“Process development for the production of *Alternaria* toxins in a bioreactor”

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„Durch Wissenschaft wird bewiesen, durch Intuition aber entdeckt.“

Henri Poincare (1854 - 1912)

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Abstract

Mycotoxins are secondary metabolites of small molecular weight formed by a wide diversity of different moulds. They are found worldwide as contaminants of food and their effects on humans and animals can be significant. Besides the health risk economic losses due to mycotoxin contamination are rather high since up to 25 % of the world´s food crops are affected. The black mould *Alternaria alternata*, the most common *Alternaria* species in harvested fruits and vegetables, produces five major *Alternaria* toxins including alternariol (AOH), alternariol monomethylether (AME) and tenuazonic acid (TA). Although numerous toxicological studies were conducted to clarify the effects of *Alternaria* toxins, an unambiguous result was not obtained and a risk assessment is not available. The best way to exclude serious health risks due to mycotoxins is the prevention of mycotoxin spoilage in foods and food raw materials. Therefore, a detailed knowledge about toxin formation is necessary.

In a first approach the influence of different carbon and nitrogen sources on mycotoxin production in shaken and static culture was investigated. The experiments showed a clear dependency between nitrogen limitation and the production of the mycotoxins AOH and AME whereas TA production appeared to be growth associated and independent on nitrogen depletion and source. By selecting different carbon and nitrogen sources mycotoxin production was enhanced significantly or inhibited completely. Both mycotoxin production and composition was dependent on cultivation conditions. Highest concentrations of all mycotoxins were detected when cultivated statically with glucose as carbon source and phenylalanine as nitrogen source. The use of acetate as carbon source resulted in the sole production of AOH independent on cultivation condition (**section 4.1**).

For the elucidation of mycotoxin formation a reliable and robust test system in a bioreactor was established. The process proved to be highly reproducible and consumption and formation rates were achieved by logistic fitting. By altering process parameters their influence on mycotoxin formation was observed. Different aeration rates (2 vvm – 0.013 vvm) were evaluated to enhance mycotoxin production. By lowering the aeration rate to 0.013 mycotoxin concentrations were increased. Furthermore, promising carbon and nitrogen sources from the medium optimization experiments were tested. Results from shaking flask experiments were confirmed and mycotoxin production was enhanced (**section 4.2**).

The regulation of secondary metabolite formation is very complex; among other things carbon to nitrogen (C:N) ratio and feedback inhibition play an important role. Therefore, in a third attempt the effects of C:N ratio on mycotoxin formation were observed by changing glucose concentrations while the nitrogen content was kept constant. First results in shaking flasks showed that the increase from 10 g/L to 30 g/L of initial glucose concentration enhanced AOH production remarkably. However, a clear production peak was observed in shaking flask experiments at day 7 followed by an abrupt decrease of AOH concentration. Thus, to elucidate possible feed-back inhibition mechanisms or degradation processes feeding experiments with AOH were performed. They revealed that feeding of AOH up to a certain concentration can enhance the production of all monitored mycotoxins. Higher initial AOH concentrations showed no further effect on mycotoxin production. Decreasing AOH concentrations were also observed in feeding experiments (**section 4.3**).

The biosynthesis of the mycotoxins AOH and AME is possibly a multi-enzyme process, but the genes are not known. The knowledge about biosynthetic genes offers new possibilities in the elucidation of regulatory mechanisms. As the genes for fungal secondary metabolite production are usually clustered the identification of one gene promotes the identification of the whole gene cluster. In a fourth approach one enzyme of the cluster, the AOH-*O*-methyltransferase, which catalyzes the methylation reaction from AOH to AME is characterized and partially purified. Two strategies were pursued; in a genetic attempt several fungal *O*-methyltransferases were compared and conserved regions were used for primer design. In the second biochemical attempt the protein was purified from protein crude extract by chromatography methods (**section 4.4**).

In this work, new approaches for the elucidation of *Alternaria* toxin production were developed and established. Particularly, the development of the bioreactor process enables high reliability and comparability of single experiments and provides a platform for further studies revealing influences on mycotoxin production.

Zusammenfassung

Mykotoxine sind sekundäre Stoffwechselprodukte von kleiner molekularer Masse, die von diversen Schimmelpilzgattungen gebildet werden. Mykotoxine wurden weltweit als Kontaminanten in Lebensmitteln nachgewiesen und entfalten ihre in Menschen und Tieren vielgestaltige, meist schädliche Wirkung. Neben gesundheitlichen Risiken verursachen sie zusätzlich enormen wirtschaftlichen Schaden, da bis zu 25 % der weltweiten Getreideerträge mit Mykotoxinen kontaminiert sind. Der Schwarzschimmel *Alternaria alternata*, die auf geernteten Obst und Gemüse am häufigsten verkommende *Alternaria* Art, produziert hauptsächlich fünf *Alternaria*-Gifte, darunter Alternariol (AOH), Alternariolmonomethylether (AME) und Tenuazonsäure (TA). Trotz zahlreicher toxikologischer Studien, die das Ziel hatten, mögliche Gesundheitsschäden durch *Alternaria*-Gifte aufzuklären, konnte diesbezüglich kein eindeutiges Ergebnis gewonnen werden. Eine Risikobewertung dieser Toxine ist auf Grund dessen nicht erfolgt. Die beste Möglichkeit, gesundheitlichen Risiken vorzubeugen, ist demnach die Vermeidung von Lebensmittelverunreinigungen durch *Alternaria*-Toxine. Um dies realisieren zu können, ist jedoch detailiertes Fachwissen über die Mykotoxinbildung in *A. alternata* notwendig.

In einem ersten Ansatz wurde der Einfluss verschiedener Kohlenstoff- und Stickstoffquellen auf die Mykotoxinbildung sowohl in Schüttel- wie auch in Standkultur untersucht. In diesen Experimenten zeigte sich ein Zusammenhang zwischen Stickstofflimitierung und der Bildung der Mykotoxine AOH und AME, wohingegen die Bildung von TA nicht von der Art der Stickstoffquelle beeinflusst wurde und vermutlich wachstumsassoziiert und unabhängig von der Stickstofflimitierung auftrat. Durch die Wahl der jeweiligen Kohlenstoff- oder Stickstoffquelle konnte die Bildung der Mykotoxine sowohl signifikant erhöht, als auch komplett verhindert werden. Zusätzlich dazu wurden Mykotoxinmenge und -zusammensetzung durch die Kultivierungsbedingungen beeinflusst. Die höchsten Konzentrationen an allen Mykotoxinen wurden in Standkultur mit Glucose als Kohlenstoffquelle und Phenylalanin als Stickstoffquelle erhalten. Die Kultivierung mit Acetat als Kohlenstoffquelle resultierte in der alleinigen Bildung von AOH (**siehe Kapitel 4.1**).

Um die Mykotoxinbildung weiter aufklären zu können, wurde ein zuverlässiges und robustes Testsystem im Bioreaktor etabliert. Der entwickelte Bioprozess erwies sich als äußerst reproduzierbar; Bildungs- und Verbrauchsraten ließen sich daraus durch ein logistisches Fitting errechnen. Durch Variation einzelner Prozessparameter wurde deren Einfluss auf die Mykotoxinbildung ermittelt. Um die Ausbeute an Mykotoxin weiter zu steigern, wurden

diverse Begasungsraten im Bereich von 2 vvm bis 0.013 vvm getestet; dies konnte durch die Verringerung der Begasung auf 0.013 vvm erreicht werden. Desweiteren wurde jeweils eine vielversprechende Kohlenstoff- und Stickstoffquelle aus den Medienoptimierungsversuchen (Kapitel 4.1) im Reaktormaßstab getestet. Das Ergebnis aus dem Schüttelkolben konnte übertragen und so die produzierte Mykotoxinmenge in der Kulturbrühe erhöht werden (**siehe Kapitel 4.2**).

Die Regulation der Sekundärmetabolitbildung ist sehr komplex. Neben weiteren Faktoren spielen aber unter anderem das Verhältnis von Kohlenstoff- und Stickstoffmengen im Medium (C:N Verhältnis) sowie mögliche Feedback-Inhibierungen eine Rolle. Aus diesem Grund wurde in einem dritten Ansatz der Einfluss des C:N Verhältnisses auf die Mykotoxinbildung untersucht, indem bei gleichbleibender Stickstoffmenge die Glucosekonzentration im Medium variiert wurde. Erste Ergebnisse aus Schüttelkolbenexperimenten zeigten, dass die Erhöhung der anfänglichen Glucosekonzentration von 10 g/L auf 30 g/L eine deutliche Steigerung der AOH Menge zur Folge hatte. Zusätzlich konnte ein klares Konzentrationsmaximum an AOH nach 7 Kultivierungstagen beobachtet werden; danach sank die AOH Konzentration abrupt ab. Desweiteren wurden Zufütterungsversuche mit AOH durchgeführt, um mögliche Degradationsprozesse und/oder Feedback-Inhibierungen aufzuklären. Diese Versuche zeigten, dass eine AOH-Zufütterung bis zu einer bestimmten Konzentration eine weitere AOH Bildung fördert. Über diesen Punkt hinaus hatte die Zufütterung keine weitere Auswirkung. Dennoch zeigte sich auch in diesen Experimenten eine Abnahme der AOH Konzentration im Medium (**siehe Kapitel 4.3**).

Die Biosynthese der beiden Mykotoxine AOH und AME wird wahrscheinlich durch einen Multi-Enzym-Komplex katalysiert, jedoch sind dessen Gene nicht bekannt. Die Aufklärung der Biosynthesegene würde allerdings neue Möglichkeiten bei der Erforschung der Regulationsmechanismen eröffnen. Da die Biosynthesegene pilzlicher Sekundärmetabolite meist in Genclustern organisiert vorliegen, kann die Identifikation eines Genes innerhalb des Cluster die Aufklärung der anderen Gene stark voranbringen. In einen vierten Ansatz wurde deshalb ein Enzym aus dem Biosynthesecluster, die AOH-*O*-Methyltransferase, die die Methylierungsreaktion von AOH zu AME katalysiert, näher charakterisiert und teilweise aufgereinigt. Dabei wurden zwei Strategien verfolgt: in einem genetischen Ansatz wurden zahlreiche pilzliche *O*-Methyltransferasen miteinander verglichen und auf konservierte Regionen innerhalb der Proteine untersucht. Diese Regionen dienten daraufhin für die

Generierung von Primern. Im zweiten, biochemischen Ansatz wurde das Enzym aus dem Proteinrohextrakt von *A. alternata* mittels chromatographischer Methoden angereichert (**siehe Kapitel 4.4**).

In dieser Arbeit wurden neue Ansätze zur Untersuchung der Mykotoxinbildung in *A. alternata* entwickelt und etabliert. Die Prozessentwicklung im Bioreaktor gewährleistet insbesondere eine hohe Reproduzierbarkeit und ermöglicht die Vergleichbarkeit einzelner Experimente. Somit wurde dadurch eine Plattform geschaffen, um weitere Einflussfaktoren der Toxinbildung zu ermitteln.

Contents

I. Introduction

The aim of the present work was the establishment of a reliable and reproducible biotechnological process for the production of *Alternaria* mycotoxins. This process enables the identification and elucidation of factors influencing mycotoxin production and helps to understand mycotoxin production in *A. alternata* which is still largely unknown. The following chapters will introduce to moulds in general and to the black mould *A. alternata* in particular, biotechnological chances and challenges of fungal fermentation and will finally summarize the existing knowledge about *Alternaria* mycotoxins.

1.1 Moulds

Moulds are filamentous fungi which do not belong to a specific phylogenetic group. Moulds can therefore be found in the fungal divisions Ascomycota, Deuteromycota (Fungi imperfecti) and Zygomycota. Typical representatives are members of the genera *Mucor*, *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (figure 1).

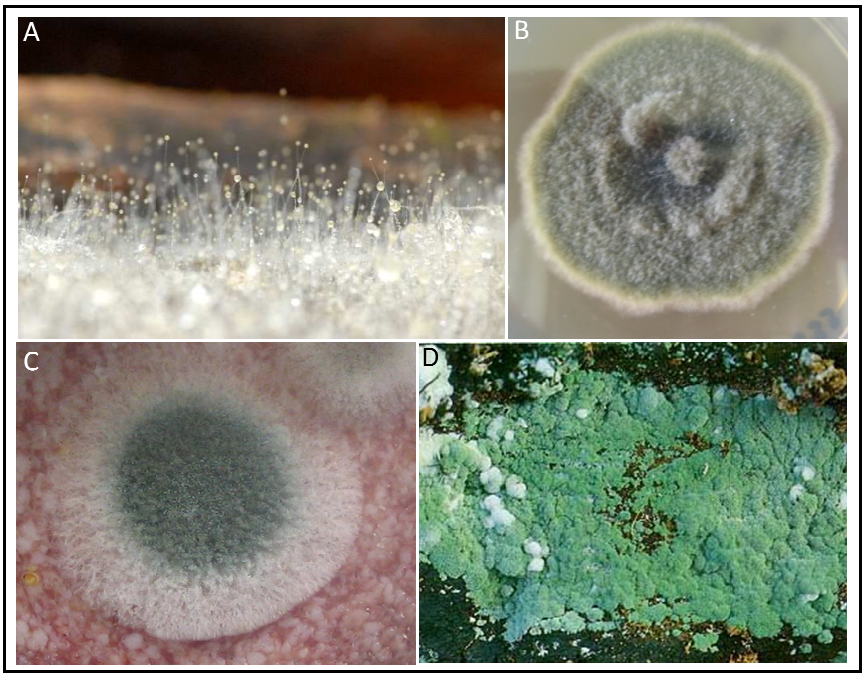

Figure 1: Typical representative genera of moulds. **A.** *Mucor* sp. (http://www.commanster.eu/commanster/Mushrooms/Asco/Mucoraceae.html). **B.** *Alternaria* sp. **C.** *Penicillium* sp. (http://de.academic.ru/pictures/dewiki/83/Schimmel_050904.jpg). **D.** *Aspergillus* sp. (http://www.skn.ac.th/skl/skn422/nature/fun5.jpg).

Moulds are ubiquitous in nature; they are heterotrophic organisms which derive their energy from organic matter. The mycelium often builds a wide network (Kück *et al*., 2009). For utilization of complex compounds in the soil moulds are secreting hydrolytic enzymes. In this way, complex polysaccharides such as starch, lignin or cellulose can be degraded and utilized by the fungus. On account of this, saprophytes play a major role in decomposition and recycling of organic material. Besides of saprophytic lifestyle moulds can also act as opportunistic pathogens in humans and livestock or as plant pathogens or decay foodstuffs and textiles (Mücke and Lemmen, 2004).

Fungal reproduction is mainly mediated by asexually produced spores or conidia (Petrini and Petrini, 2008). These spores can be uninucleate or multinucleate, were produced in large numbers and are resistant to dryness, heat and UV-radiation. Due to their little weight they are spread easily through air and germinate where the conditions are favorable. Inhaled spores are mainly responsible for fungal allergies and asthmas. Although the diversity of species causing allergies can be rather high, the most prevalent genera are *Cladosporium*, *Alternaria*, *Aspergillus* and *Penicillium* (Jürgensen and Madsen, 2009).

Many moulds are able to produce mycotoxins. Mycotoxins are toxic secondary metabolites of low molecular weight, but are not necessary for growth or development (Fox and Howlett, 2008). They can be released in the substrate and therefore spoil foodstuffs.

1.2 The genus *Alternaria*

Alternaria species are wide spread black moulds and belong to the division of Deuteromycota (Bottalico and Logrieco, 1998). The genus *Alternaria* was founded in 1917 by Nees von Esenbeck with *Alternaria alternata* (former name: *A. tenuis*) as main species (Rotem, 1994). *Alternaria alternata* (Fr.) Keissler is the most common species but it is referred to as an unresolved species group and not as a single species (Barkai-Golan, 2008). By now over 100 species can be differentiated, but there is much nomenclature confusion (Kwasna, 1992; Rotem, 1994). The taxonomy of the genus *Alternaria* is not well defined and has been extensively discussed (Neergaard, 1945; Ellis, 1971, 1976; Kwasna, 1992; Simmons, 1992; Rotem, 1994). A combined approach of morphological and genetic features as well as secondary metabolite profiles could be the key for a better discrimination and identification of species (Logrieco *et al*., 2009). The reproduction of *Alternaria* species is exclusively

mediated by asexual conidia; the corresponding sexual reproductive stage (teleomorph) is the genus *Lewia*.

Characteristically the mycelium and conidia of all *Alternaria* species are of a dark black or greenish colour (Pitt and Hocking, 2009). The mulitcelled conidia are organized in long chains which are produced from simple, sometimes branched conidiophores. Figure 2 shows the morphology of *A. alternata* and its conidia.

Figure 2: Morphology of *A. alternata*. **A.** Growing culture of *A. alternata* on Potatoe-Dextrose-Agar plates. **B.** Microscopic picture of conidia (http://www.mycology.adelaide.edu.au/). **C.** Schematic picture of multicelled conidia developing on the conidiophores (www.sci.muni.cz/mikrob/Miniatlas/alt.htm).

Alternaria species are common saprophytes found on decaying organic materials world-wide. The genus *Alternaria* includes also opportunistic plant-pathogens affecting many cultivated plants in the fields and stored fruits and vegetables during post-harvest (Guo *et al*., 2004). The most common species of the genus *Alternaria, A. alternata,* contains seven different pathogenic variants which produce host-specific toxins and causes necrotic diseases on different plants (Nishimura and Kohmoto, 1983; Kohmoto *et al*., 1995; Hatta *et al*., 2002). Therefore, it is the principle causative agent for brown spot on citrus (Kohmoto *et al*., 1979), brown necrotic lesions on foliage, black pit disease on potatoes (Droby *et al*., 1984) and late blight on California pistachios (Aradhya *et al*., 2001).

The optimal growth temperature for *Alternaria* is between 22 °C and 28 °C which enables growth at room temperature in various climate regions. However, *Alternaria* species can grow even at low temperatures; the minimal developmental temperature is -3 °C (Sommer, 1985). That is why contamination of refrigerated foodstuffs during transport and storage is possible (Ozcelik *et al*., 1990). Therefore, *Alternaria* species are able to develop on cabbage, celery and other vegetables stored at 0 °C. In particular, tomatoes stored under 8 - 12 °C are extremely sensitive to *Alternaria* infestations (Barkai-Golan, 2001). But, it should be noticed,

that *A. alternata* is not able to penetrate undamaged plant tissue. Invasion requires openings of the host tissue like calyx scars or wounded tissues which are often generated during harvest and handling (Barkai-Golan, 2008). Table 1 reviews natural infestations of *Alternaria* species on agricultural commodities.

Table 1: Pre- and postharvest infestations of *Alternaria* species

Agricultural commodity	***Alternaria* species**	**References**
Grains		
Barley	*A.* spp.	Kosiak *et al*., 2004
	A. alternata	Medina *et al*., 2006
Maize	*A. alternata*	Broggi *et al*., 2007
	A. alternata, A. radicina, *A. tenuissima, A. infectoria*	Torres *et al*., 1998
Oat	*A.* spp.	Kosiak *et al*., 2004
Rice	*A. alternata*	Broggi *et al*., 2007
Rye	*A.* spp.	Semaskiene *et al*., 2005
Sorghum/ragi	*A. infectoria*	Broggi *et al*., 2007
	A. alternata	Ansari and Shrivastava, 1990
Wheat	*A. alternata, A. infectoria*, *A. tenuissima*	Broggi *et al*., 2007
	A. alternata	Li and Yoshizawa, 2000
	A. alternata	Li *et al*., 2001
	A. spp.	Kosiak *et al*., 2004
Fruits		
Apples	*A.* spp.	Granado *et al*., 2008
	A. alternata	Robiglio and Lopez, 1995
	A. alternata	Vinas *et al*., 1992
Blueberries	*A.* spp.	Tournas and Katsoudas, 2005
	A. spp.	Stinson *et al*., 1980
Grapes	*A. alternata*	Swart and Holz, 1994
Lemons	*A.* spp.	Tournas and Katsoudas, 2005
Mandarins	*A.* spp.	Tournas and Katsoudas, 2005
Mangos	*A. alternata*	Prusky *et al*., 1983

Melons	*A.* spp.	Yang *et al*., 2003
Olives	*A. alternata*	Visconti *et al*., 1986
Oranges	*A.* spp.	Tournas and Katsoudas, 2005
Papaya	*A. alternata*	Barkai-Golan, 2001
Pears	*A. alternata*	Lockhart and Forsyth, 1974
Pecans	*A. alternata*	Schroeder and Cole, 1976
Persimmon	*A. alternata*	Prusky *et al*., 1981
Raspberries	*A. humicola*	White and Fabian, 1953
Strawberries	*A.* spp.	Tournas and Katsoudas, 2005
Vegetables		
Carrots	*A. alternata, A. radicina*	Solfrizzo *et al*., 2005
Dried beans	*A.* spp.	Mislivec *et al*., 1975
Eggplant	*A. alternata*	Barkai-Golan, 2002
Peppers	*A. alternata*	Barkai-Golan, 2002
Potatoes	*A. alternata*	Droby *et al*., 1984
Red kidney beans	*A.* spp.	Sanchis *et al*., 1988
Tomatoes	*A. alternata* *A. alternata* *A. alternata* *A. tenuissima, A. longipes*	Barkai-Golan, 2002 Hasan, 1995 Harwig *et al*., 1979 Pose *et al*., 2004
Others		
Amaranth	*A. alternata, A.* spp.	Bresler *et al*., 1995
Cottonseed	*A. alternata, A. tenuissima*	Davis *et al*., 1977
Oilseed rape	*A. alternata*	Vinas *et al*., 1994
Soybean	*A. alternata*	Broggi *et al*., 2007
Sunflower grains	*A. alternata*	Pozzi *et al*., 2005
Tobacco	*A. alternata*	Lucas *et al*., 1971

Besides of the economical loss due to harvest and post-harvest decay *A. alternata* is able to produce toxic secondary metabolites, the *Alternaria* toxins (Andersen *et al*., 2006; Logrieco *et al*., 2009; Patriarca *et al*., 2007). Since *Alternaria* species infect naturally a wide variety of fruits and vegetables even under refrigeration, contamination of these goods with *Alternaria* toxins is possible. Therefore *Alternaria* toxins can be considered as toxic contaminant of our everyday food (Barkai-Golan, 2008) (see also section 1.5).

1.3 Fungi in biotechnology

1.3.1 Products

Since thousands of years moulds (mainly *Aspergillus* and *Penicillium* species) were applied for the production and refinement of food e.g. fermentation of alcoholic/non-alcoholic beverages or bread baking. In the last 50 years the purposeful application of fungi for commercially important products has increased rapidly (Papagianni, 2004). The modern industrial mycology was founded in the 1940s with the development of the submerged production of penicillin. Nowadays fungi are used in many industrial processes for the production of enzymes, pigments, vitamins, polyhydric alcohols, polysaccharides, lipids, glycolipids and secondary metabolites. Additionally fungi are extremely useful in biotransformation processes (Adrio and Demain, 2003). Table 2 gives an overview of some commercially important products and their sources. An extensive list of industrial fungal processes and products is given by the "ATCC names of industrial fungi" list (Jong *et al*., 1994).

Table 2: Examples of fungal products of commercial interest

Product	Organism	Production process	Reference
Enzymes			
α-Amylase	*Aspergillus oryzae*	SSF	Ramachandran *et al*., 2004
Cellulase	*Trichoderma reesii*, *Aspergillus niger*	SSF	Lee *et al*., 2011
Invertase	*Aspergillus niger*	SSF	Aranda *et al*., 2006
Laccase	*Trametes pubescens*	SmF, b.	Galhaup *et al*., 2002
Lipase	*Aspergillus niger*	SmF, b.	Macris *et al*., 1996
Pectinase	*Aspergillus niger*	SSF	Couto and Sanromán, 2006

Proteases	*Aspergillus oryzae*	SSF/SmF b.	Sandhya *et al*., 2005
Antibiotics/Pharmaceuticals			
Cephalosporins	*Acremonium chrysogenum*	SSF	Adinarayana *et al*., 2003
Cyclosporins	*Tolypocladium niveum*	SmF, b.	Isaac *et al*., 1990
Griseofulvin	*Penicillium griseofulvum*	SSF	Saykhedkar and Singhal, 2004
Lovastatin	*Aspergillus terreus*	SmF, f-b.	Porcel *et al*., 2007
Penicillins	*Penicillium chrysogenum*	SmF, f-b.	Elander, 2003
Paclitaxel	*Fusarium solani*	SmF, b.	Chakravarthi *et al*., 2008
Organic acids			
Citric acid	*Aspergillus niger*	SSF/SmF	Grewal and Kalra, 1995
Fumaric acid	*Rhizopus oryzae*	SmF, b.	Zhou *et al*., 2002
Gluconic acid	*Aspergillus niger*	SmF, f-b.	Ramachandran *et al*., 2006
Itaconic acid	*Aspergillus terreus*	SmF, b.	Willke and Vorlop, 2001
Kojic acid	*Aspergillus oryzae*	SmF, b./i.	Kwak and Rhee, 1992
Mycotoxins			
Ergot alkaloids	*Claviceps purpurea*	SmF, b.	Tudzynski *et al*., 2001
Zearalenone	*Gibberella zeae*	SmF, b.	Woodings, 1972
Gibberellic acid	*Gibberella fujikuroi*	SSF	Machado *et al*., 2004
Polyunsaturated fatty acids			
γ-linolic acid	*Mortierella alpine*	SmF, b.	Shimizu *et al*., 1989
Arachidonic acid	*Mortierella alpina*	SmF, b.	Eroshin *et al*., 2000

SSF: solid state fermentation; SmF: submerged fermentation; b.: batch; f-b.: fed batch; i.: immobilized cells.

With the development of molecular biological techniques new possibilities for the homologous and heterologous protein production were provided. Host strains are chosen on the basis of production yields and regulatory issues but also on holding the "GRAS" (Generally Recognized As Safe) status which is attained by the U.S. Food and Drug Administration (FDA) (Adrio and Demain, 2003). Filamentous fungi are able to produce large amounts of proteins but the production yield of heterologous expressed, non-fungal proteins is usually low (Punt *et al*., 2002). One major reason for this is the abundant production of secreted proteases of the fungal host. Therefore much research was done on this topic and

several improved protease-deficient strains were developed (Mattern *et al*., 1992; van den Hombergh *et al*., 1997). Further improvements to enhance production yields were achieved by the use of strong promoters, the introduction of large numbers of gene copies, the use of strong secretion signals and the fusion with a fungal well-secreted protein (Archer *et al*., 1994; Gouka *et al*., 1997; Gouka *et al*., 1999; Moralejo *et al*., 1999; Punt *et al*., 2002; Verdoes *et al*., 1995). Nevertheless, the production yield of non-fungal proteins keeps low; therefore, most of the recombinant food-grade proteins are of fungal origin (Archer, 2000; Pariza and Johnson, 2001). The continuously increasing list of heterologous fungal enzymes which are improved for food application is given at http://www.enzymetechnicalassoc.org.

