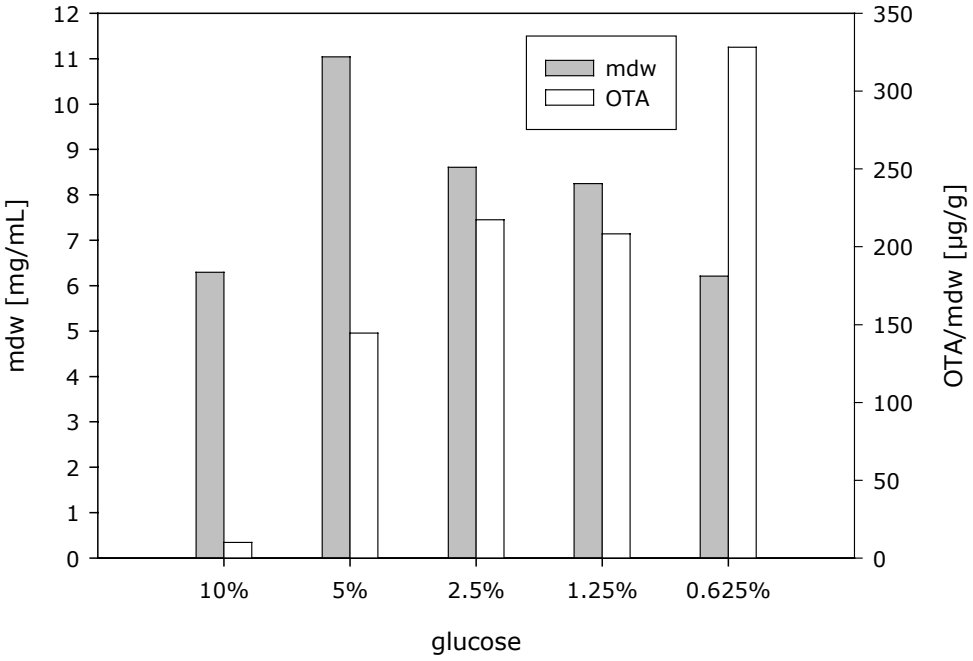


Fig. 3.11: Biomass and OTA production by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium with different concentrations of glucose supplied at initial pH 6.5 after 120 h of incubation, mdw: mycelial dry weight

When converted into OTA/biomass, however, it became clear that OTA production reacted to lowering glucose concentration inverse to biomass production. The most efficient OTA production per mycelial biomass was obtained at the lowest concentration of glucose tested, 0.625% (Fig. 3.11). Amount of OTA produced in culture media supplemented with 0.625% glucose increased most rapidly and continued to increase to 2.2 mg/L within an incubation period of 360 h, even after glucose in those culture media was depleted after 120 h of incubation.

In order to determine a possible cross link between availability of trace metals and carbon source, effect of trace metal supply on OTA production was tested in cultures supplemented with 2.5% and 0.625% glucose. Results of these experiments were similar to those observed with 5% glucose: adding no Fe^{3+} to culture media led to a slight decrease in biomass and an increase in OTA/ biomass production. Omitting Zn^{2+} from the culture media supplemented with 2.5% or 0.625% glucose, respectively, resulted in significantly reduced OTA yields (up to 50% compared to media with all trace metals added).

b) Influence of Various Carbon Sources on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 6.5

5% glucose, sucrose, lactose or fructose, respectively, were added to AM liquid medium fully supplemented with trace metals and adjusted to initial pH 6.5, in order to determine the influence of varying carbon sources on OTA production by A. *ochraceus* NRRL 3174.

Exchange of standard carbon source resulted in different amounts of OTA/mL medium depending on the carbon source supplied (Fig. 3.12). Even higher amounts of OTA/mL AM culture medium were produced in cultures supplemented with sucrose than in those with the standard carbon source glucose. A considerably smaller amount of OTA was produced in cultures supplemented with fructose, and still less in those with lactose.

Varying the carbon source also led to a shift in incubation time before start of OTA production (Fig. 3.12). Onset of OTA production was delayed for about 20 h in cultures supplemented with sucrose and delayed for over 40 h in cultures supplemented with fructose compared to cultures supplemented with glucose or lactose, respectively.

Regarding OTA/biomass production depending on carbon source supply, however, results differed slightly from those observed concerning OTA/mL medium (Fig. 3.13). Equally high yields in biomass and OTA/biomass (220 µg OTA/g mdw and 240 µg OTA/g mdw) could be detected after 120 h of incubation in cultures supplemented with glucose and sucrose, respectively. When lactose was utilized as single carbon source, 160 µg OTA/g mdw were produced after 120 h of incubation despite of substantial reduction of mycelial dry weight (40% mdw compared to cultures supplemented with glucose). With fructose as single carbon source, biomass was slightly reduced compared to glucose, whereas OTA was significantly reduced to 75 µg OTA/g mdw.

To verify the result of varying trace metal supply on OTA production observed in AM media supplemented with glucose with other carbon sources, effect of Fe^{3+} and Zn^{2+} concentration was tested in cultures supplemented with sucrose. Results obtained in these experiments were similar to those with glucose: omitting Fe^{3+} from culture media supplemented with sucrose led to a slight decrease in biomass and an increase in OTA/biomass. Omitting Zn^{2+} resulted in significantly reduced OTA yields (up to 50% compared to media with all trace metals added).

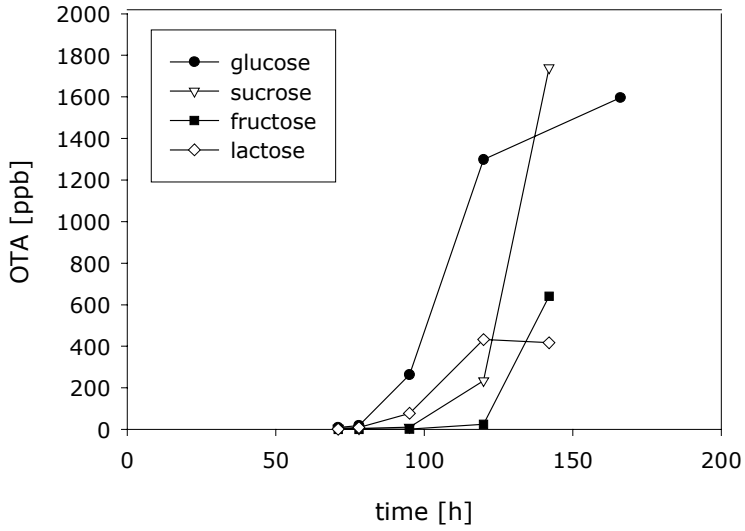


Fig. 3.12: OTA production by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium at initial pH 6.5 with different carbon sources supplied

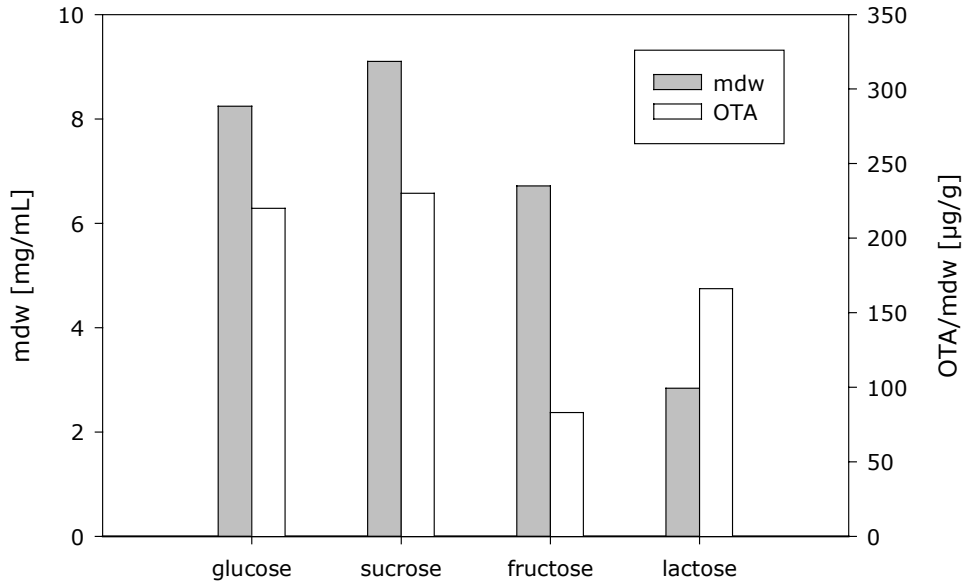


Fig. 3.13**:** Biomass and OTA production by Aspergillus ochraceus NRRL 3174 in AM liquid medium at initial pH 6.5 after 120 h of incubation with different carbon sources supplied, mdw: mycelial dry weight

c) Influence of Nitrogen Source on OTA Production by *A. ochraceus* NRRL 3174 in AM Liquid Medium at Initial pH 6.5

The culture medium used in this study was modified from a synthetic liquid medium optimized for aflatoxin production by *A. flavus* by Adye and Mateles (1964). Originally $(NH_4)_2SO_4$ was supplied as inorganic nitrogen source. Utilizing this medium for ochratoxin production by *A. ochraceus* NRRL 3174, Lai *et al*. (1970) established NH_4NO_3 as nitrogen source.

In this study, standard AM medium was supplemented with 3 g/L NH_4NO_3 as nitrogen source, a molecule in fact presenting two separate possible nitrogen sources. In order to determine the influence of both single compounds on OTA production by *A. ochraceus* NRRL 3174 in AM liquid medium supplemented with a full set of trace metals and 5% glucose, adjusted to pH 6.5, they were provided separately in equimolar concentration as $(NH_4)_2SO_4$ or KNO_3, respectively.

Supply of different nitrogen sources did not influence biomass production, but resulted in variations in OTA production after 120 h of incubation (Fig. 3.14). Supplementing AM medium with $(NH_4)_2SO_4$ resulted in 25% lower OTA/biomass production compared to NH_4NO_3 (175 µg/g mdw). Providing KNO_3 induced an increase in OTA/biomass production of 25% to 320 µg OTA/g mdw.

When KNO_3 was supplied as nitrogen source, pH of the AM medium remained unchanged at pH 6.5 during the entire cultivation period of 360 h. $(NH_4)_2SO_4$ induced a pH drop to 3.5 after 200 h of incubation.

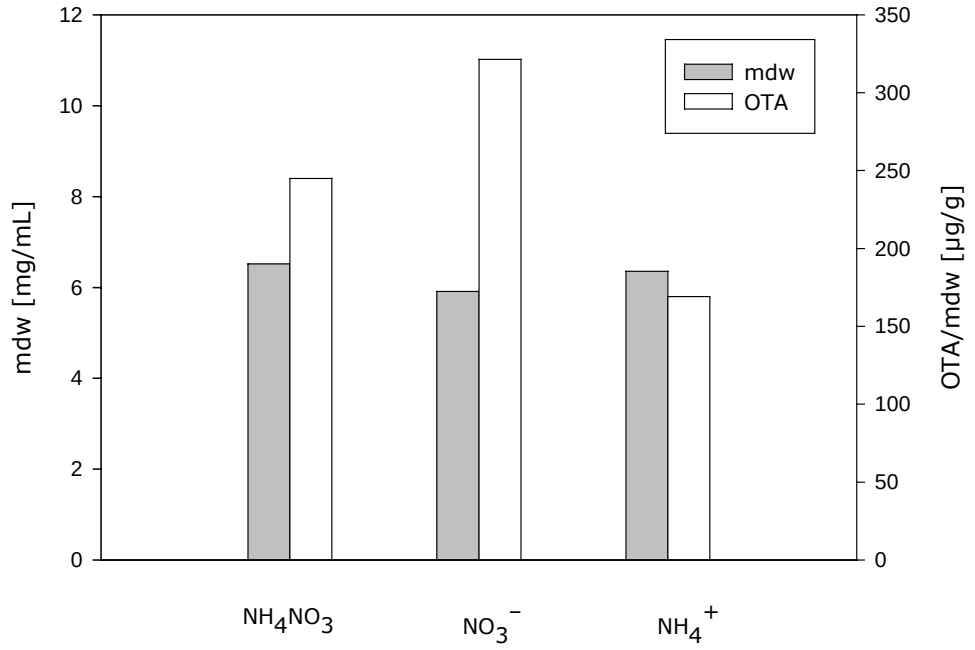


Fig. 3.14: Biomass and OTA production *Aspergillus ochraceus* NRRL 3174 in AM liquid medium at initial pH 6.5 after 120 h of incubation with different nitrogen sources supplied mdw: mycelial dry weight

3.2 Protein Purification from Submerse Cultures of OTA Producing *Aspergillus* spp.

One of the approaches on elucidating key genes of OTA biosynthesis used in this study was purification of the putative OTA synthetase catalyzing the final step of the OTA biosynthetic pathway: formation of an amide bond between the proposed direct OTA precursors ochratoxin α (OTα) and phenylalanine (Phe).

Earliest indications for existence of an OTA synthetase were given by Ferreira and Pitout (1969). In a preliminary study (Zapf, 1999), *in vitro* OTA synthesis in crude protein extracts of *A. ochraceus* mycelia after addition of OTα and Phe could be confirmed. However, these results, as well as the earliest of those presented below, were obtained from experiments with mycelia of *A. ochraceus* A8 and NRRL 3174 grown as submerse culture in YES liquid medium at room temperature. As described in chapter 3.1.1, considerable instabilities in OTA production occurred under these circumstances. Therefore, accomplishing stable OTA production in laboratory culture was a vital requirement for continuation of enzyme purification experiments. With establishing predictable and controllable OTA production by *A. ochraceus* NRRL 3174 in AM liquid medium adjusted to initial pH 6.5, these prerequisites for further research were met.

3.2.1 Measurement of OTA Formation in Activity Tests

In vitro OTA synthesis activity of crude extracts and $(NH_4)_2SO_4$ precipitated protein prepared as described in 2.2.5 was identified in activity tests in reaction tubes (2.2.6.2a)). Formation of OTA was verified by HPTLC after overnight incubation. These setups were chosen because the high protein concentrations necessary for synthesis of detectable OTA amounts disturbed fluorescence measurements. Dilution of those protein samples was not possible because of already low relative concentration of each protein in crude extracts as well as in $(NH_4)_2SO_4$ fractions. Overnight incubation was required for formation of OTA amounts detectable by HPTLC (>100 ng OTA).

Formation of OTA in activity tests using gel filtration fractions was measured in a fluorescence photometer (2.2.6.2b). Low absolute protein concentrations in these fractions but high relative concentration of each separated protein favored the photometric method in these setups. In order to estimate the amount of OTA formed in activity tests, an OTA/fluorescence calibration curve was set up. OTα solution (6 ng OTα/µL TBS) showed background fluorescence of approx. 1000 fluorescence units. Increasing concentrations of OTA (0.1 ng/µL - 5 ng/µL) added to OTα solution resulted in increased fluorescence at 448±5 nm (Fig. 3.15). Because of the background fluorescence of OTα as well as protein solution added to the activity tests, definite detection of OTA formation in these activity test setups was only feasible with concentrations >0.67 ng OTA/µL, equivalent to conversion of >10 % OTα.

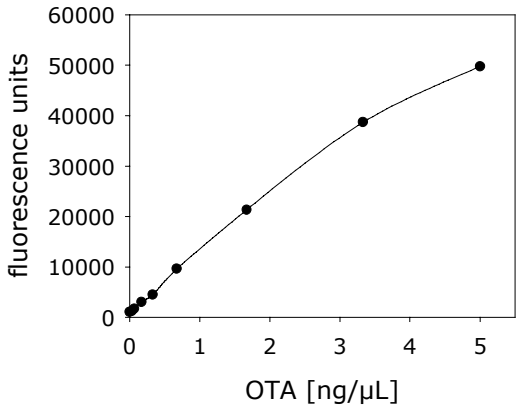


Fig. 3.15: Fluorescence calibration curve of increasing OTA concentrations in OTα solution (6 ng/µL) (excitation: 380±10 nm, emission: 448±5 nm)

3.2.2 Protein Purification from OTA Producing Cultures of *A. ochraceus* A8 Grown in YES Liquid Medium

Crude protein extract of *A. ochraceus* A8 mycelia was prepared using a mortar and pestle cooled with liquid nitrogen. Total cell protein displaying OTA formation in activity tests was precipitated using 60% $(NH_4)_2SO_4$. Protein factions were gradually redissolved in decreasing ammonium sulfate concentrations, resulting in activity within 25% to 35% $(NH_4)_2SO_4$ fractions. Therefore, in further experiments total cell protein was fractioned using ascending 20%, 40% and 60% concentrations of ammonium sulfate.

Proteins in the active 40% $(NH_4)_2SO_4$ fraction were further separated by gel filtration (Fig. 3.16). Resulting gel filtration fractions were tested for *in vitro* OTA synthesis utilizing a fluorescence photometer, resulting in a 6% increase in fluorescence in gel filtration fraction 23 (Fig. 3.17). Although fluorescence increase was too small for unambiguous detection of OTA formation, this result could be confirmed in each of two parallel experiments in two separate activity assays.

Elution of active fraction 23 occurred together with the salt peak of the running buffer, indicating molecular interactions with the column matrix (Superdex™ 200 prep grade, Amersham Biosciences, Freiburg). Therefore, molecular size of the proteins within this fraction could not be estimated.

Active fractions from the two parallel setups were pooled and protein was concentrated by ultra centrifugation (Centriprep YM-30, Millipore, Bedford, USA). Native PAGE and SDS-PAGE (2.2.7) were utilized for further separation of concentrated protein samples. Native gels were overlaid with a 1% agar layer containing 6 ng/µL OTα and 4 ng/µL Phe and incubated up to 24 h at room temperature. No fluorescence shift indicating OTA formation could be detected in these in-gel activity tests.

Using SDS PAGE, three distinct protein bands were visible in ultra filtration residue on silver stained PAA gels (Fig. 3.18). Those proteins were clearly accumulated in the ultra filtration residue of protein fractions showing *in vitro* OTA formation in photometric activity tests, compared to crude extracts and 40% $(NH_4)_2SO_4$ protein fraction. However, not enough protein could be collected for sequence analysis of these proteins separated on SDS PAGE gels.