1.3.2 Challenges of fungal fermentation

The development of submerged production and therefore the establishment of fungal biotechnology in the 1940s created great production potentials. However, the fungal morphology appeared to be problematic. Filamentous fungi are morphological complex organisms. They undergo different developmental stages in which morphology is changed during the life cycle. In nature, fungi are growing in long branched hyphae in surface culture creating a wide spread mycelium consisting of a mass of hyphal filaments. In submerged culture different morphological forms are possible ranging from dispersed mycelial filaments to dense pellets which assemble due to strong interactions between hyphae. The morphology is influenced by certain process parameters, i.e. chemical (medium components), physical (pH, temperature) and culturing conditions (nature of inoculum) (Kossen, 2000; Schügerl *et al*., 1998), but it is in turn affected by the process. Filamentous growth results in high viscosity which has a negative impact on the transport of O_2, CO_2, heat and nutrients. In contrast pellet growth effects in low viscosity, but the inner part of larger pellets is often limited in nutrients and undergoes autolysis whereas the degree of limitation is dependent on the pellet density (Elmayergi, *et al*., 1973). Figure 3 gives an overview of the relationship between morphology and process conditions (according to Kossen, 2000).

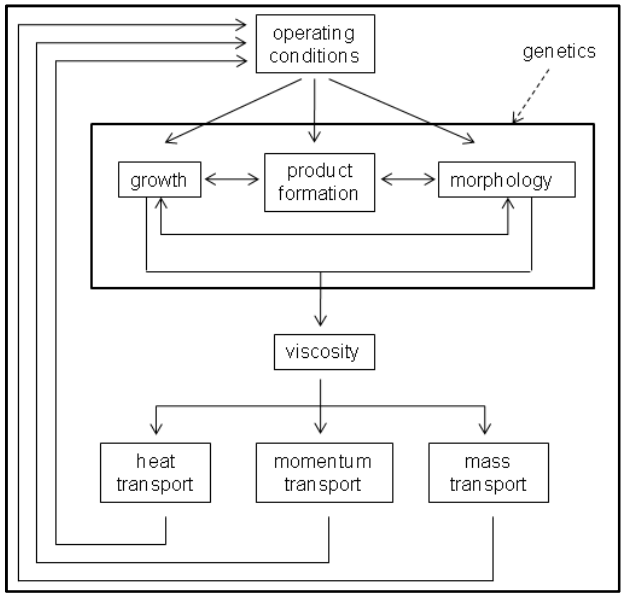

Figure 3: Relationship between morphology and process conditions (according to Kossen, 2000).

Fungal morphology is an important factor concerning production yields. Therefore, in some fermentation processes the maximum product yield can only be achieved when the fungus is growing in a particular morphological form. Bermek *et al*. (2004) investigated the influence of morphology on the production of ligninolytic enzyme production in *Trichophyton rubrum*. The best results were obtained when the fungus was growing in small pellets with extending filaments. In case of secondary metabolite production, e.g. mycotoxins, the formation of the product is independent of growth and not associated with increasing biomass (Megee *et al*., 1970). The production of different metabolites requires a unique set of physiological conditions which has to be determined for each metabolite and fungus for the exploitation of the full production potential. Therefore, for each product the precise physiological conditions and the correct stage of development have to be identified (Papagianni, 2004). On account of this Schügerl *et al*. (1998) summarized extensively the process parameters which influence xylanase production by *Aspergillus awamori*. They could show that a large interrelationship exists between the key parameters reactor type (shake flasks, stirred tank and airlift tower loop reactor), medium composition (semisynthetic and complex medium with solid particles of different sizes, different concentrations of phosphate), the specific power input (stirrer speed) and growth, morphology, physiology and productivity of the fungus. The authors stated that published results may appear contradictory when not all relevant parameters were considered.

Considering the challenges of fungal submerged fermentation due to morphological aspects solid state fermentation appears to be advantageous. Although efficient fungal strains are available which are well adapted to submerged fermentation by genetic engineering, cultivation in free water is not the natural habitat of fungi. Therefore, submerged cultivation may impair metabolic efficiency (Hölker *et al*., 2004). Solid state fermentation is carried out on moist solid supports which acts either as inert carrier or is additionally used as nutrient source (Barrios-Gonzáles *et al*., 2005). Free water is nearly absent in this kind of fermentation which mimics the natural environment of the organisms. Due to the tight contact of the cultivated fungi to the substrate highest substrate concentrations can be achieved (Hölker *et al*., 2004). Solid state fermentations in a small scale provide several advantages compared to submerged fermentation: Due to the low water demand the desired end product is highly concentrated. Additionally, less energy is required, aeration is easier and anti-foam chemicals are not needed. As can be seen from table 2 many process optimizations of well established submerged fermentations were conducted using solid state fermentation. Nevertheless, in industry enzymes and secondary metabolites are mainly produced under submerged cultivation conditions due to the simplified scale-up and greater control of parameters, i.e. pH, heat and nutrient conditions (Robinson *et al*., 2001). Although the importance of solid state fermentation especially for the production of secondary metabolites was recognized by Barrios-Gonzáles and Mejia (1996) and Robinson *et al*. (2001), the production on industrial scale is hampered by the unavailability of suitable bioreactors (Balakrishnan and Pandey, 1996).

With respect to the production of mycotoxins, other secondary metabolites or any other products with *Alternaria* spp. submerged fermentation protocols were not developed yet. For the establishment of a reproducible process for *Alternaria* toxin production all the above mentioned important physiological factors have to be considered and optimized which will help for the development of further production processes of other interesting metabolites of *Alternaria* spp.

1.4 Mycotoxins

Mycotoxins are secondary metabolites of small molecular weight formed by a wide diversity of different moulds. Well-known mycotoxin producers are *Aspergillus*, *Fusarium*, *Alternaria*, *Trichoderma*, *Trichothecium* and *Penicillium* species (Smith *et al*., 1983). Today about 650 different mycotoxins are known which belong to approximately 25 chemical groups (Klaffke, 2010). Mycotoxins like other secondary metabolites are usually formed subsequent to the growth phase (trophophase) in the idiophase. The formation of mycotoxins is subjected to complex regulation mechanisms, but is often induced by nutrient limitations (Demain, 1986). The production of mycotoxins is neither necessary for growth nor for development. Therefore, the reason for their formation is not known (Fox and Howlett, 2008). A role as chemical defense mechanisms against other microbial organisms may be conceivable to improve the environmental conditions enabling further proliferation.

Due to the release of mycotoxins into the surrounding substrate, contamination of agricultural products and foodstuff is possible. In this way mycotoxins can reach the food chain and can be ingested by both humans and livestock (Andersen and Frisvad, 2004). Based on estimations of the Food and Agriculture Organization (FAO) of the United Nations approximately 25 % of the world´s food crops are affected by mycotoxin producing fungi and global losses of foodstuffs due to mycotoxins are in the range of 1000 million tons per year (http://www.fao.org/ag/agn/agns/chemicals_mycotoxins_en.asp; Mücke and Lemmen, 2004). The occurrence of mycotoxins in foods and feeds is depending on intrinsic and extrinsic factors. Extrinsic factors connected to storage conditions are controllable other than climate or intrinsic factors like fungal strain specificity, strain variation, and instability of toxigenic properties (Hussein and Brasel, 2001). Therefore, it is not possible to predict their presence or to prevent their occurrence entirely during preharvest, storage and processing operations (Wood, 1992). Mycotoxins are responsible for various acute or chronic effects on humans and animals (mycotoxicosis) (Hussein and Brasel, 2001). Some of the versatile toxic effects include the increased risk of cancer (Mücke and Lemmen, 2004), organ damages of liver and kidney (Keweloh, 2006), the influence on hormone system, damage of the central nervous system and teratogenic and mutagenic properties (Schön, 2005). The severity of a mycotoxicosis depends on the mycotoxin itself as well as on the amount and the duration of exposure, but also the age, sex and health of the poisoned individual may play a role. Additionally, mycotoxicoses can heighten vulnerability to microbial diseases, worsen the

effects of malnutrition, and interact synergistically with other toxins (Bennet and Klich, 2003).

The versatile toxicological effects of mycotoxins can be partly explained by their diverse chemical characteristics. Table 3 gives an overview of the major mycotoxicological groups.

Table 3: Major groups of mycotoxins (according to Bhatnagar *et al*., 2002; Bennet and Klich, 2003)

Group of mycotoxin	**Main producers**	**Toxic effect**	**Liturature**
Aflatoxins	*Aspergillus parasiticus* *Aspergillus flavus*	acute toxicity (death), hepatotoxic, carcinogenic	Goldblatt, 1969; Cary *et al*., 2000; Cullen *et al*., 1987; Eaton and Groopman, 1994.
Ochratoxins	*Aspergillus ochraceus* *Aspergillus alliaceus*, *Aspergillus auricomus*, *Aspergillus carbonarius*, *Aspergillus glaucus*, *Aspergillus melleus* *Aspergillus niger* *Penicillium verrucosum*	nephrotoxic, carcinogenic, dermatotoxic hepatotoxic, teratogenic, immuno-suppressive	Abarca *et al*., 1994; Bayman *et al*., 2002; Ciegler *et al*., 1972; Creppy, 1999; Kuiper-Goodman and Scott, 1989; van Egmond and Speijiers, 1994.
Citrinin	*Aspergillus ochraceus* *Aspergillus niveus* *Aspergillus terreus* *Aspergillus oryzae* *Penicillium citrinum* *Penicillium camemberti* *Monascus ruber* *Monascus purpureus*	nephrotoxic, yellow rice disease	Blanc *et al*., 1995; Hetherington and Raistrick, 1931; Manabe, 2001; Saito *et al*., 1971; Jordan *et al*., 1978.
Ergot alkaloids	*Claviceps* species	gangrene, abortion, convulsions, hypersensitivity, ataxia, lactation suppressive, hallucinogenic	Flieger *et al*., 1997; Lorenz and Hoseney, 1979.
Patulin	*Penicillium griseofulvum* *Penicillium expasum*	antibacterial, antiviral, antiprotozoal, carcinogenic, contact edema, hemorrhage	Ciegler, 1977; Ciegler *et al*., 1971; Trucksess and Tang, 2001.

Fusarium toxins			
Fumonisins	*Fusarium verticillioides*	phytotoxic, Leuko-encephalomalacia, pulmonary edema, hydrothorax, hepatotoxic, carcinogenic, apoptosis in liver.	Bezuidenhout *et al.*, 1988; Marasas, 2001; Gelderblom *et al.*, 1988; Harrison *et al.*, 1990; Ross *et al.*, 1992; Pozzi *et al.*, 2001; Tseng and Liu, 1997; Wan Norhasima *et al.*, 2009.
Zearalenones	*Fusarium graminearum*	estrogenic	Abid-Essefi *et al.*, 2009.
Trichothecenes	*Fusarium* species	inhibit eukaryotic protein synthesis, hemorrhage, vomiting, dermatitis.	Marasas *et al.*, 1984; Sudakin, 2003); Cundliffe *et al.*, 1974); Chu, 1997; Wu *et al.*, 2010.
Alternaria toxins	for details see section 1.5		

Regulations concerning mycotoxins in foods and feeds have been established in many countries to protect the consumer from the effects. Since the discovery of the first mycotoxins (aflatoxins) in 1960 the number of countries with known specific mycotoxin regulation increased to 100 in 2003. Currently 13 mycotoxins or groups of mycotoxins are under concern. Their regulations are based on scientific opinions of authoritative bodies like the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JECFA) and the European Food Safety Authority (EFSA) (van Egmond *et al.*, 2007).

In the EU legal limits for mycotoxins were harmonized and national regulations were replaced. From five out of the nine mycotoxin groups given in table 3 regulatory limits exist. In the commission regulation from 2006 updated in 2010 maximum levels for mycotoxins in foodstuff were set as follows (http://eurlex.europa.eu/; Köppen et al., 2010):

Aflatoxins: The maximum amount of aflatoxin B1 in foodstuffs is set to 2 µg/kg and the sum of aflatoxins B1, B2, G1 and G2 to 4 µg/kg. Dairy product may contain 0.05 µg/kg aflatoxin M1. Maximum amounts of aflatoxins in dietary foodstuffs for babies and small children are lower and were set to 0.1 µg/kg.

Ochratoxin A: The maximum tolerable weekly intake (TWI) of ochratoxin A was set to 120 ng/kg body weight (b.w.). The maximum amounts in foodstuffs are between 2 µg/kg (for wine and grape juices) and 10 µg/kg (for raisins and instant coffee).

Patulin: For patulin a provisional maximum tolerable daily intake (PMTDI) of 0.4 µg/kg b.w. is endorsed. The maximum amount in foodstuffs is set to 50 µg/kg in apple juice and to 25 µg/kg in apples and other solid apple products.

Fusarium toxins: With regard to *Fusarium* toxins a temporary tolerable daily intake (TDI) of 0.2 µg/kg b.w. is set for zearalenone and of 2 µg/kg b.w. for fumonisins.

In addition regulations for further *Fusarium* toxins (desoxynivalenol, nivalenol, T-2 and HT-2) exist. Taken together, limits for around 50 mycotoxin-food combinations are defined. In the following years a limit will probably also be established for ergot alkaloids. For the other mycotoxins including *Alternaria* toxins a risk assessment is currently not available.

1.5 *Alternaria* Toxins

Alternaria species are capable to produce a wide diversity of secondary metabolites belonging to different chemical groups including dibenzopyrones, tetramic acids, lactones, quinones and cyclic peptides. More than 120 secondary metabolites of *Alternaria* species are known; a quarter of that is designated as mycotoxins (Panigrahi, 1997).
However, only five major toxins of the known 30 are common natural contaminants of consumable goods: the benzopyrene derivatives alternariol (AOH), alternariol monomethylether (AME), altenuene (ALT), the tetramic acid tenuazonic acid (TA) and the perylene derivative altertoxin I (ATX I) (figure 4).

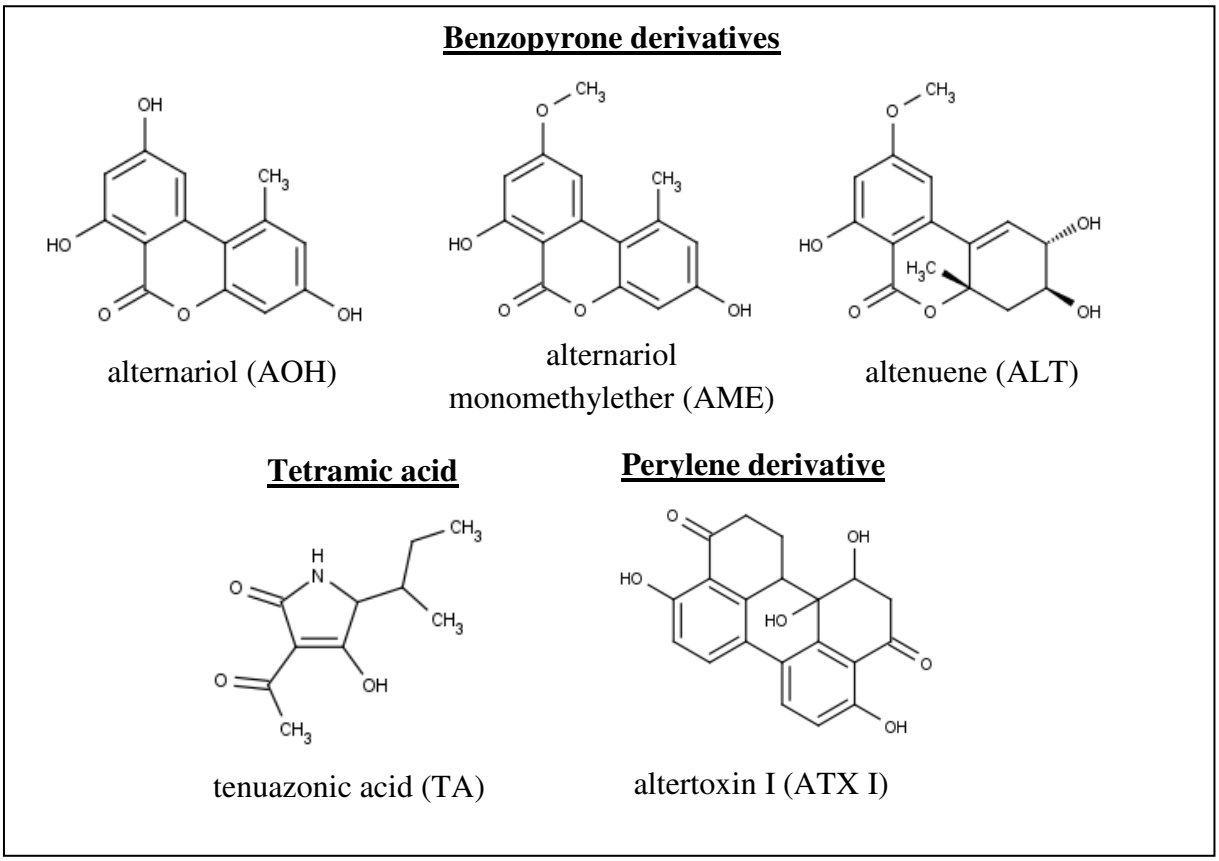

Figure 4: Chemical structures of the five major *Alternaria* toxins alternariol, alternariol monomethylether, altenuene, tenuazonic acid and altertoxin I (Barkai-Golan, 2008).

In the last years *Alternaria* toxins have received increasing attention: e.g. from 2000 to 2004 the European Union carried out the multi-disciplinary project: "Safe organic vegetables and vegetable products by reducing risk factors and sources of fungal contaminants throughout the production chain: the carrot – *Alternaria* model" for the development of strategies for a safe organic food supply. The project included the establishment of methods for the detection of *Alternaria* spp. on carrot, the establishment of analytical methods for *Alternaria* toxins, the determination of the toxigenic potential of *Alternaria* spp. isolated from carrot and the monitoring of mycotoxin accumulation in the production chain (http://www.seedcentre.wur.nl/UK/Projects/Safe_Organic_Vegetables/). The detection and discrimination of *Alternaria* spp. contaminating carrots will mainly be achieved by morphological characteristics and by using of selective media. A PCR method for the differentiation of *A. alternata*, *A. dauci* and *A. radicina* is available but proved to be not always reliable. HPLC-based analytical methods for artificially on rice produced mycotoxins were developed. Although *A. alternata* and *A. radicina* isolates from carrot and wild carrot showed a high virulence and a high mycotoxigenic potential when grown on rice, *A. alternata* toxins were not detected in naturally infected carrots. This fact was also confirmed by

Solfrizzo *et al*. (2005). The results will be used by EU regulatory and standard bodies and can be at least partly transferred to other organic crops and food products which are affected by *Alternaria* spp.

Nevertheless, based on the current data a risk assessment for *Alternaria* toxins is not possible, which was recently stated by the German Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung, BfR, 2003). According to the BfR only little toxicological data is available just for seven out of the 30 known *Alternaria* mycotoxins which is insufficient for an assessment of the health risk for the consumer. This opinion was supported 2007 by the Czech Scientific Committee on Food (CSCF, 2007).

1.5.1 Producers of *Alternaria* toxins

Alternaria alternata, the most common *Alternaria* species in harvested fruits and vegetables, produces the five major *Alternaria* toxins and additionally tentoxin, altenuisol, alteniric acid, altenusin and dehydroaltenusin (Chelkowski and Visconti, 1992; Bottalico and Logrieco, 1998). The ability of *A. alternata* to produce several different mycotoxins renders it interesting for mycotoxicologists (Scott, 2004).

Nevertheless, other *Alternaria* species are also able to produce mycotoxins. AOH and AME were found to be produced by *A. tenuissima, A. brassicae, A. capsici-annui, A. citri, A. cucumerina, A. dauci, A. kikuchiana, A. longipes, A. porri, A. solani* and *A. tomato* (Bottalico and Logrieco, 1998; Andersen and Frisvad, 2004; Pose *et al*., 2004). The tetramic acid TA is not only produced by *A. alternata* but also by *Phoma sorghina* and *Pyricularia oryzae* and other *Alternaria* species including *A. capsici-annui, A. citri, A. japonica, A. kikuchiana, A. longipes, A. porri, A. radicina, A. tenuissima* and *A. tomato* (Iwasaki *et al*., 1972; Meronuck *et al*., 1972; Steyn and Rabie, 1976; Bottalico and Logrieco, 1998; Pose *et al*., 2004). The perylene derivative ATX I and the related altertoxin II and III were also formed by *A. tenuissima, A. mali, A. radicina* and *A. tomato* (Bottalico and Logrieco, 1998). Additionally the plant pathogen *Alternaria alternata* f. sp. *lycopersici* produces the host specific AAL toxin which is similar to fumonisins (Abbas and Riley, 1996; Mirocha *et al*., 1992). Table 4 summarizes the *Alternaria* species which are able to produce *Alternaria* toxins (according to Ostry, 2008).

Table 4: Main *Alternaria* toxins produced by *Alternaria* species according to Ostry (2008)

Alternaria species	Mycotoxins	References
A. alternata	AOH, AME, ALT, ATX I, TA	Chelkowski and Visconti, 1992; Bottalico and Logrieco, 1998.
A. tenuissima	AOH, AME, ATX I, -III, TA	Davies *et al*., 1977; Young *et al*., 1980; Bottalico and Logrieco, 1998.
A. brassicae	AOH, AME	Bottalico and Logrieco, 1998.
A. capsici-annui	AOH, AME, TA	Bottalico and Logrieco 1998.
A. citri	AOH, AME, TA	Freeman, (1966); Kinoshita *et al*., 1972.
A. cucumerina	AOH, AME	Raistrick *et al*., 1953; Freeman, 1966.
A. dauci	AOH, AME	Raistrick *et al*., 1953; Freeman, 1966.
A. kikuchiana	AOH, AME, TA	Torikata *et al*., 1969; Kinoshita *et al*., 1972; Kameda *et al*., 1973.
A. longipes	AME, TA	Mikami *et al.*, 1971; Bottalico and Logrieco 1998.
A. porri	AME, TA	Bottalico and Logrieco, 1998.
A. solani	AOH, AME, TA	Stoessl, 1969; Pollock *et al.*, 1982; Bottalico and Logrieco, 1998.
A. tomato	AOH, AME, TA ATX I, -II, -III	Bottalico and Logrieco, 1998.
A. japonica	TA	Kinoshita *et al*., 1972.
A. radicina	ATX I, -II, -III, TA	Bottalico and Logrieco, 1998; Solfrizzo *et al*., 2005.
A. mali	ATX I, -II, -III, TA	Kinoshita *et al*., 1972.
A. cassiae	ATX I, -II	Hradil *et al*., 1989.
A. oryzae	TA	Kinoshita *et al*., 1972.

AOH: alternariol; AME: alternariol monomethylether; TA: tenuazonic acid; ATX: altertoxin.

1.5.2. Occurrence of *Alternaria* toxins

Alternaria species infest naturally diverse fruits and vegetables. Therefore, it is not surprising that *Alternaria* toxins can be detected in these goods. However, mycotoxin content and composition strongly depends not only on the fungal species or strain but also on the substrate meaning the host species or cultivar and the environmental conditions. These facts are represented by apparently contradictory publications: Ozcelik *et al*. (1990) detected AOH and AME in apples and tomatoes. The maximum concentrations were 1161 µg/g AOH and 323 µg/g AME in tomatoes and accordingly 372 µg/g AOH and 32 µg/g AME in apples. TA was not observed. In contrast, in earlier studies by Harwig *et al*. (1979) and by Stinson *et al*. (1980; 1981) TA was the major mycotoxin found in inoculated and naturally infested tomatoes with maximum concentrations of 1373 µg/g TA in fruit tissue.

Similarly, Logrieco *et al*. (1990) isolated two different *A. alternata* strains from a natural black or gray "heart rot" of mandarin. The two kinds of *Alternaria* heart rot differed in coloring of the mycelia and both qualitatively and quantitatively in the production of mycotoxins. The black rot samples were able to produce TA, AOH and AME whereas TA was the only detectable mycotoxin in the grey rot sample. The toxin profile changed when both samples were growing on rice kernels. The overall production of all mycotoxins in both isolates was higher and additionally ALT and ATX I were detectable.

Considering all agricultural commodities which are naturally infested by *Alternaria* species the potential of spoilage by *Alternaria* mycotoxins appears to be high. Several studies investigated both the natural occurrence of *Alternaria* toxins in foodstuffs and the mycotoxigenic potential of *Alternaria* isolates subsequently inoculated on their natural substrate. Although *Alternaria alternata* is able to produce high yields of mycotoxins on different substrates under artificial conditions, *Alternaria* toxins were detected only in few commodities in nature. Table 5 gives an overview about the natural occurrence of *Alternaria* mycotoxins in foodstuffs.

Table 5: Natural occurrence of *Alternaria* mycotoxins in foodstuff

Commodity	Mycotoxin (max. level)	References
Apples	AOH (58.8 µg/g) AME (2.3 µg/g) ALT (0.5 µg/g) TA (0.5 µg/g)	Stinson *et al*., 1981,
	TA (9.6 µg/g)	Singh and Sumbali, 2004.
Barley	AOH 0.13 µg/g)	Kütt *et al*., 2010
Mandarins	AOH (5.2 µg/g) AME (1.4 µg/g) TA (173.9 µg/g)	Logrieco *et al*., 1990
Olives	AOH (2.9 µg/g) AME (2.3 µg/g) ALT (1.4 µg/g) TA (0.3 µg/g)	Visconti *et al*., 1986
Pecans	no information	Schroeder and Cole, 1976
Peppers	AOH (440 µg/g) AME (294 µg/g) ALT (103 µg/g) TA (342 µg/g)	Scott, 2001
Sorghum	AOH + AME (7.9 µg/g)	Seitz *et al*., 1975
Sunflower grain	AOH (170.9 ng/g) AME 108.6 (ng/g)	Pozzi *et al*., 2005
Tomatoes	AOH (5.3 µg/g) AME (0.8 µg/g) ALT (1.1 µg/g) TA (139.0 µg/g)	Stinson *et al*., 1981
	TA (70 µg/g)	Stack *et al*., 1985
	AOH (1.3 µg/g) AME (0.3 µg/g) TA (7.2 µg/g)	Bottalico and Logrieco, 1998
Wheat	AOH (0.731 µg/g), AME (1.426 µg/g), TA (6.432 µg/g).	Li and Yoshizawa, 2000
	AOH (1.388 µg/g) AME (7.451 µg/g) TA (8.841 µg/g)	Azcarate *et al*., 2008

AOH: alternariol; AME: alternariol monomethylether; ALT: altenuene; TA: tenuazonic acid.

Based on this data a risk assessment in the daily nutrition is problematic. Most of the samples contaminated with *Alternaria* toxins were visibly moldy, i.e. the products were obviously not suitable for consumption. Therefore, an intentional consumption of moldy products would be unlikely. But mycotoxins are not restricted to the rotten part. Robiglio and Lopez (1995) detected mycotoxins in the surrounding tissues of rotten fruits which were not visibly affected by the fungus. Furthermore, mycotoxins can be detected in cell-free culture supernatants as well as in asymptomatic tissues of inoculated fruits. Occurrence of mycotoxins in apparently unaffected fruits is therefore possible and has to be considered (Barkai-Golan, 2008).

The natural occurrence of mycotoxins in processed food may pose a serious risk for the human health. As other mycotoxins, *Alternaria* toxins are chemically stable during storage and processing, even after cooking or autoclaving. Combina *et al*. (1999) evaluated the heat stability of AOH, AME and TA in sunflower flour. While AOH and AME concentrations were not significantly decreased after 90 min of heating at 100 °C, TA concentration was reduced to 50 % after heat treatment. The combination of heat and pressure while autoclaving proved to be more effective: the 90 min treatment at 121 °C and 0.1 MPa resulted in 75 % decontamination of AOH and a 100 % decontamination of AME. TA concentration was reduced to 33 % compared to the initial concentration. On account of this it is not surprising to detect *Alternaria* toxins in several fruit juices, wine and edible oils (table 6).