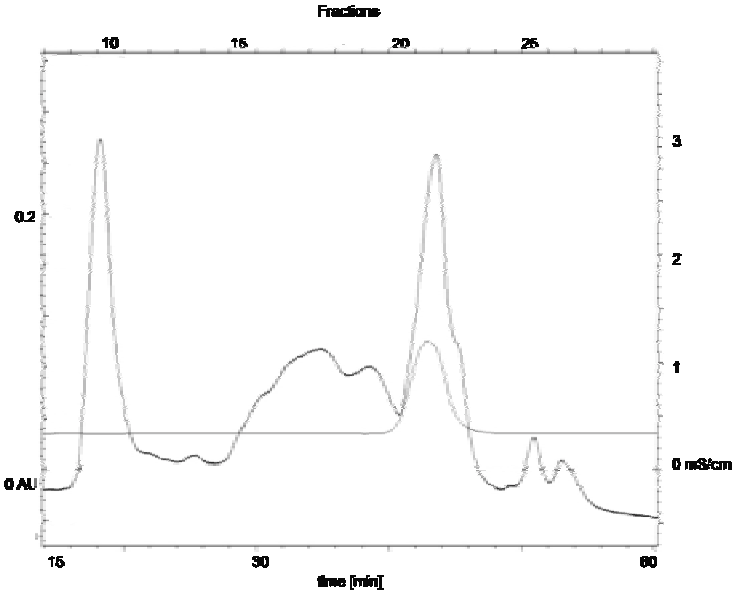


Fig. 3.16: Gel filtration chromatogram of 40 % $(NH_4)_2SO_4$ fraction of crude protein extracts from *A. ochraceus* A8 after 96 h of incubation in YES media, — UV absorption of proteins, ····Conductivity of running buffer

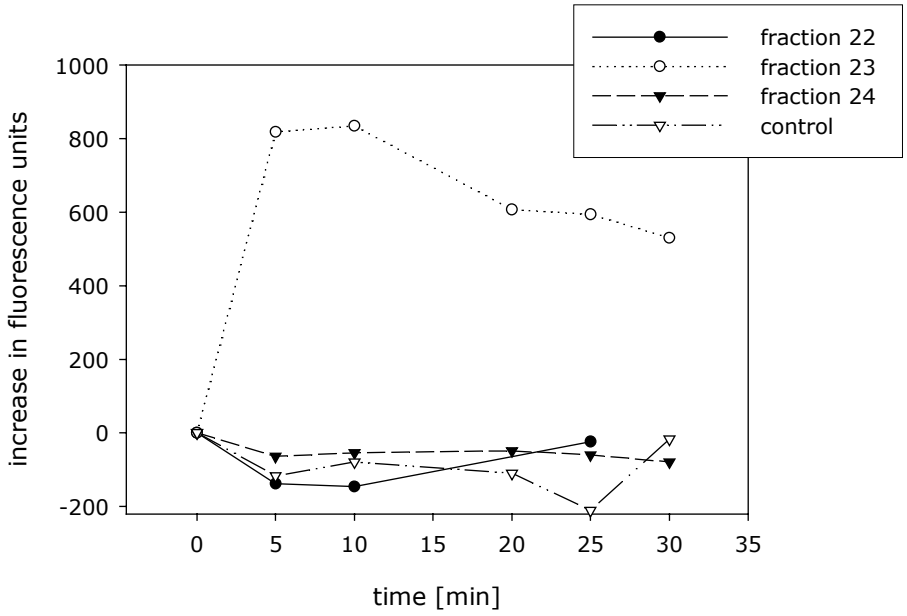


Fig. 3.17: Increase in fluorescence (exitation:360 ± 10 nm, emission: 465 ± 5 nm) in *in vitro* OTA synthesis activity tests, testing gel filtration fractions of protein extracted from mycelia of *A. ochraceus* A8 after 96 h of incubation in YES media.

For reproduction and extension of the results illustrated above, protein extracts were prepared according to the purification protocol described above from several OTA producing mycelia of *A. ochraceus* A8. Most of these protein extracts did not show *in vitro* OTA synthesis in continuing purification steps. Furthermore, considerable instability occurred in OTA production by cultures of *A. ochraceus* A8 in YES complex media. Therefore, growth experiments were conducted in order to find culture conditions favoring consistent OTA production (chapter 3.1). Mycelia of *A. ochraceus* NRRL 3174 reliably producing OTA after 72 h of incubation at 25°C in AM synthetic media adjusted to initial pH 6.5 were chosen as basis for further investigations.

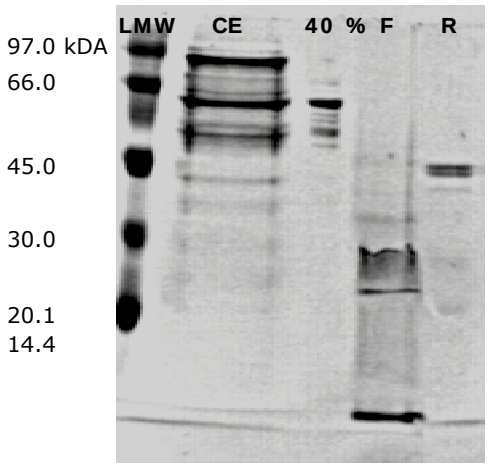


Fig. 3.18: Silver stained SDS PAGE of continuing purification steps of protein extracts from OTA producing mycelia of *A. ochraceus* A8. LMW: low molecular weight protein marker.
CE: crude extract. 40 %: protein fraction precipitated using 40 % $(NH_4)_2SO_4$. F: filtrate in ultra filtration. R: residue in ultra filtration.

3.2.3 Protein Purification from OTA Producing Cultures of *A. ochraceus* NRRL 3174 Grown in AM Liquid Medium

In order to overcome the difficulties arising from instability of OTA production by *A. ochraceus* A8 in YES media, further experiments were conducted using *A. ochraceus* NRRL 3174 cultivated in AM media.

3.2.3.1 Protein Purification from OTA Producing Cultures of *A. ochraceus* NRRL 3174 Grown in AM Media Adjusted to Initial pH 6.5

Mycelia of *A. ochraceus* NRRL 3174 were harvested after 96 h of incubation in AM media at initial pH 6.5. Crude protein extract was precipitated with increasing concentrations of ammonium sulfate and 40 % $(NH_4)_2SO_4$ fraction was separated by gel filtration (Fig. 3.19).

Gel filtration fractions were subjected to photometric activity tests without addition of ATP. Increase in fluorescence occurred in fraction 23 within the first 5 min of incubation at 30 °C. Results presented in Fig. 3.20 are the mean from two experiments conducted separately. However, this slight increase (850 FU, equaling 0.3 ng OTA/µL) was to small to confirm OTA formation. Doubling the amount of enzyme added to activity tests only raised background fluorescence and did not improve percentage of fluorescence increase.

After the first 5 min of incubation, no further fluorescence increase was detected. This observation indicated, that conditions for *in vitro* OTA formation were not yet met and residual essential compounds in the protein sample were used up. Further tests to determine necessity of several activity test components and additives like ATP did not produce any results in photometric measurements.

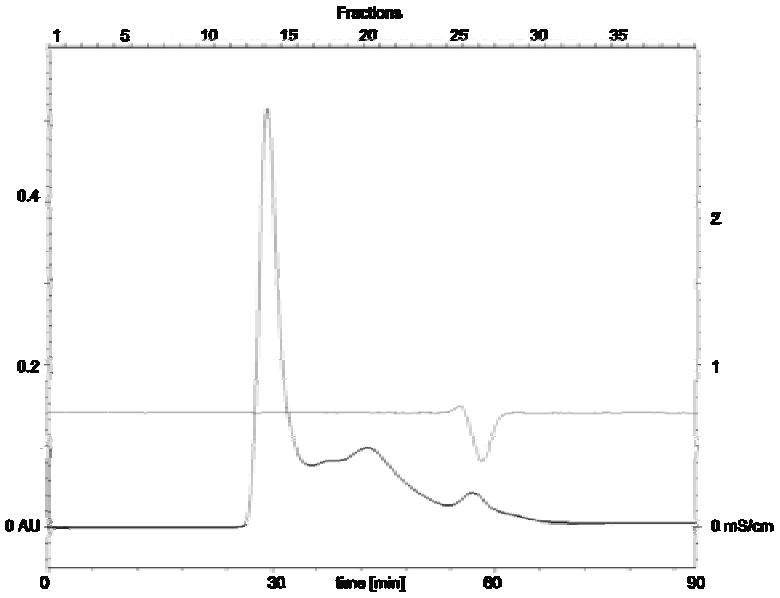


Fig. 3.19: Gel filtration chromatogram of 40 % $(NH_4)_2SO_4$ fraction of crude protein extracts from *A. ochraceus* NRRL 3174 after 96 h of incubation in AM media at initial pH 6.5, — UV absorption of proteins, ·····Conductivity of running buffer

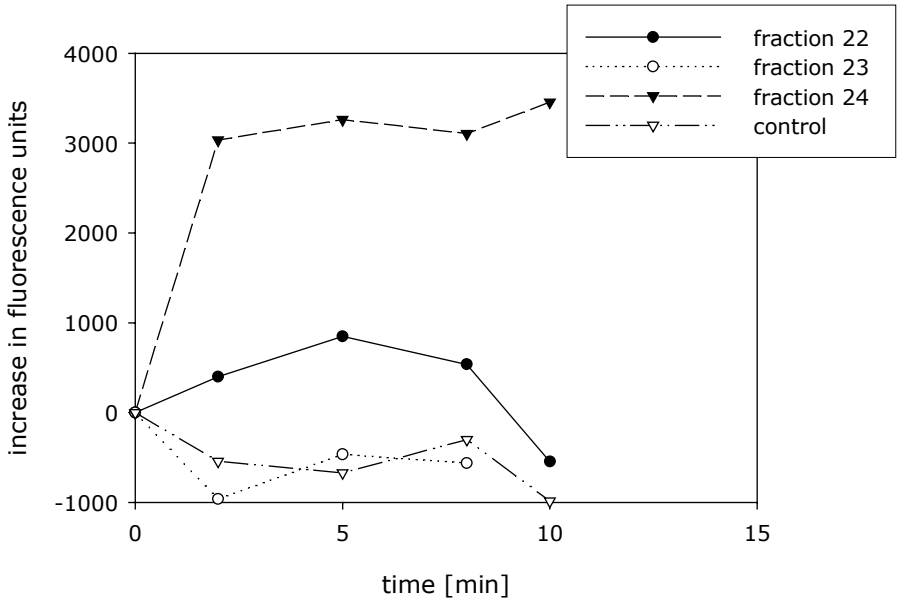


Fig. 3.20: Increase in fluorescence (exitation:380 ± 10 nm, emission: 448 ± 5 nm) in *in vitro* OTA synthesis activity tests, testing gel filtration fractions of protein extracted from mycelia of *A. ochraceus* NRRL 3174 after 96 h of incubation in YES media

In parallel to ammonium sulfate precipitation and gel filtration, crude extract samples extracted from mycelia *A. ochraceus* NRRL 3174 grown for 96 h in AM liquid medium adjusted to initial pH 6.5 were separated by IEC. No *in vitro* OTA synthesis was identified in any of the fractions tested.

In order to reproduce and expand these results, several OTA producing mycelia of *A. ochraceus* NRRL 3174 harvested after 96 h of incubation in AM medium adjusted to pH 6.5 were subjected to the purification protocol described above. The greater part of crude protein extracts from those mycelia did not show *in vitro* OTA synthesis. Some that did, lost OTA formation capability early during the purification procedure, i.e. after ammonium sulfate precipitation or gel filtration.

Despite the fact, that *A. ochraceus* NRRL 3174 consistently produced OTA in AM liquid medium adjusted to initial pH 6.5 after 72 h of incubation at 25°C, *in vitro* OTA synthesis capability of processed protein samples extracted from mycelia harvested after 96 h of incubation was very weak and instable. Harvesting mycelia of *A. ochraceus* NRRL 3174 after 72 h of incubation, no *in vitro* OTA synthesis was detected in gel filtration fractions and crude protein extracts.

Furthermore, determination of stabilizing conditions (e.g. buffer composition, activity test components) and further characterization of proteins was not achievable due to rapid aging of protein preparations.

3.2.3.2 Protein Purification From OTA Producing Cultures of *A. ochraceus* NRRL 3174 after pH Adjustment of AM Media

In the setups described above, instability of *in vitro* OTA synthesis activity in protein fractions prepared from several OTA producing mycelia of *A. ochraceus* NRRL 3174 was experienced. Probably, this was due to reduced enzyme activity in mycelia harvested after 96 h of incubation (24 h after onset of OTA production).

However, using mycelia harvested after 72 h, at the time point of emerging of detectable OTA in culture media, no *in vitro* OTA synthesis could be measured. As harvesting after 3 d resulted in very low enzyme yields, this setup might have presented too little biomass for detection of *in vitro* OTA synthesis.

OTA production in cultures of *A. ochraceus* NRRL 3174 was determined by initial pH of culture media and onset of OTA production could be controlled by pH adjustment (3.1.3.1). This pH effect on OTA production was utilized in the following experiments to improve yields of mycelia harvested shortly after onset of OTA production.

AM media adjusted to initial pH 5.0 were inoculated with spores of *A. ochraceus* NRRL 3174 and incubated for 48 h. After pH adjustment to pH 6.5 and 48 h of further incubation at 25 °C, mycelia were harvested. Crude protein extract was precipitated with increasing concentrations of ammonium sulfate and 40 % $(NH_4)_2SO_4$ fraction was subjected to gel filtration. Despite of the changes in culture conditions, the gel filtration chromatogram was similar to preceding ones (Fig. 3.21).

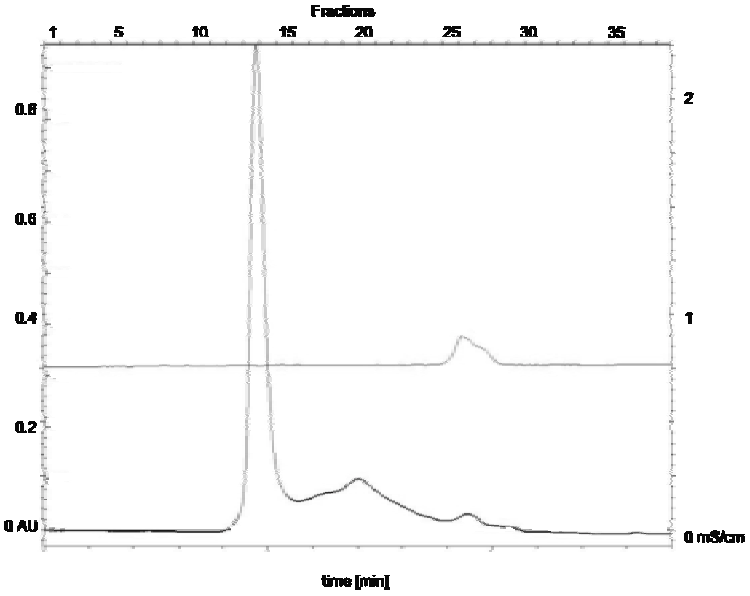


Fig. 3.21: Gel filtration chromatogram of 40 % $(NH_4)_2SO_4$ fraction of crude protein extracts from *A. ochraceus* NRRL 3174 after 48 h of incubation in AM media at initial pH 5.0 and further 48 h of incubation after pH adjustment to pH 6.5, — UV absorption of proteins, ····Conductivity of running buffer

Gel filtration fractions were tested for *in vitro* OTA synthesis in activity assays with 0.1 µmol ATP. No increase in fluorescence at 448±5 nm was detected in activity tests after short term incubation, therefore incubation times were prolonged to 72 h and 90 h. Only fraction 26 of one of two mycelia tested showed significant fluorescence increase after 72 h and 90 h of incubation, respectively (Fig. 3.22). Compared to the OTA/fluorescence calibration curve, this indicates a slow increase of OTA concentration to 1.5 ng OTA/µL in activity test setups containing 50 µL of gel filtration fraction 26 within 90 h of incubation. In contrast to short term incubation, these extended activity tests produced definite evidence for *in vitro* OTA synthesis. However, because of the experienced instability of OTA synthesis activity in protein preparations, this prolonged incubation time was unfavorable to further purification and characterization procedures.

In order to test which assay components were required for OTA synthetic activity, portions of crude protein extract, 40 % $(NH_4)_2SO_4$ fraction as well as active gel filtration samples were subjected to activity tests with and without ATP, OTα and/or Phe, respectively. In those assays, no *in vitro* OTA synthesis was detected, most likely due to rapid aging of the enzyme preparations.

Changing the culture conditions to increase biomass yields at harvesting almost immediately after onset of OTA production did not improve reliability of *in vitro* OTA synthesis in activity tests. Furthermore, incubation time in activity assays was too extended for following additional characterization of protein fractions. Therefore, no further enzyme purification experiments were conducted using cultures of *A. ochraceus* NRRL 3174.

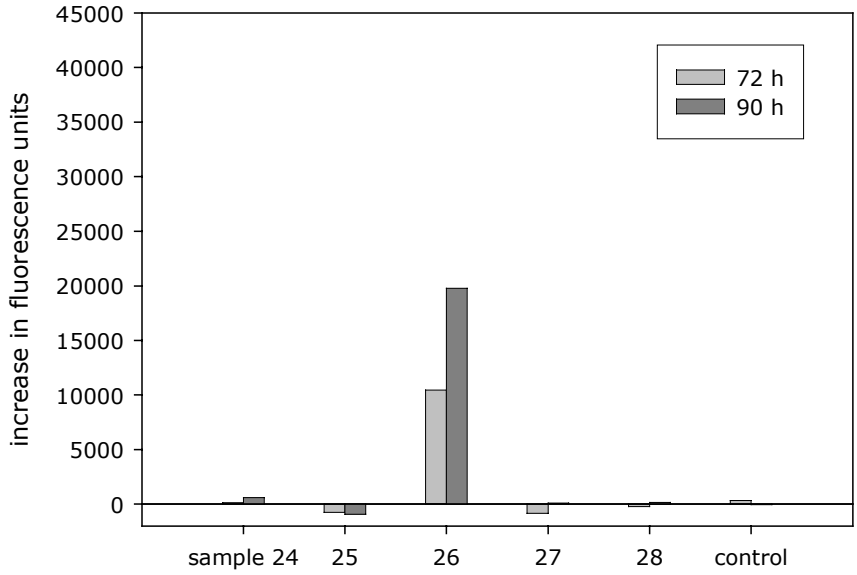


Fig. 3.22: Increase in fluorescence (exitation:380 ± 10 nm, emission: 448 ± 5 nm) in *in vitro* OTA synthesis activity tests after 72 h and 90 h of incubation at room temperature, testing gel filtration fractions of protein extracted from mycelia of *A. ochraceus* NRRL 3174 after 48 h of incubation in AM media at initial pH 5.0 and further 48 h of incubation after pH adjustment to pH 6.5

3.2.4 Protein Purification from OTA Producing Cultures of *A. niger* var. *niger* CBS 101697 Grown in AM Liquid Medium Adjusted to pH 6.5

In order to test whether *in vitro* OTA synthesis activity was more reliable in protein extracts from other OTA producing *Aspergillus* spp., enzyme purification experiments were set up using *A. niger* var. *niger* CBS 101697 which was found to be a reliable producer of OTA in the preceding experiments.

Mycelia of *A. niger* var. *niger* CBS 101697 were harvested after 96 h of incubation in AM media at pH 6.5. Crude protein extract was precipitated with increasing concentrations of ammonium sulfate and 40% $(NH_4)_2SO_4$ fraction was subjected to gel filtration (Fig. 3.23).

Gel filtration fractions were tested for *in vitro* OTA synthesis in activity tests. Characteristic fluorescence increase was measured in fractions 25-27 after 44 h and 72 h of incubation at room temperature (Fig 3.24). OTA concentrations within 72 h of incubaton increased to 1.5 ng OTA/µL in fraction 25, 4 ng OTA/µL in fraction 26 and 2.2 ng OTA/µL in fraction 27.

Although fluorescence increase occurred earlier and was more obvious than observed using protein preparations from cultures of *A. ochraceus* NRRL 3174, no *in vitro* OTA synthesis occurred in further assays on necessity of test components incubation increased to 1.5 ng OTA/µL in fraction 25, 4 ng OTA/µL in fraction 26 and 2.2 ng OTA/µL in for OTA synthesis. Also, not enough protein could be accumulated using gel filtration for further analysis of proteins separated on SDS PAGE gels.

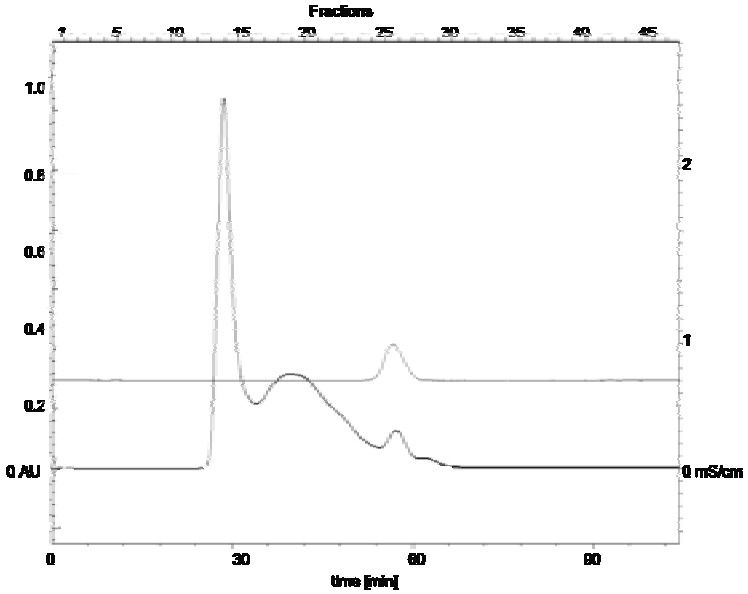


Fig. 3.23: Gel filtration chromatogram of 40 % $(NH_4)_2SO_4$ fraction of crude protein extracts from *A. niger var. niger* CBS 101697 after 96 h of incubation in AM media at initial pH 6.5, — UV absorption of proteins, ····Conductivity of running buffer

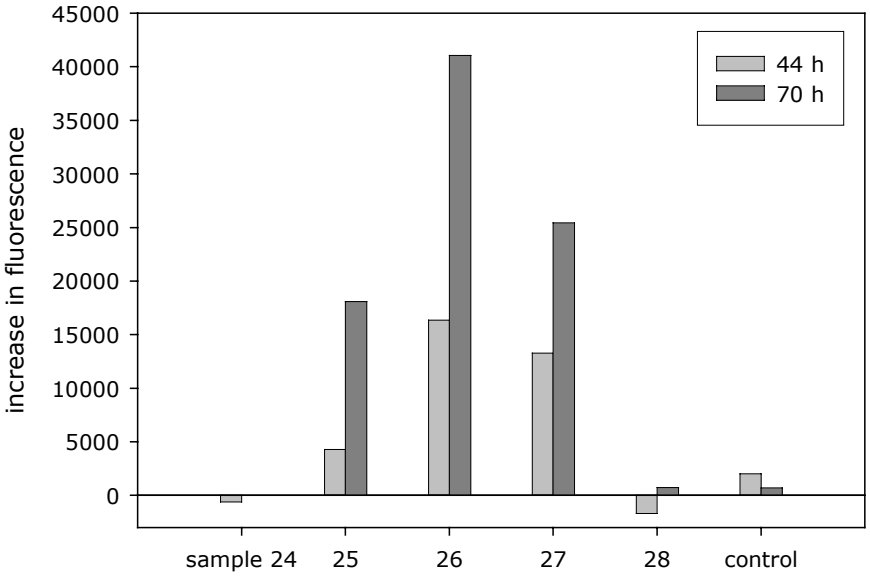


Fig. 3.24: Increase in fluorescence (exitation:380 ± 10 nm, emission: 448 ± 5 nm) in *in vitro* OTA synthesis activity tests after 44 h and 70 h of incubation at room temperature, testing gel filtration fractions of protein extracted from mycelia of *A. niger var. niger CBS 101697* after 96 h of incubation in AM media at initial pH 6.5

After separation of proteins by IEC, no *in vitro* OTA synthesis could be detected in any of the fractions tested. Harvesting mycelia of *A. niger* var. *niger* CBS 101697 after 72 h of incubation presented no positive results in activity tests with and without 0.1 µmol ATP.

As instabilities and other difficulties described above in enzyme purification setups could not be overcome in the extent and with the means of this study, it became clear that these attempts were not suitable to accomplish the aim to find genes connected to OTA biosynthesis. An alternative approach on revealing those genes was chosen: molecular genetic experiments comparing gene expression patterns under OTA permissive and nonpermissive culture conditions.

3.3 Characterization of Genes Connected to OTA Biosynthesis Using cDNA-AFLP

The decisive prerequisite for elucidation of key genes of OTA biosynthesis, apart from stability of OTA production in laboratory culture, was to have a reliable means to induce or suppress OTA production in culture by altering no more than one growth factor.

Such a tool suitable as a basis for further research on OTA biosynthesis was acquired conducting the growth experiments described in chapter 3.1. With adjustment of initial pH of AM culture media to pH 6.5 or pH 5.0, respectively, OTA permissive and nonpermissive growth conditions could be created only changing a single growth parameter.

In order to find genes connected to OTA biosynthesis, gene expression patterns of cultures of *A. ochraceus* NRRL 3174 from growth stages before (<72 h incubation time) and after onset of OTA production under OTA-permissive (initial pH of AM culture medium pH 6.5) and nonpermissive (initial pH of AM culture medium pH 5.0) conditions were compared using cDNA-AFLP.

3.3.1 Preparation of cDNA from Mycelia of *A. ochraceus* NRRL 3174 Grown in AM Liquid Medium under OTA Permissive and Nonpermissive Conditions

Cultures of *A. ochraceus* NRRL 3174 were grown for different periods in AM liquid medium supplemented with 5% glucose, 3g/L NH_4NO_3 and a full set of trace metals. In order to create OTA permissive and nonpermissive growth conditions, media were adjusted to initial pH 6.5 or 5.0, respectively. To be able to compare growth stages before and after onset of OTA production, mycelia were harvested after continuing hours of incubation –48 h, 56 h, 72 h, 76 h and 80 h– according to 2.2.4. Samples were stored at –80 °C until further use. Total RNA was isolated from each of these semi-dried mycelia (2.2.8). Residual DNA in the RNA samples was digested in a DNase incubation step. From each total RNA sample, mRNA was transcribed into double stranded cDNA using oligo$(dT)_{12}$ primers as described in 2.2.9 (Table 3.3). Quality and quantity of RNA and cDNA samples were determined according to 2.2.10 and 2.2.11. cDNA samples were stored at –20 °C.

Table 3.3: Numbers of cDNA samples isolated from *A. ochraceus* NRRL 3174 mycelia harvested after several periods of growth in AM liquid medium adjusted to initial pH 6.5 or pH 5.0, respectively

Sample Number	Incubation Time	Initial pH of AM Culture Medium
1	48 h	pH 6.5
2	56 h	pH 6.5
3	72 h	pH 6.5
4	76 h	pH 6.5
5	80 h	pH 6.5
6	96 h	pH 6.5
7	48 h	pH 5.0
8	56 h	pH 5.0
9	72 h	pH 5.0
10	76 h	pH 5.0
11	80 h	pH 5.0
12	96 h	pH 5.0

3.3.2 cDNA-AFLP with cDNA Samples from Mycelia of *A. ochraceus* NRRL 3174 Grown in AM Liquid Medium under OTA Permissive and Nonpermissive Conditions

Following translation into double stranded cDNA, all samples were subjected to cDNA AFLP (2.2.12): cDNA was restricted with *Mse* I and ligated specifically to *Mse*-AFLP adapters at the restriction sites. All cDNA fragments with two adapters were amplified by a preamplification PCR using non-selective primer *Mse* I. In order to limit emerging cDNA patterns to a manageable number of fragments, specific amplification of a selection of cDNA fragments was accomplished in an amplification reaction with primers containing 1–3 selective bases at the 3' end. Resulting amplified cDNA fragments were separated on PAA gels and silver stained.

After several preliminary experiments with different specific primers in the amplification reaction, a setup using primer *Mse* I in preamplification reactions and primer MAC in subsequent amplification reactions was utilized. Four separate fragments could be distinguished in this setup only detectable in cDNA prepared after onset of OTA production (>72 h of incubation time) from cultures of *A. ochraceus* NRRL 3174 grown under OTA permissive conditions (samples 3 – 6 in Fig. 3.26).

3.3.3 Characterization of cDNA Fragments

In the cDNA-AFLP experiment as described above, four of the observed fragments were only detectable in cDNA preparations from cultures of *A. ochraceus* NRRL 3174 after onset of OTA production under OTA permissive conditions. These four fragments were subjected to further characterization as described in 2.2.13.

3.3.3.1 Cloning and Sequencing of Reamplified cDNA Fragments Only Detectable in cDNA Samples Isolated from OTA Synthesizing Mycelia

cDNA fragments were cut out of the silver stained PAA gel and redissolved in TE buffer. After separate reamplification of all four fragments in an AFLP-PCR with primer MAC, PCR products were ligated into vector pSTBlue-1 (AccepTor™ Vector, Novagen, Madison, USA). Resulting plasmids were transformed into *E. coli* DH5α. Plasmid DNA was reisolated from ampicillin resistant white clones of *E. coli* DH5α, that could only be found in transformations of vectors ligated to fragment 3 or 4, respectively. Plasmids were checked for insert size by reamplification of possible inserts using AFLP-PCR and subsequent gel electrophoresis (Fig. 3.25).
Two plasmids containing an insert of approx. 500 bp and 600 bp, respectively, were subjected to sequencing reactions. Resulting sequences of cDNA fragments 3 and 4 are presented in Fig. 3.27.

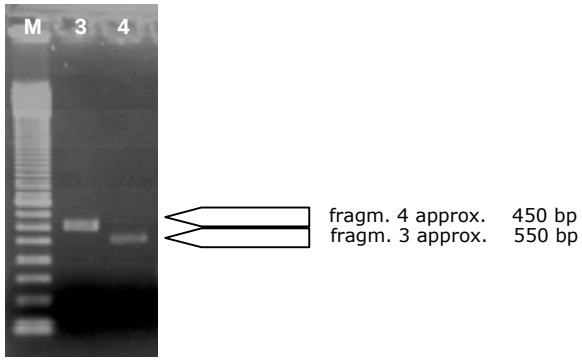
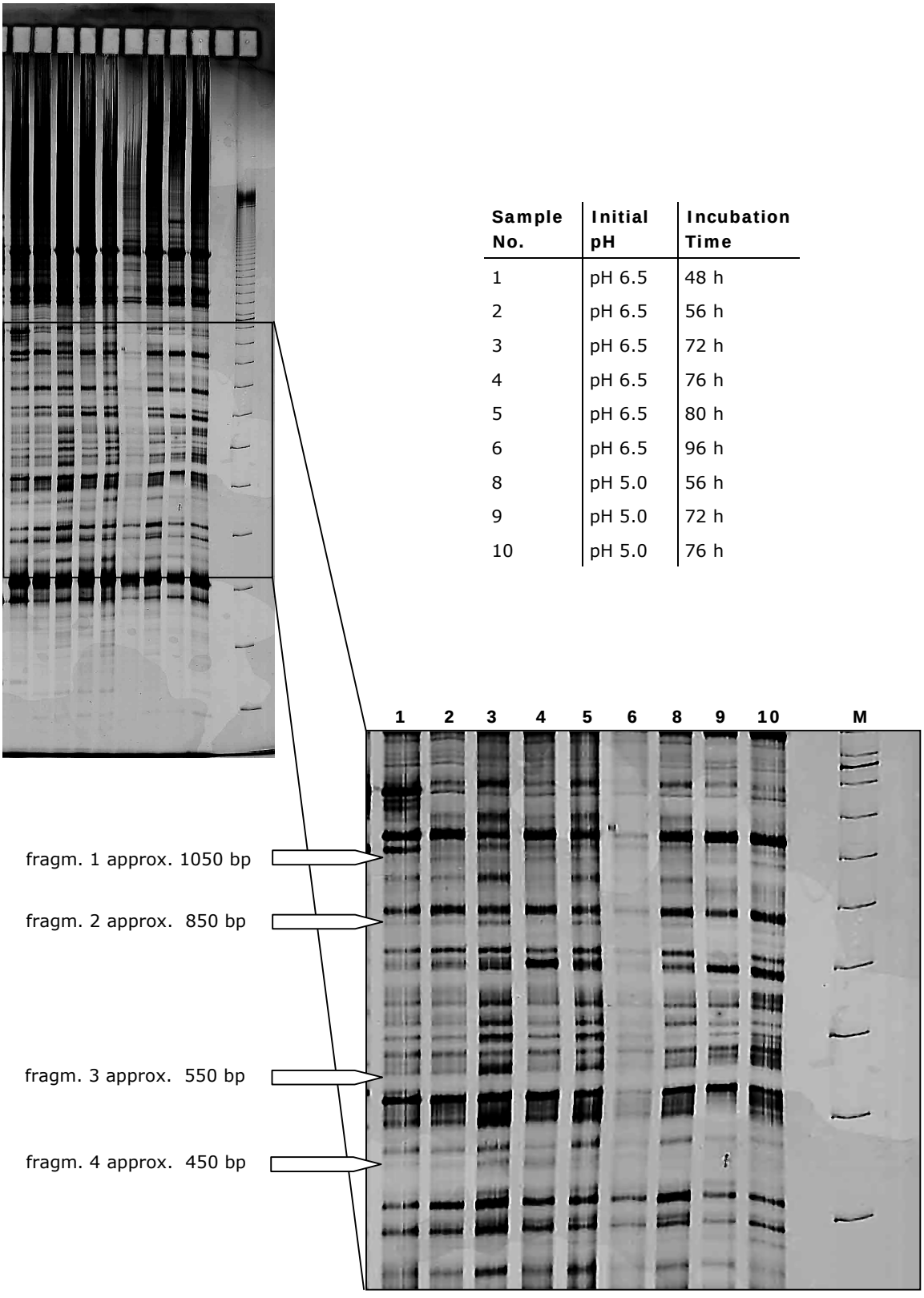


Fig. 3.25: Agarose gel electrophoresis of DNA fragments amplified from plasmids (vector pSTBlue-1 + insert: fragment 3 or fragment 4, resp.) isolated from *E.coli* DH5α in an AFLP-PCR with primer MAC, M: 100 bp ladder



Sample No.	Initial pH	Incubation Time
1	pH 6.5	48 h
2	pH 6.5	56 h
3	pH 6.5	72 h
4	pH 6.5	76 h
5	pH 6.5	80 h
6	pH 6.5	96 h
8	pH 5.0	56 h
9	pH 5.0	72 h
10	pH 5.0	76 h

Fig. 3.26: Silver stained PAA gel loaded with AFLP PCRs of cDNA obtained from 9 different mycelia: *Aspergillus ochraceus* KA103 was cultivated under OTA permissive (pH 6.5) and nonpermissive (pH 5.0) conditions for 48 h to 96 h. Arrows indicated cDNA fragments only to be found in cDNA preparations from OTA producing mycelia. M: DNA size marker.