Table 6: Occurrence of *Alternaria* toxins in processed food

Product	Mycotoxin (max. level)	Reference
Tomato puree	AOH (8.756 µg/g) AME (1.734 µg/g) TA (4.021 µg/g)	Terminiello *et al*., 2006
	TA (76 ng/g)	Motta and Valente Soares, 2001
Tomato pulp	TA (110 ng/g)	Motta and Valente Soares, 2001
Tomato paste	TA (100 ng/g)	Scott and Kanhere, 1980
Apple juice concentrate	AOH (5.42 ng/ml) AME (1.71 ng/ml)	Delgado and Goméz-Cordovéz, 1998
Apple juice	AOH (5 ng/ml)	Scott *et al*., 1997
	AOH (2.4 ng/ml)	Lau *et al*., 2003

	AME (0.43 ng/ml)	
Raspberry juice	AOH (0.84 ng/ml)	Lau *et al*., 2003
Cranberry nectar	AOH (5.6 ng/ml) AME (0.7 ng/ml)	Lau *et al*., 2003
Cranberry juice	AOH (0.04 ng/ml) AME (0.003 ng/ml)	Scott *et al*., 2006
Prune nectar	AOH (5.5 ng/ml) AME (1.4 ng/ml)	Lau *et al*., 2003
Grape juice	AOH (1.6 ng/ml) AME (0.23 ng/ml)	Lau *et al*., 2003
Red grape juice	AOH (0.46 ng/ml) AME (39.5 ng/ml)	Scott *et al*., 2006
Red wine	AOH (1.9 ng/ml)	Lau *et al*., 2003
(Canadian)	AOH (5.02 ng/ml) AME (0.21 ng/ml)	Scott *et al*., 2006
(Italian)	AOH (19.4 ng/ml) AME (0.19 ng/ml)	Scott *et al*., 2006
White wine	AOH (1.48 ng/ml) AME (0.06 ng/ml)	Scott *et al*., 2006
Sunflower seed meal[#]	AOH (180 ng/g) AME (100 ng/g) TA (1900 ng/g)	Nawaz *et al*., 1997
Oilseed rape meal[#]	AOH (68 ng/g) AME (55 ng/g) TA (730 ng/g)	Nawaz *et al*., 1997
Olive oil*	AOH (793 ng/ml) AME (285.7 ng/ml)	Visconti *et al*., 1986

* oil produced in laboratory from the most contaminated olives.
[#] for animal feed purposes
AOH: alternariol; AME: alternariol monomethylether; TA: tenuazonic acid.

Detection of *Alternaria* toxins ranged from 50 % of 32 samples of commercial apple juice as shown by Delgado and Goméz-Cordovéz (1998) to sporadic samples of other fruit beverages

as shown by Lau *et al*. (2003). The absence of TA in most of the fruit beverages may be explained by the lower heat stability compared to AOH and AME (Combina *et al*., 1999). A decontamination of TA due to pasteurization of the fruit juices would be conceivable. In contrast to TA both AOH and AME are able to withstand the pasteurization procedure without apparent losses and are stable over weeks in apple juice as reported by Scott and Kanhere (2001). TA was not tested in this study.

Considering the data shown in table 6 an unintentionally intake of AOH and AME in the range of several µg per day appears to be thoroughly probable. A daily consumption of one liter apple juice is quite common and may contain AOH between 2.4 µg/L (Lau *et al*., 2003) and 5.42 µg/L (Delgado and Goméz-Cordovéz, 1998). In case of processed food the consumer needs to rely on the care of the manufacturer in the choice of fruits and vegetables because the presence of moldy raw-materials in the end-product is not identifiable by the consumer. It is therefore urgent to clarify the risk potential of *Alternaria* toxins and to set maximum allowable amounts in food.

1.5.3 Toxicity and biological effects of *Alternaria* toxins

Compared to other mycotoxins, e.g. aflatoxins, the *Alternaria* toxins have a low acute toxicity. Therefore, an acute toxicosis of humans by *Alternaria* toxins caused by food supply seems unlikely. But low-level exposure over long terms is an additional serious hazard. Vinas *et al*. (1992) considered tenuazonic acid as the most important toxic compound produced by *Alternaria*. Alternariol, alternariol monomethylether, altenuene and altertoxin I are more interesting for their mutagenic potential. For the determination of the *Alternaria* toxin toxicity both single mycotoxins and complex extracts with unknown composition were used.

***Alternaria* extracts**

The exposure to *Alternaria* toxins has been associated with certain adverse health effects. Pero *et al*. (1973) tested the toxicity of both crude extracts and single mycotoxins to mice by intraperitoneal injections. The lethal dose of *Alternaria* extract was 300 mg/kg body weight. Sauer *et al*. (1978) reported on feeding experiments with *Alternaria* spoiled corn and rice to rats and chickens. Toxic effects on chicken were observed when the feed contained 50 % of spoiled corn and rice. Necrosis and hemorrhages could be observed in the gizzard and isthmus of dead birds. Lethality was observed on rats fed with the 50 % spoiled feeding mixture. Dead animals showed hemorrhages in the stomach and intestines. In the study of Schrader *et al*.

(2001) the mutagenicity of *Alternaria* extracts was tested using the Ames test. A direct mutagenic activity capable to induce frameshifts and base pair mutations was observed in *Salmonella* strains TA98 and TA100. Liu *et al*., (1991) examined the mutagenic and tumorigenic properties of *Alternaria* extracts. They observed reverse mutations in *E. coli* cells, abnormal DNA replication events in several mammalian cell lines and transformation of NIH-3T3 fibroblast cells. Of particular health concern is the incidence of esophageal cancer in Linxin, China. The etiology of this cancer was connected with the contamination of cereal grains with *A. alternata* (Dong *et al*., 1987; Liu *et al*., 1992). As stated by Pero *et al*. (1973), the toxicity of complex extracts is much higher than of the single tested mycotoxins which suggest synergism between single components.

Alternariol and derivatives

As stated by Pero *et al*. (1973), Pollock *et al*. (1982) and Olson and Visconti (1988) the benzopyrone derivatives AOH, AME and ALT are not very acutely toxic, but AOH and AME have been reported to act mutagenic and genotoxic (Scott and Stoltz, 1980; DiCosmo and Straus, 1985; An *et al*., 1989; Davis and Stack, 1994; Schrader *et al.*, 2001, 2006; Brugger *et al.*, 2006). The LD_{50} for AOH and AME is higher than 400 mg/kg bodyweight for mice (Pero et al., 1973). Panigrahi and Dallin (1994) measured the toxicity of TA, AOH, ALT and ATX-I on brine shrimp larvae (*Artemia salina* L.). The determined LD_{50} values were 75 g/ml, 100 g/ml, 375 g/ml and 200 g/ml for TA, AOH, ALT and ATX-I, respectively. AME was not found to produce any mortality.

Brugger *et al*. (2006) investigated the mutagenic potential of AOH on two different gene loci in the Chinese hamster cell line V79 and in mouse lymphoma cells L5178Y $tk^{+/-}$. The treatment with 10 µM AOH and higher resulted in a significant and concentration-dependent induction of mutations in the gene-loci of both cell lines. The morphology of treated mouse lymphoma cells was indicative for extensive chromosomal deletions and the induction of micronuclei. The authors stated that the mutagenic potential of AOH is about 50 fold lower than the established mutagen 4-nitroquinoline-*N*-oxide but may be the reason for the cancerogenicity of this mycotoxin.

Pero *et al*. (1973) elucidated the cytotoxic potential of AOH, AME and ALT against HeLa cells and *Bacillus mycoides*. In the *Bacillus*-test the lowest concentrations of the toxins for a measurable inhibition zone were 60 µg/disc, 500 µg/disc and 125 µg/disc for AOH, AME and ALT, respectively. In the test with HeLa cells the ID_{50} values were 6 µg/ml, 14 µg/ml and 28 µg/ml for AOH, AME and ALT, respectively. In the *Bacillus*-test a clear synergism of AOH

and AME was observed: Only 0.25 µg of each AOH and AME in a 1:1 mixture was necessary to elicit an inhibition zone. In the HeLA cells test a similar synergism was not observed. Furthermore a fetotoxic effect of AOH was examined when administered to rats at a concentration of 100 mg/kg b.w.

AME was found to act mutagenic in the *E. coli* ND160 reverse mutation assay (An *et al*., 1989). These assay measures the frameshift revertants at a *lac*Z mutation (Clarke and Wade, 1975).

But there are also contradictory studies concerning the mutagenicity of AOH and AME. Using the Ames-Test both AOH and AME were found to be not mutagenic for the *Salmonella typhimurium* strains TA98 and TA100 which are indicative for reversions at GC sites (Scott and Stoltz, 1980; Davis and Stack, 1994; Schrader *et al*., 2001). The weak mutagenicity of AME towards the strain TA98 observed by Scott and Stoltz (1980) was explained by possible contamination with highly mutagenic compounds by Davis and Stack (1994). However, Schrader *et al*. (2006) observed a weak mutagenicity of both AOH and AME for the *S. typhimurium* strains TA102 and TA104 which are indicative for AT mutations. As stated by Schrader *et al*. (2006) an additional mechanism involved in mutagenic activation may be possible. The low mutagenicity of single mycotoxins detected in the Ames-Test seems to be unable to account for the proposed carcinogenicity.

Concerning the genotoxic properties AOH seems to exhibit a more pronounced genotoxicity than AME. In the study of Fehr *et al*. (2009) both AOH and AME increased the rate of DNA strand breaks at micromolar concentrations in human carcinoma cells, but did not enhance oxidative DNA damage. Furthermore, a substantial affinity of AOH to the minor groove of the DNA could be demonstrated. Interaction studies confirmed that AOH, as the most DNA-damaging *Alternaria* metabolite, potently inhibited DNA relaxation and stimulated DNA cleavage activities of topoisomerase I, IIa and IIb. Therefore, AOH was characterized as a poison of topoisomerase I and II.

The DNA strand breaking ability observed by Fehr *et al*. (2009) confirmed the studies of Pfeiffer *et al*. (2007). In the work of Pfeiffer *et al*. (2007) the treatment of cultured Chinese hamster V79 cells, human liver HepG2 cells and human colon HT-29 cells with AOH and AME caused a concentration-dependent induction of DNA strand breaks at concentrations ranging from 5 to 50 µM. They demonstrated that the genotoxic activity is carried out by the compounds themselves and was abolished by glucuronidation. Therefore, they concluded that the activity of a cell for the formation of glucuronides may play a critical role in the health risk posed by *Alternaria* toxins.

In the study of Lehmann *et al*. (2006) the estrogenic activity, the effect on cell proliferation and the genotoxic potential of AOH was examined *in vitro* in Ishikawa cells (human endometrial adenocarcinoma cell line) and in V79 cells. The estrogenic potential found for AOH was approximately 10,000 fold weaker than the endogenous hormone E2, but was comparable with the phytoestrogen Daidzein or the environmental xeno-estrogen Bisphenol A. Although the possible exposure to AOH, e.g. due to apple-juice consummation, is very low (100 fold lower than the determined EC_{50} value in the experiments), the authors suggested that it is likely to exceed the exposure to the weak estrogen Bisphenol A which has raised considerable toxicological concerns (Markey *et al*., 2001). With respect to the genotoxic and cytotoxic potential the authors noticed an induction of micronuclei in both cell lines and a cell-cycle arrest in the G2 and S phase which is a common response of eukaryotic cell to genotoxic challenges. Nevertheless, the *in vivo* relevance of these results has to be questioned due to the high concentrations needed for the observed effects.

Tenuazonic acid

As shown by several studies TA is more toxic as AOH, AME and ALT. Griffin and Chu (1983) tested the toxicity of *Alternaria* toxins in the chicken embryo essay. In contrast to the other mycotoxins TA elicited dose-related mortality responses over the range of 150–1500 µg/egg. The calculated LD_{50} value was 548 µg/egg.

When consumed orally, TA induced the collapse of the cardiovascular systems of mouse, dog and monkey. Furthermore, an increase in salivary secretion, nausea and death were observed in these animals (Smith *et al*., 1968).

When fed to young chicken over a period of three weeks, a dose of 1.25 – 1.5 mg TA/kg/day caused weight loss and nutritional irregularities. No death occurred, but microscopic and macroscopic lesions in various tissues were observed (Giambrone *et al*., 1978).

In the study of Yekeler *et al*. (2001) the effect of AME (50/100 mg/kg/day) and TA (25 mg/kg/day) on the esophagus of mice was examined when fed daily over a period of 10 months. After 10 month of mycotoxin feeding the esophageal epithelium of both AME and TA fed mice displayed inflammatory responses. In TA-fed animals severe developmental changes of the mucosa cells (dysplasia) was observed. The highest number of precursor lesions was present in TA-fed animals and precancerous changes of the esophageal mucosa were evident. Furthermore, sorghum grains spoiled by *Phoma sorghina* containing TA were associated with the human haematological disorder “Onyalai” (Steyn and Rabie, 1976).

Tested for the mutagenic potential the Ames Test showed no mutagenic activity to *Salmonella* strains TA98, TA100 and TA104 (Scott and Stoltz, 1980; Schrader *et al*., 2001, 2006), but was slightly mutagenic in strains TA97 and TA102 (Schrader *et al*., 2006).
Zhou and Qiang (2008) demonstrated the inhibition of cell proliferation in the cell lines 3T3 mouse fibroblasts, Chinese hamster lung cells (CHL) and human hepatocytes (L-O2) with $EC_{50(72h)}$ values of 31.22, 35.73 and 41.84 µg/ml, respectively. The inhibition was dependent on exposure time and increased with the extension of toxin exposure. Furthermore, a decrease in the total protein content of all three cell lines was observed.
As previously discovered by Shigeura and Gordon (1963) TA inhibits the incorporation *in vivo* and *in vitro* of amino acids into proteins and acts therefore as a potent protein biosynthesis inhibitor. Furthermore, it exhibits antitumor, cytotoxic and antibacterial activities (Gitterman, 1965). Miller *et al*. (1963) demonstrated that the sodium salt of TA inhibited the cytopathic effect of a wide spectrum of viruses.

Although numerous toxicological studies were conducted to clarify the effects of *Alternaria* toxins, an unambiguous result was not obtained. Rather the opposite appears to be the case; the reported results are mainly contradictory. Experiments with *Alternaria* extracts are reasonable because it corresponds to the situation when spoiled foodstuff is ingested. Nevertheless, the extracts are complex, the ingredients are not defined. Additionally, the cited studies are not comparable in any way because extracts of different *A. alternata* isolates/strains or even another *Alternaria* species were used which were obtained by cultivation on different substrates. The risk potential was furthermore studied in dissimilar test systems. However, the studies revealed a serious mutagenic potential due to DNA damage which is verified by the cancer incident in China (Dong *et al*., 1987; Liu *et al*., 1992). Nevertheless, tests with single toxins or defined toxin mixtures are an indispensable requirement to assess the mutagenic/toxigenic potential of single toxins and to evaluate synergisms. In recent studies commercially available single toxins or chemically synthesized toxins were applied. Although different test systems were used, the results appear to be more comparable and reliable. For the induction of significant mutations or DNA damages AOH and AME concentrations in the low µM-range are necessary. Brugger et al. (2006) observed first mutagenic effects at concentrations between 5 µM and 10 µM (1.3 mg/L – 2.6 mg/L) AOH, whereas Fehr et al. (2009) detected the strand-breaking ability of AOH and AME at concentrations of 1 µM (0.26 mg/L and 0.27 mg/L, respectively). Comparing these studies with detected mycotoxin concentrations in processed food (see section 1.5.2, table 6) the concentration needed to observe effects in cell culture are 1,000 fold higher than found in e.g.

apple juice. Nevertheless, studies with long-term exposure were not conducted and it is not clear if toxin accumulation in cells or tissues takes place. Therefore, coordinated and systematical long-term studies have to be conducted before the mutagenic potential can be fully assessed. For these tests pure toxins in the mg-range were needed which can be promoted by a cost-efficient production process.

1.5.4 Formation of *Alternaria* toxins

The best way to exclude serious health risks due to mycotoxins is the prevention of mycotoxin spoilage in foods and food raw materials. Therefore, a detailed knowledge about toxin formation is necessary. In the following sections the biosynthesis pathway and the conditions for mycotoxin formation are summarized.

1.5.4.1 Biosynthesis of *Alternaria* toxins

Although AOH is thought to be formed by a polyketide synthetase (Light, 1970), the biosynthetic genes are not known and the enzymatic pathway has still to be elucidated. Isotope labeling experiments of Gatenbeck and Hermodsson (1965) revealed that a single chain is formed by a head to tail condensation of one molecule acetyl-CoA and six molecules of malonyl-CoA followed by a subsequent cyclization to the aromatic compound AOH. Stinson and Moreau (1986) identified and partially purified a putative *O*-methyltransferase which catalyzes the methylation of AOH to AME, named alternariol-*O*-methyltransferase. The methyl donor S-adenosyl-L-methionine (SAM) and Mg^{2+} were found to be Co-factors. The experiments showed that the enzyme is located in the cytosol and is not associated with the AOH-synthetase. Hiltunen and Söderhäll (1992) further purified the enzyme and determined its molecular mass to 43 kDa. AOH was considered as precursor of many benzopyrones of the genus *Alternaria*. Figure 5 gives a hypothetical scheme of the formation of benzopyrone derivatives (adapted from Stinson, 1985). Based on AME ALT may be formed via the intermediates altenusin and dehydroaltenusin by further hydroxylations, oxidations and reductions.

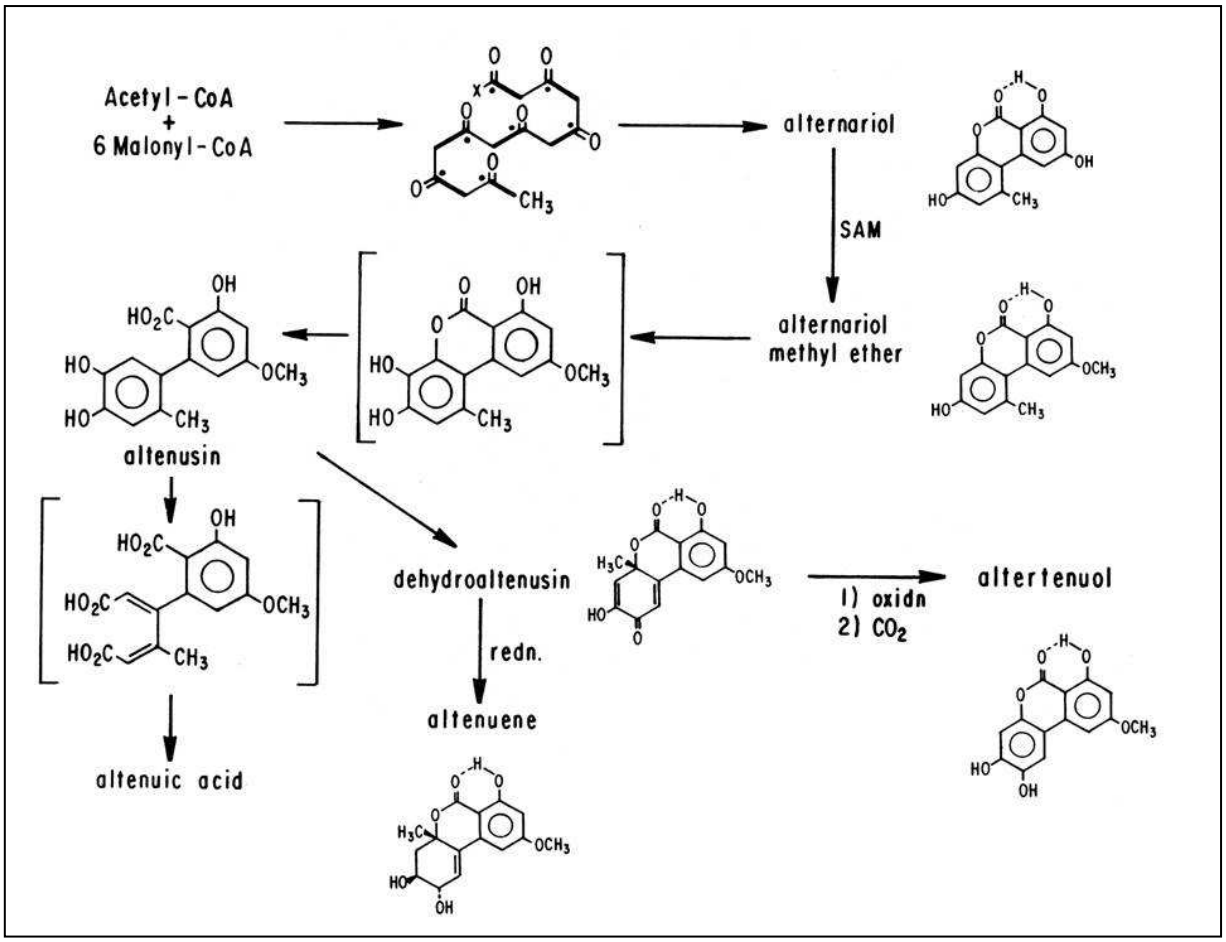

Figure 5: Hypothetical biosynthetic pathway of alternariol its derivatives (adapted from Stinson, 1985). SAM: S-adenosyl-L-methionine; redn: reduction; oxidn: oxidation.

The tetramic acid TA is biosynthetically derived from L-isoleucine and acetate (Stickings, 1959; Stickings and Townsend, 1961). Gatenbeck and Sierankiewicz (1973) demonstrated in their experiments that the initial step of TA formation is N-aceto-acetylation of L-isoleucine followed by the formation of the five-membered ring. Just like with the AOH biosynthesis, the genes of TA biosynthesis are not known.

As the genes for fungal secondary metabolite production are usually clustered (Keller and Hohn, 1997), the identification of the biosynthetic gene cluster for AOH production (and its derivatives) could be essentially promoted by the identification of a single gene of this cluster. The purification of the alternariol-*O*-methyltransferase is one aim of this doctoral thesis and will help to identify further genes of the cluster. The genome of *A. alternata* was sequenced by the group of Chris Lawrence, Virginia Bioinformatics Institute, but the sequence is not freely accessible. The access to the genomic data and subsequent annotation will be a great help for the identification of the other biosynthetic genes.

1.5.4.2 Environmental conditions affecting mycotoxin production

Mycotoxin production varies with fungal strain, the substrate and environmental growth conditions. As with other secondary metabolites, mycotoxin formation is not coupled with fungal growth and starts in the late exponential growth phase (Söderhäll *et al*., 1978). Therefore, despite similar mycelial growth the mycotoxin production is influenced by different culture conditions (Söderhäll *et al*., 1978; Burroughs *et al*., 1976). This includes factors like water activity, temperature, pH-value, light and substrate. In general according to Schmidt-Heydt *et al*. (2008) mycotoxin production can be regarded as an adaptation to imposed abiotic or other stresses of the mycotoxigenic species.

Moulds need a certain amount of free water for germination and growth which is expressed as water activity (a_w) of the substrate. The a_w value is defined as the vapor pressure of a liquid or of the substrate divided by the vapor pressure of pure water at the same temperature. The minimum a_w for growth of *A. alternata* was determined between 0.84 and 0.88, while the optimum is between 0.98 and 1.00 (Magan and Lacey, 1984; Rowan *et al*., 1999; Pose *et al*., 2009). Pose *et al*. (2010) determined the optimum a_w value and temperature for the *Alternaria* toxin production using a synthetic tomato medium. Independent of the tested temperature AOH and AME production were favored at an a_w of 0.954 which is lower than the optimal a_w value for growth. In contrast to this TA concentration increased with the a_w; the maximum TA level was detected at an a_w of 0.982 which matches with the optimal a_w for growth. Contradictory, Magan *et al*. (1984) observed highest production of AOH, AME and ALT at an a_w of 0.98 both on wheat extract agar and wheat grains. Similarly, Oviedo *et al*. (2010) revealed the best combination of water activity and temperature for the production of AOH and AME by two *A. alternata* strains isolated from soybean on soybean agar. Both strains produced highest amounts of AOH at an a_w value of 0.98, but for AME production the optimal a_w value differed: 0.92 and 0.94 for the different strains, respectively. According to Sanchis and Magan (2004) the absolute a_w limit required for mycotoxin (AOH, AME and ALT) production is 0.88 – 0.89.

Besides of the water activity incubation temperature is also an important environmental factor. In many studies the influence of both water activity and temperature on mycotoxin production is analyzed together. For germination and growth the optimal temperature varies between 25°C to 30°C depending on the strain (Magan and Lacey, 1984). As for the water activity optimal temperatures for mycotoxin varies significantly with different strains and media. Hasan (1995) reported on different optimal temperatures for the different mycotoxins:

28°C for AOH and AME production, 21°C for TA production and 14°C for altertoxin production. Magan *et al*. (1984) detected mycotoxins in nearly all combinations of a_w value and temperatures tested in the range of 5°C to 30°C and water activity of 0.9 to 0.98, respectively. Independent of the a_w value highest concentrations of AOH, AME and ALT were obtained at 25°C. In the study of Pose *et al*. (2010) the optimal temperatures were 21°C for AOH and TA production and 35°C for AME production. Nevertheless, it should be noted that the cited studies did not examine mycotoxin production at the same temperatures nor used equal temperature intervals which makes the studies roughly comparable.

The pH value of the growth substrate plays an important role on germination, growth and secondary metabolite production. *A. alternata* is capable to grow in a pH range of 2.7 – 8.0 with an optimum of pH 5.4 (Hasija, 1970). The influence of pH on the mycotoxin production of *A. alternata* was not studied yet. In general mycotoxin formation seems to be favored at acidic pH values as shown for aflatoxin and sterigmatocystin by Keller N.P. *et al*. (1997) and for fumonisin B1 by Keller S.E. *et al*. (1997).

Light influences filamentous fungi with regard to growth and mycotoxin production. In fungi, three light-sensing systems have been described at the molecular level: blue light-sensing achieved by a flavin-based photoreceptor, red light-sensing achieved by a phytochrome and a retinal-based opsin-system with unclear function (Purschwitz *et al*., 2006).
For the determination of the influence of light on *A. alternata* four studies were conducted. According to Söderhäll *et al*. (1978) and Häggblom and Niehaus (1986) light had no influence on fungal growth in drop culture when exposed to a light intensity of 180 W/m^2 or 15 W/m^2, respectively, but inhibited mycotoxin production. Mycotoxin production was strongly reduced when the culture was illuminated continuously and was totally inhibited when the culture was treated during the exponential growth phase. Instead of mycotoxin production a red-brown pigment was observed in light treated cultures (Söderhäll *et al*., 1978). In the study of Häggblom and Niehaus (1986) detailed experiments for the detection of sensitive time-periods and the influence of the illumination length were conducted. Two different light responses were detected: the first response during the early growth period immediately after inoculation which required a longer light period (6 h and more) and inhibited subsequent AOH production and the second response during the AOH production phase which required only short exposure (1 min) and inhibited further AOH production. Häggblom and Unestam (1979) compared the influence of white, blue and red light on the mycotoxin production of seven *A. alternata* isolates with different AOH and AME production

abilities. They confirmed the inhibition of mycotoxin production at continuously light exposure for all strains tested. Whereas blue light inhibited the production of AOH and AME up to 73 %, the treatment with red light had no effect on AOH and AME formation. Furthermore, the illumination with blue light enhanced the total lipid content of the mycelium and red-brown pigments were produced as well as after exposure with white light. Since AME production was always inhibited to the same extent as AOH production the authors concluded that the methylation step for AME formation was not affected by light. Schmidt-Heydt *et al*. (2011) confirmed recently the reduction of AME formation by blue-light.