Fragment 3

```
1    5'CGGTATCTCCTGTCATTTAGAGTAATAAGGCTGGCTAGAAGATAGAAAGGTAAAAAGTCACTGGAG   66
67   TCGTTAGGGCTGTCCTGACCGATGATGTAATAGTTGCTGCCTTAGGCTTCCACTTCGGCAGCTCCCAG  134
                3for
135  AACAGCTAGCGCCAGGTGTCGGCCGGGCATGTGAAAGGCCTCCAGAATCCTCGTCCGTTGAGTCTTTT  202
                              3rev1
203  GCATCTCCGATAAGCAGTCCCATTCCAAATTCCCCCATCGTTTGACAGCCTGTGATCGTCCCACTCGG  270
271  TTGCACTTCTATTGTCTGCACGGACTGTGTTTCGGAGACATGCTCACCTGGCAAGTTCGGTAATCTTC  338
339  AACCCCCACGACTAGCTTTCAAGCATGGAAAGGTTTACTCAGTTTCGGGATCGAGGTTCGCTCCATCT  406
407  CCCTCTAGTGACCCTGGTTCAGACTAACGCGCTTTCTAATGATGCAGGGTCGGGCATCGCCCCCTTTC  474
475  TCCCCGTTCCCTCTGAACCGCTCAGTTTTCAGCTGCCGTTATGCGTCTTTCTCTTCTTCCTCCGCCTC  542
                                                                3rev2
543  CCGTTCTTCATCTTCGTCT 3'                                                561
```

Fragment 4

```
1    5' CGCATGGCAGCTTTTTCATTCTATATTTGACGACGGTGTTCTCGGAAACCCAGAACAGCTAGCTAC   66
                                       4for
67   CTTGTTTAGCAAACAGGTGGAGTATTACCATCGTCTGCTTTACAATGACGTATCAGATTGTCTAGCAG   134
135  AAATTGGCCGCAGAACGATCGCTTTTCGGCCAGCTGGACAGCTAGCATAACCTTCTGGCCCGAGCATT   202
203  ATTTGGCCTCTGGAACCCCTATTGGGTCCCGCATGAAGGACGGATTAGAAACACGTCGACACCGGTGT   270
                          4rev1
271  AATTGTCAATGTGGATAGACGATCCCAACCGGTCTACTCCGTACTCGCATAAATGTATGCTGGGCTCC   338
339  AGCGGAGGCCAATGCCGACTCGGCATCCCACCCAATGCCATGCCACTACCAAGTCGCTTCGGCCGTCC   406
407  AACCCCTCCTTGCCGATTGAACTCTCGCCCGGAGGAAGCGAAAGCAATAAATGTGGGGCATTGT 3'    470
                          4rev2
```

Fig. 3.27: DNA sequences of fragments 3 and 4 reamplified from a silver stained PAA gel loaded with AFLP PCRs of cDNA obtained *Aspergillus ochraceus* NRRL 3174 cultivated under OTA permissive and conditions for 72, 76, 80 and 96 h, respectively (see Fig. 3.26). Gray boxes indicate position of binding sites for specific primers

3.3.3.2 Sequence Analysis of cDNA Fragments 3 and 4

(Translated) DNA sequences of the two fragments were compared with a wide variety of other DNA or protein sequences for possible homologies using the BLAST tool (Altschul, 1997; Altschul *et al.*, 1990). No significant similarities to nucleotide or amino acid sequences registered in the BLAST library could be detected in any reading frame applied to the DNA sequence.

Nucleotide sequences were translated into amino acid sequences in the 6 possible reading frames using the DNASIS Software Version 2.5 (Hitachi Software Engineering Company, Ltd.). Resulting peptide sequences, however, did not show a continuous open reading frame (ORF) in neither of the cDNA fragments in any of the 6 possible reading frames.

3.3.3.3 Analysis of Expression Patterns of mRNA Represented by cDNA Fragments 3 and 4 by Quantitative PCR

As described above, four distinct cDNA fragments were only detectable in samples 3, 4, 5 and 6 separated on a PAA gel after AFLP amplification. Samples 3 – 6 represented mRNA isolated from *A. ochraceus* NRRL 3174 cultures after onset of OTA production under OTA permissive conditions.

Two of these four cDNA fragments that were cut out of the PAA gel were reamplified and sequenced. In order to determine whether the cDNA fragments 3 and 4 were in fact representing differentially expressed genes, quantitative real time PCR was conducted with all cDNA samples listed in Table 3.3 using primer pairs 3for/3rev1 and 4for/4rev1, respectively. In order to determine absolute cDNA concentration of the samples tested, a separate reaction with primers Bt2a/Bt2b was set up for each cDNA sample. For DNA quantification, a dilution series of genomic DNA of *A. ochraceus* NRRL 3174 was subjected to quantitative PCR with primer pair 4for/4rev1. From the fluorescence curves of the diluted genomic DNA a linear regression was calculated for determination of the DNA content of the cDNA samples (Fig. 3.28).

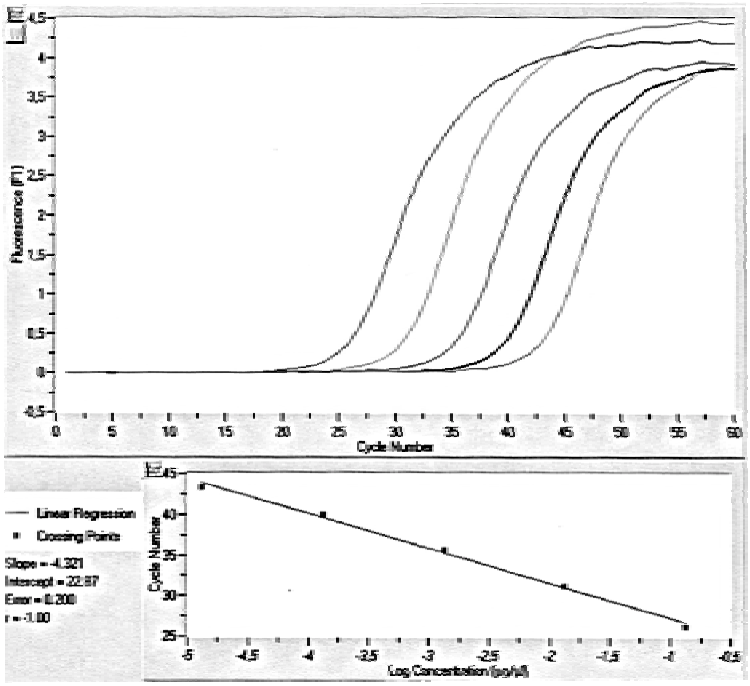


Fig. 3.28: Calibration curve and linear regression of a genomic DNA dilution series of *A. ochraceus* NRRL 3174 amplified with 4for/4rev1

Fig. 3.29 shows fragments amplified with each of the three primer pairs from cDNA or DNA samples from *A. ochraceus* NRRL 3174. The occurrence of an 208 bp intron within fragment amplified from the β-tubulin gene allows differentiation between DNA and cDNA in real time PCR experiments: amplification of only one PCR product with a melting point of 86,7°C indicates that the cDNA samples were not contaminated with genomic DNA (Fig. 3.30).

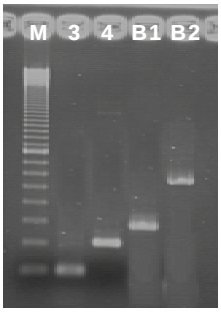


Fig. 3.29: Agarose gel electrophoresis of DNA fragments amplified from *A. ochraceus* NRRL 3174 cDNA samples with primer pairs 3for / 3rev1 (**3**, 101 bp), 4for / 4rev1 (**4**, 204 bp) and Bt2a / Bt2b (**B1**, 287 bp), respectively, and genomic DNA from *A. ochraceus* NRRL 3174 with Bt2a / Bt2b (**B2**, 495 bp), M: DNA size marker

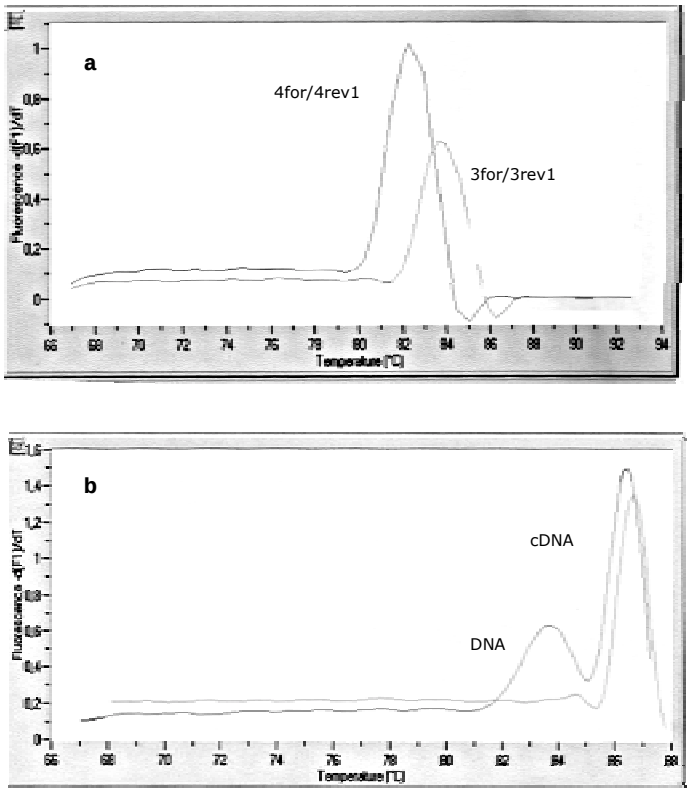


Fig. 3.30: Melting curves of real time PCR products from amplifications of cDNA sample 12 (see Table 3) with 3for / 3rev1 and 4for / 4rev1, respectively (**a**) and genomic DNA of *A. ochraceus* NRRL 3174 and cDNA sample 12 with Bt2a / Bt2b (**b**).

Real time PCR experiments showed that both cDNA fragments were regulated in a similar way, although apparently at different overall concentrations compared to the constitutively expressed β-tubulin gene: expression of genes represented by fragment 3 and 4 increased shortly before onset of OTA production. Fragment 3 and fragment 4 were present up to elevenfold in mRNA preparations from OTA permissive, derepressed mycelia of *A. ochraceus* NRRL 3174 (initial pH 6.5, 72 h of incubation; cDNA sample 3) compared to OTA permissive, repressed stages (initial pH 6.5, <72 h incubation time; cDNA sample 1,2) and nonpermissive conditions (initial pH 5.0; cDNA samples 7-12) (see Table 3.3).

Fig. 3.31 and Fig. 3.32 compare mean results of two separate LightCycler™ quantification experiments using primers 3for/3rev1 and 4for/4rev1, respectively, and cDNA samples of cultures of *A. ochraceus* NRRL 3174 grown for increasing incubation times in AM liquid media adjusted to initial pH 6.5 or pH 5.0, respectively, with OTA/biomass production in these cultures.

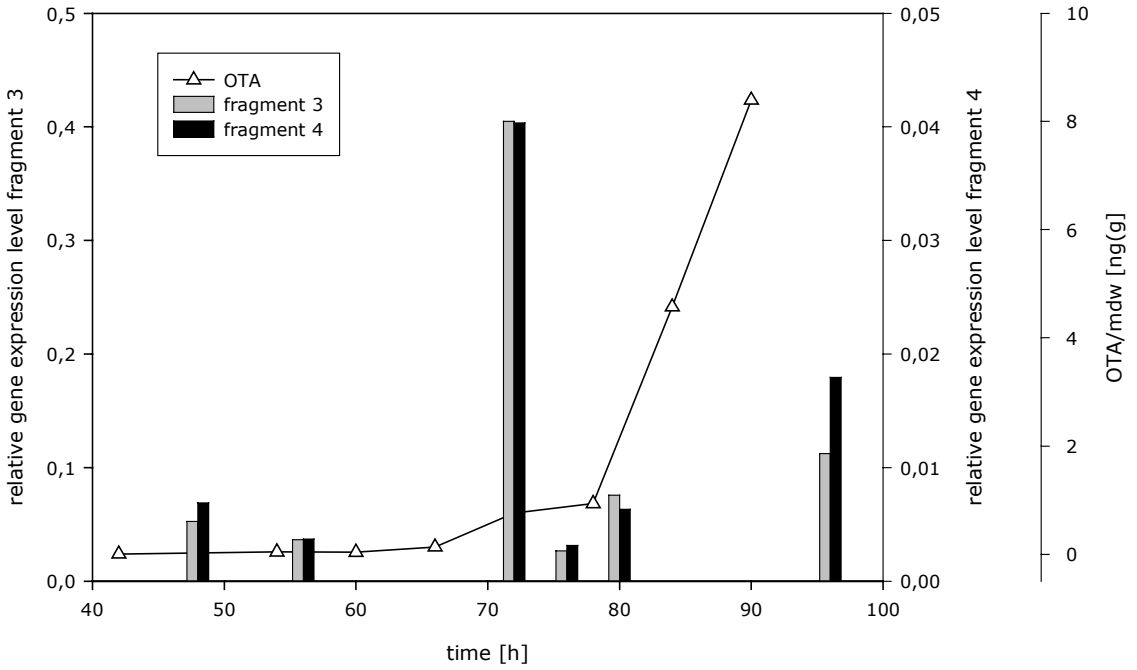


Fig. 3.31: Relative expression of genes represented by fragment 3 and 4 in cDNA samples compared to OTA/biomass production by *A. ochraceus* NRRL 3174 in AM liquid medium at initial pH 6.5, mdw: mycelial dry weight

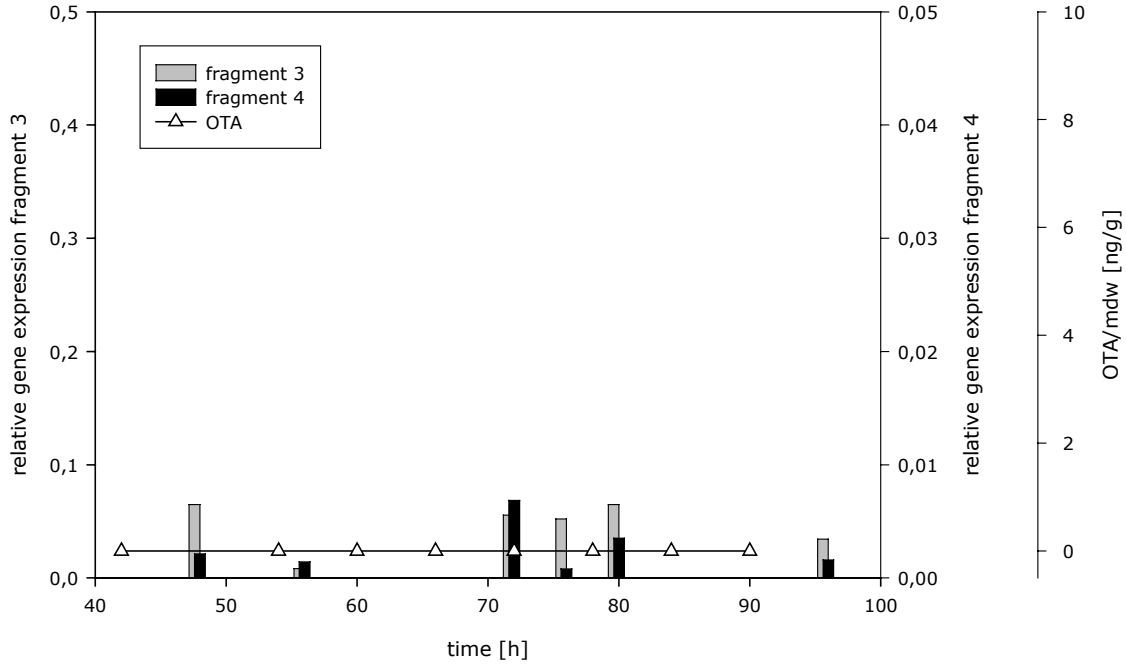
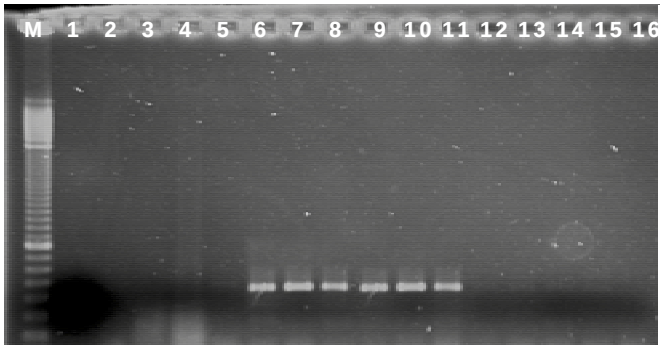


Fig. 3.32: Relative expression of genes represented by fragment 3 and 4 in cDNA samples compared to OTA/biomass production by *A. ochraceus* NRRL 3174 in AM liquid medium at initial pH 5.0, mdw: mycelial dry weight

3.3.3.4 Detection of other OTA Producing *Aspergillus* spp. with primers derived from differentially expressed cDNA fragments

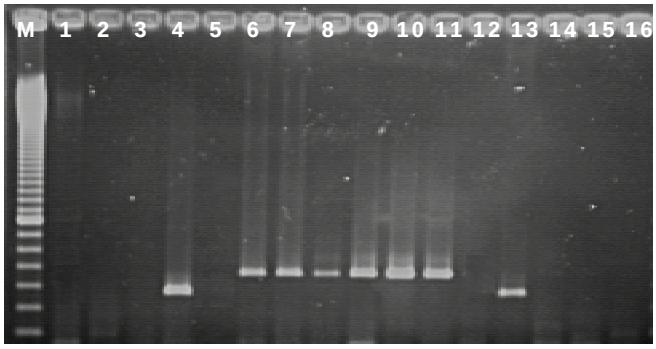
Occurrence of possible genes represented by cDNA fragments 3 and 4 in cDNA samples and genomic DNA of *A. ochraceus* NRRL 3174 was demonstrated in regular PCR and LightCycler™ PCR using primer pairs derived from cDNA fragment 3 and 4, respectively (see Fig. 3.29). Several other OTA producing and non-producing *Aspergillus* spp. were tested for occurrence of similar nucleotide sequences in their genomic DNA in PCRs with primer pairs 3for/3rev2 or 4for/4rev2, respectively.