Besides abiotic factors the substrate or nutritional factors affect mycotoxin production as well. Burroughs *et al*. (1976) observed AOH, AME and ALT production of three different *A. alternata* isolates on various sterile grain substrates. Best results were achieved on rice. However, substrate preferences are strongly strain-dependent and cannot be generalized. In contrast nitrogen starvation seems to be a more general inducer of mycotoxin biosynthesis as shown for various mycotoxigenic species like fumonisin production in *Fusarium proliferatum* (Kohut *et al*., 2009) and ochratoxin A in *Aspergillus spp*. (Medina *et al*., 2008). Mycotoxin production was examined in different studies in both synthetic and complex media supplemented with various carbon / nitrogen sources. But, as said before, results are strongly dependent on fungal strain and additionally on the basal medium supplemented. A systematical approach for the determination of the influence of carbon and nitrogen sources in a semi-synthetic medium on *Alternaria* toxin production has not been carried out yet and is part of this thesis.

As mentioned above, Schmidt-Heydt *et al*. (2008) concluded their studies that mycotoxin production might be a stress adaptation. Therefore, from the food safety point of view, conditions imposing stress have to be avoided during storage and transport to lower the risk of mycotoxin spoilage. This might also include the use of preservatives (Schmidt-Heydt *et al*., 2007) and the use of protective atmospheres (Magan and Lacey, 1984b).

Since the discovery of AOH and AME in 1953 by Raistrick *et al*. (1953) many physiological and toxicological studies were conducted. Unfortunately, these studies were rarely systematic and are roughly comparable due to the utilization of different strains, cultivation methods, media and experimental conditions. Therefore, a profound and deep knowledge about regulation and production of *Alternaria* toxins was not obtained in nearly 60 years of research. With increasing attention on the part of the European Union and other authoritative

bodies and with the availability of sensible analytical methods and commercially available standards a first step towards directed, comparable and standardized research was done. Nevertheless, there is still a lot of work to be done until a risk assessment for *Alternaria* toxins will be available. The present work is therefore concerned with the establishment of a reproducible production process which enables a systematic research of process parameters affecting mycotoxin formation and the directed production of single mycotoxins. Furthermore first experiments for the purification of a protein involved in mycotoxin biosynthesis were presented which will help to identify the biosynthetic gene cluster.

II. Research Proposal

Mycotoxins are found worldwide as contaminants of food and their effects on humans and animals can be significant. Besides the health risk economic losses due to mycotoxin contamination are rather high since up to 25 % of the world´s food crops are affected. Regulations concerning mycotoxins in foods and feeds have been established in many countries to protect the consumer from the effects. Nevertheless, based on the current data a risk assessment for *Alternaria* toxins is not possible. Therefore, no-observed-adverse-effect-levels (NOAEL) and benchmark doses (BMD) are not established for *Alternaria* mycotoxins and consequently exposure limits as tolerable daily intake (TDI) or even a provisional maximum tolerable daily intake (PMTDI) are not defined by the Joint FAO / WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA).

Since prevention is better than cure, the aims of this project were to determine factors which affect (positively or negatively) the mycotoxin production by *Alternaria alternata* DSM 12633. The establishment of a bioprocess for mycotoxin production enables monitoring of the mycotoxin production in detail dependent on different factors and the production of single mycotoxins or mycotoxin mixtures for further toxicological studies. To realize these aims the present work was focused on following aspects:

- Screening for an appropriate defined medium to elucidate nutritional factors affecting mycotoxin production.
- Optimization of the production medium by supplementation with different carbon and nitrogen sources.
- Establishment of a submerged bioprocess for the production of *Alternaria* mycotoxins in a small-scale bioreactor.
- Monitoring of the process and detection of important factors for the mycotoxin production by altering process parameters.
- Optimization of the process by using the results of the medium optimization experiments and by determination of the optimal C/N ratio.
- Identification of the gene cluster for the production of the polyketide mycotoxins AOH, AME and ALT by characterization and purification of the alternariol-*O*-methyltransferase catalyzing the methylation of AOH yielding AME.

III. Characteristics of the Bioreactor Minifors

The characteristics of the bioreactor have to be considered for constant and reproducible mycotoxin production.

Fermentations of *Alternaria alternata* DSM 12633 were performed in the bench-scale bioreactor system Minifors (Infors AG, Bottmingen, Switzerland) (figure 5).

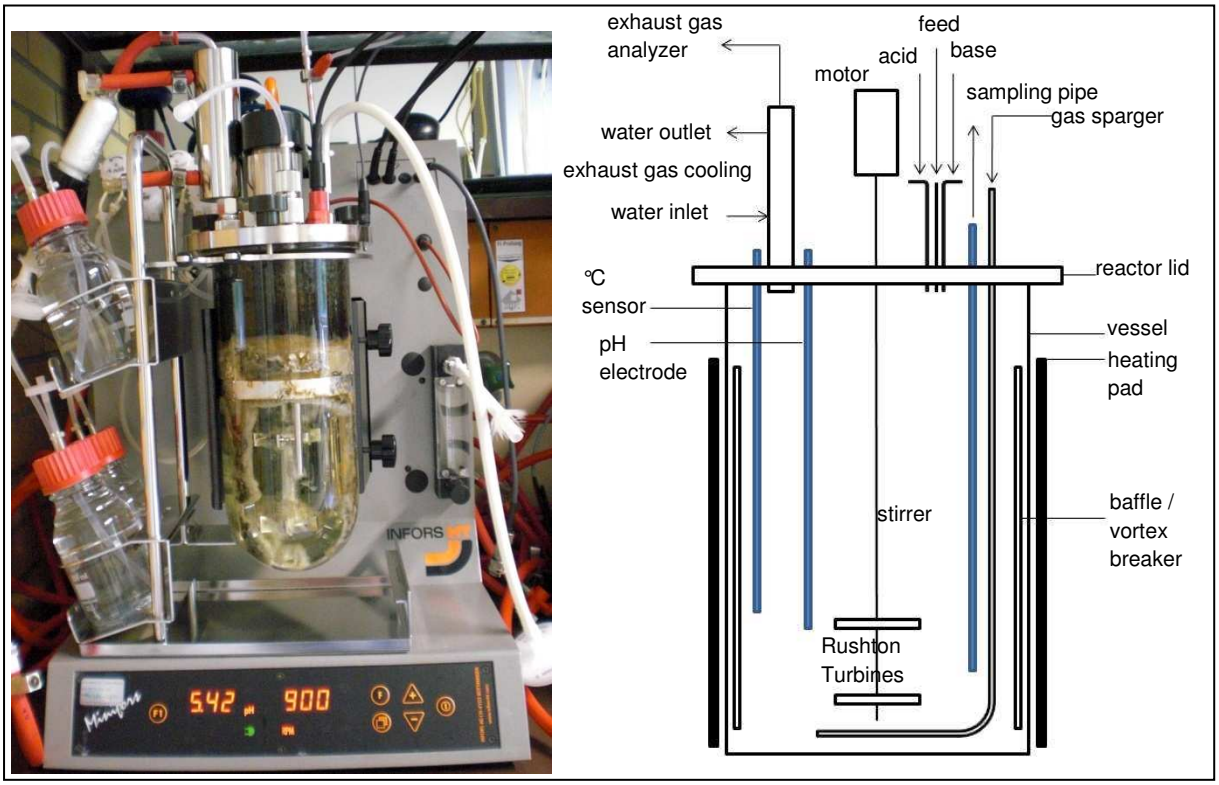

Figure 5: Photo (left) and scheme (right) of the Minifors bench-scale bioreactor system.

The bioreactor comprises of a total volume of 2.5 L. With a height of 275 mm and diameter of 110 mm the reactor possess a slenderness ratio of 2.5. It can be controlled by a digital measuring and control unit operated via the software IRIS which is responsible for data acquisition, process instrumentation and control. All acquired online data are saved and displayed graphically during cultivation. This includes stirrer speed, pH, temperature, titration of base and acid and exhaust gas analytics. Therefore, it is possible to infer from the acquired data on the growth phase and substrate consumption of the fungus, e.g. titration of base occurs while consumption of ammonium. Figure 6 shows exemplarily an online cultivation course.

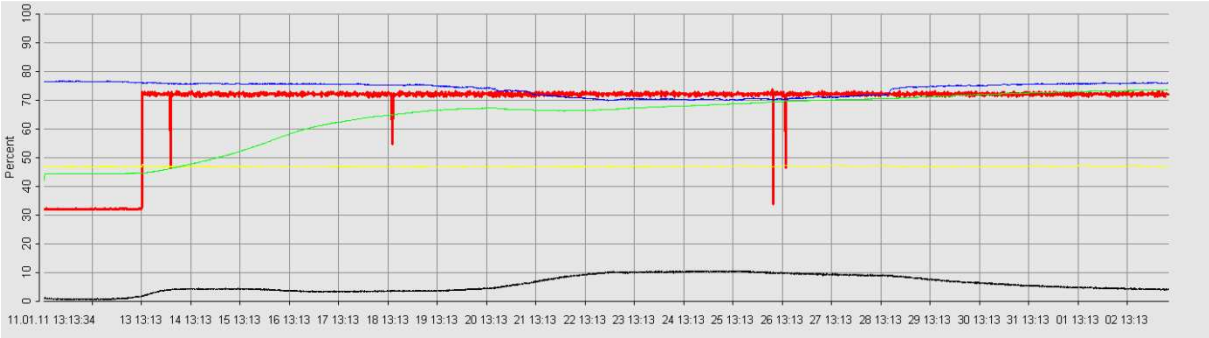

Figure 6: Exemplary cultivation course of a 22 day fermentation without pH regulation displayed by IRIS software. Temperature course (yellow), stirrer speed (red), pH value (green), O_2 amount (blue) and CO_2 amount (black) are illustrated.

To ensure the best possible comparability and reproducibility of all cultivations the reactor installations have to match in each experiment. Therefore, the positions of stirrer and flow breakers were kept constant in the vessel as well as the installation of the electrodes and sensors in the reactor lid. The adjustment of the installations on the lid is shown in figure 7 and further described in table 7.

Figure 7: Reactor lid of Minifors bioreactor with numbered connections (see also table 7).

Table 7: Connections of the reactor lid

#	Connection plug	Company
1	vessel mount	Infors AG
2	blind pipe for temperature sensor Pt100	Infors AG
3	gas sparger	Infors AG
4	anti-foam sensor	Infors AG
5	pH electrode 405-DPAS-SC-K8S/225	Mettler-Toledo
6	sampling pipe	Infors AG
7	dummy plug / pO_2- electrode Oxyferm 225	Infors AG Hamilton
8	tapping membrane	Infors AG
9	exhaust gas cooler	Infors AG
10	inlet for acid, base and feed	Infors AG
11	stirrer	Infors AG

For process control the reactor is equipped with a temperature sensor, a pH- and a pO_2-electrode. The temperature of the medium inside the bioreactor is controlled by two heating pads which are tempered by cooling water circulation. In all fermentation the pO_2- electrode is not used due to invasive growth of the fungus on the electrode membrane which precludes data acquisition. Therefore the corresponding connection plug is sealed by a dummy and oxygen consummation will be monitored via exhaust gas analyzing. Anti-foam reagent is not necessary due to low foam formation during fermentation and is not used either; therefore, the sensor is fixed above the medium level and the inlet for anti-foam reagent is sealed with a piece of flexible tube. For stirring and a fine dispersion of gas the reactor is equipped with two six-bladed Rushton Turbines assembled in a distance of 6 cm on the stirrer shaft. The system is aerated by using filter sterilized compressed air regulated by a rotameter. The exhaust gas is water-cooled and filtered through glass wool before it is conducted through the exhaust gas analyzer (Infors AG). The acquired data are processed by the IRIS software. The pH value of the medium is monitored by the pH electrode (Mettler-Toledo) during the cultivation. Due to consumption of substrate and secretion of metabolites the pH can be altered. The desired pH value is kept constant by titration with 2 M NaOH or 2 M H_3PO_4. The addition of base and acid occurs pulsed and the pH is measured before adding the next portion; the pump speed and duration can be set via the software. For sterilization the bioreactor can be removed from the control unit and is autoclaved 20 min at 121 °C and 1 bar

overpressure. To ensure complete darkness during the whole fermentation the glass vessel is muffled with black felt. The medium is inoculated via the tapping membrane: conidia suspension is drawn in a syringe and injected through the membrane.

IV. Publications and Manuscripts

This doctoral thesis is based on following publications and manuscripts which are specified in detail in the following sections (chapters 4.1 – 4.4).

Chapter 4.1:

Running title: Influence of carbon and nitrogen sources on mycotoxin production.
Brzonkalik K., Herrling T., Syldatk C., Neumann A., (2011). The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*.
International Journal of Food Microbiology 147: 120-126. DOI: 10.1016/j.ijfoodmicro.2011.03.016.
(Parts of the experimental work were done within the diploma thesis of Tanja Herrling.)

This article describes: 1.) Mycotoxin production starts after nitrogen source depletion. 2.) Mycotoxin production and composition is also dependent on nitrogen source as well as on carbon source. 3.) Mycotoxin production and composition is altered by cultivation conditions.

Chapter 4.2:

Running title: Mycotoxin production in a bioreactor.
Brzonkalik K., Herrling T., Syldatk C., Neumann A., (2011). Process development for the elucidation of mycotoxin formation in *Alternaria alternata*.
Submitted to Applied Microbiology and Biotechnology.
(Parts of the experimental work were done within the diploma thesis of Tanja Herrling.)

This article contains: 1.) The establishment of a reproducible process in a bioreactor for the production of *Alternaria* mycotoxins. 2.) The influence of aeration rate on the production of *Alternaria* mycotoxins. 3.) Process optimization using alternative nitrogen and carbon sources.

Chapter 4.3:

Running title: Optimization of the carbon/nitrogen ratio and AOH feeding experiments.

Brzonkalik K., Syldatk C., Neumann A. Determination and optimization of process parameters for mycotoxin production by submerged cultivation of *Alternaria alternata*: carbon/nitrogen ratio and product inhibition.

(manuscript in preparation)

This manuscript specifies: 1.) Growth kinetics and parameters of *A. alternata* cultivated submerged in a semi-synthetic medium in shake flasks. 2.) Influence of initial glucose concentration on biomass production and mycotoxin formation. 3.) Influence of different concentration of the product alternariol on mycotoxin formation.

Chapter 4.4:

Running title: Enzymes of mycotoxin biosynthesis: AOH-*O*-methyltransferase.

Brzonkalik K., Syldatk C., Neumann A. Identification of putative *O*-methyltransferases and characterization of the alternariol-*O*-methyltansferase of *Alternaria alternata*.

(manuscript in preparation).

This manuscript describes: 1.) Comparison of fungal *O*-methyltransferase sequences and identification of conserved domains. 2.) Amplification of four putative *O*-methyltransferase genes of *A. alternata* using degenerated primers. 3.) Amplification of one full-length gene using TAIL-PCR method and expression of this gene in *E.coli* and the yeast *Kluveromyces lactis* 4.) Characterization of the AOH-*O*-methyltransferase in protein crude extract, determination of pH and salt stability and first purification attempts.

4.1 Influence of carbon and nitrogen sources on mycotoxin production

The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*

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Abstract

The aim of this study was to determine the influence of different carbon and nitrogen sources on the production of the mycotoxins alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) by *Alternaria alternata* at 28 °C using a semi-synthetic medium (modified Czapek-Dox broth) supplemented with nitrogen and carbon sources. Additionally the effect of shaken and static cultivation on mycotoxin production was tested. Initial experiments showed a clear dependency between nitrogen depletion and mycotoxin production. To assess whether nitrogen limitation in general or the type of nitrogen source triggers the production, various nitrogen sources including several ammonium/nitrate salts and amino acids were tested. In static culture the production of AOH/AME can be enhanced greatly with phenylalanine whereas some nitrogen sources seem to inhibit the AOH/AME production completely. TA was not significantly affected by the choice of nitrogen source. In shaken culture the overall production of all mycotoxins was lower compared to static cultivation. Furthermore tests with a wide variety of carbon sources including monosaccharides, disaccharides, complex saccharides such as starch as well as glycerol and acetate were performed. In shaken culture AOH was produced when glucose, fructose, sucrose, acetate or mixtures of glucose/sucrose and glucose/acetate were used as carbon sources. AME production was not detected. The use of sodium acetate resulted in the highest AOH production. In static culture AOH production was also stimulated by acetate and the amount is comparable to shaken conditions. Under static conditions production of AOH was lower except when cultivated with acetate. In static cultivation 9 of 14 tested carbon sources induced mycotoxin production compared to 4 in shaken culture. This is the first study which analyses the influence of carbon and nitrogen sources in a semi-synthetic medium and assesses the effects of culture conditions on mycotoxin production by *A. alternata*.

Keywords:

Alternaria alternata, Mycotoxin, Nitrogen source, Carbon source

1. Introduction

Mycotoxins are secondary metabolites of low molecular weight formed by a wide diversity of different moulds. Well-known mycotoxin producers are *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* species. Fungi of the genus *Alternaria* contaminate fruits, vegetables and cereals both as plant-pathogens and saprophytes. Besides the economic loss due to pre-harvest and post harvest decay, *Alternaria* mycotoxin contaminated food can be ingested by both livestock and humans (Andersen *et al*., 2006; Logrieco *et al*., 2009; Patriarca *et al*., 2007). *Alternaria alternata* produces several different mycotoxins of which alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) are best studied (Coombe *et al*., 1970; Montemurro and Visconti, 1992; Pero *et al*., 1973; Rosett *et al*., 1957; Visconti *et al*., 1986). *Alternaria* is known to produce mycotoxins when growing on wheat (Li and Yoshizawa, 2000), sunflower seeds (Nawaz *et al*., 1997; Pozzi *et al*., 2005), oilseed rape (Nawaz *et al*., 1997), pecans (Schroeder and Cole, 1976), fruits and fruit juices (Delgado *et al*., 1996; Lau *et al*., 2003), carrots (Solfrizzo *et al*., 2004), tomato products (Andersen and Frisvad, 2004; da Motta and Valente Soares, 2001; Ozcelik *et al*., 1990) and grains (Broggi *et al*., 2007; Scott, 2001). *Alternaria alternata* can grow at low temperatures so that contamination of refrigerated foodstuffs during transport and storage is possible (Ozcelik *et al*., 1990). *Alternaria* toxins are connected to certain health disorders (Woody and Chu, 1992). The acute toxic effects of AOH and AME are quite weak: their LD_{50} is higher than 400 mg/kg of bodyweight for mice. However, AME is cytotoxic and shows synergistic effects with AOH (Pero *et al*., 1973). There are only a few studies which investigate the effects of *Alternaria* toxins in mammalian cells, but Brugger *et al*. (2006) showed that AOH is mutagenic in cultured cell lines. Furthermore Pfeiffer *et al*. (2007) demonstrated *in vitro* the DNA strand-breaking ability of AOH and AME. Because of the oncogenic potential of AOH (Liu *et al*., 1992) a connection to the incidence of oesophageal cancer in Linxian, China was suggested by Dong *et al*. (1987).

Tenuazonic acid is produced not only by *Alternaria* species but also by *Phoma* and *Pyricularia* species (Iwasaki *et al*., 1972; Meronuck *et al*., 1972; Steyn and Rabie, 1976). Davies *et al*. (1977) reported the toxicity of TA to chicken embryos and rats where it caused haemorrhage and death. It is thus essential to investigate factors which induce or inhibit mycotoxin production. An increasing number of studies have demonstrated the role of environmental factors in mycotoxin production in many filamentous fungi (Georgianna and Payne, 2009; Schmidt-Heydt *et al*., 2008). Schmidt-Heydt *et al*. (2008) demonstrated that

abiotic factors such as temperature, pH and water activity exert influence at the transcriptional level and enhance the expression of the biosynthetic genes. Water activity and temperature in particular are shown to influence the production of *Alternaria* mycotoxins (Hasan, 1995; Oviedo *et al*., 2010; Pose *et al*., 2010). Besides abiotic factors nutritional factors seem to affect mycotoxin production as well. Nitrogen-starvation induces the expression of genes for the biosynthesis of fumonisin in *Fusarium proliferatum* and ochratoxin in *Aspergillus* spp. (Kohut *et al*., 2009; Medina *et al*., 2008) whereas sufficient amounts of nitrogen caused a repression of fumonisin production (Shim and Woloshuk, 1999). In the same way other culture conditions such as composition of culture media, trace elements and carbon source markedly influenced ochratoxin production in *Aspergillus* species (Medina *et al*., 2004, 2008; Mühlencourt *et al*., 2004). Some authors studied the influence of carbon sources on ochratoxin production but the result depended on strain and medium used (Ferreira and Pitout, 1969; Lai *et al*., 1970; Medina *et al*., 2008). Nevertheless Medina *et al*. (2008) showed that differences in ochratoxin production can be related to the nature of the sugar.

Although AOH is thought to be formed by a polyketide synthase (PKS) (Gatenbeck and Hermodsson, 1965; Light, 1970; Wittowski, 1984), the biosynthetic genes are not known and the enzymatic pathways have still to be elucidated. AME is formed by methylation of AOH. The putative AOH-*O*-methyltransferase was partially purified and characterised by Stinson and Moreau (1986). The corresponding gene is still unknown. The tetramic acid TA is proposed to be formed from acetyl-CoA and isoleucine (Gatenbeck and Sierankiewicz, 1973). As well as the lack of knowledge of genetic controls, there are few studies on the influence of culture conditions on *Alternaria* toxin formation. The scope of this study is to examine the influence of nitrogen and carbon sources and different culture conditions on *Alternaria* toxin production in a semi-synthetic medium. This may be helpful in providing more information about the physiology and the metabolic pathways of mycotoxin production in *A. alternata* and it could provide a basis for the development of a process to produce AOH in larger amounts.

2. Materials and methods

2.1. Reagents and standards

Standards of alternariol, alternariol monomethyl ether, altenuene and tenuazonic acid were purchased from Sigma-Aldrich (Germany). All reagents, fine-chemicals and solvents were obtained from Roth (Germany), Becton, Dickinson and Company (BA, Germany) or Sigma-Aldrich (Germany).

2.2. Fungi and medium

A. alternata DSM 12633 was obtained from the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). All cultures of *A. alternata* were routinely grown on PDA (39 g/l potato dextrose agar, pH 5.6). Conidia were harvested from plates that were incubated 7 days at 28 °C with 25% glycerol and filtered through Miracloth (Calbiochem). Conidia were counted in a Thoma counting chamber and diluted to a concentration of 1×10^6 conidia per ml. Aliquots were stored at −80 °C. For the experiments modified Czapek-Dox medium was used (modified after Gatenbeck and Hermodsson, 1965): 10 g/l glucose (anhydrous), 0.06 g/l NH_4Cl, 0.25 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.25 g/l NaCl, 0.25 g/l KCl, 0.01 g/l $FeSO_4{\cdot}7H_2O$, 0.01 g/l $ZnSO_4{\cdot}7H_2O$, 1 g/l yeast extract, pH 5.5. Carbon sources and nitrogen sources were prepared separately and added after autoclaving. For the carbon test glucose was replaced by other carbon sources (see Section 2.4) and for the nitrogen test the mixture of ammonium chloride and sodium nitrate was replaced by other nitrogen sources (see Section 2.3). Carbon and nitrogen tests were both prepared in shaken and in static culture. For both culture conditions a volume of 20 ml of the respective medium was used. All cultures were inoculated with 850 conidia per ml medium. Static cultures were performed in standard disposable Petri dishes (Ø 85 mm), and the shaken cultures in 100 ml glass shake-flasks with baffles. For both culture conditions a time course was performed. Static cultures were kept for 5–12 days in the dark at 28 °C. Shaken cultures were cultivated on a rotary shaker at 140 rpm for 5–8 days at 28 °C in the dark. All conditions were performed in triplicate. Each sample was prepared in an individual flask or Petri dish and the entire contents used for analysis. Results were presented for the static cultures after 8 days, and for the shaken cultures after 7 days.

For the fermentation experiments 1.5 l of the basal modified Czapek-Dox medium at pH 5.5 was used. The process was operated in the small-scale bioreactor (vessel volume 2.0 l)

Minifors (Infors, Switzerland) for 14 days at 28 °C in the dark. The medium (1.5 l) was inoculated with 1×10^6 conidia. Stirrer speed was increased from 400 rpm to 900 rpm after 48 h, and aeration rate was 0.013 vvm. For titration 0.2 M sodium hydroxide and 0.2 M phosphoric acid were used.

2.3. Nitrogen sources

For the nitrogen tests the nitrogen sources ammonium chloride and sodium nitrate of the modified Czapek-Dox medium were replaced. In these tests glucose was added as carbon source. The basal medium had a total nitrogen concentration of 56.8 mg/l. To ensure the same conditions for all samples the amount of all tested nitrogen sources was adjusted to a total nitrogen concentration of 56.8 mg/l. Apart from ammonium and nitrate salts several amino acids and urea were investigated: NH_4Cl + $NaNO_3$ (0.06 g/l + 0.25 g/l), NH_4Cl (0.217 g/l), $NaNO_3$ (0.345 g/l), NH_4NO_3 (0.162 g/l), $(NH_4)H_2PO_4$ (0.467 g/l), NH_4CH_3COO (0.313 g/l), KNO_3 (0.411 g/l), glycine (0.305 g/l), serine (0.427 g/l), proline (0.467 g/l), phenylalanine (0.671 g/l), arginine (0.177 g/l), asparagine (0.305 g/l), aspartate (0.54 g/l), glutamate (0.597 g/l) and urea (0.122 g/l). To elucidate the influence of yeast extract in the medium a control sample without additional nitrogen source was performed. For the determination of ammonium and nitrate the photometrical assays “Ammonium-Test” (Spectroquant®, Merck, Germany) and “Nitrat-Test” (Spectroquant®, Merck, Germany) were used.

2.4. Carbon sources

For the carbon tests glucose was replaced by other carbon sources and the mixture of ammonium chloride and sodium nitrate was added as nitrogen source. In all samples the total carbon amount was 4 g/l and the concentration of all carbon sources was adjusted to this. A wide range of different carbon sources was tested: D-glucose (10 g/l), L-arabinose (10 g/l), D-xylose (10 g/l), D-galactose (10 g/l), L-rhamnose (10.11 g/l), D-fructose (10 g/l), D-maltose (10 g/l), D-sucrose (9.5 g/l), lactose monohydrate (10 g/l), starch (10 g/l), cellulose (10 g/l), glycerol (10.22 g/l), D-sorbitol (10.11 g/l) and sodium acetate trihydrate (22.66 g/l). In shaking flask experiments a combination of glucose/sucrose and glucose/sodium acetate was tested additionally. The total carbon concentration remained constant; the ratio of applied carbon was 1:1 (5 g/l glucose+4.25 g/l sucrose) and 25:1 (9.62 g/l glucose + 0.91 g/l sodium acetate). To determine the influence of yeast extract an additional control sample without

carbon source was carried out. The glucose concentration during fermentation was monitored with the photometrical anthrone assay (Pons *et al*., 1981).

2.5. Detection and measurement of *Alternaria* mycotoxins

A 10 ml aliquot of cell-free culture broth was acidified with 10 µl conc. HCl (32%, 10.32 M) and extracted twice with ethyl acetate. At each extraction step the mixture was vigorously vortexed and centrifuged at 4600 g for 5 min. The ethyl acetate supernatants from both extraction steps were combined and evaporated to dryness in a vacuum centrifuge. More than 90% of the mycotoxins could be extracted by this method. The residue was redissolved in 200 µl methanol (HPLC grade) and used for HPLC analyses.

Mycotoxin standard solutions of 0.2 mM (AOH), 0.1 mM (AME) and 10 mM (TA) in methanol were diluted with methanol and used for calibration. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a 25 cm reversed phase column (Luna 5 µm C18(2), Phenomenex, Germany) at 30 °C. Mobile phase solution was methanol/0.1 M NaH_2PO_4 pH 3.2 (2:1) at a flow rate of 0.7 ml/min (according to Shephard *et al*., 1991). Mycotoxins were monitored with a UV detector at 280 nm. Retention times were 5.3±0.1 min for TA, 10.2±0.1 min for AOH and 23.3 ±0.1 min for AME. Detection limits for this method were 16 ng of injected AOH, 33 ng of injected AME and 12 ng of injected TA. To quantify the mycotoxin concentration in the culture broth the peak area in each sample was plotted against the standard curve. In addition to the mycotoxin concentration in the culture broth mycotoxin contents were normalized to biomass and expressed as µg mycotoxin per g dry weight. To determine dry biomass, fungal mycelium was transferred in a weighed tube and dried completely at 60 °C. The weight was determined on a standard balance.

3. Results

3.1. Nitrogen limitation leads to AOH and AME production

Several media were tested for the production of mycotoxins. The production of mycotoxins was significantly higher in all tested complex or undefined media (e.g. autoclaved rice, rice-flour liquid medium according to Misra and Sinha, 1979) than in the tested defined media (e.g. Czapek-Dox medium and Vogel's medium) (results not shown). However, for elucidating the effects of carbon or nitrogen sources, a more defined medium is necessary so that medium components can be exchanged and to exclude, as far as possible, combinatorial effects with undefined medium components. The semi-synthetic modified Czapek-Dox medium combines good fungal growth with acceptable mycotoxin yields. To produce AOH in larger amounts a bioprocess was developed (detailed results for process development will be published elsewhere). A typical process scheme is shown in Fig. 1.

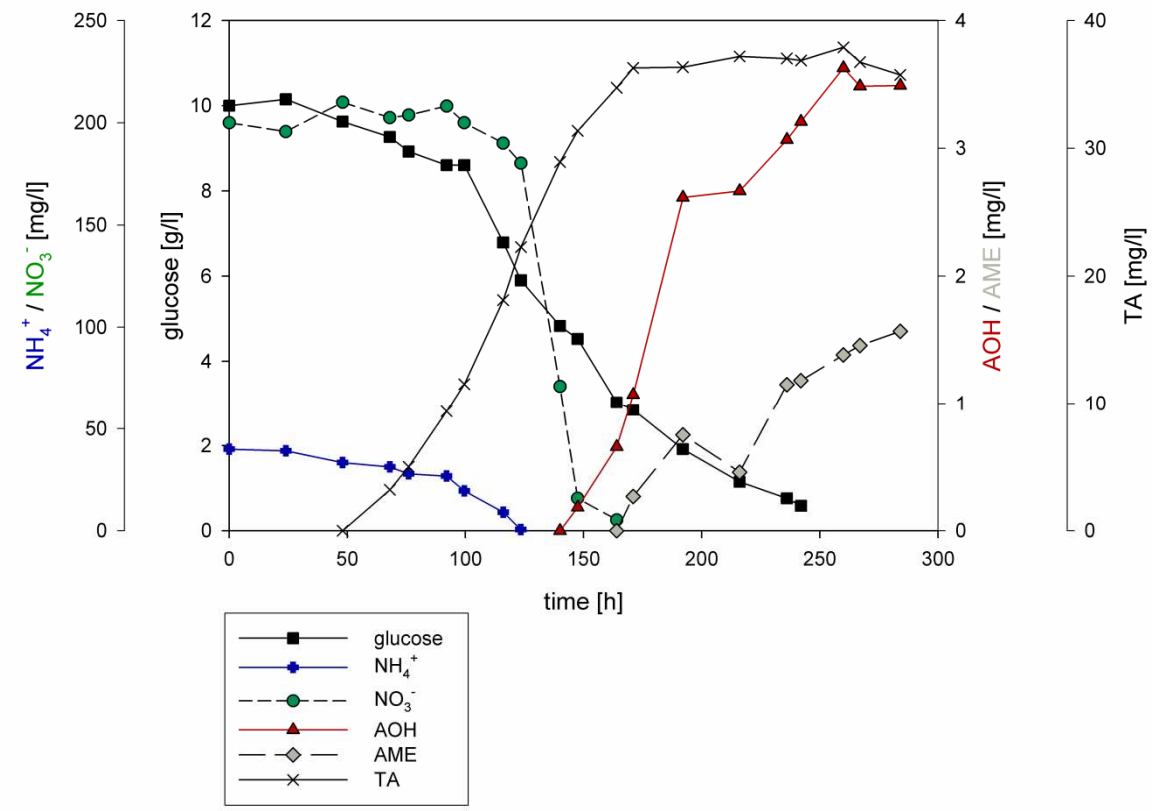

Fig. 1. Fermentation of *A. alternata* in modified Czapek-Dox medium (glucose 10 g/l, NH_4Cl 0.06 g/l, $NaNO_3$ 0.25 g/l, KH_2PO_4 1 g/l, NaCl 0.25 g/l, KCl 0.25 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, yeast extract 1 g/l), pH 5.5 at an aeration rate of 0.013 vvm and an agitation rate from 400 rpm to 900 rpm after 48 h.

Glucose was consumed at a constant linear rate during the whole process with half of the amount (5 g/l) being depleted after 140 h. Nitrogen consumption followed a different pattern. After total exhaustion of ammonia, depletion of nitrate took place. AOH was first detected after 150 h. At this point only traces of nitrate were still measurable. AME production started after 170 h. TA production began almost immediately at 50 h during the first fungal growth phase. The typical mycotoxin yields in this process were 3.5–3.6 mg/l AOH, 1.4– 1.5 mg/l AME and ~37 mg/l TA. To elucidate whether the AOH production depended on a nitrogen limitation in general or on the applied nitrogen source, several experiments were conducted in shaken flasks and static culture.

3.2. Impact of nitrogen sources on toxin production in shaken and static culture

3.2.1. Static culture

The cultivation of *A. alternata* was performed in Petri dishes with media supplemented with different nitrogen sources. Growth was observed in all media, but the appearance of mycelium was influenced markedly depending on the supplemented nitrogen source (see Fig. A, supplementary data). Differences in shape, color, texture and tightness were observed. Dry biomass after 8 days of growth was more or less the same except in samples grown with ammonium chloride or ammonium dihydrogen phosphate where dry biomass was lower. Table 1 shows the mycotoxin levels at day 8 expressed in mg per liter culture broth and normalized to biomass as mg per g dry weight (data not shown for days 5–7 and 9–12). TA was detected without significant differences in concentration in all samples irrespective of the nitrogen source, whereas the production of the polyketide mycotoxins AOH and AME was strongly influenced by the nitrogen source. Media containing ammonium chloride, sodium nitrate, ammonium dihydrogen phosphate, potassium chloride or arginine seemed to inhibit the production of these mycotoxins. In contrast media containing ammonium nitrate, aspartate or phenylalanine elicited a high mycotoxin yield up to 27 mg/l for phenylalanine, an enhancement of 300 fold compared to the basal medium. When inorganic salts were used as the nitrogen source a combination of ammonium and nitrate was adequate for reasonable mycotoxin production. The application of either single ammonium or nitrate salts seemed to inhibit the production of polyketide mycotoxins. Furthermore the ratio of both salts appeared to be important. The ratio of the basal medium was 1:2.6 (NH_4^+ : NO_3^-) whereas the ratio in ammonium nitrate is 1:1. The use of ammonium nitrate increased AOH production ~40 fold. The levels produced with the majority of tested amino acids were higher than the initial

medium (between ~2 fold for proline and ~20 fold for glutamate) whereas quite high levels could be obtained with aspartate (~40 fold) and phenylalanine in particular (~300 fold).

Table 1
Mycotoxin production after 8 days by *Alternaria alternata* cultured statically in modified Czapek-Dox medium supplemented with different N sources

N source[a]	AOH		AME		TA	
	[mg/l]	[mg/g]	[mg/l]	[mg/g]	[mg/l]	[mg/g]
None added	0.01±0.02	0.002±0.003	ND	ND	17.07±0.87	3.31±0.17
NH_4Cl and $NaNO_3$	0.09±0.03	0.02±0.01	ND	ND	25.58±1.39	5.68±0.31
NH_4Cl	ND	ND	ND	ND	22.79±4.68	4.70±0.97
$NaNO_3$	ND	ND	ND	ND	21.94±0.36	3.43±0.06
NH_4NO_3	3.73±0.86	0.62±0.14	1.75±0.26	0.29±0.04	29.95±0.57	4.99±0.09
$(NH_4)H_2PO_4$	ND	ND	ND	ND	25.07±1.60	5.22±0.33
NH_4Acetate	0.81±0.65	0.13±0.11	0.22±0.16	0.04±0.03	24.71±1.04	4.08±0.17
KNO_3	ND	ND	ND	ND	20.16±0.45	3.36±0.47
Urea	0.62±0.28	0.10±0.05	0.23±0.10	0.04±0.02	25.45±1.70	4.17±0.28
Gly	0.54±0.28	0.25±0.13	0.17±0.14	0.08±0.07	21.18±1.13	4.15±0.22
Ser	0.36±0.16	0.06±0.03	0.17±0.07	0.03±0.01	25.79±1.05	4.41±0.18
Pro	0.21±0.01	0.04±0.002	0.12±0.006	0.02±0.001	23.27±0.72	3.85±0.12
Phe	27.54±2.48	4.44±0.40	15.16±2.13	2.45±0.34	37.95±1.36	6.12±0.22
Arg	ND	ND	ND	ND	23.19±0.18	3.74±0.03
Asn	0.55±0.17	0.07±0.02	0.32±0.09	0.04±0.01	27.11±0.25	3.39±0.03
Asp	3.96±0.98	0.81±0.20	1.77±1.06	0.36±0.22	30.83±0.11	6.29±0.02
Glu	1.76±0.49	0.30±0.08	0.75±0.23	0.13±0.04	30.90±1.01	5.19±0.17

Results are mean of three replicates ± standard deviation and given in mg mycotoxin per litre culture broth (mg/l) and mg mycotoxin related to g biodrymass (mg/g).
[a] N source added to modified Czapek Dox medium without nitrogen source (1 g/l KH_2PO_4, 0.25 g/l NaCl, 0.25 g/l KCl, 0.5 g/l $MgSO_4$ * 7 H_2O, 0.01 g/l $FeSO_4$ * 7 H_2O, 0.01 g/l $ZnSO_4$ * 7 H_2O, 1 g/l yeast extract) supplemented with 10 g/l glucose, inital pH 5.5.
ND: not detected.

3.2.2. Shaken culture

In shaken culture the morphology of *A. alternata* was not dependent on the supplemented nitrogen source. The black mycelium adhered to the wall above the liquid level and grew also in pellet form in the medium. Highest production yields were obtained after 7 days (data not shown for days 3–6 and 8). Table 2 shows the results of the shaken flask experiments and displays only nitrogen sources which resulted in AOH/AME production.

Table 2

Mycotoxin production after 7 days by *Alternaria alternata* cultured in shaking flasks in modified Czapek-Dox medium supplemented with different N sources

N source[a]	AOH		AME		TA	
	[mg/l]	[mg/g]	[mg/l]	[mg/g]	[mg/l]	[mg/g]
None added	0.33±0.08	0.08±0.02	0.15±0.01	0.04±0.002	5.56±1.04	1.37±0.22
NH_4Cl and $NaNO_3$	1.31±0.42	0.25±0.08	0.55±0.34	0.11±0.06	13.00±0.89	2.49±0.13
NH_4NO_3	3.65±0.12	0.73±0.01	1.24±0.33	0.25±0.08	19.75±0.28	3.93±024
KNO_3	1.01±0.20	0.19±0.03	0.60±0.09	0.12±0.02	9.53±0.006	1.82±0.06
Phe	2.52±1.66	0.45±0.31	1.58±1.30	0.28±0.24	23.09±0.27	4.10±0.18
Arg	0.71±0.29	0.13±0.05	0.44±0.14	0.08±0.03	10.85±2.32	2.02±0.56
Asn	0.77±0.19	0.15±0.04	0.34±0.16	0.07±0.03	17.05±2.20	3.34±0.60
Asp	4.00±2.60	0.95±0.63	0.64±0.10	0.15±0.03	23.66±3.25	5.59±0.69
Glu	3.49±0.37	0.75±0.09	1.24±0.31	0.26±0.06	19.05±2.28	4.07±0.44

Results are mean of three replicates ± standard deviation and given in mg mycotoxin per liter culture broth (mg/l) and mg mycotoxin related to g biodrymass (mg/g).
[a] N source to modified Czapek Dox medium without nitrogen source (1 g/l KH_2PO4, 0.25 g/l NaCl, 0.25 g/l KCl, 0.5 g/l $MgSO_4$ * 7 H_2O, 0.01 g/l $FeSO_4$ * 7 H_2O, 0.01 g/l $ZnSO_4$ * 7 H_2O, 1 g/l yeast extract) supplemented with 10 g/l glucose, intial pH 5.5.

AOH/AME production was not observed in media supplemented with ammonium chloride, sodium nitrate, ammonium dihydrogen phosphate, ammonium acetate, urea, glycine, serine or proline. In response to the altered cultivation method (shaken vs static) AOH and AME production was different for some N-sources. Whereas mycotoxin production was obtained with ammonium nitrate and aspartate with nearly the same yields in shaken as in static culture, production with phenylalanine was strongly reduced. Compared with static culture, mycotoxin levels increased in the basal medium with the combination of ammonium chloride and sodium nitrate, but were still lower compared to a controlled fermentation in a bioreactor.

Nevertheless the production levels were increased around 3 fold by using ammonium nitrate or aspartate compared to the basal medium which could provide a good basis for the process optimisation.

3.3. Impact of the carbon source on toxin production in shaken and static culture

3.3.1. Static culture

Biomass and morphology were strongly influenced by carbon source (see also Fig. B, supplementary data). Media which contained starch, cellulose, glycerol, sodium acetate or no carbon source resulted in reduced growth with low biomass. AOH was detected in media containing glucose, xylose, fructose, rhamnose, sorbitol, maltose, sucrose, starch or sodium acetate, whereas AME was detected only after cultivation with fructose, sorbitol, rhamnose and sucrose. TA was produced in alternating concentrations in nearly all media excluding those that contained no carbon source, sodium acetate or cellulose. Cultivation with most of the carbon sources did not enhance AOH production significantly compared to glucose, rhamnose and sodium acetate being the exception. Cultivation with rhamnose increased both AOH and AME production to ~1.7 mg/l of AOH (24 fold enhancement) and ~1.6 mg/l of AME (AME was not detected in medium with glucose) whereas cultivation with sodium acetate only raised AOH production to ~10.5 mg/l (150 fold enhancement). Highest yields of TA were achieved with maltose and sucrose which resulted in 26.8 mg/l and 24.6 mg/l TA, respectively (Table 3).

Table 3
Mycotoxin production after 8 days by *Alternaria alternata* cultured statically in modified Czapek-Dox medium supplemented with different C sources

C source[a]	AOH		AME		TA	
	[mg/l]	[mg/g]	[mg/l]	[mg/g]	[mg/l]	[mg/g]
None added	ND	ND	ND	ND	ND	ND
Glucose	0.07±0.02	0.01±0.003	ND	ND	20.92±1.37	3.48±0.23
Arabinose	ND	ND	ND	ND	9.96±0.29	1.99±0.06
Xylose	0.09±0.08	0.013±0.01	ND	ND	15.60±1.25	2.40±0.20
Fructose	0.07±0.08	0.01±0.01	0.09±0.08	0.01±0.01	16.09±5.66	2.68±0.94
Galactose	ND	ND	ND	ND	16.56±0.70	3.01±0.13
Rhamnose	1.69±0.06	0.31±0.01	1.60±0.09	0.29±0.02	5.41±0.67	0.98±0.12
Sorbitol	0.06±0.05	0.009±0.008	0.09±0.01	0.01±0.002	15.20±1.55	2.34±0.24
Maltose	0.12±0.02	0.02±0.003	ND	ND	26.78±0.78	4.46±0.13
Sucrose	0.12±0.11	0.02±0.02	0.06±0.05	0.01±0.009	24.63±2.18	4.48±0.40
Lactose	ND	ND	ND	ND	10.85±0.94	2.41±0.21
Starch	0.13±0.07	/*	ND	/*	1.22±0.87	/*
Cellulose	ND	/*	ND	/*	ND	/*
NaOAc	10.52±0.96	3.51±0.32	ND	ND	ND	ND
Glycerol	ND	ND	ND	ND	8.81±0.37	3.52±0.15

Results are mean of three replicates ± standard deviation and given in mg mycotoxin per liter culture broth (mg/l) and mg mycotoxin related to g biodrymass (mg/g).
[a] C source added to modified Czapek Dox medium without carbon source (0.06 g/l NH_4Cl, 0.25 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.25 g/l NaCl, 0.25 g/l KCl, 0.5 g/l $MgSO_4$ * 7 H_2O, 0.01 g/l $FeSO_4$ * 7 H_2O, 0.01 g/l $ZnSO_4$ * 7 H_2O, 1 g/l yeast extract), pH 5.5.
/* biodrymass not determined.
ND: not detected.

3.3.2. Shaken culture

All carbon sources tested in static culturewere also tested in shaken culture. Additionally two combinations of carbon sources (glucose/sucrose and glucose/acetate)were examined. AOH and AME production was not detected in media with most of the tested carbon sources. AOH was produced only in the presence of glucose, fructose, sucrose, sodium acetate and with both carbon source combinations. In Table 4 all carbon sources are listed which induced AOH production.

Table 4
Mycotoxin production after 7 days by *Alternaria alternata* cultured in shaking flasks in modified Czapek-Dox medium supplemented with different C sources

C source[a]	AOH		AME		TA	
	[mg/l]	[mg/g]	[mg/l]	[mg/g]	[mg/l]	[mg/g]
None added	ND	ND	ND	ND	ND	ND
Glucose	0.60±0.17	0.11±0.03	ND	ND	14.83±1.8	2.65±0.35
Fructose	0.15±0.07	0.03±0.01	ND	ND	18.63±0.1	3.45±0.03
Sucrose	0.18±0.99	0.03±0.02	ND	ND	23.31±1.69	4.45±0.22
NaOAc	4.66±2.24	1.16±0.45	ND	ND	ND	ND
Gluc.+sucr.	0.47±0.21	0.09±0.04	ND	ND	25.69±3.95	4.66±0.71
Gluc.+ac.	0.16±0.08	0.03±0.02	ND	ND	3.74±1.49	0.69±0.27

Results are mean of three replicates ± standard deviation and given in mg mycotoxin per liter culture broth (mg/l) and mg mycotoxin related to g biodrymass (mg/g).
[a] C source added to modified Czapek Dox medium without carbon source (0.06 g/l NH_4Cl, 0.25 g/l $NaNO_3$, 1 g/l KH_2PO4, 0.25 g/l NaCl, 0.25 g/l KCl, 0.5 g/l $MgSO_4$ * 7 H_2O, 0.01 g/l $FeSO_4$ * 7 H_2O, 0.01 g/l $ZnSO_4$ * 7 H_2O, 1 g/l yeast extract), initial pH 5.5.
ND: not detected; Gluc.+sucr.: glucose + sucrose (1:1); Gluc.+ac.: glucose + sodium acetate (10:1).

Highest mycotoxin concentrations were achieved after 7 days (data not shown for days 3–6 and 8) and were similar or slightly higher than the static culture with the exception of sodium acetate which was half that of the static culture. For both of the C-source combinations AOH production did not increase compared with the levels obtained with the respective C-sources alone. Nevertheless sodium acetate enhanced AOH production around 7 fold and seemed to inhibit the production of the other mycotoxins. This may be useful for the purification of AOH and is a good basis for process optimization.

4. Discussion

In this study a semi-synthetic medium was used to determine the influence of carbon and nitrogen sources on the production of mycotoxins in *A. alternata*. This semi-synthetic medium was chosen to exclude, as far as possible, the effects of abundant nutrients and growth factors and to enable the exchange of carbon and nitrogen sources. Yeast extract (1 g/l) present in the modified Czapek-Dox medium provided the required of vitamins and micronutrients. According to the manufacturer (Becton, Dickinson and Company) the yeast extract that was used for these experiments contained 11% (w/w) nitrogen. To determine the influence of yeast extract, controls without an additional nitrogen or carbon source were made. Although *A. alternata* grew fairly well with yeast extract as sole nitrogen source, the production of the mycotoxins was very low. Without an additional carbon source growth with just yeast extract was reduced and mycotoxins were not detected. Therefore it can be concluded that the observed effects regarding mycotoxin formation can be assigned to the supplemented nitrogen and carbon sources.

Considering all data from this study it can be assumed that mycotoxin production is regulated not only by nutritional factors but also by cultivation conditions. Static culture appeared to be supportive for mycotoxin production since higher concentrations of mycotoxins were detected with many more of the tested carbon and nitrogen sources in static culture than in shaken culture. In general the production of the polyketide mycotoxins seemed to be influenced by both carbon and nitrogen sources whereas TA production was more influenced by carbon sources. The production of TA was apparently connected to fungal growth since it started very early in the exponential growth phase.

Most nitrogen sources elicited different results in mycotoxin production in static and shaken culture. In the basal medium with sodium nitrate and ammonium chloride, very low yields of AOH (0.09 mg/l) were obtained in static culture. In shaken culture the yield was ~15 fold higher and during fermentation in the bioreactor a ~38 fold enhancement could be achieved compared to static culture. For phenylalanine this tendency was inverted: 27 mg/l were produced in static culture whereas the yields in shaken culture were 11 fold lower. In contrast to this, the varying culture conditions seemed not to influence mycotoxin production in media containing either ammonium nitrate or aspartate. One reason for these observations could be the formation of gradients. In shaken culture and in the bioreactor all substances are well mixed and gradients of nutrients should not form, whereas in static culture nutrients must diffuse and localized nutrient limitation may occur. Kohut *et al*. (2009) showed that nitrogen

starvation and other stresses triggered the expression of the fumonisin biosynthetic genes and therefore the production of the mycotoxin fumonisin. For *Alternaria alternata* some studies exist which investigated the influences of abiotic factors, e.g. temperature and water activity (Hasan, 1995; Tournas and Stack, 2001), but only one study examined the impact of nitrogen on mycotoxin production. Orvehed *et al*. (1988) tested the effect of sodium nitrate, glutamate and urea on AOH and AME production. They found that the production dramatically decreased when 6 or 12mM of these nitrogen sources were added to a modified Czapek-Dox medium 72 h after inoculation. Nitrate repression of mycotoxin production was shown for aflatoxin intermediates in *Aspergillus parasiticus* (Kacholz and Demain, 1983) or for ochratoxin in *Aspergillus ochraceus* (Abbas et al., 2009). In our study AOH and AME could not be detected in media containing sodium or potassium nitrate in static culture. Also in shaken culture AOH and AME were not produced in medium with sodium nitrate and in the bioreactor AOH production only started after nitrate consumption. So repression due to nitrate can be hypothesized. In contrast to the study of Orvehed *et al*. (1988) AOH was produced in the presence of glutamate and urea. In the study of Orvehed *et al*. (1988), however, the nitrogen sources were added during the exponential growth phase and not at the beginning. Furthermore much more nitrogen in total (up to 12 mM) was used in the Orvehed study so the inhibitory effects observed could simply be caused by the high amounts of nitrogen in the medium.

With respect to the carbon sources different monosaccharides, disaccharides, complex saccharides and smaller carbon sources were tested methodically. However, a general scheme for the regulation of *Alternaria* toxins was not observed. As with the nitrogen source experiments, mycotoxin yields varied considerably in shaken and static culture and in shaken culture only four of all tested carbon sources induced the production of AOH. Nevertheless highest amounts of AOH were obtained with sodium acetate in static culture (10.5 mg/l) as well as in shaken culture (4.6 mg/l). Interestingly TA was totally repressed using sodium acetate as carbon source. There are several studies that have investigated the influence of carbon sources on mycotoxin production fungi other than *A. alternata*. They show that the best carbon source has to be determined for each mycotoxin and is dependent on the strain used and the medium which is supplemented. Ochratoxin production in *A. ochraceus* HP was supported by lactose (Abbas *et al*., 2009) but in *A. ochraceus* (Aso 2) by a mixture of sucrose and glucose (Medina *et al*., 2008). Aflatoxin production in *A. parasiticus* NRRL 2999 was enhanced by glucose, fructose, sucrose or sorbitol (Buchanan and Stahl, 1984) whereas sucrose, kestose or nystose increased trichothecene production in different *Fusarium*

graminearum isolates (Jiao *et al*., 2008). The production of polyketide mycotoxins seems to be under complex regulation since sodium acetate, for example, enhanced AOH production but greatly repressed AME production. Due to the lack of knowledge about the regulation of the AOH production it is difficult to speculate why acetate appeared to be the best C-source. Although the same amount of carbon was available in each experiment, acetate is more easily oxidised than glucose and other monosaccharides tested. Less energy can be provided because less NADH is produced due to direct insertion into the citrate cycle. Besides the lack of energy, differences in redox status because of lower NADH levels or a direct stimulation of the first biosynthetic genes of AOH production by acetate may be possible reasons for this observation.

Considering the results presented here, first insights into physiological control mechanisms were obtained and a good basis was provided for process optimization. The differences between static and shaken culture show the importance of a controlled and homogenous system in providing information on the influence of nitrogen or carbon sources on mycotoxin production. Analogous experiments in a bioreactor are currently in progress in our laboratory. Controlled cultivation in a bioreactor enables further investigation of the influence on mycotoxin production of important parameters such as temperature and strain which could not be considered in this study.