Specific amplification of a 470 bp fragment with genomic DNA from *A. ochraceus* strains was achieved using primer pair 3for/3rev2, as shown in Fig. 3.33. Using primer pair 4for/4rev2, a 410 bp fragment with genomic DNA of *A. ochraceus* strains was amplified. Furthermore, species of the *A. carbonarius* group were detected by a smaller fragment of about 350 bp (Fig. 3.34).



1. *A. alliaceus* DSM 813
2. *A. awamori* CBS 101704
3. *A. carbonarius* M335
4. *A. carbonarius* M336
5. *A. fresenii* CBS 550.65
6. *A. ochraceus* A8
7. *A. ochraceus* CBS 588.68
8. *A. ochraceus* CBS 589.68
9. *A. ochraceus* CCT 6819
10. *A. ochraceus* M 337
11. *A. ochraceus* NRRL 3174
12. *A. niger* CBS 101698
13. *A. niger var. niger* CBS 101697
14. *A. usamii* var. *shiro-usamii* CBS 101700
15. *Penicillium verrucosum* CBS 603.14
16. *Fusarium culmorum* LWL F85-5

Fig. 3.33: Agarose gel electrophoresis of 470 bp DNA fragments amplified from indicated fungal genomic DNA samples with primer pair 3for/3rev2. M: DNA size marker.



1. *A. alliaceus* DSM 813
2. *A. awamori* CBS 101704
3. *A. carbonarius* M335
4. *A. carbonarius* M336
5. *A. fresenii* CBS 550.65
6. *A. ochraceus* A8
7. *A. ochraceus* CBS 588.68
8. *A. ochraceus* CBS 589.68
9. *A. ochraceus* CCT 6819
10. *A. ochraceus* M337
11. *A. ochraceus* NRRL 3174
12. *A. niger* CBS 101698
13. *A. niger var. niger* CBS 101697
14. *A. usamii* var. *shiro-usamii* CBS 101700
15. *P. verrucosum* CBS 603.14
16. *F. culmorum* LWL F85-5

Fig. 3.34: Agarose gel electrophoresis of 410 bp and 350 bp DNA fragments amplified from indicated fungal genomic DNA samples with primer pair 4for/4rev2.
M: DNA size marker.

Table 3.4 summarizes the amplification results of both primer pairs with genomic DNA preparations of several OTA producing and non-producing fungi. All strains of *A. ochraceus* were detected by primer pair 3for/3rev2. All strains of *A. ochraceus* as well as *A. carbonarius* using primers 4for/4rev2. In both cases, detection was regardless of the OTA production ability given by the supplier.

Table 3.4: Occurrence of an amplification product with genomic DNA preparations of several OTA producing and non-producing fungi in PCRs using primer pair 3for/3rev2 or 4for/4rev2, respectively.
OTA: OTA production ability as desribed by the supplier (see Table 2.1).

Template genomic DNA of			**Amplification product with**		
			3for / 3rev2	**4for / 4rev2**	
species	**strain**	**OTA**	**470 bp**	**410 bp**	**350 bp**
Aspergillus alliaceus	TMW 4.1077	–	–	–	–
A. awamori	CBS 101704	+	–	–	–
A. carbonarius	M 324	+	+	–	+
A. carbonarius	M 325	+	–	–	+
A. carbonarius	M 335	+	–	–	+
A. carbonarius	M 336	+	–	–	+
A. elegans	CBS 310.80	–	–	–	–
A. flavus	CBS 113.32	–	–	–	–
A. foetidus	CBS 114.49	–	–	–	–
A. fresenii	CBS 550.65	+	–	–	–
A. fumigatus	TMW 4.0624	–	–	–	–
A. ochraceus	A 8	+	+	+	–
A. ochraceus	A 91	–	+	+	–
A. ochraceus	CBS 263.67	+	+	+	–
A. ochraceus	CBS 588.68	+	+	+	–
A. ochraceus	CBS 589.68	+	+	+	–
A. ochraceus	CCT 6794	+	+	+	–
A. ochraceus	CCT 6796	+	+	+	–
A. ochraceus	CCT 6810	–	+	+	–
A. ochraceus	CCT 6815	–	+	+	–
A. ochraceus	CCT 6816	–	+	+	–
A. ochraceus	CCT 6817	+	+	+	–
A. ochraceus	CCT 6819	–	+	+	–
A. ochraceus	CCT 6820	+	+	+	–
A. ochraceus	CCT 6837	–	+	+	–
A. ochraceus	CCT 6847	–	+	+	–
A. ochraceus	CCT 6901	–	+	+	–
A. ochraceus	CCT 6911	–	+	+	–
A. ochraceus	CCT 6916	+	+	+	–
A. ochraceus	M337	+	+	+	–
A. ochraceus	NRRL 3174	+	+	+	–
A. niger	CBS 101698	+	–	–	–
A. niger	CCT 6809	–	–	–	–
A. niger var. *niger*	CBS 101697	+	–	–	+
A. scleotiorum	CBS 549.65	–	–	–	–
A. usamii var. *shiro-usamii*	CBS 101700	+	–	–	–
Fusarium graminearum	TMW 4.0449	–	–	–	–
F. poae	TMW 4.0042	–	–	–	–
Penicillium verrucosum	CBS 603.14	+	–	–	–
P. verrucosum	CBS 815.96	+	–	–	–
Stachybotrys chartarum	CBS 222.46	–	–	–	–

4 Discussion

The aim of the present study was to devise an adequate basis allowing for the setup of a molecular diagnostic tool for detection of ochratoxigenic fungi on feed and foodstuffs. In order to simultaneously detect as many *Aspergillus* spp. with OTA production potential as possible, these tools ought to be based on special genetic features of OTA producers, meaning that they should either be species or group specific or aimed at genes of the biosynthetic pathway leading to OTA formation.

Within this study, elucidating key genes of OTA biosynthesis was first attempted using enzyme purification methods. This approach was aimed at enzymes involved in the OTA biosynthetic pathway, particularly the putative OTA synthetase catalyzing the final step of this pathway.
An alternative molecular genetic method was used in this study in order to discover genetic markers for ochratoxigenic *Aspergillus* spp. based on either group specificity or genes connected to OTA biosynthesis. For the latter setup, gene expression was compared in cultures grown under OTA submissive or non-submissive conditions, respectively.

For both approaches on elucidation of key genes of OTA biosynthesis, consistency of OTA production was a vital requirement. Especially for the genetic setup, availability of a reliable means to induce or suppress OTA production in culture by altering no more than one growth factor was another important prerequisite.

4.1 Ochratoxin A Production by *Aspergillus* spp.

Working with mycelia of *A. ochraceus* A8 grown in YES complex medium, considerable fluctuation in OTA biosynthesis and even apparent complete loss of OTA production capability was observed. However, in order to provide the prerequisites for further research on key genes of OTA biosynthesis, consistency and predictability of OTA production in laboratory culture was imperative. In preliminary experiments, dependence of OTA production on selected growth parameters did not appear to coincide with literature data.

Therefore, detailed knowledge was required about factors determining OTA production in the specific setup used in this study. For this purpose, a series of growth experiments was set up to study the regulation of OTA production by *A. ochraceus* NRRL 3174 in AM synthetic medium by several growth parameters.

4.1.1 Dependence of OTA Production on Several Growth Parameters

According to literature data, OTA production by any ochratoxigenic *Aspergillus* spp. is dependent on external growth factors like a_w value, temperature, pH, nutrient supply, matrix, aeration etc. The effects of variations in the growth factors mentioned above observed by several authors were as diverse as the conditions applied e.g. isolate studied, composition of culture medium and shaken or stationary incubation. A general scheme to regulation of OTA production could not be derived. Therefore it was essential to characterize OTA biosynthesis patterns in the particular setup used in this study:
A. ochraceus NRRL 3174 (obtained as *A. ochraceus* KA 103 from J. Chelkowski, Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland) was cultivated for up to 15 d as submerse culture (shaken at 120 rpm) in standard AM liquid medium supplemented with 5% glucose, 3 g/L NH_4NO_3 adjusted to an initial pH of 6.5 prior to inoculation. Within this setup, influence of pH, trace metals, carbon and nitrogen source on OTA production by *A. ochraceus* NRRL 3174 and other ochratoxigenic aspergilli was tested.

Production of OTA by *Aspergillus ochraceus* NRRL 3174 and *A. niger* var. *niger* CBS 101697 in AM synthetic liquid medium was determined by initial external pH. The amount of OTA produced per fungal biomass of *A. ochraceus* NRRL 3174, *A. ochraceus* CCT 6819, *A. niger* var. *niger* CBS 101697 and *A. fresenii* CBS 550.65 varied with supply of trace metals, especially Zn. Nature and concentration of carbon and nitrogen source influenced OTA production by *A. ochraceus* NRRL 3174.
Results of this study are compared to literature data on dependence of OTA production in the following chapters. However, comparability of a number of previously published results is limited because no information about OTA/biomass production but only about OTA produced per mL culture medium was specified.

4.1.1.1 pH Dependence of OTA Production

Whereas *A. ochraceus* NRRL 3174 grew in AM synthetic medium adjusted to pH 3.5 to 9.0 prior to inoculation, OTA production only occurred within a range of pH 5.5-8.0. The pH dependent regulation of OTA biosynthesis appeared to be stringent, as there was either no OTA production at < pH 5.5 and > pH 8.0, respectively, or otherwise similar levels of OTA production between pH 5.5 and pH 8.0 (starting pH of the fungal culture). This absolute regulation of OTA production by initial medium pH could be verified with two different species representing two separate taxonomic units within the genus *Aspergillus*: the yellow *A. ochraceus* NRRL 3174 and the black *A. niger* var. *niger* CBS 101697.

Aziz and Moussa (1997) on the other hand, observed OTA production by an *A. ochraceus* strain down to starting pH 4.0 with a maximum at pH 5.5–6.0 using unbuffered YES liquid medium, but no OTA production at pH 8.0. Lai *et al.* (1970) observed growth and OTA production of A. *sulphureus* NRRL 4077 in an Adye and Mateles medium adjusted to pH values as low as pH 3.0 within 8 d of incubation. These observations indicate, that pH dependence of OTA production might not be absolute for all species and strains and/or under all medium conditions.

Table 4.1:Literature data on dependence of OTA production by *Aspergillus* spp. on medium pH

Authors	Organism	Medium	pH	OTA
Aziz and Moussa, 1997	*A. ochraceus*	YES, 4 % sucrose, 25 °C, stat., 10 d	4.0 4.5 5.0 **5.5** **6.0** 6.5 7.0 7.5 8.0	1 mg/L 1.4 1.8 **3.2** **3.2** 2.6 1.6 0.8 0
Lai *et al.*, 1970	*A. sulphureus* NRRL 4077	Adye-Mateles medium, 5 % glucose, 22-27 °C, 8-12 d	3.0 **6.0** 7.8	500 µg/mg **200** 100

In the current work, onset of OTA production in AM synthetic liquid medium was affected by initial but not final pH of the culture media. During the incubation period of up to 360 h, pH dropped in all setups studied. In cultures of *A. ochraceus* NRRL 3174 fully supplemented with trace metals, pH was balanced to pH 4, regardless of the starting pH and OTA production status. On the other hand, pH of cultures adjusted to initial pH 6.5 not supplemented with Zn^{2+} showing reduced OTA production, only dropped to pH 6.

OTA production by *A. ochraceus* NRRL 3174 and *A. niger* var. *niger* CBS 101697 could be "switched on" by pH adjustment of AM culture media within the exponential growth phase of the fungal culture. The occurrence of a "lag time" of about 40 h in onset of OTA synthesis following a pH shift from pH 5.0 to pH 6.5 indicates that pH affected gene expression rather than enzyme activity.

As reported by numerous authors, trace metal supply is one of the major factors influencing mycotoxin production (see 4.1.1.2). Therefore experiments were set up in order to test whether the regulatory effect of medium pH was due to reduced bioavailability of metal ions like Zn^{2+} and Fe^{3+} at pH 6.5 compared to lower pH values. However, the "pH effect" appears to affect other regulatory mechanisms, as no significant OTA production could be observed when Zn^{2+}, Zn^{2+} and Fe^{3+} or all trace metals were omitted from the culture medium at pH 5.0. Adding up to tenfold more Fe^{3+} or Zn^{2+} to the liquid medium did also not result in increased OTA production.

4.1.1.2 Dependence of OTA Production on Trace Metals

Concentration of trace metals had a strong influence on the amount of OTA produced by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium adjusted to pH 6.5. Addition of Zn^{2+} to the culture medium had a stimulatory effect on both biomass and OTA production. OTA was also produced in the absence of Zn^{2+} from the culture medium, but at a 50% lower rate than in media supplemented with 0.2 mg/L Zn^{2+}. In contrast, Fe^{3+} seemed to have a suppressive effect on OTA production, at least in the concentration used in the current study (0.12 mg/L). Completely omitting Fe^{3+} from culture media resulted in an up to 40% increase in OTA production whereas biomass production remained almost unaffected with and without Fe^{3+}. When both Fe^{3+} and Zn^{2+} were not added to culture media, the "Zn effect" dominated: biomass and OTA production decreased to 50%. Although the data were not presented in detail, omission of Cu^{2+} from AM culture media adjusted to initial pH 6.5 had no influence on OTA production by *A. ochraceus* NRRL 3174. In culture media with no trace metals added, biomass reduction was comparable to setups without Zn^{2+}, but OTA production was marginal. This indicates dependence of OTA production on sufficient supply of nutrients, at least trace metals, that are necessary for growth. Zn^{2+} is on of the major triggers of OTA production, but also at least one of the other metal ions added to AM medium -except Fe^{3+} and Cu^{2+}- appears to have a stimulatory influence on OTA production, most likely in combination with Zn^{2+}.

Similar gradual regulation of OTA production was observed with other *Aspergillus* species and strains. Although to varying levels, a stimulatory effect of Zn^{2+} as well as a restraining effect of Fe^{3+} on OTA production by *A. ochraceus* CCT 6819 and *A. niger* var. *niger* CBS 101697 was detected. On the other hand, in cultures of *A. fresenii* CBS 550.65 both Zn^{2+} and Fe^{3+} had a slight stimulatory effect on OTA production.

Comparison of the results presented in this study to literature data confirmed that the effect of trace metal concentrations in culture media on OTA production levels depended on medium, pH, strain, temperature and aeration (Table 4.2).

In contrast to this study, Steele *et al.* (1973) reported trace element dependence of OTA biosynthesis to be stringent and addition of Zn^{2+}, Fe^{3+} and Cu^{2+} to stimulate OTA production. Steele *et al.* (1973) observed maximum OTA production by *A. ochraceus* NRRL 3174 in Ferreira medium with 0.4 mg/L of Zn^{2+} (80 mg OTA/L), 4.1 mg/L Fe^{3+} (50 mg OTA/L) and 0.08 mg/L Cu^{2+} (40 mg OTA/L), respectively. No OTA was produced when either trace element was absent. Although the authors observed no correlation between amount of mycelial dry weight and OTA produced, OTA synthesis correlated with rapid utilization of sucrose and decrease in medium pH.

Table 4.2: Literature data on dependence of OTA production by *Aspergillus* spp. on trace metals added to culture media

Authors	Organism	Medium	pH	Additives	OTA
Aziz and Moussa, 1997	*A. ochraceus*	YES, 4 % sucrose, 25 °C, stat., 10 d	5.5	0 mg/L Fe^{2+} 0.8 1.2 1.6 0 mg /L Cu^{2+} 1.9 2.8 3.8 0 mg/L Zn^{2+} 0.45 0.9 1.35 1.8	3.2 mg/L 3.4 **3.6** 2.4 3.2 3.2 **3.4** 1.8 3.3 3.4 3.6 **3.8** 1.4
Steele *et al*., 1973	*A. ochraceus* NRRL 3174	Ferreira medium, sucrose, 0,3 % glutamic acid, 28 °C, 150 rpm, 7 d	6.3 7.2 6.6 7.0 6.7 7.3	0 mg / L **Zn^{2+}** 0.08 **0.4** 0.8 8.9 44.4 0 mg / L **Cu^{2+}** **0.08** 0.3 0.8 0 mg / L **Fe^{3+}** 0.08 0.4 **4.2** 41.7	0 mg / L 60 **80** 40 20 10 0 **40** 20 20 0 10 40 **50** 40
Lai *et al*., 1970	*A. sulphureus* NRRL 4077	Adye-Mateles medium, 5% glucose, 22-27 °C, stat., 8-12 d	6.3	none 0.08 mg / L **Cu^{2+}** 2.2 Fe^{3+} 3.94 Zn^{2+} **all**	470 µg /mg 540 400 470 550
	A. ochraceus NRRL 3174			none 0.08 mg / L Cu^{2+} 2.2 Fe^{3+} 3.94 Zn^{2+} **all**	0 0 0 trace 90
	A. melleus NRRL 3520			none 0.08 mg / L Cu^{2+} 2.2 Fe^{3+} 3.94 **Zn^{2+}** all	2 5 2 **65** 17
	A. melleus NRRL 3529			none 0.08 mg / L Cu^{2+} 2.2 Fe^{3+} 3.94 Zn^{2+} **all**	0 0 0 4 **13**

Aziz and Moussa (1997) also observed stimulatory effects of Zn^{2+}, Fe^{3+} and Cu^{2+} on OTA production, with concentrations of 1.35 mg/L Zn^{2+}, 1.2 mg/L Fe^{3+} and 2.8 mg/L Cu^{2+}, respectively, optimal for OTA production (3.8 mg/L, 3.6 mg/L and 3.4 mg/L). After 10 d of incubation of an unspecified *Aspergillus ochraceus* strain at 25 °C in stationary culture using unbuffered liquid YES medium adjusted to initial pH 5.5, maximum OTA production correlated with maximum biomass yields. Adding no Zn^{2+}, Fe^{3+} or Cu^{2+} as well as adding more than the concentrations considered optimal, reduced biomass and OTA production only slightly. Yet, estimation of overall trace metal concentration remains difficult because of the underlying YES complex medium.