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Supplemental material

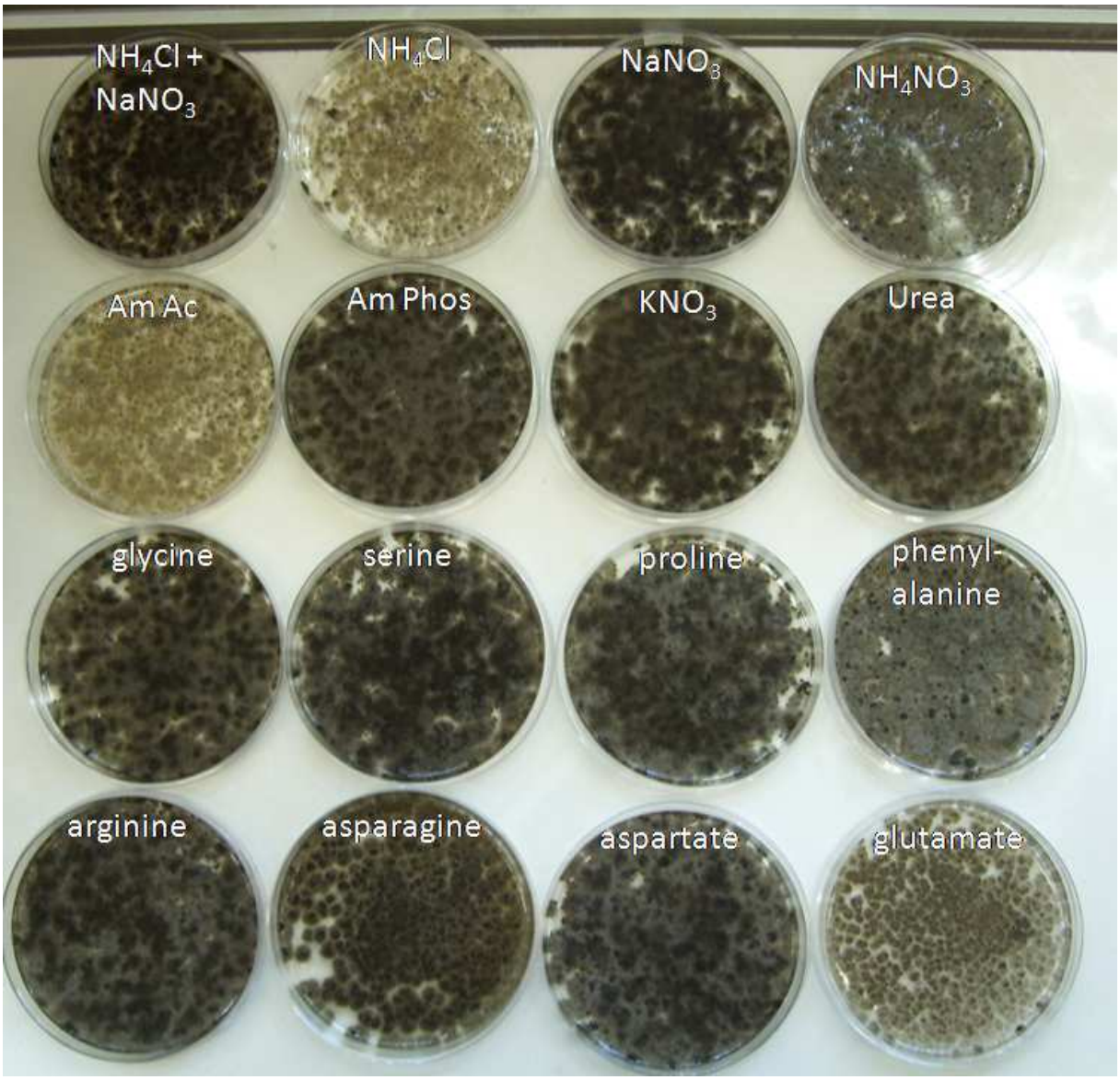

Fig. A Effect of nitrogen sources on growth of *A. alternata* in static culture after 8 days. Modified Czapek-Dox medium without additional nitrogen source (glucose 10 g/l, KH_2PO_4 1 g/l, NaCl 0.25 g/l, KCl 0.25 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, yeast extract 1 g/l) was supplemented with the indicated nitrogen source. All samples were inoculated with 1.7×10^4 conidia.

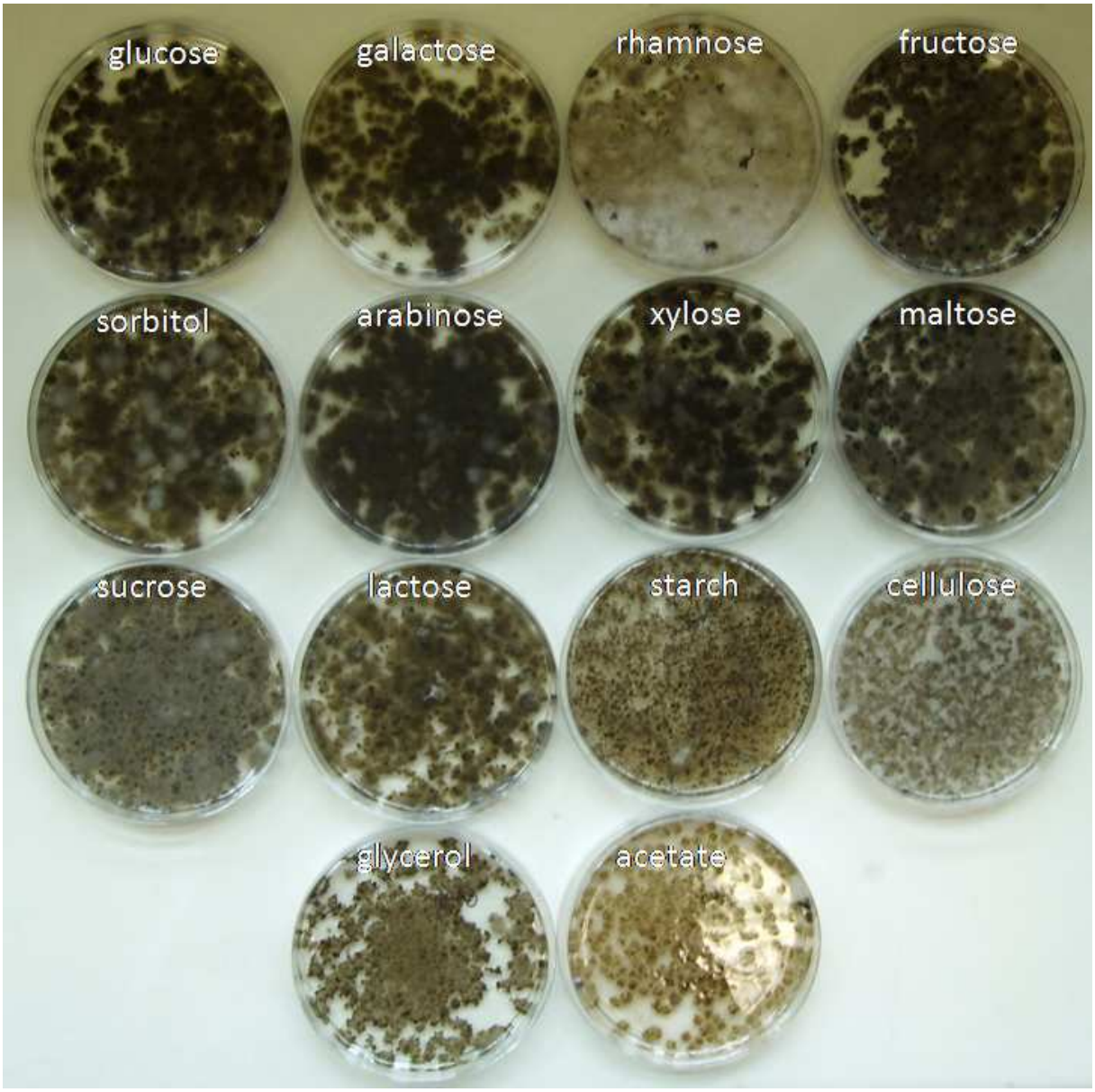

Fig. B Effect of carbon sources on growth of *A. alternata* in static culture after 8 days. Modified Czapek-Dox medium without additional carbon source (NH_4Cl 0.06 g/l, $NaNO_3$ 0.25 g/l, KH_2PO_4 1 g/l, NaCl 0.25 g/l, KCl 0.25 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, yeast extract 1 g/l) was supplemented with the indicated carbon source. All samples were inoculated with 1.7×10^4 conidia.

4.2 Mycotoxin production in a bioreactor

Process development for the elucidation of mycotoxin formation in *Alternaria alternata*

Submitted to

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Abstract

The black mould *Alternaria alternata* produces a wide diversity of mycotoxins which are of particular health concern. Since no maximum allowable limits are set for *Alternaria* toxins in food and feed, prevention of *Alternaria* infestations and mycotoxin spoilage is the only way to avoid health risks. Thus, the understanding of mycotoxin biosynthesis is essential. For that purpose, a reliable batch process in a 2 L bioreactor was established which enables the study of several parameters influencing the production of the mycotoxins alternariol (AOH), alternariol monomethylether (AME) and tenuazonic acid (TA) by *A. alternata* DSM 12633. Modified Czapek-Dox medium was used with glucose as carbon source and ammonium and nitrate as nitrogen sources. Consumption of carbon and nitrogen sources as well as formation of the three mycotoxins were monitored; the average data of five independent fermentations was plotted and fitted using a logistic equation with four parameters. Maximum mycotoxin concentrations of 3.49 ± 0.12 mg/L AOH, 1.62 ± 0.14 mg/L AME and 38.28 ± 0.1 mg/L TA were obtained.

In this system the effect of different aeration rates (0.53 vvm - 0.013 vvm) was tested which exerted a great influence on mycotoxin production. The use of the semi-synthetic Czapek-Dox medium allowed the exchange of carbon and nitrogen sources for acetate and aspartic acid. The use of acetate instead of glucose resulted in the sole production of alternariol whereas the exchange of ammonium and nitrate for aspartate enhanced the production of both AOH and AME while TA production was not affected.

Keywords:

Alternaria alternata, Mycotoxin, Batch process, Aeration rate.

1. Introduction

Mycotoxins are secondary metabolites of low molecular weight produced by filamentous fungi. Since the discovery of the first mycotoxins, the aflatoxins, in 1960 which caused the death of 10,000 turkeys many new mycotoxins have been identified in the last 50 years. Today 300 to 400 compounds are designated as mycotoxins (Bennett and Klich, 2003). As other secondary metabolites mycotoxins are formed subsequently to the growth phase and are not necessary for growth or development (Fox and Howlett, 2008). Mycotoxin formation is subjected to a complex regulation, but it is often induced by nutrient limitation (Demain, 1986). Mycotoxins are released by the fungus in the surrounding substrate and contamination of agricultural products is therefore possible. They are connected to certain health disorders and elicit acute toxic, mutagenic, teratogenic, carcinogenic and sometimes estrogenic properties (Bhatnager *et al.*, 2002). Based on estimations of the Food and Agriculture Organization (FAO) of the United Nations approximately 25 % of the world´s food crops are affected by mycotoxin producing fungi and global losses of foodstuffs due to mycotoxins are in the range of 1000 million tons per year (http://www.fao.org/ag/agn/agns/chemicals_mycotoxins_en.asp).

Alternaria species are wide spread black moulds which belong to the division of Deuteromycota (Bottalico and Logrieco, 1998) and are common saprophytes found on decaying organic material world-wide. The genus *Alternaria* includes also opportunistic plant-pathogens affecting many cultivated plants in the fields and stored fruits and vegetables during post-harvest (Guo *et al.*, 2004). *Alternaria* species are capable to produce a wide diversity of secondary metabolites belonging to different chemical groups including dibenzopyrones, tetramic acids, lactones, quinones and cyclic peptides. More than 120 secondary metabolites of *Alternaria* species are known; a quarter of that are designated as mycotoxins (Panigrahi, 1997). Five major *Alternaria* toxins can be found as natural contaminants in foodstuffs: the benzopyrene derivatives alternariol (AOH), alternariol monomethylether (AME), altenuene (ALT), the tetramic acid tenuazonic acid (TA) and the perylene derivative altertoxin I (ATX I) (Barkai-Golan, 2008). These toxins were detected in apples (Stinson *et al.*, 1981), tomatoes (Stinson *et al.*, 1981), wheat (Azcarate *et al.*, 2008; Li and Yoshizawa, 2000), olives (Visconti *et al.*, 1986), sunflower seeds (Pozzi, 2005), fruit juices (Lau *et al.*, 2003) and tomato products (Motta and Valente Soares, 2001; Terminiello *et al.*, 2006). Therefore, *Alternaria* toxins can be considered as toxic contaminant of our everyday food (Barkai-Golan, 2008).

Although the acute toxicity of *Alternaria* toxins is low (LD_{50} of *Alternaria* extracts: 300 mg/kg body weight in mice, LD_{50} of AOH: > 400 mg/kg body weight of mice (Pero *et al.*, 1973)), they are connected to certain health disorders. *Alternaria* extracts have been described as mutagenic and tumorigenic (Liu *et al.*, 1991; Schrader *et al.*, 2001). Of particular health concern is the incidence of esophageal cancer in Linxin, China. The etiology of this cancer was connected with the contamination of cereal grains with *A. alternata* (Dong *et al.*, 1987; Liu *et al.*, 1992). As stated by Pero *et al.* (1973), the toxicity of complex extracts is much higher than of the single tested mycotoxins which suggest synergism between single components. Although numerous toxicological studies were conducted to clarify the effects of *Alternaria* toxins a risk assessment for *Alternaria* toxins is not possible. According to the German Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung, BfR, 2003) only little toxicological data is available just for seven out of the 30 known *Alternaria* mycotoxins which is insufficient for an assessment of the health risk for the consumer.

As long as maximum allowable limits in food for *Alternaria* toxins were not defined prevention of *Alternaria* infestations and mycotoxin spoilage is the best way to avoid health risks. Therefore, knowledge about factors which enhance or inhibit mycotoxin production and its regulation is crucial. Mycotoxin production varies with fungal strain, the substrate and environmental growth conditions. This includes factors like water activity, temperature, pH-value and light. According to Schmidt-Heydt *et al.* (2008) mycotoxin production can be regarded as an adaptation to imposed abiotic or other stresses of the mycotoxigenic species. Whereas the influence of water activity, temperature and light was extensively studied for different *A. alternata* strains and media (Hasan, 1995; Magan *et al.*, 1984; Pose *et al.*, 2010; Schmidt-Heydt *et al.*, 2011; Söderhäll *et al.*, 1978), the effects of pH and nutritional factors were neglected. Additionally, all these studies use different kinds of media and culture conditions, e.g. solid agar-media, liquid surface culture or drop cultures, which render direct comparisons difficult. The intention of this work is therefore the establishment of a reproducible system which enables the elucidation of all important influences on mycotoxin production in *A. alternata*. For optimal reproducibility and comparability of single experiments a process in a bioreactor was developed allowing direct monitoring and prevention of nutrient or oxygen limitations. With respect to the production of mycotoxins with *Alternaria* spp. submerged fermentation protocols were not developed yet. To the knowledge of the authors only one protocol for submerged fermentation of *Alternaria* spp. for the production of the new antibiotic altersetin (Hellwig *et al.* 2002) and another solid state fermentation protocol for the production of mycoherbicidal agents with *A. alternata* (Singh *et*

al., 2010) were published. The primary purpose of the presented process is to enhance the knowledge of regulatory mechanisms for *Alternaria* toxin production but additionally it may be helpful for the development of further production processes of other interesting secondary metabolites of *Alternaria* spp.

2. Materials and methods

2.1 Strain, media and processsing

A. alternata DSM 12633 was obtained from DSMZ culture collection ("Deutsche Sammlung von Mikroorganismen und Zellkulturen", Braunschweig, Germany). All cultures of *A. alternata* were routinely grown on PDA (Roth, Germany). Conidia were harvested from plates that were incubated seven days at 28°C with 25 % glycerol and filtered through Miracloth (Calbiochem). Conidia were counted in a Thoma counting chamber and diluted to a concentration of $1*10^6$ condia per ml. Aliquots were stored at -80°C.

For the fermentation experiments 1.5 L of modified Czapek-Dox medium (modified after Gatenbeck and Hermodsson, 1965) at pH 5.5 were used: 10 g/L glucose, 0.06 g/L NH_4Cl, 0.25 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ * 7 H_2O, 0.25 g/L NaCl, 0.25 g/L KCl, 0.01 g/L $FeSO_4$ * 7 H_2O, 0.01 g/L $ZnSO_4$ * 7 H2O, 1 g/L yeast extract. For all experiments the same amounts of carbon (4 g/L) and nitrogen (56.8 mg/L) were used: Glucose was exchanged for 22.66 g/L sodium acetate trihydrate and the mixture of ammonium chloride and sodium nitrate was exchanged for 0.54 g/L aspartic acid. The carbon sources were prepared separately and were added after autoclaving. The process was operated in the small-scale bioreactor (vessel volume 2.0 L) Minifors (Infors, Bottmingen, Switzerland) for the indicated time period (table 2) at 28°C in the dark. The medium was inoculated directly with $1*10^6$ conidia, a pre-culture was not used. The bioreactor was equipped with two 6-blade Rushton Turbines; stirring speed was enhanced from 400 rpm to 900 rpm after 48 h. The aeration rate was 0.013 vvm if not indicated otherwise. For pH adjustment 0.2 M sodium hydroxide and 0.2 M phosphoric acid were used.

2.2 Analytical methods

2.2.1 Data analysis

Nutrient consumption and mycotoxin production were fitted using a logistic equation with four parameters in a scientific data analysis and graphing software (Sigma Plot 9.0, Systat, San Jose, USA). The used equation was:

$$y(x) = y_0 + \frac{a}{1+(\frac{x}{x_0})^b} \qquad (1)$$

The four parameters are the following: y_0 indicates the minimum concentration of the respective nutrient or mycotoxin; a indicates the maximum nutrient/mycotoxin concentration; x_0 indicates the process time when half of the nutrient amount is consumed or half of the maximum mycotoxin concentration is produced; b is a shape parameter and difficult to explain biologically (Erkmen and Alben, 2002). Ammonium and AME concentration lapse were derived analogously with a logistic equation with three parameters (cf. Eq. 1, but excluding y_0). Derivation of the fitting was used for the determination of absolute consumption and production rates.

2.2.2 Detection of mycotoxins

Alternariol (AOH), alternariol monomethylether (AME) and tenuazonic acid (TA) were analyzed simultaneously by HPLC. The standard HPLC device (Agilent 1100 Series, Agilent, Waldbronn, Germany) was equipped with a 25 cm reversed phase column (Luna 5 µm C18(2), Phenomenex, Aschaffenburg, Germany). Analyses were performed at 30 °C and a flow rate of 0.7 ml/min. Mobile phase solution was methanol/0.1 M NaH_2PO_4 (2:1), pH 3.2 (according to Shephard *et al.*, 1991). Mycotoxins were monitored with a UV detector at 280 nm. For quantification a standard curve with mycotoxin standard solutions was prepared. The standards were purchased from Sigma-Aldrich (Munich, Germany) and solved in methanol.

Mycotoxins were extracted twice with equal amounts of ethyl acetate from 5 ml culture broth after acidifying with 5 µl conc. HCl. The supernatants were combined and evaporated to dryness in a vacuum centrifuge. The residue was dissolved in methanol and used for HPLC analyzes. Retention times were 5.3 ± 0.1 min (TA), 10.2 ± 0.2 min (AOH) and 23.3 ± 0.1 min (AME).

2.2.3 Quantification of nutritional components and biomass

The glucose concentration during the fermentation process was monitored with the photometrical anthrone assay (Pons *et al.*, 1981).

Ammonium and nitrate were determined with the photometrical assays “Ammonium-Test” (Spectroquant®, Merck, Germany) and “Nitrat-Test” (Spectroquant®, Merck, Darmstadt, Germany).

For biomass quantification fungal mycelium was transferred from the bioreactor at the end of fermentation to a weighed tube and dried completely at 60°C. The weight was determined on a standard balance.

3. Results

3.1 Process parameters of *A. alternata* fermentation in a 2 L bioreactor system

A first approach for the biotechnological production of *Alternaria* toxins was published previously by Brzonkalik *et al.* (2011). To elucidate the reproducibility of the system five independent fermentations were performed. The following results represent the average data of all five fermentations (figure 1). Consumption of the nutrients glucose, ammonium and nitrate showed characteristic logistic decrease and were fitted according to Eq. 1. The root squares for the consumption curve fittings were ≥0.98. Formation of the mycotoxins could be described logistically and were also fitted according to Eq. 1. The root square for the formation curve fittings of TA and AOH were ≥0.99 and of AME ≥0.97. The maintained parameters for the fittings are displayed in table 1.

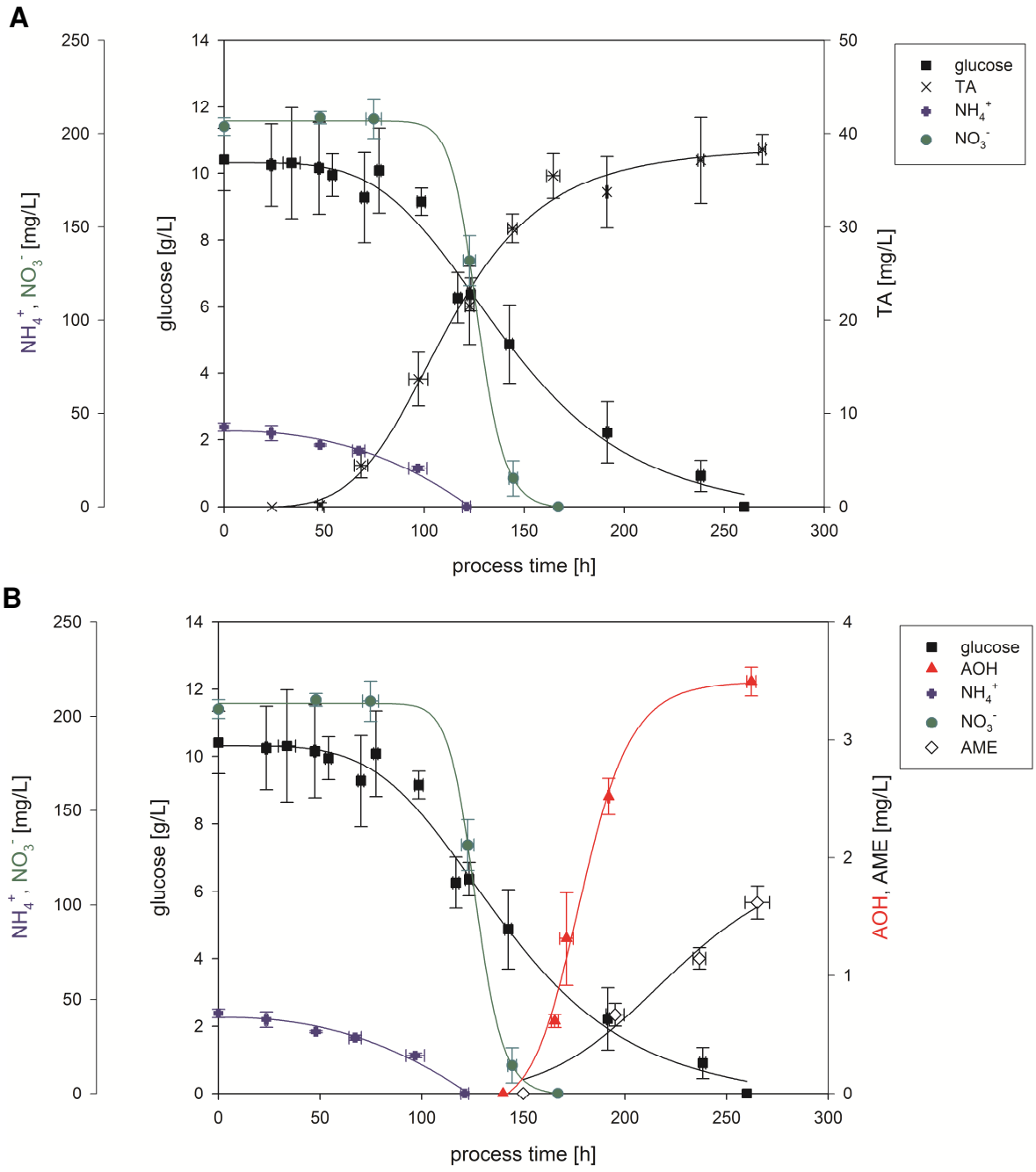

Figure 1: Production of the mycotoxins tenuazonic acid (TA) (**A**), alternariol (AOH) and alternariol monomethylether (AME) (**B**) and consumption of the nutrients glucose, nitrate and ammonium (**A**, **B**) with *A. alternata* DSM 12633 in a 2 L bioreactor. Measured glucose, nitrate, ammonium, TA, AOH and AME concentrations are given as averages of five independent fermentations. All lines represent logistic fittings of the concentrations based on Eq. 1.

Table 1: Parameters of logistic fittings based on Eq. 1 of nutrient consumption and mycotoxin formation in a bioreactor cultivation with *A. alternata*

	y_0	a	x_0	b	R^2	Max. consumption/ production rate r_i [mg/(L*h)]
Glucose	-0.3964	10.7147	141.2869	4.2033	0.9891	84.38
Nitrate	-1.174	207.9278	126.501	18.6103	0.9997	0.742
Ammonium	0	39.0676	89.0597	4.9735	0.9828	7.67
TA	-0.0448	38.7128	112.0248	-4.5425	0.9936	0.412
AOH	-0.1304	3.6254	178.5357	-14.4819	0.9933	0.078
AME	0	2.0951	225.0668	-6.8872	0.9775	0.017

TA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethylether.
Parameters were maintained from five experiments.

Both nitrogen sources and glucose were consumed completely during the process. The consumption of glucose and ammonium did not start immediately most probably due to a germination phase of approximately 24 h and the presence of yeast extract in the medium. After 50 h of cultivation first TA concentrations of 0.92 mg/L were quantified. TA production continued until the end of fermentation (260 h) but was slowed down with decreasing glucose concentrations. A maximum TA concentration of 38.28 ±1.61 mg/L was achieved. The nitrogen sources were depleted subsequently; after total exhaustion of ammonia consumption of nitrate started. With exhaustion of nitrate first AOH concentrations could be detected and reached a maximum concentration of approximately 3.49 ± 0.12 mg/L at the end of fermentation. AME production started delayed after AOH production and reached a maximum concentration of 1.62 ± 0.14 mg/L.

Absolute consumption and production rates were obtained by derivation of the respective fitting with the maxima indicated in table 1. All rates were normalized and the relative rates of glucose and the mycotoxins TA, AOH and AME are shown in figure 2.

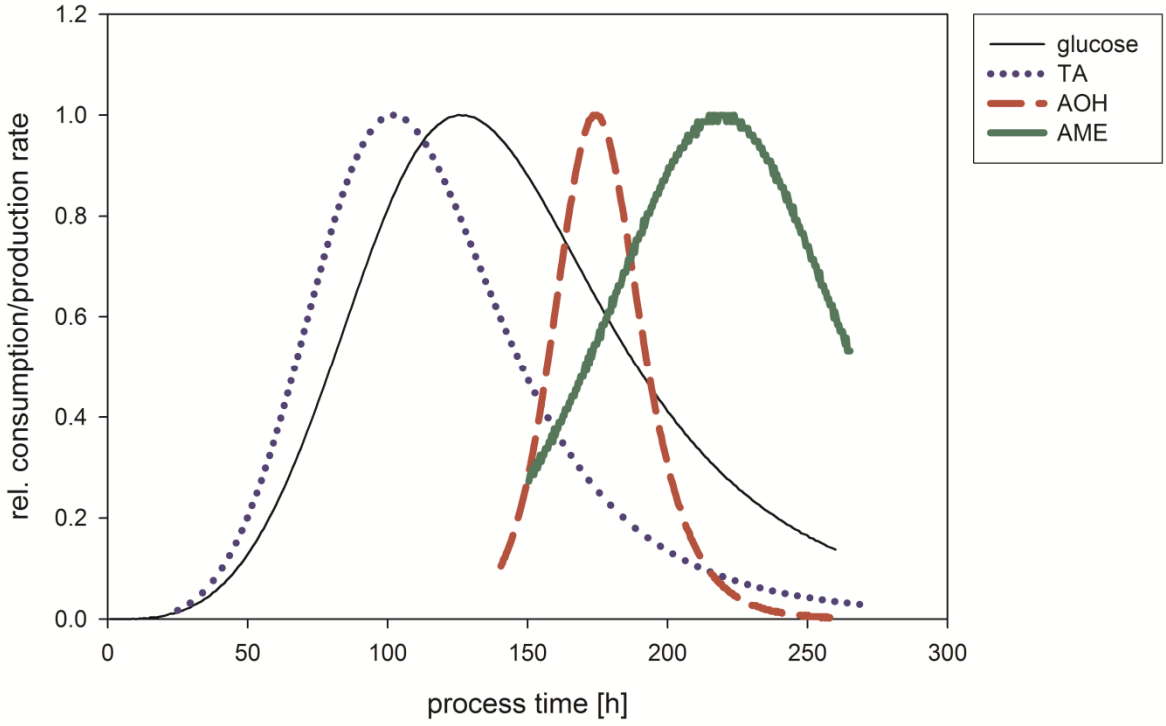

Figure 2: Calculated averaged relative glucose consumption rate and mycotoxin production rates of five independent fermentations of *A. alternata* DSM 12633 in a 2 L bioreactor.
TA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethylether.

The maxima of the TA, AOH and AME production rates were calculated for 100 h, 175 h and 215 h of cultivation, respectively, whereas the maximum of the glucose consumption rate was determined for 125 h of cultivation. The maximum of glucose consumption rate indicates high metabolic activity and probably high biomass increase. Therefore, TA production appeared to growth related while AOH and AME production is not growth-related indicative for a typical behavior of secondary metabolites.

3.2 Influence of aeration rate on mycotoxin production

For the process development different aeration rates were tested. Figure 3 shows the effect of different aeration rates on mycotoxin production. In these experiments measurement of pO_2 was not possible due to invasive fungal growth on the electrode. At higher aeration rates (2 vvm – 0.53 vvm) *A. alternata* was not growing in pellet form, but was clinging on the flow-breaker and other fixtures very tightly. Only low concentrations of AOH (0.67 ± 0.31 mg/L) and TA (23.52 ± 6.43 mg/L) could be detected in the culture broth, AME was not detectable. A reduction of the aeration rate to 0.067 vvm resulted in an increase of all mycotoxins to 1.81 ± 1.40 mg/L AOH, 0.74 ± 1.05 mg/L AME and 37.87 ± 0.88 mg/L. A further enhancement of

mycotoxin production was achieved by lowering the aeration rate to 0.013 vvm: 3.1 ± 0.06 mg/L AOH, 1.78 ± 0.23 AME and 38.35 ± 1.22 mg/L could be detected. Due to the decrased aeration rates the morphology of *A. alternata* changed: At 0.067 vvm less mycelium was clinging at the vessel wall, total biomass was reduced and pellets occurred in the culture broth. When the aeration rate was lowered to 0.013 vvm the biomass was further reduced and more mycelium was present freely in the broth in form of pellets or filaments. Inherent to the design further decrease of the aeration rate was not possible. Therefore, a gas mixture was used consisting of 5 % oxygen and 95 % nitrogen to decrease the oxygen supply while keeping the aeration rate at 0.013 vvm. The aeration with the gas mixture caused a drastic decrease of the polyketide mycotoxins (0.24 ± 0.35 mg/L AOH, AME was not detected) but did not affect TA production (34.60 ± 1.58 mg/L) significantly. In a final experiment the aeration was stopped completely after 48 h at 0.013 vvm (designated as "anaerobic" in fig. 3). The production of the polyketide mycotoxins seemed to be inhibited; AOH and AME were not detected, TA production was reduced to a maximum concentration of 8.04 ± 0.52 mg/L.

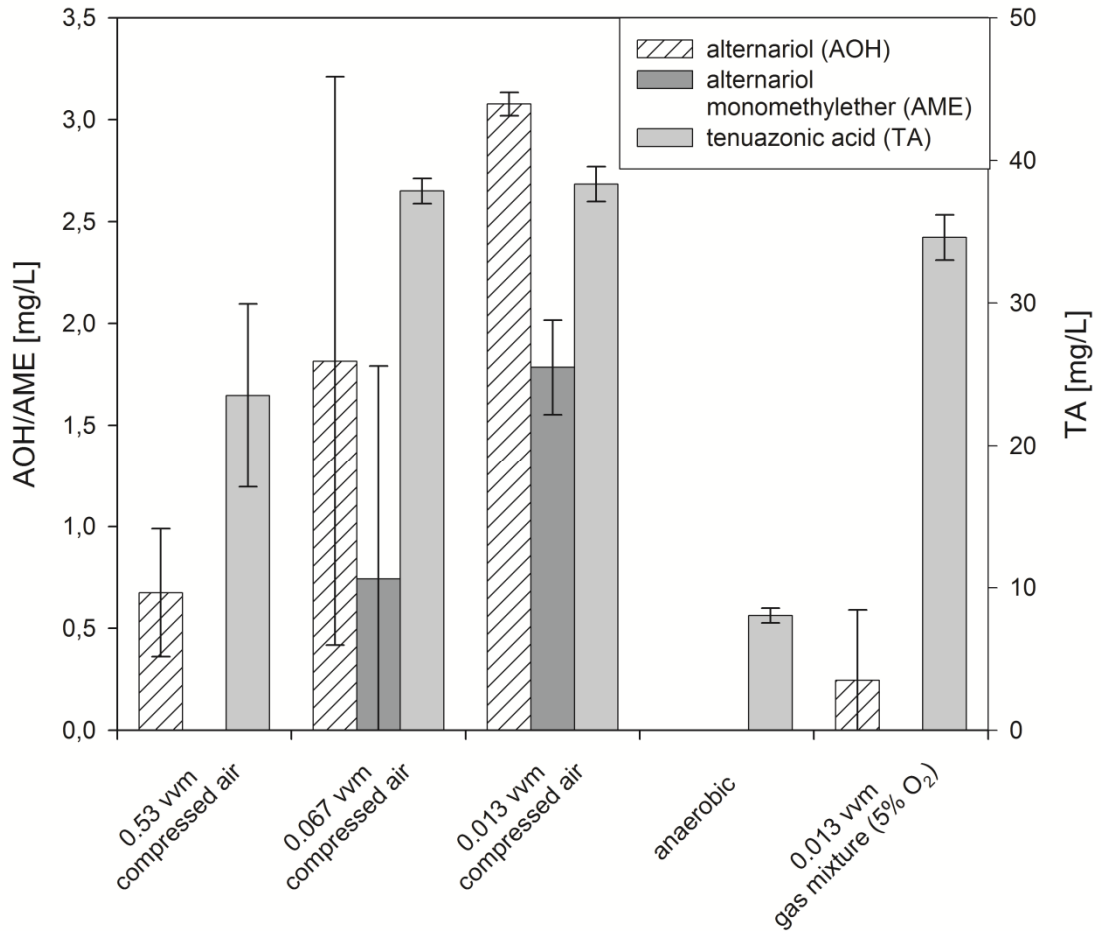

Figure 3: Mycotoxin production with *A. alternata* in a 2 L bioreactor system using different aeration rates. Results are mean of two replicates.
TA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethylether.

3.3 Fermentation with alternative carbon and nitrogen sources

As shown previously for ochratoxin (Abbas *et al.*, 2009; Medina *et al.*, 2008), aflatoxin (Buchanan and Stahl, 1984), trichothecene (Jiao *et al.*, 2008) and *Alternaria* toxins (Brzonkalik *et al.*, 2011), mycotoxin production depends on nitrogen and carbon source. In the study of Brzonkalik *et al.* (2011) the carbon source acetate and the nitrogen source aspartic acid were promising candidates for an enhancement of *Alternaria* toxin production in static cultivation and shaking flask experiments. Consequently, fermentation experiments were performed with the described process with different combinations of carbon and nitrogen sources and are displayed in table 2.

Table 2: Mycotoxin production in a 2 L bioreactor by *A. alternata* depending on carbon and nitrogen source at an aeration rate of 0.013 vvm

carbon source	nitrogen source	AOH [mg/L]	AME [mg/L]	TA [mg/L]	process time [h]	BDM [g/L]
[a]Glucose	NH_4Cl, $NaNO_3$	3.49 ± 0.121	1.62 ± 0.142	38.28 ± 1.61	260	3.3 ± 0.1
[b]Glucose	aspartic acid	7.75 ± 0.064	4.81 ± 0.014	36.54 ± 0.81	350	3.49 ± 0.27
[b]Na-acetate	NH_4Cl, $NaNO_3$	6.64 ± 0.010	ND	ND	400	1.98 ± 0.06
Na-acetate	aspartic acid	3.66	ND	ND	400	2.62

[a] Results are mean of 5 replicates ± standard deviation.
[b] Results are mean of 2 replicates ± standard deviation.
Acetate fermentations were conducted without pH control, glucose fermentations were performed at pH 5.5. Values display maximal detected mycotoxin concentration. Process time gives the earliest time point when the maximum concentration was achieved.
AOH: alternariol; AME: alternariol monomethylether; TA: tenuazonic acid; BDM: biodrymass; ND: below detection limit of < 0.001 mg/L.

The exchange of ammonium and nitrate for aspartic acid resulted in a 2.2 fold increase of the AOH maximum concentration to 7.75 mg/L and enhanced AME production to 4.81 mg/L. Maximum TA concentration was not affected compared to the fermentation with ammonium and nitrate. While the biomass concentration was not altered significantly, process time had to be prolonged to 350 h to reach abovementioned mycotoxin concentrations.

The exchange of glucose for acetate in combination with ammonium and nitrate seemed to inhibit the formation of TA and AME. Only AOH was detected and its maximum concentration was enhanced 1.9 fold to 6.64 mg/L. Biomass production was decreased to 1.98 g/L, but the process was slowed down again and had to be prolonged to nearly 400 h. This may be explained by the slow consumption of acetate which took 300 h to total depletion. Keeping the pH at 5.5 in the acetate fermentation did result in an inhibition of conidia

germination, therefore, the initial pH was set to 6.5 and was not controlled throughout the process. A pH optimization of this fermentation could probably reduce fermentation time. A combination of acetate and aspartic acid did not result in any further increase of AOH maximum concentration compared to the combination of glucose and ammonium/nitrate, but again TA and AME were not detected.

When AOH content is normalized to biomass (expressed as mg mycotoxin per g biomass) maintained concentrations were the following: 1.06 mg/g (glucose/ammonium and nitrate), 2.22 mg/g (glucose/aspartic acid), 3.35 mg/g (acetate/ammonium and nitrate) and 1.40 mg/g (acetate/aspartic acid).

4. Discussion

It was shown, that *Alternaria* toxins can be produced reproducibly in a bioreactor system under controlled conditions. Consumption of nutrient and mycotoxin formation can be characterized with logistic equations. The semi-synthetic Czapek-Dox broth is perfectly suitable for the elucidation of nutritional influences as shown previously (Brzonkalik *et al.*, 2011). Therefore, this medium was chosen for the fermentation experiments, but a further enhancement of mycotoxin production can be achieved by using other complex media.

Literature about mycotoxin production in bioreactor systems is rare; most studies were conducted in shaking flasks or solid media which cannot ensure optimal mixing, pH control and uniform supply with nutrients. Regulation of mycotoxin formation is very complex; fungal morphology and culture conditions have a great impact on mycotoxin production. As shown by Brzonkalik *et al.* (2011) mycotoxin formation was different in static and in shaken culture although the same production strain and the same medium were used. Several different nitrogen and carbon sources were tested but whether mycotoxin production was higher in static or in shaken cultivation differed with each tested C or N source. With respect to the basal modified Czapek-Dox medium containing glucose and ammonium/nitrate cultivation in a bioreactor seems to be favorable since the maintained AOH concentrations are ~3 fold higher than in the shaking flask experiments mentioned by Brzonkalik *et al.* (2011) and the standard deviations of detected mycotoxin concentrations were lower. However, biomass detection during the process remained difficult. The mycelium was not dispersed homologuesly in the culture broth. Therefore, reliable biomass determination during sampling was not possible and total biomass could only be quantified at the end of the process. Nevertheless, glucose consumption showed a typical logistic lapse which may be used as an

indirect method for biomass determination as suggested for mammalian cells growing in packed-bed reactors (Meuwly *et al.*, 2007). In case of *Alternaria* fermentations less comparable data exist. To the knowledge of the authors only one process in a stirred tank reactor was described in literature. Hellwig *et al.* (2002) reported the production of the new antibiotic altersetin. Due to structure similarities the author presumed that altersetin might be a derivative of TA. Furthermore, its formation was inhibited when nitrogen was restricted. Formation of TA during their process was mentioned but detected concentrations were not given. Optimization of stirrer speed and aeration rate in the bioreactor enhanced altersetin production considerably from 1.5 mg/L up to 25 mg/L. Bioreactor experiments do therefore not only provide more constant results, they offer also the possibility to study more parameters compared to shaking flasks, e.g. aeration. The aeration rate influences fungal morphology directly (Mantzouridou *et al.*, 2002; Pfefferle *et al.*, 2000; Stasinopolous and Seviour, 1992; Wecker and Onken, 1991) and fungal morphology in turn plays an important role in metabolism during fermentation (Cho *et al.*, 2002; Metz and Kossen, 1997). As shown in this study, decreased aeration rates led to an increase of free mycelium in form of pellets or filaments and to higher mycotoxin concentrations. The optimal aeration rate was found to be 0.013 vvm in combination with an agitation rate of 900 rpm. A high agitation was necessary in combination with low aeration rates to prevent blocking of the air sparger due to fungal growth. For altersetin production a higher aeration rate (0.3 vvm) combined with lower agitation (100 rpm) was found to be optimal, but altersetin concentrations were not given for lower or higher agitation rates (Hellwig *et al.*, 2002). Aflatoxin production with *Aspergillus flavus* was optimized by testing aeration rates at a constant stirring speed of 100 rpm (Hayes *et al.*, 1966). The authors detected a nearly 20 fold increase in aflatoxin production when the aeration was enhanced from 0.6 vvm to 0.9 vvm. A further increase to 1.2 vvm resulted in decreasing aflatoxin concentrations. The effect of two aeration rates (0.5 vvm and 0.05 vvm) on fumonisin production in *Fusarium proliferatum* was studied by Keller *et al.* (1997). The higher aeration with 0.5 vvm at an agitation with 500 rpm caused an increase fumonisin production compared to the lower aeration rate. Although the comparability between all these studies is limited, it can be stated that the aeration rate has a considerable impact on fungal metabolites production. With respect to the optimal aeration rate for mycotoxin production a general statement cannot be given, but all studies found aeration rate below 1.0 vvm to be supportive for their processes, but for each process agitation has to be taken into account. An enhanced stirrer speed results in an increased dispersity of gas bubbles and therefore in an increased oxygen transfer rate and dissolved oxygen content in the culture broth.

Consequently, high agitation rates enable lower aeration rates. Additionally, the type of stirrer influences shear forces and gas dispersity, but stirrer types were not specified in the abovementioned studies.

As mentioned before, mycotoxin regulation is complex and many factors are influencing their formation, this includes nutritional factors. Mycotoxin production is affected by carbon and nitrogen metabolism mediated by global regulators like the Cys_2His_2 zinc finger transcription factors AreA (nitrogen metabolism) and CreA (carbon metabolism) and their homologues. Additionally, availability of precursor units for mycotoxin production may play a role (Yu and Keller, 2005). However, regulation mechanisms of *Alternaria* toxin formation are not known and the biosynthetic gene clusters have not been identified yet.

Nevertheless, acetate serves as precursor for all three mycotoxins: TA is formed from isoleucine and acetate (Stickings, 1959; Stickings and Townsend, 1961), the polyketides AOH and AME develop from a head to tail condensation of one molecule acetyl-CoA and six molecules of malonyl-CoA followed by a subsequent cyclization (Gatenbeck and Hermodsson, 1965). Unsurprisingly, fermentation with acetate resulted in highest AOH concentration when normalized to biomass but inhibited formation of TA and AME. As shown in figure 2 production of TA production appears to be growth-related in contrast to the polyketide mycotoxins. Simply feeding of precursor units did not enhance but inhibit TA production indicating further regulation mechanisms. The fact that acetate allows AOH but not AME production indicates for an independent regulation of the polyketide synthase and the methyltransferase enzyme which catalyzes the methylation reaction of AOH to AME (Stinson and Moreau, 1986). An independent regulation of both enzymes was already presumed by Orvehed *et al.* (1988). From a biotechnological point of view the possibility to produce single mycotoxins is desirable because less purification steps are necessary.

Considering the results of this study a defined process was successfully established enabling the elucidation of the effect of aeration rate, carbon and nitrogen sources on mycotoxin production. The presented process suits perfectly for further investigations of parameters influencing mycotoxin production and facilitates the comparability of different experiments.

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4.3 Optimization of carbon/nitrogen ratio and AOH feeding experiments

Determination and optimization of process parameters for mycotoxin production by submerged cultivation of *Alternaria alternata*: carbon/nitrogen ratio and product inhibition

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Abstract

Effects of carbon/nitrogen ratios on production of the mycotoxins alternariol (AOH) and tenuazonic acid (TA) and fungal biomass by submerged cultivation of the black mould *Alternaria alternata* DSM 12633 were investigated in shake flasks. Glucose was used as carbon source and its concentration was altered while nitrogen concentration was kept constant. Growth kinetics and mycotoxin production parameters were studied depending on carbon/nitrogen ratios in the range of 24-96. With increasing initial glucose concentration fungal biomass did increase but the maximum specific growth rate was not influenced. Highest titres of AOH, i.e. 1.95 mg/L ± 0.03, and TA, i.e. 22.25 mg/L ± 1.17, were obtained with 30 g/L glucose which corresponds to a C/N ratio of ~72. Furthermore AOH feeding experiments were conducted to investigate possible product inhibitions and mycotoxin metabolization. When smaller AOH concentrations (1 mg/L, 5 mg/L) were present mycotoxin production was stimulated. Higher AOH concentrations had no effect and mycotoxin yields were comparable to the control.

Keywords:

Alternaria alternata, mycotoxins, C/N ratio, Alternariol feeding experiments

1. Introduction

Mycotoxins are harmful secondary metabolites produced by a wide variety of moulds. Black moulds of the genus *Alternaria* are able to form several mycotoxins of different chemical classes whereas the polyketide mycotoxins alternariol (AOH) and its methylated derivative alternariol monomethylether (AME) as well as the tetramic acid tenuazonic acid (TA) are best studied (Coombe *et al*., 1970; Logrieco *et al*., 2009; Patriarca *et al*., 2007). These compounds have been identified in food products contaminated with *Alternaria* species: wheat (Li and Yoshizawa, 2000) and other grains (Broggi *et al*., 2007), sunflower seeds (Nawaz *et al*., 1997; Pozzi *et al*., 2005), oilseed rape (Nawaz *et al*., 1997), pecans (Schroeder and Cole, 1976), fruit and fruit juices (Delgado *et al*., 1996; Lau *et al*., 2003), tomato products (Andersen and Frisvad, 2004; Motta and Valente Soares, 2001; Ozcelik *et al*., 1990) and olives (Visconti *et al*., 1986).

While the acute toxicity of *Alternaria* toxins is low compared to other mycotoxins as e.g. aflatoxins low-level exposure over long terms can be a serious problem. AOH posses an oncogenic potential and was connected to an incidence of oesophageal cancer in Lixian, China (Dong *et al*., 1987). Although *Alternaria* toxins received increased attention over the last years, e.g. the multidisciplinary project "Safe organic vegetables and vegetable products by reducing risk factors and sources of fungal contaminants throughout the production chain: the carrot – *Alternaria* model" carried out by the European Union from 2000 – 2004, a risk assessment based on the current data is still not possible. In addition to the insufficient toxicological data not much is known about biosynthesis and regulation of the mycotoxins either. Hypothetical biosynthesis pathways for AOH/AME and TA have been suggested (Gatenbeck and Hermodsson, 1965; Gatenbeck and Sierankiewicz, 1973; Hiltunen and Söderhäll, 1992; Stinson, 1985; Light, 1970) but the genes are not known. Environmental factors have a great impact on *Alternaria* toxin production. Mycotoxin production varies with fungal strain, substrate and other growth conditions. While TA production seems to be connected to fungal growth (Brzonkalik *et al*., 2011), production of AOH and AME is not coupled with fungal growth and starts in the late exponential phase (Söderhäll *et al*., 1978). As shown by Magan *et al*. (1984), Oviedo *et al*. (2010) and Pose *et al*. (2010) temperature and water activity are important factors influencing AOH/AME and TA production in *Alternaria alternata*. Furthermore light exert a great influence on AOH production and can reduce mycotoxin formation significantly compared to yields obtained in the dark (Häggblom and Niehaus, 1986; Häggblom and Unestam, 1979; Söderhäll *et al*., 1978). Brzonkalik *et al*. (2011) studied the influence of different carbon and nitrogen sources in a semi-synthetic

medium in static and shaking culture on mycotoxin production in *A. alternata*. They revealed that AOH and AME production is regulated by nitrogen and starts after total nitrogen consumption. The influence of the nitrogen source is higher than of the carbon source. Choice of cultivation condition, carbon and nitrogen source can change mycotoxin composition and determines mycotoxin amount.

The best way to avoid health risks due to ingestion of contaminated food is the prevention of food spoilage by *Alternaria* toxins. Therefore, profound knowledge about mycotoxin formation is absolutely essential. Detailed information of influencing factors and regulatory mechanisms may additionally be helpful for enhanced production of mycotoxin for further toxicological studies. The present paper reports on mycotoxin production and biomass formation of *A. alternata* DSM 12633 in a semi-synthetic medium. By changing glucose concentration while keeping the amount of nitrogen constant the effects of C:N ratio on mycotoxin formation were observed. Furthermore feeding experiments with AOH were performed to elucidate possible feed-back inhibition mechanisms or degradation processes.

2. Materials and methods

2.1 Organism and cultivation

A. alternata DSM 12633 was obtained from the DSMZ culture collection ("Deutsche Sammlung von Mikroorganismen und Zellkulturen", Braunschweig, Germany) and was routinely grown on PDA (Roth, Germany). Conidia were harvested with 25 % glycerol from plates that were incubated for 7 days at 28 °C and filtered through Miracloth (Calbiochem). Conidia were counted and diluted to 10^6 conidia per ml. Aliquots were stored at -80 °C.

For all experiments modified Czapek-Dox medium (modified after Gatenbeck and Hermodsson, 1965) at an initial pH of 5.5 was used: 0.06 g/L NH_4Cl, 0.25 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ x 7 H_2O, 0.25 g/L NaCl, 0.25 g/L KCl, 0.01 g/L $FeSO_4$ x 7 H_2O, 0.01 g/L $ZnSO_4$ x 7 H_2O, 1 g/L yeast extract. The used yeast extract contained 11 % (w/w) nitrogen according to the manufacturer (Becton, Dickinson and Company). Glucose was added separately after autoclaving in final concentrations of 10 g/L, 20 g/L, 30 g/L and 40 g/L which corresponds to a C:N ratio of approx. 24, 48, 72 and 96, respectively. 100 ml shaking flasks with baffles were filled with 20 ml of the respective medium and inoculated with 1.7 x 10^4 conidia. Mycotoxin production, biomass formation, glucose and nitrogen consumption

were observed over 14 days. Shaking flasks were incubated at 28 °C on a rotary shaker at 140 rpm in the dark. Samples were taken after 3, 5, 7, 10, 12 and 14 days in triplicates. For each sample an individual flask was prepared which was harvested completely.

For the feeding experiment a stock solution of 400 mg/L AOH in methanol was prepared. AOH was added to final concentrations of 1 mg/L (50 µl stock solution), 5 mg/L (250 µl stock solution) and 10 mg/L (500 µl stock solution) to the medium. Cultivation conditions remained the same but samples were taken only after 7 and 14 days in triplicates.

2.2 Analytical methods

2.2.1 Biomass dry weight, glucose and nitrogen concentration

At a given sampling time fungal mycelium was completely removed from the shaking flask and transferred to a pre-weight tube. Biomass was dried in an oven at 60 °C and its weight was determined on a standard balance.

Glucose concentration was monitored with the photometrical anthron assay (Pons *et al*., 1981).

For the determination of ammonium and nitrate the photometrical assays "Ammonium-Test" (Spectroquant®, Merck, Germany) and "Nitrat-Test" (Spectroquant®, Merck, Germany) were used.

2.2.2 Mycotoxins

A 10 ml aliquot of cell-free culture broth was acidified with 10 µl conc. HCl (32%, 10.32 M) and extracted twice with 10 ml ethyl acetate. At each extraction step the mixture was vigorously vortexed and centrifuged at 4,600 g for 5 min. The ethyl acetate supernatants from both extraction steps were combined and evaporated to dryness in a vacuum centrifuge. The residue was redissolved in 200 µl methanol (HPLC grade) and used for HPLC analyses. Standards of AOH, AME and TA were purchased from Sigma-Aldrich (Germany). Mycotoxin standard solutions of 0.2 mM (AOH), 0.1 mM (AME) and 10 mM (TA) in methanol were diluted with methanol and used for calibration. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a 25 cm reversed phase column (Luna 5 µm C18(2), Phenomenex, Germany) at 30 °C. Mobile phase solution was methanol/0.1 M NaH_2PO_4 pH 3.2 (2:1) at a flow rate of 0.7 ml/min (according to Shephard *et al*., 1991). Mycotoxins were monitored with a UV detector at 280 nm. Retention times were 5.3±0.1 min for TA, 10.2±0.1 min for AOH and 23.3 ±0.1 min for AME.

To quantify the mycotoxin concentration in the culture broth the peak area in each sample was plotted against the standard curve.

2.2.3 Data analysis

Glucose consumption and biomass production were fitted using a logistic equation with four parameters in a scientific data analysis and graphing software (Sigma Plot 9.0, Systat, San Jose, USA). The used equation was:

$$y(x) = y_0 + \frac{a}{1+(\frac{x}{x_0})^b} \qquad (1)$$

The four parameters are the following: y_0 indicates the minimum concentration of the glucose or biomass; a indicates the maximum glucose/biomass concentration; x_0 indicates the process time when half of the glucose amount is consumed or half of the maximum biomass concentration is produced; b is a shape parameter and difficult to explain biologically (Erkmen and Alben, 2002). Derivation of the fitting was used for the determination of absolute consumption and production rates.

3. Results and discussion

3.1 Growth kinetics

Carbohydrates are not only important as energy and carbon source for cell growth; they can even influence secondary metabolite profiles. As shown previously by Brzonkalik *et al.* (2011) glucose proved to be an appropriate carbon source for elucidation of AOH and TA production in submerged culture whereas other carbon sources seemed to inhibit mycotoxin production. For the determination of the optimal C:N ratio the initial glucose content was varied while the quantity of nitrogen in the medium was held constant (167.8 mg/L). In all experiments nitrogen was the limiting nutrient and was consumed after 5 days (data not shown). The time point of total nitrogen consumption and the beginning of nitrogen limitation was indicated as a red line in all figures. Figure 1 displays glucose consumption and fungal growth expressed in dry biomass.