Lai *et al*. (1970) used Adye and Mateles medium adjusted to pH 6.3 and incubated stationary at room temperature for 8–12 d. As summarized in Table 4.2, addition of several trace metals to cultures of several *Aspergillus* spp. had divergent effects: Adding Zn^{2+} to cultures of *A. ochraceus* NRRL 3174 or *A. melleus* NRRL 3529 increased OTA production slightly, but only with all trace elements supplied, significant amounts of OTA/biomass were produced. Apparently, both *Aspergillus* spp. required the combination of two or more of these trace metals for OTA production. The authors speculated on Zn^{2+} being one of the crucial triggers of OTA biosynthesis within this combination. On the other hand, *A. melleus* NRRL 3520 only needed Zn^{2+} for maximum OTA production, whereas OTA production in cultures of *A. melleus* NRRL 3520 and *A. sulphureus* NRRL 4077 also occurred without addition of trace metals. In contrast to all results described above, addition of Zn^{2+} had no effect on OTA/biomass production. While 0.08 mg/L Cu^{2+} stimulated OTA production by 15%, adding 2.2 mg/L Fe^{3+} to cultures of *A. sulphureus* NRRL 4077 even reduced OTA/biomass production by 15%.

Evidently, influence of trace metals like zinc and iron on OTA production and whether a gradual or absolute regulatory effect was caused by those trace metals, depended on medium, species and strain used in the particular study.

Furthermore, considerable differences were observed in the amount of OTA produced by various *Aspergillus* spp. and even by the same strain under different culture conditions (see Table 4.3; Davis *et al*., 1969)

4.1.1.3 Dependence of OTA Production on Carbon and Nitrogen Source

Another growth factor influencing OTA production by *A. ochraceus* NRRL 3174 was concentration of carbon source in the culture medium. In this study, OTA production increased with decreasing amounts of glucose supplied. While restricting glucose concentration to 1/8 of the standard concentration reduced mycelial dry weight to 50%, more than twice the amount of OTA per biomass was produced after 120 hours of incubation. In contrast to results obtained from variations in trace metal concentration, this indicates a reciprocal correlation between biomass and OTA production dependent on concentration of the carbon source. In all setups, OTA production occurred within the exponential growth phase and was connected to rapid glucose utilization and a steady decrease in pH. Using unbuffered YES medium adjusted to starting pH 6.0, Davis *et al*. (1969) found a maximum overall OTA production after 12 d with 4% sucrose (290 mg/L). However, expressed as OTA/biomass, maximum OTA production in that study occurred at 1% sucrose (18.2 mg OTA/g mdw) rather than at 4% (15.8 mg OTA/g mdw). Though higher concentration of sucrose led to an increase in fungal biomass, they also induced a decrease in OTA/biomass production. Ferreira (1967) observed the strongest increase in OTA concentration within the exponential growth phase of *A. ochra*ceus K804 in Czapek medium connected with rapid utilization of carbon source and stable medium pH. The author did not observe changes in OTA production with concentration of carbon source in the medium (100 mg OTA/L with 2–6% sucrose).

Ferreira (1967) also tested the influence of various carbon sources on OTA biosynthesis. He observed highest yields (100 mg OTA/L) with sucrose and galactose (75 mg OTA/L), considerably less with glucose and maltose (2 mg OTA/L), none with fructose and none with lactose. Lai *et al*.

(1970) observed that in AM medium at pH 7.0 highest OTA production by *A. sulphureus* NRRL 4077 occurred with glucose and sucrose as carbon source, respectively (200 mg OTA/L). They detected no growth on lactose as sole carbon source. In our experiments, *A. ochraceus* NRRL 3174 produced equally high yields of biomass and OTA with glucose and sucrose, respectively. Though supplying lactose as single carbon source mycelial dry weight was strongly reduced (40% compared to glucose), yields of OTA/biomass were only reduced by 70% compared to glucose. Biomass production was only slightly reduced with fructose as single carbon source, but OTA production was reduced to 50% compared to glucose. Therefore, no correlation appeared to exist between biomass and OTA production in cultures grown on different carbon sources. OTA production rather was influenced by the nature of the carbon source itself, dependent on strain, medium and culture conditions applied.
Amounts of OTA produced by *A. ochraceus* NRRL 3174 also depended on the nitrogen source supplied. Providing NH_4^+ only, as originally published by Adye and Mateles (1964), OTA/biomass production was reduced by 25% without any significant change in mycelial dry weight compared to standard AM medium supplemented with NH_4NO_3.

Table 4.3: Literature data on dependence of OTA production by *Aspergillus* spp. on carbon and nitrogen source

Author(s)	Organism	Medium	pH	Carbon source	Nitrogen source	OTA
Davis *et al*., 1969	*A. ochraceus* NRRL 3174	YES, 25 °C, 12 d	6	0 % sucrose		0.3 µg /mg
				0.5		12.2
				1		**18.2**
				2		16.3
				4		15.8
				8		7.3
				16		3.9
Lai *et al*., 1970	*A. sulphureus* NRRL 4077	Adye-Mateles medium, 22-27 °C, 8 -12 d	7	5 % **glucose**		**200** mg / L
				sucrose		200
				maltose		140
				mannose		135
				galactose		80
Ferreira, 1967	*A. ochraceus* K 804	Ferreira medium, 25 °C, 250 rpm, 6 d	6.0–6.2	1 % sucrose	2 g/ L NH_4NO_3	40 mg / L
				2		**100**
				3		**100**
				6		**100**
				3 % glucose		2
				fructose		0
				galactose		**75**
				maltose		2
				lactose		0
				mannose		0
				glycerol		0
			7	3 % sucrose	2 g / L $NaNO_3$	0
					2 $(NH_4)_2SO_4$	0
					2 NH_4Cl	0
					10 NH_4Ac	50
					5 Asn	2
					10 Asp	5
					10 Glu	**100**
					8 Pro	**100**
					3 Gly	2
					12 Phe	0

Supply of NO_3^- as single nitrogen source increased OTA/biomass production by 25%, also without changing biomass production. Amount of OTA produced in standard AM medium seemed to be the result of some kind of balance between influence of ammonia and nitrate on OTA production. Ferreira (1967) observed the best yields (100 mg/L) either using NH_4NO_3 or organic nitrogen sources such as glutamic acid or proline. In contrast to results presented in this study, experiments conducted by Ferreira using $NaNO_3$ or $(NH_4)_2SO_4$, respectively, resulted in no OTA production.

4.1.2 Regulation of Ochratoxin Production

The divergent and in some cases conflicting results on regulation of OTA biosynthesis by particular growth parameters as described above, suggest that OTA production by *Aspergillus* strains is either much more dependent on the interplay of several environmental conditions rather than on a single growth factor. Or if such a single superordinate parameter determining OTA production exists, it has not yet been discovered. However, it has become clear that OTA biosynthesis by *Aspergillus* spp. is regulated quite differently from OTA production by *Penicillium* spp., despite of the common end product to the biosynthetic pathway.

4.1.2.1 OTA Production by *Penicillium* spp.

In contrast to OTA production in *Aspergillus* spp., OTA production in the genus *Penicillium* seems to be much more stable and/or evenly distributed than in *Aspergillus* spp. Also, the dependence of OTA production by *Penicillium* spp. on trace metals, carbon and nitrogen source appears to be notably different from that observed with *Aspergillus* spp. Filtenborg *et al.* (1990) found that additional supply of Zn^{2+} or Cu^{2+} remained without any effect on OTA production in *Penicillium verrucosum* on YES agar plates, while adding Mg^{2+} greatly enhanced OTA yields. Importance of Zn^{2+} for OTA production by *Aspergillus* spp. as well as irrelevance of Mg^{2+} supply was demonstrated in several experiments, including this study. Furthermore, in *Penicillium* spp. OTA production could be switched on or off depending on the combination of nitrogen and carbon source supplied: OTA production could be triggered when supplying the fungal cultures with NH_4^+ and glycerol, whereas it was suppressed when adding NO_3^- and glucose to solid growth media (R. Geisen, pers. comm.). In *Aspergillus* spp. on the other hand, glucose and nitrate proved to be one of the "best combinations" for high OTA yields. Supply of ammonium as nitrogen source only reduced OTA production by *A. ochraceus* NRRL 3174 by 50%. No growth did occur with glycerol as single carbon source. Despite the fact that both groups of organisms obviously produce the same mycotoxin, and most likely by a similar biosynthetic pathway, regulation patterns seem to be widely diverging. Differently from *Penicillium* spp., OTA production in *Aspergillus* spp. did not display a uniform response to variation of single growth parameters.

Furthermore, OTA production in *Penicillium nordicum* strains appears to be common and relatively stable. OTA producing and non-OTA producing strains could be grouped into two distinct clusters by molecular typing methods (Larsen *et al.*, 2001; Castella *et al.*, 2002). OTA producing ability was not correlated to taxonomical grouping of *A. ochraceus* strains using several DNA fingerprinting techniques (Schmidt *et al.*, 2003, Varga *et al.*, 2000). Also taxonomical grouping of aflatoxinogenic *Aspergillus* spp. did not display homogenous distribution of toxigenic strains (Peterson, 2000; Peterson *et al.*, 2000). This might be a sign of heterogeneity occurring in aflatoxin or OTA production by closely related strains, respectively. Also data concerning OTA production might not in all cases be reliable because of instabilities in OTA production by *Aspergillus* strains as well as complex regulation patterns (see Table 3.1).

4.1.2.2 Mycotoxin Production by *Aspergillus* spp.

As already indicated above, OTA biosythesis in *Aspergillus* spp. and Penicillium spp. is regulated differently despite of the common end product On the other hand, production of ochratoxins in *Aspergillus* spp. appears to be as complexly regulated as biosynthesis of aflatoxin, likewise polyketide-derived mycotoxins produced by *Aspergillus* spp. Comparably to OTA production, effects of pH, trace metal supply, carbon and nitrogen source on aflatoxin production by *Aspergillus flavus* have been studied extensively by many authors. As observed in experiments on influence of several growth parameters on OTA production, results were often divergent or even contradicting. However, abstracting from all those results, aflatoxin production turned out to be triggered by combinations of several parameters, connected to a general deficiency status of the aflatoxin producing mycelia.

a) Aflatoxin Production by *Aspergillus* spp.

Effects of initial pH on aflatoxin production have been reported by several authors. Highest aflatoxin yields, however, were observed at different pH values: While some authors observed highest amounts of aflatoxin at pH 5.5–6.0 (Buchanan and Ayres, 1975; Cotty, 1988; Aziz and Moussa, 1997), other authors reported highest levels of aflatoxin at pH 3.5–4.0 (Joffe and Lisker, 1969; Mashaly and El-Deeb, 1981, Klich, 2000). Jarvis and Mason (1971) observed highest aflatoxin concentrations at pH 7. Davis *et al*. (1966) concluded from these divergent results, that a pH effect on aflatoxin production depended on strain, media and incubation conditions.

The same diversity of results was observed concerning effects of trace metals on aflatoxin production: Zn^{2+} was considered essential for aflatoxin production by Maggon *et al*. (1977) and Gupta *et al*. (1977). Mateles and Adye (1965) reported that aflatoxin production was stimulated by Zn^{2+}, unchanged by Fe^{3+}, and repressed by Cu^{2+}. Maggon *et al*. (1973) found Zn^{2+} and Cu^{2+} to stimulate aflatoxin production at low concentrations, and Fe^{2+} having no effect on aflatoxin/biomass production. Aziz and Moussa (1997) observed that addition of Fe^{2+}, Zn^{2+} and Cu^{2+} stimulated aflatoxin production. Whereas Davis *et al*. (1967) concluded that trace elements like Zn^{2+} display their influence on aflatoxin production indirectly through their general importance for mycelial growth, Marsh *et al*. (1975) observed no correlation between biomass and aflatoxin production.

Aflatoxin production by *A. flavus* in submerse culture has been reported to start 18–20 h after inoculation, considerably earlier than OTA production (Yu *et al*., 1995). On the other hand, aflatoxin biosynthesis seemed to be closely linked to depletion of nitrogen or trace elements (apart from Zn^{2+} which has a stimulatory effect on aflatoxin biosynthesis) while carbon sources remain available (Wiseman and Buchanan, 1987). Many carbohydrates were tested for their ability to induce aflatoxin production (Buchanan and Stahl, 1984); glucose appeared to be the most potent inducer of aflatoxin biosynthesis (Abdollahi and Buchanan, 1981a,b; Davis and Diener, 1968).

Whereas Kachholz and Demain (1983) reported nitrate repression of aflatoxin production, other authors could not verify this repressing effect upon supply of nitrate as single nitrogen source (Cotty, 1988; Davis *et al*., 1966). Another important trigger of aflatoxin production was oxidative stress (Jayashree and Subramanyam, 2000; Hayes *et al*., 1966). Under these circumstances, accompanied by accumulation of acetylCoA and limited NADPH availability, precursors of the fatty acid biosynthesis are shifted to polyketide synthesis (Brown *et al*., 1999).
Nevertheless, these conditions did not always trigger aflatoxin production or if they did, not always to the same extent. Instead, aflatoxin production in strains of *Aspergillus flavus* has been observed to be instable within a certain species (Kale *et al*., 1994; Lemke *et al*., 1989) as well as in laboratory isolates from one strain (Teren *et al*., 1997; Egel *et al.,* 1994; Diener and Davis 1974, 1966). Furthermore, degradation of aflatoxin in cultures of *A. flavus* and *A. parasiticus* was reported (Huynh and Lloyd, 1984; Ciegler *et al*. 1966).

b) Ochratoxin Production by *Aspergillus* spp.

In fully supplemented AM media adjusted to initial pH 6.5, *A. ochraceus* NRRL 3174 produced OTA after 72 h of incubation at 25 °C, 120 rpm. During the following incubation period, a steady increase of OTA concentration in the culture medium could be observed. Similar patterns of OTA production were reported by Ferreira (1967): A steady increase in OTA concentration after detection of OTA 3 d after onset of biomass production. OTA production occurred during or near the end of the exponential growth phase (Harris and Mantle, 2001; Saxena *et al*., 2001). On the other hand, cultures of *A. niger* var. *niger* CBS 101697 displayed earlier onset of OTA production and a sigmoid course of OTA concentration in the culture medium, most likely due to high amounts of ochratoxin in conidia (Skaug *et al*., 2001) and degradation of OTA by the fungal mycelium (Varga *et al*., 2000). Independent of these different production patterns, OTA biosynthesis by several *Aspergillus* spp. could be influenced within this study varying medium pH and nutrient supply. In contrast to results published by other authors, initial medium pH had a stringent regulatory effect on OTA production, whereas the amount of OTA produced was only gradually influenced by trace metal, carbon and nitrogen source supply. Nevertheless, some parallels with literature data could be derived within the gradual regulation of OTA production: In accordance to data published on OTA and aflatoxin production, OTA production was stimulated by Zn^{2+} (Aziz and Moussa, 1997). This effect was even more pronounced in combination with one of the other trace metals added to AM media (also: Lai *et al*., 1970). On the other hand, a slight repression of OTA production by Fe^{2+} was observed, agreeing with facts on aflatoxin rather than OTA production (Maggon *et al*., 1973; Mateles and Adye, 1966). Unlike aflatoxin production, high levels of glucose in the culture medium were no prerequisite for OTA production. In fact, more OTA/biomass was produced with decreasing levels of glucose in this study as well as in an earlier study by Davis *et al*. (1969). Also in contrast to observations described for aflatoxin production, nitrate rather had a stimulatory effect on OTA production.

Furthermore, no correlation between biomass production and OTA synthesis could be observed. OTA production seemed to be completely independent from the growth status of the fungal culture. Conditions favorable for growth did not always correspond to conditions optimal for OTA production or vice versa. Growth at pH 5.0 was almost unchanged compared to pH 6.5, but no OTA was produced. Experiments with varying concentrations of trace elements suggested that conditions favorable for growth were also triggering maximum OTA production. On the other hand, growth experiments with different amounts of glucose suggested an inverse correlation between OTA and biomass production. Results observed in experiments with different carbon and nitrogen sources showed no correlation between biomass and OTA production at all. These findings are in accordance with observations of almost all authors working on aflatoxin or OTA biosynthesis (Marsh *et al*., 1975; Davis *et al*., 1969).

However, OTA production is apparently subjected to a different regulation scheme only partly in agreement with that identified for aflatoxin production. Not only no concluding regulation pattern (as described for aflatoxin production) could be verified within this study, but also regulation patterns varied depending on growth conditions as well as *Aspergillus* strain used. The results obtained from the current study and from the literature suggest that it is necessary to determine specific conditions determining OTA production for each *Aspergillus* spp. strain. Even the character of regulation of OTA production might change with strain, pH, media or nutrients applied: under some conditions pH is only a gradual regulator of OTA biosynthesis, merely influencing the amount of OTA produced. In the current study, on the other hand, it was a means to absolutely trigger or suppress onset of OTA biosynthesis by *Aspergillus ochraceus* NRRL 3174 in AM liquid medium.

Differences in observations reported by several authors could also be a result of variable production levels of OTA by different mycelia, even originating from the same spore suspension. Röschenthaler *et al*. (1984) observed varying amounts of OTA produced in single spore cultures isolated from one

Aspergillus mycelium. Another explanation for divergent results might be instability of OTA production in laboratory cultures, as observed in the current study, and even irreversible loss of ability to produce OTA (Harwig, 1974), a phenomenon also known from *A. flavus* aflatoxin production (Leiach and Papa, 1974). This could also be observed in the current study, e.g. different amounts of OTA produced in cultures of *A. ochraceus* NRRL 3174 grown under the same culture conditions for identical incubation periods (see Fig. 3.11 and Fig. 3.13).
Several strains described as OTA producing by the supplier (incubation conditions not specified) did not produce OTA on solid malt extract or liquid AM medium after up to 15 d of incubation in our laboratory. *A. carbonarius* M 335 and M 336 both produced OTA when grown on coffee cherries and were fluorescence positive on coconut cream agar (Joosten *et al.*, 2001). Only one of the two strains produced OTA on MEA and no OTA production was observed in AM liquid medium. Chelack *et al.* (1991) speculated on a connection between OTA production and cell differentiation dependent on the kind and phase of mycelial growth. Such a dependence of mycotoxin production on the "mycelial habit" has been shown recently by Pazouki and Panda (2001). With most *Aspergillus* strains, better OTA production on solid substrates was observed (Moss, 1991, 1984). The reverse was demonstrated in the current work regarding *A. ochraceus* CCT 6819: no OTA production on malt extract agar but high OTA yields in AM liquid medium.
Therefore, it seems obvious that OTA biosynthesis of any strain within the ochratoxigenic *Aspergillus* spp. is rather determined by environmental conditions than by OTA production ability of the organism. If any *Aspergillus* strain tested produced no OTA under a set of given conditions, that does not justify a predication about general OTA production ability of this organism. Also any grouping into "OTA producers" and "non-producers" based on these data might be misleading. Differentiation of OTA producing and non-producing species and strains within the *A. niger* and *A. ochraceus* group has been undertaken several times (Bayman *et al.*, 2002; Accensi *et al.*, 2001; Abarca *et al.*, 1997, 1998), mostly based on physiological data gathered by "easy screening method[s] for OTA production in pure cultures" (Bragulat *et al.*, 2001).
Moreover, adjusting storage conditions or food preservation may not be enough to guarantee mycotoxin-free feed and foodstuffs. For one, minor changes in these conditions could lead to an onset of OTA production by previously "silent" *Aspergillus* spp. On the other hand, storage or preservation conditions deployed to forestall OTA production by certain species might not also be sufficiently suppressing OTA production by any other *Aspergillus* spp. Therefore it is vital to have a molecular tool to be able to detect any ochratoxigenic aspergilli in feed and foodstuffs to sufficiently ensure food safety. Establishing consistency of OTA production by *A. ochraceus* NRRL 3174 in fully supplemented AM liquid medium adjusted to initial pH 6.5, preconditions for further research on enzymes of the OTA biosynthesis pathway were accomplished. Furthermore, employing the regulative tool of culture medium pH as established in the current study it was feasible to investigate genes connected to OTA biosynthesis by comparison of gene expression in *A. ochraceus* NRRL 3174 under OTA permissive and nonpermissive conditions.

4.2 Biosynthesis of Ochratoxin A by a Putative "OTA Synthetase System"

Ferreira and Pitout (1969) first assumed the existence of an "OTA synthetase" responsible for *in vitro* OTA formation from its precursors ochratoxin α (OTα) and phenylalanine (Phe) in cell free extracts of *A. ochraceus* Wilh. CSIR 804. In order to further characterize this putative OTA synthetase in the present study, protein was prepared from OTA producing cultures of *A. ochraceus* A8, *A. ochraceus* NRRL 3174 and *A. niger* var. *niger* CBS 101697.

4.2.1 Partial Characterization of a Putative "OTA Synthetase"

Protein purification experiments were based on a method published by Ferreira and Pitout (1969), including extraction buffers and initial activity tests. Prior to enzyme purification experiments, cultures of *A. ochraceus* NRRL 3174 were grown in fully supplemented AM liquid media adjusted to initial pH 6.5. As previously reported by Ferreira and Pitout, *in vitro* OTA formation was observed in 40 % $(NH_4)_2SO_4$ fractions. Proteins in OTA producing 40 % $(NH_4)_2SO_4$ fractions were further purified by gel filtration.

Some of these enzyme preparations from OTA producing mycelia of *Aspergillus* spp. displayed *in vitro* formation of OTA in photometric activity tests. Characteristic fluorescence increase was only detectable in one to three distinct fractions after gel filtration and each time in similar fractions emerging from different OTA producing mycelia. Separating these fractions on SDS PAGE gels showed discrete protein bands at about 45 kDa accumulated from the crude protein extracts. Therefore it seems likely that an enzyme was involved in *in vitro* OTA formation. On the other hand, elution of an "OTA forming agent" in fractions near the salt peak might suggest responsibility of small molecules for *in vitro* formation of OTA. However, this does not account for the inconsistency of OTA formation capability in those fractions. All things considered, it appears plausible that a so far uncharacterized type of enzyme is required for OTA formation in the fungal cells as well as in *in vitro* activity tests.

A major drawback experienced in this study was considerable inconsistency of *in vitro* OTA formation capability. Only a small number of several similar enzyme preparations from OTA producing mycelia of *Aspergillus* spp. displayed measurable OTA formation in activity tests. Within these setups, explicit OTA formation could be detected after >40 h of incubation. *In vitro* synthesis of OTA could not be retrieved in further experiments with any of these active fractions. Apparently, protein in these preparations was degraded during the long incubation periods.

The apparent randomness of the enzyme activity observed during this study might represent a general instability of the OTA synthetase. Clearly, the appropriate settings for conservation of the enzyme activity could not be provided. Otherwise, the optimal reaction conditions for *in vitro* activity testing have not been met in all cases. One possible explanation might be the lack of important components or optimal reaction parameters (temperature, oxidation status, pH *etc.*). Another possibility explaining the observed unpredictability of *in vitro* OTA formation would be unrecognized differences in enzyme preparations, e.g. residual activated precursors essential for OTA formation. However, *in vitro* OTA formation without addition of ATP and Mg^{2+} was observed, suggesting that either activation of the precursors is not catalyzed by the OTA synthetase or the activation reaction is not dependent on ATP.

4.2.2 Reaction Mechanism of a Putative "OTA Synthetase System"

The enzymatic reaction attributed to the putative OTA synthetase is the formation of an amide bond between the carboxy group of the polyketide OTα and the amino group of Phe. As reaction mechanism of this final step in OTA biosynthesis, Huff and Hamilton (1979) proposed an acyl replacement reaction after acyl activation of both precursors. However, these catalytic steps are merely a theoretical scheme that has not yet been demonstrated.

Following this reaction scheme, the polyketide had to be activated in order to provide the energy necessary for the acyl transfer reaction. For this purpose, the carboxy group of OTα would be converted to the acyl form. This kind of acyl activation of carboxylic acids is commonly used in biosynthetic pathways, e.g. in fatty acid biosynthesis. In order to participate in such a replacement reaction, Phe would need to change its physiological state as *zwitter* ion by formation of an acyl derivative. Huff and Hamilton suggested esterification of Phe, since the ethyl ester of OTA occurs as natural structure analogue OTC (Steyn and Holzapfel, 1967). This activation of the Phe moiety also

applies to other amino acids like serine, lysine and hydroxyproline, leading to the formation of structural analogues of OTA (Hadidane *et al.*, 1992).

An example for occurrence of such an acyl transfer reaction within the mycotoxins is one of the early enzymatic steps within the biosynthesis of viridicatin (Turner, 1971). Viridicatin is a quinoline alkaloid formed by *P. cyclopium* and *P. viridicatum* from anthranilic acid, Phe and the methyl group of L-methionine (Nover and Luckner, 1969; Luckner, 1967). The first reaction in this biosynthetic pathway is catalyzed by a "cyclopeptine synthetase system" (Lerbs and Luckner, 1985). Although no information is available about number, composition or type of enzyme(s) involved, the catalytic principle of an anthranilate adenylyltransferase has been demonstrated in *in vitro* and *in vivo* experiments (Voigt *et al.*, 1978). An adenylyltransferase activity within the cyclopeptine synthetase system forms an amide bond between AMP-activated anthranilic acid and AMP-activated Phe. During this reaction, a linear intermediate is formed as an enzyme bound thioester. After cyclization the first stable intermediate cyclopeptine is released (El Azzouny *et al.*, 1977; Framm *et al.*, 1973).

Another mycotoxin initially synthesized by a polyketide pathway, also established within the genus *Penicillium*, is penicillic acid. The first stable intermediate of the biosynthetic pathway leading to the formation of penicillic acids, a ACV linear tripeptide, is also synthesized by *A. nidulans* (Aharonowitz *et al.*, 1992). The ACV linear tripeptide results from condensation of three amino acids by an enzyme that functions similarly to peptide synthetases involved in nonribosomal biosynthesis of peptide antibiotics (Smith *et al.*, 1990). As observed by Lerbs and Luckner (1985) for the cyclopeptine synthetase system, these reaction also involves linkage of AMP-activated amino acid precursors by an adenylyltransferase activity. Molecular size, structure and reaction mechanism of nonribosomal peptide synthetases have been extensively investigated:

The "thiotemplate multienzymic mechanism" of peptide synthetases has less similarity with ribosomal peptide synthesis than with fatty acid and polyketide biosynthetic pathways (Stachelhaus and Marahiel, 1995). The reaction starts with the ATP-dependent activation of amino acid precursors. The enzyme-stabilized adenylates are subsequently attached to the enzyme as thioesters. Formation of amide bonds between the amino acid residues is then carried out by 4'-phosphopantetheine cofactors. The enzyme consists of several domains, each with a covalently bound cofactor. Sequence of amino acids depends on the structure of the multienzyme complex. The amino terminus of those linear peptides can be modified in many ways, one of that being acylation with a carboxylic acid (Kleinkauf and van Döhren, 1990).

All the catalytic steps of nonribosomal peptide biosynthesis are performed by a multienzyme complex or a single multifunctional enzyme with several domains of approx. 70 kDa (Zuber, 1991). Conserved sequences have been detected within the adenylation and thioester module with high degrees of similarity between different enzymes (Stachelhaus and Marahiel, 1995). However, the domains of peptide synthetases like ACV synthetase integrates more activities than only oligomerization of amino acids, e.g. a hydrolase and an invertase activity (Martin, 1998; Schofield *et al.*, 1997). As a putative "OTA synthetase (system)" catalyzes a considerably less complex reaction, only one or two domains or subunits would be needed in OTA formation from OTα and Phe, capable of AMP-activation and linkage of both molecules.

Potential parallels of reaction mechanisms can be used in further research on the putative "OTA synthetase system", e.g. stabilization of *in vitro* OTA synthesis by providing appropriate precursor substances and/or fulfilling the requirements of an enzyme complex performing a multi-step reaction. In case part of this reaction scheme also applies to a putative "OTA synthetase system", inconstancy of *in vitro* OTA formation would most likely be a consequence of varying remains of acyl activated precursors in the protein preparations. This kind of precursor activation would not be achievable by addition of ATP to the reaction mixture unless the complex activation reactions would also be catalyzed by the OTA synthetase or all enzymes involved in a "OTA synthetase system" are present in the same gel filtration fraction. Whether the lack of activated precursor

substances or enzymes involved in precursor activation was responsible for inconsistency of *in vitro* OTA formation, was tested in this study by addition of crude cell free extracts to gel filtration fractions. However, attempts to determine further components required for *in vitro* OTA synthesis activity resulted in no measurable OTA formation. These observations indicate that either the reaction principle described above does not apply to the putative OTA synthetase or enzymes and/or precursors were rapidly degraded.

Another possibility to make use of an existing similarity of reaction principles is a heterologous genetic approach based on the common active regions of enzymes known to perform formation of an amide/peptide bond. However, both examples presented above are embedded in biosynthetic pathways leading to the formation of cyclic peptides consisting of two or more amino acids. In contrast, the ochratoxins are a group of unique acyclic combinations of an amino acid with a complex carboxylic acid linked via an amide bond. Therefore, the reaction mechanism might as well be substantially different from the biosynthesis of viridicatin or penicillic acid.

4.3 Characterization of Genes Connected to OTA Biosynthesis

As described above, no stable enzymatic activity could be observed despite of culture conditions suitable for persistent OTA production. However, those conditions established for enzyme purification purposes could be furthermore exploited for detection of genes connected to OTA biosynthesis via a genetic approach. For this purpose, gene expression in cultures grown under OTA submissive or non-submissive conditions, respectively, was compared using cDNA-AFLP, resulting in establishing suitable genetic markers for detection of ochratoxigenic *Aspergillus* spp.

4.3.1 Use of cDNA-AFLP for Detection of Genes Connected to OTA Biosynthesis

The most important prerequisite for the genetic setup was met with establishing constancy and predictability of OTA production by *A. ochraceus* NRRL 3174 in fully supplemented AM liquid medium adjusted to initial pH 6.5, also an indispensable requirement for enzyme purification attempts. Monitoring biomass and OTA production by *A. ochraceus* NRRL 3174 in AM culture media revealed two clearly discernable stages in OTA biosynthesis: a repressed status before and a derepressed status after onset of OTA production at 72 h of incubation (Fig. 3.3). Consistent accessibility of two distinct stages in OTA production could subsequently be exploited in gene expression experiments. A similar approach on cloning of genes connected to mycotoxin production was used by Appleyard *et al.* (1995): Gene expression patterns of repressed (after 16 h of incubation) and derepressed (after 65 h of incubation) gibberellin biosynthesis were compared using differential display resulting in detection of 100 fragments not present under repressed conditions or at least noticeably less prominent.

In order to distinguish differentially expressed fragments associated with differences in growth status from those connected to OTA biosynthesis, OTA permissive and nonpermissive conditions were additionally integrated into a multifactor comparison. A reliable means to induce or suppress OTA production in culture was available with pH adjustment of AM culture media prior to inoculation to pH 6.5 or pH 5.0, respectively (Fig. 3.4). By altering no more than a single growth factor to induce or suppress OTA biosynthesis, culture conditions were sufficiently similar, thus restricting the differentially transcribed mRNA to the smallest possible number. For comparison of gene expression patterns under permissive and nonpermissive culture conditions in combination with repressed and derepressed stages, cDNA was prepared from cultures of *A. ochraceus* NRRL 3174 grown for increasing incubation periods in AM medium adjusted to initial pH 5.0 or 6.5, respectively.

cDNA-AFLP was chosen as molecular genetic method for comparison of gene expression patterns because of its potential of simultaneous examination of multiple cDNA samples (Bachem *et al*., 1998) and because it is more rapid and robust compared to "classical" mRNA differential display (Gellatly *et al*., 2001). Furthermore, using suitable adapter and primer combinations, emerging cDNA fragment patterns could be restricted to a manageable number, which is especially important in setups with eukaryotic cDNA. Using a suitable primer combination in cDNA-AFLP reactions with cDNA samples from cultures of *A. ochraceus* NRRL 3174 grown for increasing incubation periods in AM medium adjusted to initial pH 5.0 or 6.5, respectively, four fragments could be distinguished after separation on PAA gels, that were only present (fragments 1 and 4) or at least plainly more prominent (fragments 2 and 3) under OTA permissive, derepressed conditions (Fig. 3.26).

4.3.2 Further Characterization and Determination of Expression Patterns

2 of the 4 fragments only detectable in cDNA preparations from OTA producing cultures were excised from the PAA gel, reamplified, cloned and sequenced. No significant similarities to any entry listed in the BLAST libraries was found. Although it was proven in real time PCR experiments that both fragments are in fact derived from mRNA and not residual DNA (Fig. 3.30), no continuous ORF could be detected on fragment 3 or 4, respectively. Whether this might be due to transcription mistakes by *Taq* polymerase accumulated in several reamplification reactions prior to cloning, alternative codon usage or seizing of premature mRNA, remains unknown.

Primers 3for/3rev1 and 4for/4rev1 designed from fragments 3 and 4, respectively, were utilized in real time PCR with all cDNA samples listed in Table 3.3: Numbers of cDNA samples isolated from *A. ochraceus* NRRL 3174 mycelia harvested after several periods of growth in AM liquid medium adjusted to initial pH 6.5 or pH 5.0, respectively

. Gene expression of both fragments was compared to the β-tubulin house-keeping gene to compensate variability in overall cDNA concentration (see Sweeney *et al*., 2000). Results of the cDNA quantification experiments showed similar expression patterns for both fragments, although at different overall concentration levels (Fig. 3.31). This indicates, that either both cDNA fragments were part of one gene, or both genes represented by fragments 3 and 4 were co-regulated, even though gene expression occured on different levels. Detection of different expression levels does not necessarily mean divergent *in vivo* expression but might be a question of differing amplification capabilities of both primer pairs used in LightCycler™ experiments. Further molecular genetic research will be necessary to clarify the connection between fragment 3 and 4.

Furthermore, real time PCR experiments verified that the gene(s) represented by fragments 3 and 4 were in fact expressed more strongly under OTA permissive, derepressed culture conditions compared to nonpermissive and derepressed conditions, respectively: fragments 3 and 4 were found in 7-11 fold concentration in cDNA sample 3, prepared from a culture of *A. ochraceus* NRRL 3174 grown for 72 h in AM medium adjusted to initial pH 6.5. Elevated relative amount of fragments 3 and 4 in mRNA populations was detected shortly before onset of OTA production was measurable. Apparently, expression of gene(s) represented by these two fragments was in some way connected to OTA biosynthesis.

However, increased expression levels of both fragments were only detected at one point of sampling time (72 h) and decreased in the subsequent samples (76 h, 80 h, 96 h). In a similar approach, Sweeney *et al*. (2000) used primers designed from two genes of the aflatoxin biosynthetic pathway in RT-PCR reactions for monitoring of aflatoxin production by *A. parasiticus* 439. Transcripts of genes of aflatoxin biosynthesis were detected after 48 h of incubation under aflatoxin-permissive conditions and remained constant from 72 h to 120 h of incubation compared to the β-tubulin house keeping gene used as means to assess gene transcription levels. This indicates, that the

connection of gene(s) represented by fragments 3 and 4 to OTA biosynthesis is rather indirect, meaning that those genes are not directly involved in the OTA biosynthetic pathway. On the other hand, this might also imply that gene expression patterns of key genes of OTA biosynthesis are different from aflatoxin production. As no function or significant domain similarity could so far be assigned to the gene fragments 3 and 4, the effective connection to OTA biosynthesis remains to be determined.

Edwards *et al*. (2002) found two sequences from separate genes in *A. ochraceus* ATCC 22947 with homology to fungal ketosynthases employing a heterologous genetic approach to identify the putative polyketide synthase involved in OTA biosynthesis. Yet, in reverse transcription experiments, gene expression could be detected under OTA permissive (Yeast Extract Sucrose) and non-permissive (Yeast Extract Peptone) conditions with higher levels of transcription under non-permissive conditions. Therefore, the authors concluded that those *pks* genes are expressed in *A. ochraceus* ATCC 22947 but are not necessarily connected to OTA biosynthesis. In contrast, gene(s) represented by fragments 3 and 4 acquired in the current study using cDNA-AFLP were connected to OTA biosynthesis in terms of gene expression levels and therefore may be used as anonymous markers for molecular genetic detection of OTA production in known ochratoxigenic *Aspergillus* spp.

4.3.3 Detection of Ochratoxigenic *Aspergillus* spp.

The ultimate objective of elucidation of key genes of OTA biosynthesis is to have a reliable molecular diagnostic tool for detection of all ochratoxigenic fungi in raw materials, feeds and foodstuffs. Ideally, this molecular detection method should also be capable to distinguish between OTA producing and non-producing individuals within this group. This important aim is currently hampered by two central facts: First of all, no genes involved in OTA biosynthesis have been discovered yet. Therefore, at this stage of research, detection of ochratoxigenic fungi in foodstuffs can only be based on group specific markers for ochratoxigenic fungi. Most likely this would be an anonymous group specific markers with an uncertain connection to OTA biosynthesis. General efficacy of such a marker, on the other hand, is limited because OTA is produced by a heterologous group of *Aspergillus* spp.: yellow aspergilli with *A. ochraceus* and related ochratoxigenic species like *A. fresenii* (synonym: *A. sulphureus)* as well as the black aspergilli divided into the *A. niger* group comprising ochratoxigenic species like *A. niger*, *A. awamori*, *A. usamii* and the *A. carbonarius* group with the ochratoxigenic species *A. carbonarius*, *A. niger* var. *niger*.

During the present study, two primer pairs were acquired with different detection spectrum for ochratoxigenic *Aspergillus* spp. Primer pair 3for/3rev2 derived from fragment 3 was suitable for specific detection of the species *A. ochraceus*. This was demonstrated using several *A. ochraceus* strains as well as other *Aspergillus* spp. (Fig. 3.33). A similar PCR specific for the species *A. ochraceus* based on an anonymous genetic marker sequence was set up by Schmidt *et al*. (2003): Following AFLP experiments, bands unique for *A. ochraceus* strains were cloned and sequenced. Primers OCA-V/OCA-R derived from the fragment H10 were tested against a set of *Aspergillus* spp. and showed high specifity for *A. ochraceus* strains. With establishing this PCR system it is now feasible to detect a large group of ochratoxigenic fungi, considered the main OTA producer on green coffee (Urbano *et al*., 2001). However, both PCR systems mentioned above have no potential for detection of other ochratoxigenic species than *A. ochraceus*. In addition, those primers are not suitable to distinguish between producing and non-producing strains within the species *A. ochraceus*.

Primer pair 4for/4rev2 derived from fragment 4 was applicable for detection and discrimination of several ochratoxigenic strains within the species *A. ochraceus* and the *A. carbonarius* group. However, similar to the other PCR systems for detection of ochratoxigenic fungi, this primer pair was not suitable for detection of all ochratoxigenic species within our outside these groups (e.g. *A.*

fresenii, *P. verrucosum*; see Fig. 3.34) or distinguishing between OTA producing and non-producing strains. Both disadvantages are a natural consequence of the fact that the primers are not based on key genes of the OTA biosynthetic pathway, the only background suitable for detection of all ochratoxigenic species at once and the simultaneous differentiation between producing and non-producing strains. Furthermore, any grouping of OTA producing and non-producing strains so far established might not reflect the genetic background OTA biosynthesis ability of all those strains (see 4.1.2.2).

On the other hand, when genes of the biosynthetic pathway leading to the formation of a mycotoxin are known, detection systems specific for one or more of those genes can be set up, capable of detecting all toxigenic species at once. Experiences from molecular detection of aflatoxigenic fungi in foodstuff show that even with detailed knowledge about the biosynthesis genes it is not easy to distinguish between producers and non-producers within these species: McAlpin and Mannarelli (1995) constructed a species specific DNA probe for differentiation of *A. flavus* strains based on an anonymous genetic marker derived from repetitive DNA sequences. Several authors used standard or triplex PCR to detect the aflatoxigenic species *A. flavus* and *A. parasiticus* on foodstuffs (Färber *et al.*, 1997; Geisen, 1996; Shapira *et al.*, 1996). However, no differentiation between aflatoxigenic and non-aflatoxigenic strains within the *A. flavus* group was possible using these methods. The only exception was the distinction of *A. oryzae*, a non-aflatoxigenic subgroup of *A. flavus* strains missing the *nor*-1 gene of aflatoxin biosynthesis pathway. Criseo *et al.* (2001) accomplished differentiation of aflatoxin producers and non-producers in the *A. flavus* group using a multiplex PCR with 4 different primers. Most non-producers could be distinguished by an incomplete pattern obtained in PCR. However, there was also a certain percentage of non-aflatoxigenic strains with positive signals due to simple mutations not detectable in PCR.

All methods described above only provide qualitative data on presence or absence of toxigenic fungi. Using primers specific for genes of mycotoxin biosynthetic pathways in real time PCR, toxigenic fungi in food samples can be quantified. Such an approach has already been established for trichothecene producing *Fusarium* spp. based on *tri5* (Schnerr *et al.*, 2001) and more recently for aflatoxigenic *Aspergillus* spp. based on the *nor*-1 gene (Mayer *et al.*, 2003). In case of *A. flavus*, only the combination of both approaches gives rise to a clear and accurate statement on the amount of toxigenic strains in food and feedstuff.

PCR based diagnostics based on anonymous genetic markers as presented in this study and in the work of Schmidt *et al.* (2003) constitute an approach on detection of ochratoxigenic fungi in raw materials, feed and foodstuffs that is feasible at the current state of research on OTA biosynthesis. For further enhancement of molecular diagnostic tools, detailed knowledge about enzymes and genes involved in the OTA biosynthetic pathway is required in order to simultaneously detect all ochratoxigenic species and distinguish producing and non-producing strains, as discussed with aflatoxigenic *Aspergillus* spp.

5 Summary

Early detection of ochratoxigenic fungi on raw materials, feed and foodstuffs is a vital prerequisite in order to ensure safety of these agricultural products. In order to detect occurrence of organisms capable of ochratoxin A (OTA) production during further storage and processing, generic molecular diagnostic tools based on special genetic features of ochratoxigenic *Aspergillus* spp. need to be developed. These molecular methods for detection of *Aspergillus* spp. with OTA production potential would best be targeted at genes and enzymes of the biosynthetic pathway leading to OTA formation.

Important preconditions for further research was consistency of OTA production in laboratory culture as well as the availability of a reliable means to induce or suppress OTA production by altering no more than one growth factor. To be able to predict and control OTA production by *Aspergillus* spp. used in this study, detailed knowledge about the external factors affecting OTA production was gathered.

Aspergillus ochraceus NRRL 3174 reliably produced OTA after 72 h of incubation at 25°C in AM synthetic liquid medium fully supplemented with trace metals, 5% glucose and 3 g/L NH_4NO_3 adjusted to initial pH 6.5. Production of OTA in AM liquid medium depended on initial external pH. The amount of OTA produced per fungal biomass varied with supply of trace metals, especially Zn^{2+}, nature and concentration of carbon and nitrogen source.

Onset of OTA production was affected by initial but not final pH of the culture medium through a stringent regulation mechanism. It could be "switched on" by pH adjustment during the exponential growth phase of an *Aspergillus* spp. culture.

Concentration of trace metals had a strong influence on the amount of OTA produced in AM liquid medium adjusted to pH 6.5. Zn^{2+} had a stimulatory effect on both biomass and OTA production. In contrast, Fe^{3+} had a slight suppressive effect on OTA production while biomass production remained almost unaffected. Another growth factor influencing OTA production was concentration of carbon source in the culture medium. OTA/biomass production increased with decreasing amounts of glucose supplied. Quantity of OTA produced was also influenced by the nature of the carbon source supplied. *A. ochraceus* NRRL 3174 produced the highest yields of biomass and OTA with glucose and sucrose as single carbon sources. Mycelial dry weight and yields of OTA/biomass were reduced to diverging extent with lactose and fructose as single carbon source. Supply of different nitrogen sources had no influence on biomass production, but compared to NH_4NO_3, ammonium reduced OTA/biomass production by 25%. Supply of nitrate as single nitrogen source increased OTA/biomass production by 25%.

No correlation between biomass production and OTA synthesis was observed. OTA production appeared to be completely independent from the growth status of the fungal culture. Conditions favorable to growth did not always correspond to conditions optimal for OTA production or vice versa. Experiments with varying concentrations of trace metals suggested that conditions favorable to growth were also triggering maximum OTA production. On the other hand, growth experiments with different amounts of glucose suggested an inverse correlation between OTA and biomass production. Results obtained in experiments with different carbon and nitrogen sources showed no correlation between biomass and OTA production at all.

Compared to literature data on regulation of OTA production, results of this study strongly suggest that OTA biosynthesis depends on the interplay of several environmental conditions rather than on a single growth factor. Furthermore, OTA biosynthesis of any strain within the ochratoxigenic *Aspergillus* spp. is rather determined by environmental conditions than by OTA production ability of the organism.

After consistency of OTA production in laboratory culture was achieved, mycelia of *Aspergillus* spp. actively producing OTA were used in order to identify enzymes of the OTA biosynthetic pathway. Experiments conducted with enzyme preparations led to the partial characterization of the putative OTA synthetase. Further purification and characterization attempts failed, most likely because of the lack of appropriately activated precursor substances for OTA formation.

Mycelia of *A. ochraceus* NRRL 3174 at repressed (<72 h incubation time) and derepressed (>72 h incubation time) growth stages under OTA permissive (initial pH 6.5) and nonpermissive (initial pH 5.0) culture conditions were utilized for comparison of gene expression patterns. From cDNA-AFLP experiments conducted with specific cDNA samples collected after increasing incubation periods under OTA permissive or non-permisive conditions, two fragments connected to OTA biosynthesis were reamplified. Increased expression of genes represented by both fragments under OTA permissive, derepressed conditions was confirmed in LightCycler™ Real Time PCR experiments.

Two primer pairs were derived from those fragments with different detection spectra for ochratoxigenic *Aspergillus* spp. Primer pair 3for/3rev2 was suitable for specific detection of the species *A. ochraceus*. Primer pair 4for/4rev2 was applicable for detection and discrimination of several ochratoxigenic species/strains within the *A. ochraceus* group and the *A. carbonarius* group.

6 Zusammenfassung

Die Früherkennung Ochratoxin A (OTA) bildender Pilze auf Rohstoffen, Nahrung und Tierfutter ist eine unverzichtbare Voraussetzung, um die Sicherheit landwirtschaftlicher Produkte für den Verbraucher garantieren zu können. Zum Nachweis von *Aspergillus* spp., die bei weiterer Verarbeitung oder Lagerung dieser Produkte OTA bilden könnten, werden molekulargenetische Methoden benötigt, die sich an den speziellen genetischen Merkmalen der OTA bildenden Aspergillen orientieren, d.h. Gene und Enzyme, die direkt am OTA Biosyntheseweg beteiligt sind.
Die wichtigste Voraussetzung für die Erarbeitung solcher molekularen Methoden stellte die Beständigkeit der OTA-Produktion unter Laborbedingungen dar sowie die Möglichkeit, die OTA-Produktion durch die Veränderung nur eines Wachstumsfaktors zu induzieren oder zu unterdrücken. Um die OTA-Produktion bei den *Aspergillus* spp., die in dieser Arbeit verwendet wurden, derart kontrollieren zu können, wurden zunächst einige der Wachstumsfaktoren untersucht, welche die OTA-Produktion beeinflussen.

OTA-Produktion durch *Aspergillus ochraceus* NRRL 3174 zeigte sich verlässlich nach 72 h Inkubation bei 25°C in synthetischem AM Flüssigmedium, eingestellt auf pH 6,5 und versetzt mit Spurenelementen, 5% Glucose und 3 g/L NH_4NO_3. Die OTA-Produktion wurde gesteuert durch den anfänglich eingestellten pH-Wert des Kulturmediums. Die Menge des von der Pilzkultur pro g Biomasse synthetisierten OTA wurde beeinflusst durch die Versorgung der Kultur mit Spurenelementen, besonders Zink, die Art und Konzentration der Kohlenstoffquelle sowie die Stickstoffquelle.

Das Einsetzen der OTA-Produktion war abhängig vom anfänglich eingestellten pH-Wert des Kulturmediums. Durch die Einstellung eines entsprechenden pH-Wertes während der exponentiellen Wachstumsphase einer *Aspergillus* spp.-Kultur konnte die OTA-Produktion angeschaltet werden.

Die Konzentration der Spurenelemente im auf pH 6,5 eingestellten AM Kulturmedium hatte einen starken Einfluss auf die Menge des gebildeten OTA. Zink hatte einen stimulierenden Effekt auf die Biomasse-Produktion und die OTA-Biosynthese. Zugabe von Eisen zum Kulturmedium dagegen verminderte die OTA-Synthese, während die Produktion der Biomasse unverändert blieb. Ein weiterer Wachstumsfaktor, der die OTA-Produktion beeinflusste, war die Konzentration der dem Medium zugesetzten Kohlenstoffquelle. Je weniger Glucose dem Kulturmedium zugesetzt wurde, desto mehr OTA/Biomasse wurde gebildet. Die Menge an OTA, die gebildet wurde, war weiterhin abhängig von der Art des zugesetzten Zuckers. *A. ochraceus* NRRL 3174 produzierte die höchsten Ausbeuten an Biomasse and OTA mit Glucose bzw. Saccharose als Kohlenstoffquellen. Mit Lactose bzw. Fructose als Kohlenstoffquellen waren Biomasse- und OTA/Biomasse-Ausbeute in unterschiedlichem Maße reduziert. Die Zugabe verschiedener Stickstoffquellen hatte keinen Einfluss auf die Biomasse-Produktion. Im Vergleich zu NH_4NO_3 war die OTA/Biomasse-Produktion bei Zugabe von Ammonium um 25% reduziert. Bei Zugabe von Nitrat dagegen war die OTA/Biomasse-Produktion um 25% gesteigert.

Zwischen der Biomasse- und der OTA-Produktion konnte demzufolge kein direkter Zusammenhang beobachtet werden. Die OTA-Produktion schien dagegen vollständig unabhängig von dem Status der Pilzkultur zu sein. Bedingungen, die das Wachstum förderten, waren nicht in allem Fällen ebenfalls optimal für die OTA-Produktion und umgekehrt. Experimente mit unterschiedlichen Spurenelement-Konzentrationen ergaben, dass wachstumsfördernde Bedingungen auch die höchsten OTA-Ausbeuten zur Folge hatten. Andererseits zeigten Wachstumsversuche mit unterschiedlicher Glucosekonzentration anscheinend eine inverse Korrelation zwischen Biomasse- und OTA-Produktion. Ergebnisse aus Versuchen mit unterschiedlichen Kohlenstoff- und Stickstoffquellen dagegen ergaben keinerlei Zusammenhang zwischen der Produktion von Biomasse und OTA.

Im Zusammenhang mit Literaturangaben zur Regulation der OTA-Produktion konnte in dieser Studie gezeigt werden, dass die OTA-Biosynthese vielmehr von einem komplexen Zusammenspiel verschiedener Umweltfaktoren bestimmt wird als von einem einzelnen Wachstumsfaktor.

Weiterhin scheint die OTA Produktion eines Stammes innerhalb der OTA-bildenden *Aspergillus* spp. vielmehr von den äußeren Bedingungen abhängig zu sein als von der geneitsch fixierten Fähigkeit dieses Organismus, OTA zu synthetisieren.

Nachdem die Stabilität der OTA-Produktion gewährleistet war, wurden weitere Versuche zur Identifizierung von Enzymen des OTA-Biosyntheseweges mit OTA-produzierenden *Aspergillus*-Myzelien durchgeführt. Experimente mit Enzymaufreinigungen ergaben die teilweise Beschreibung der „OTA-Synthetase“. Weitergehende Aufreinigung und Charakterisierung des Enzyms konnten nicht erreicht werden, höchstwahrscheinlich aufgrund fehlender aktivierter Vorstufen, welche für die *In vitro*-OTA-Bildung benötigt wurden.

Myzelien von *A. ochraceus* NRRL 3174, die unter OTA-permissiven (Anfangs-pH 6,5) oder nicht-permissiven (Anfangs-pH 5,0) Kulturbedingungen gewachsen waren und sich in reprimierten (<72 h Inkubation) bzw. dereprimierten (>72 h Inkubation) Wachstumsstadien befanden, wurden zum Vergleich der Genexpressionsmuster herangezogen. Ausgehend von cDNA-AFLP Experimenten mit cDNA-Proben, die nach steigenden Inkubationszeiten unter OTA-permissiven bzw. nicht-permisiven Kulturbedingungen geerntet worden waren, wurden zwei Fragmente reamplifiziert, die in Zusammenhang mit der OTA-Biosynthese standen. Erhöhte Expression der Gene, welche durch diese cDNA-Fragmente repräsentiert wurden, unter OTA-permissiven und dereprimierten Kulturbedingungen wurde mittels LightCycler™ PCR-Experimenten bestätigt.

Auf der Basis der DNA Sequenz der beiden Fragmenten wurden zwei Primerpaare abgeleitet, die ein unterschiedliches Nachweisspektrum für OTA-bildende *Aspergillus* spp. zeigten. Mit dem Primerpaar 3for/3rev2 wurden spezifisch *A. ochraceus*-Stämme detektiert. Durch den Einsatz des Primerpaares 4for/4rev2 konnten verschiedene Arten bzw. Stämme innerhalb der *A. ochraceus*-Gruppe und der *A. carbonarius*-Gruppe nachgewiesen und voneinander unterschieden werden.

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