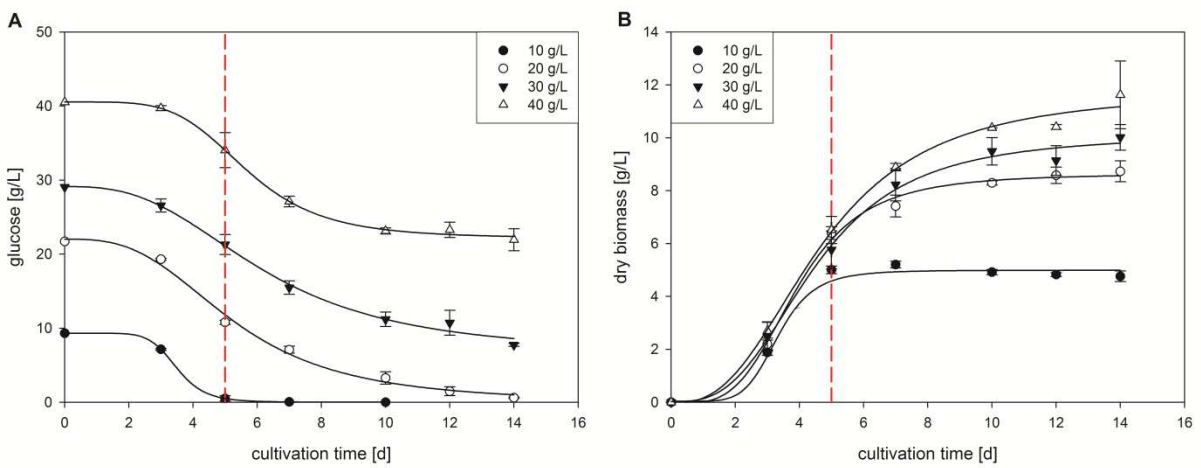

Figure 1: Glucose consumption courses (A) and fungal growth expressed as dry biomass (B) of *A. alternata* depending on initial glucose concentrations. All results are mean of three independent samples ± standard deviation. The lines are results of the fittings according to Eq. 1. The red dotted line represents total consumption of nitrogen and the beginning of nitrogen limitation.

With an initial glucose concentration of 10 g/L glucose was completely consumed after 5 days. Therefore, biomass increased only until day 7 to 5.2 g/L and kept constant from then on. It can be assumed that glucose given at an initial concentration of 10 g/L was also a limiting factor in these experiments. In all other experiments independent on initial glucose concentration approximately 20 g/L glucose were consumed over the observed 14 days and glucose consumption curves were quite similar. According to figure 1B the logistic growth phase can be located between day 3 and 5. After nitrogen consumption at day 7 the stationary growth phase was reached and only slight increases in biomass could be observed. Highest biomass yields (11.62 g/L) were achieved with initial glucose concentration of 40 g/L after 14 days. Interestingly, further biomass increase was noticed after 5 days for initial glucose concentrations ≥20 g/L although nitrogen was depleted. One possible explanation may be some sort of recycling of nitrogen containing components of older hyphae. Fungal growth occurs typically at the tip and older parts of the mycelium are used as storage compartments with little cell organelles and metabolic activity. Table 1 summarizes the effect of initial glucose concentration on fungal growth (by maximum dry weight).

Table 1: Effects of initial glucose concentrations / different C:N ratios on fungal growth of *A. alternata*

Parameter	Initial glucose concentration [g/L] (C:N ratio)			
	10 (24)	20 (48)	30 (72)	40 (96)
Maximum DW[a] [g/L]	5.20 ± 0.13	8.73 ± 0.39	10.02 ± 0.48	11.62 ±1.28
μ_{max} [h^{-1}][b]	0.045	0.039	0.032	0.029
max. growth rate r_x [g/h][b]	0.095	0.093	0.079	0.082
average growth rate [g/d]	0.74	0.62	0.72	0.83
$Y_{X/S}$ [g/g]	0.52	0.44	0.45	0.64

All results refer to day 14 excepting the results for 10 g/L glucose which refer to day 7.
[a] Results are mean of three replicates ± standard deviation.
[b] obtained from the fit according Eq. 1.
DW: dry weight of biomass.

Independent on the glucose concentration the maximum specific growth rate μ_{max} and the maximum growth rate are almost the same in all experiments and were determined to be 0.029 – 0.045 h^{-1} and 0.079 – 0.095 g/h, respectively. To the knowledge of the authors these parameters have not been determined before for *Alternaria*. Comparison of specific growth rates of other filamentous fungi proved to be difficult as the growth rate varies considerably with culture conditions and medium composition. As shown for *Monascus purpureus* it can be enhanced from 0.011 h^{-1} to 0.073 h^{-1} by lowering the culture pH from 6.5 to 4.0 in nitrogen limited medium (Chen and Johns, 1993). The growth rate of *Aspergillus terreus* in a defined medium under nitrogen limited condition was determined to be 0.052 by Hajjaj *et al*. (2001). Therefore, the determined growth rate for *A. alternata* is low but in range of the other cited data. In other studies the average growth rate is used instead of the specific growth rate. Mao *et al*. (2005) observed an average growth rate of 0.93 d^{-1} – 1.06 d^{-1} in submerged culture of *Cordyceps militaris* when initial glucose concentration was higher than 25 g/L. As with specific growth rate the average growth rate for *A. alternata* is low but in the same range when differences in medium composition are considered. However, growth yield on glucose (0.44 g/g – 0.64 g/g) appeared to be very high which suggest a high conversion of glucose to biomass. In comparison the growth yield on glucose for *A. terreus* in a complex medium was 0.52 g/g and in a defined medium 0.25 g/g (Hajjaj *et al*., 2001). In all experiments nitrogen was the limiting compound; therefore, biomass increase ought to be limited by nitrogen and

not depending on initial glucose concentration. However, the presented results suggest that $Y_{X/S}$ is slightly depending on glucose concentration. The high biomass/substrate coefficient for 40 g/L glucose may partly be explained by measurement uncertainties illustrated by the high standard deviation of the last biomass value. But the coefficient remains high even if the data of day 12 is used for calculation ($Y_{X/S}$ (12d): 0.62).

3.2 Mycotoxin formation

As for the growth kinetics mycotoxin production was observed depending on different C:N ratios. Figures 2 and 3 show the production courses of AOH and TA over 14 days as well as the highest AOH yields on day 7.

AOH was initially detected at day 5 when nitrogen was depleted. A maximum AOH concentration can be observed at day 7. These maximum was further compared in figure 2B. For the initial glucose concentrations of 10 g/L, 20 g/L and 40 g/L nearly the same AOH concentrations were detected: 0.25 mg/L ± 0.003, 0.12 mgL/ ± 0.07 and 0.33 mg/L ± 0.29, respectively. AOH production was noticeably enhanced at an initial glucose concentration of 30 g/L, 1.95 mg/L ± 0.03 could be achieved. The detected AOH amount decreased at day 10 and 12 and increased slightly at day 14.

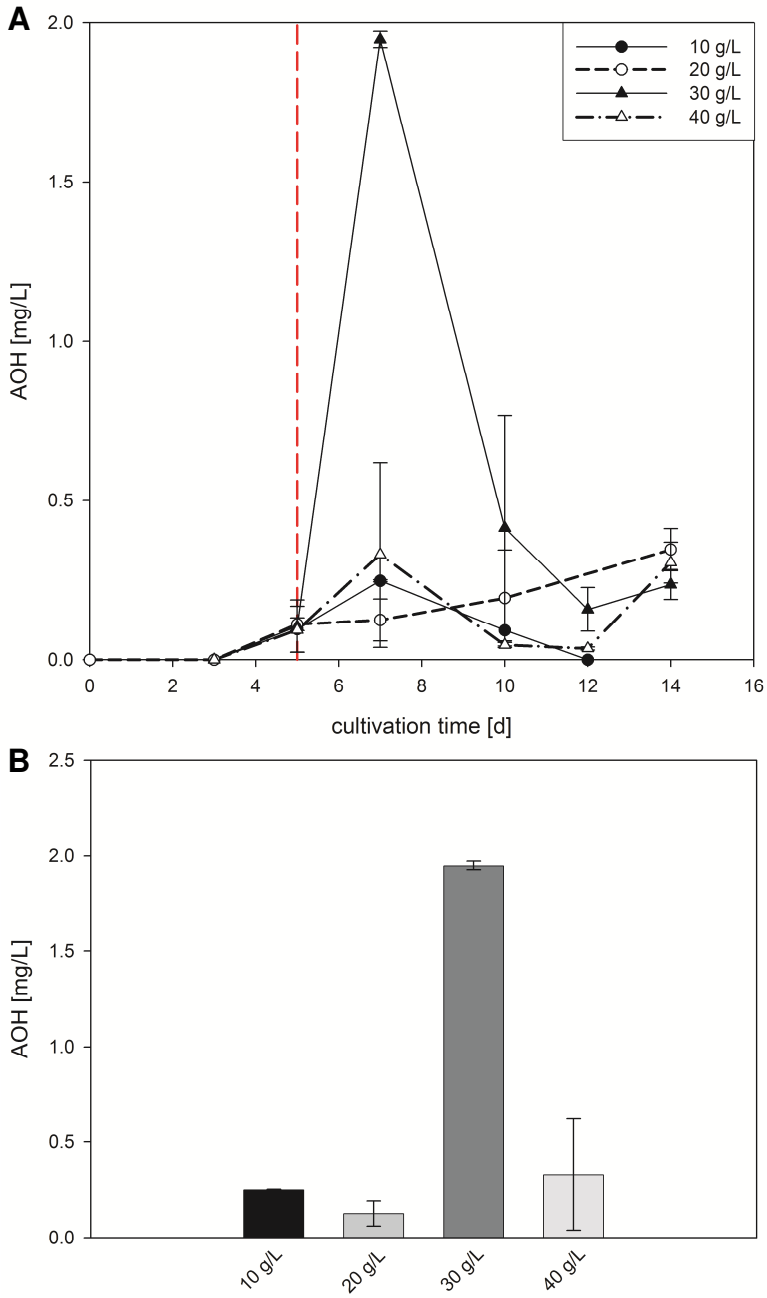

Figure 2: Alternariol (AOH) production of *A. alternata* depending on different initial glucose concentrations. Time courses of mycotoxin production are given over 14 days (A) and highest mycotoxin concentrations at day 7 were compared (B). All results are mean of 3 independent samples ± standard deviation. The dotted red line indicates nitrogen depletion.

The noticeable increase in AOH production at 30 g/L initial glucose concentration is very interesting from the biotechnological and physiological point of view. Nevertheless, due to the sampling every second day it may be possible that the production peaks of the other glucose concentrations were not recorded because of shifted production maxima to day 6 or day 8. But even at day 10 when AOH concentration dropped considerably for all glucose concentrations the highest concentrations can be detected for 30 g/L initial glucose

concentration. Therefore, this experiment is an interesting hint for further experiments but it has to be confirmed in a bioreactor system. In this system the production phase is prolonged and the production curve does not suddenly drop as in shaking flask cultivations. The reason for the sudden decrease of AOH is not clear, but was confirmed in several other shaking flask experiments. Since it is not recorded for bioreactor cultivations parameters which are not controlled during shaking flask cultivations come into question, e.g. pH and aeration.

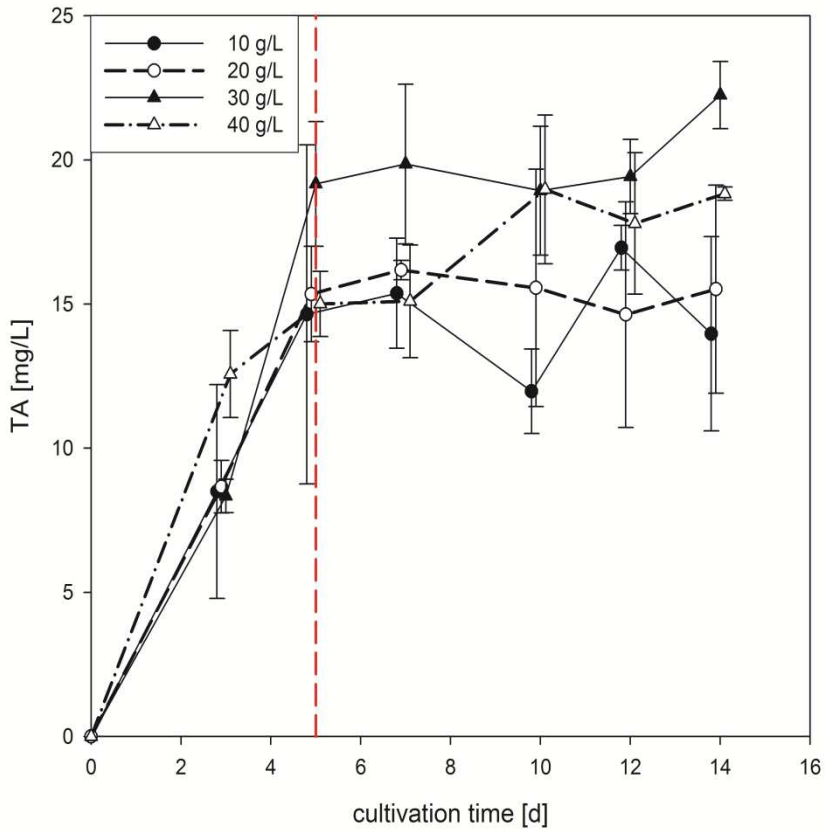

Figure 3: Tenuazonic acid (TA) production of *A. alternata* depending on different initial glucose concentrations. Time courses of TA concentrations are given over 14 days. All results are mean of 3 independent samples ± standard deviation. The dotted red line indicates nitrogen depletion.

TA was formed in the early growth phase as it was already detected at day 3. TA concentration courses followed biomass increase. Although measured TA data had fluctuations all concentration time courses were in the same range. TA production increased to day 7 and stayed almost constant like the dry weight curve. As with AOH production the initial glucose concentration of 30 g/L seemed to favor TA production, but the enhancement appeared not to be significant and may be explained by higher biomass production. Highest TA concentrations were detected for 10 g/L glucose at day 12 with 16.95 mg/L ± 0.78, for 20 g/L at day 7 with 16.18 mg/L ± 0.34, for 30 g/L glucose at day 14 with 22.25 mg/L ± 1.17 and

for 40 g/L glucose at day 10 with 18.97 mg/L ± 2.58. Table 2 summarizes the effect of initial glucose concentrations on mycotoxin formation.

Table 2: Effects of initial glucose concentrations / different C:N ratios on mycotoxin production of *A. alternata*

Parameter	Initial glucose concentration [g/L] (C:N ratio)			
	10 (24)	20 (48)	30 (72)	40 (96)
Max. AOH titre [mg/L] (day)[a]	0.25 ± 0.003 (7)	0.34 ± 0.07 (14)	1.95 ± 0.03 (7)	0.33 ± 0.29 (7)
AOH productivity [mg/(L*d)]	0.036	0.024	0.279	0.047
AOH yield on DW [mg/g]	0.048	0.038	0.237	0.037
Max. TA titre [mg/L] (day)[a]	16.95 ± 0.78 (12)	16.18 ± 0.34 (7)	22.25 ± 1.17 (14)	18.97 ±2.58 (10)
TA productivity [mg/(L*d)]	1.41	2.31	1.59	1.90
TA yield on DW [mg/g]	3.51	2.18	2.22	1.83

[a] Results are mean of three replicates ± standard deviation.
DW: dry weight of biomass; AOH: alternariol; TA: tenuazonic acid.

The comparison of the concentration curves of AOH and TA implies different regulatory mechanisms for both mycotoxins. Although both mycotoxins are originated from acetyl-CoA (Stinson, 1985; Stickings and Townsend, 1961) TA was already formed at an early growth stage whereas AOH was first detected after nitrogen depletion. Therefore AOH production seemed to be associated with the stationary growth phase due to nitrogen limitation when acetyl-CoA units from the citrate cycle can be used for polyketide formation. For AOH formation, its highest titre, productivity and yield related to biomass were obtained with 30 g/L glucose or an C:N ration of approx. 72. Higher or lower initial glucose concentrations were unfavorable.

TA is formed from acetyl-CoA and the amino acid isoleucine (Stickings and Townsend, 1961). Therefore the concentration curves for biomass and TA production resemble each other and TA production will stop under nitrogen limited conditions. Absolute TA titres enhanced with increasing biomass, but when related to biomass the highest yield of 3.51 mg/g was achieved with an initial glucose concentration of 10 g/L and decreased with higher glucose concentrations and biomass production. In contrast to AOH TA production did not

show a clear maximum and was not degraded or transformed to other metabolites when nitrogen was depleted although it could be used as nitrogen source. The reason for the drop is not clear since in bioreactor cultivations this decrease was not observed (Brzonkalik *et al*., 2011). Shake flask experiments are very helpful for preselection of promising cultivation parameters and can provide first tendencies but they are suitable only for preliminary investigations because conditions in shake flask are only loosely defined and neither the pH value nor dissolved oxygen concentration are monitored and controlled and standard deviations are usually high. Therefore fully controlled cultivations in a bioreactor are currently performed in our lab.

3.3 Alternariol feeding experiments

For the elucidation of AOH degradation and possible feed-back inhibition of AOH production feeding experiment were performed. Therefore 3 different AOH amounts (1 mg/L, 5 mg/L and 10 mg/L final concentration) were added at the beginning of the cultivation with an initial glucose concentration of 10 g/L. Cultures were extracted after 7 and 14 days and mycotoxin yields were determined (table 3).

Table 3: Mycotoxin yields of *A. alternata* after feeding with different amounts of alternariol (AOH)

Added AOH	AOH [mg/L]		AME [mg/L]		TA [mg/L]	
[mg/L] day 0	day 7	day 14	day 7	day 14	day 7	day 14
0	0.25 ± 0.003	n.d.	n.d.	n.d	15.34 ± 1.91	13.97 ±3.37
1	3.93 ± 0.12	1.25 ± 0.02	1.48 ± 0.12	0.27 ± 0.04	62.9 ± 0.62	52.07 ± 0.07
5	10.85 ± 0.47	4.79 ± 1.13	7.93 ± 1.34	3.42 ± 2.62	60.9 ± 0.01	55.97 ± 0.03
10	0.74 ± 0.41	0.56 ± 0.04	n.d	n.d	19.03 ± 0.02	17.07 ± 0.01

Results are mean of 3 independent experiments ± standard deviation. AOH stock solution was prepared in methanol and was added to 20 ml modified Czapek-Dox medium to the indicated final concentration before inoculation.
AOH: alternariol; AME: alternariol monomethylether; TA: tenuazonic acid; n.d.: below detection limit.

The addition of AOH in small amounts of 1 mg/L or 5 mg/L final concentration seemed to stimulate the AOH production of *A. alternata*. These amounts of AOH in the culture broth are not uncommon since ~5 mg/L AOH can be produced in a bioreactor with modified Czapek-

Dox medium containing 10 g/L glucose as shown previously (Brzonkalik *et al*., 2011). Furthermore AME production was initiated which was not observed in cultures without AOH addition and TA production was enhanced additionally. The addition of 1 mg/L AOH resulted in the detection of 3.93 mg/L AOH in the culture broth after 7 days which is a fourfold increase compared to the added concentration. A TA concentration of 62.9 mg/L was measured which is a fourfold increase compared to the control culture and the detected AME concentration was 1.48 mg/L. All mycotoxin concentrations decreased at day 14; The AOH and AME amounts were reduced remarkably to 32 % (1.25 mg/L) and 18 % (0.27 mg/L), respectively. TA concentration was slightly lowered to 83 % (52.07 mg/L). When 5 mg/L AOH were added AOH production was enhanced as well to 10.85 mg/L at day 7 which corresponds to a twofold enhancement compared to the added concentration. However, the AME production was improved considerably to a concentration of 7.93 mg/L at day 7. TA concentration was not further increased and was 60.9 mg/L. All mycotoxin concentrations decreased at day 14 but were only reduced to 44 % of AOH (4.79 mg/L) and 43 % of AME (3.42 mg/L). TA amount was kept almost constant. The addition of 10 mg/L AOH did not result in an increase of mycotoxin production. AOH and TA amounts were comparable to the control cultivation and AME was not detected.

The indicated data did not imply a feed-back inhibition but suggest a self-enhancement of AOH production up to a certain concentration. Interestingly, the production of the other mycotoxins AME and TA were also enhanced. Nevertheless, mycotoxin titres dropped at day 14 as well as in the other experiments shown in section 3.2. This phenomenon may be explained by the experiments conducted by Jonsson *et al*. (1987). They observed the metabolization of labeled AOH and AME in *A. alternata* cultures and suggested a possible involvement of a peroxidase in the turnover. However, the authors of the this work suggest another possibility when the hypothetical production pathway of AOH (Stinson, 1985) is considered. AOH and AME are not endproducts of this pathway; they are further modified and converted to other mycotoxins e.g. altenuene. Therefore, AOH and AME have not to be degraded necessarily but converted to metabolites which could not be identified with the used analytical methods. The involved enzymes may be stimulated by certain concentrations of precursor molecules like AOH and AME. This hypothesis cannot be proved until the gene cluster is identified. Nevertheless, metabolization seems to be dependent on culture conditions since decrease in mycotoxin concentrations was not observed in bioreactor experiments (data not shown).

4. Concluding remarks

In this work the influence of different C:N ratios on biomass and mycotoxin production of *A. alternata* was demonstrated. Biomass production increased with higher initial glucose concentrations but growth kinetic parameters stayed almost constant. AOH production showed a clear maximum at day 7 and decreased thereafter. Highest AOH concentrations (1.95 mg/L) were obtained with an initial glucose concentration of 30 g/L. The curve shape of TA production mimicked the biomass curve. TA concentration stayed constant when nitrogen was depleted. Highest TA concentrations of 22.25 mg/L were obtained with 30 g/L initial glucose concentration. Feeding experiments revealed that feeding of AOH up to a certain concentration can enhance the production of all monitored mycotoxins. The fundamental data obtained in this work may be useful for deeper understanding of mycotoxin regulation in *A. alternata* and helps for further optimization of parameters for *Alternaria* toxin production in a bioreactor.

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4.4 Enzymes of mycotoxin biosynthesis: AOH-*O*-methyltransferase

Identification of putative *O*-methyltransferases and characterization of the alternariol-*O*-methyltansferase of *Alternaria alternata*

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Abstract

Alternaria alternata produces the polyketide mycotoxin alternariol (AOH) and alterarniol monomethylether (AME) when grown in darkness. The biosynthesis of the mycotoxins is possibly a multi-enzyme process, but their genes are not known. As shown previously, AME is formed by methylation of AOH mediated by an AOH-*O*-methyltransferase. The present work describes the identification and amplification of putative *O*-methyltransferase of *A. alternata* in order to identify the AOH-*O*-methyltransferase. By comparison of fungal *O*-methyltransferase proteins three conserved domains were determined and used for primer design. Four *O*-methyltransferases fragments were amplified and one complete gene was overexpressed. In a second approach the enzyme was characterized in *A. alternata* crude extract. Salt stability and pH tolerance range was determined. In agreement with other studies the enzyme tolerated 1 M of different salts and its pH optimum was around pH 7. For purification several buffers proved to be appropriate, but highest enzyme activity was detected in phosphate-buffer. First purification steps were the precipitation of the protein with 40 % $(NH_4)_2SO_4$ and the application to DEAE-Sepharose anion-exchange column. Determination of the protein and subsequently of the gene sequence will be an important step for the elucidation of the whole biosynthesis gene cluster.

Keywords:

Alternaria alternata, mycotoxin biosynthesis, alternariol-*O*-methyltransferase

1. Introduction

Mycotoxins are harmful secondary metabolites of small molecular weight. They are produced by several mold genera including *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*. These metabolites contaminate foods and feeds and can therefore be ingested by humans and animals. The consumption of contaminated food is related to several acute and chronic health disorders like induction of cancer, mutagenicity, urogenital, vascular, kidney and nervous disorders. Besides the health risk significant economic losses due to mycotoxin contamination have to be taken into account. According to FAO estimations global losses of foodstuffs are in the range of 1000 million tons per year (http://www.fao.org/ag/agn/agns/chemicals_mycotoxins_en.asp).

The best way to overcome both health risks and economical losses is the prevention of mycotoxin contamination in foods and feeds. Therefore, a comprehensive knowledge of the regulation of biosynthesis on both genetic and physiological level is necessary.

Compared to other mycotoxins knowledge about *Alternaria* toxins is limited. Although *Alternaria* mycotoxins can be found in many foodstuffs like wheat (Li and Yoshizawa, 2000), sunflower seeds (Nawaz *et al*., 1997; Pozzi *et al*., 2005), oilseed rape (Nawaz *et al*., 1997), pecans (Schroeder and Cole, 1976), fruits and fruit juices (Delgado *et al*., 1996; Lau *et al*., 2003), tomato products (Andersen and Frisvad, 2004; Motta and Valente Soares, 2001; Ozcelik *et al*., 1990) and other grains (Broggi *et al*., 2007; Scott, 2001), and are connected to certain health disorders like the suggested connection to esophageal cancer in China (Dong *et al*., 1987), toxicity data relevant for hazard characterization are not available (Ostry, 2008). According to the German Federal Institute of Risk Assesment (Bundesinstitut für Risikobewertung BfR) in 2003 a risk assessment of *Alternaria* toxins is not possible based on the current data.

Alternaria alternata produces several different mycotoxins whereas the polyketide toxins alternariol (AOH) and alternariol monomethyl ether (AME) and the tetramic acid tenuazonic acid (TA) are best studied (Coombe *et al*., 1970; Montemurro and Visconti, 1992; Pero *et al*., 1973; Rosett *et al*., 1957; Visconti *et al*., 1986). The formation of *Alternaria* toxins is influenced by abiotic and nutritional factors. The production of the polyketide toxins AOH and AME is like other secondary metabolites not linked to fungal growth and is initiated in the late exponential growth phase (Söderhäll *et al*., 1978). In contrast to this, the formation of TA seems to be coupled to fungal growth and is similar to primary metabolites (Brzonkalik *et al*., 2011). The influence of water activity and temperature on mycotoxin production of different *A. alternata* strains grown on several media was extensively studied (Pose *et al*.,

2010; Magan *et al*., 1984; Magan and Lacey, 1984; Hasan, 1995) and was shown to be very important. Light is a further factor which influences mycotoxin production. Light inhibits the formation of AOH and AME as shown by Söderhäll *et al*. (1978) and Häggblom and Niehaus (1986). Like other mycotoxins AOH and AME production is triggered by nitrogen starvation (Brzonkalik *et al*., 2011).

The biosynthetic pathways of *Alternaria* toxins are still hypothetical since the genes are not known. Although the genome of *A. alternata* is sequenced, it is not annotated and not available to the public. However, the genome of *A. brassicicola*, a close relative to *A. alternata*, is fully sequenced and annotated (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html). Furthermore, *A. brassicicola* is also able to produce TA, AOH and AME and contains therefore the respective biosynthetic genes.

The tetramic acid TA is biosynthetically derived from L-isoleucine and acetate (Stickings, 1959; Stickings and Townsend, 1961). Gatenbeck and Sierankiewicz (1973) demonstrated in their experiments that the initial step of TA formation is N-aceto-acetylation of L-isoleucine followed by the formation of the five-membered ring. AOH is thought to be formed by a polyketide synthetase (Light, 1970) and probably subsequently methylated to the methylether AME mediated by the S-adenosylmethionine (SAM) dependent AOH-*O*-methyltransferase (Stinson and Moreau, 1986). Further derivatives like altenuene may be built by further hydroxylations, oxidations and reductions (Stinson, 1985).

As the genes for fungal secondary metabolite production are usually clustered (Keller and Hohn, 1997), the identification of the biosynthetic gene cluster for AOH (and its derivatives) production can be essentially promoted by the identification of a single gene of this cluster. After the identification of one gene of the cluster other genes can easily be isolated by PCR based methods, e.g. primer walking. This work focuses therefore on the characterization and partial purification of the (SAM) dependent AOH-*O*-methyltransferase because some basic studies were conducted previously by Stinson and Moreau (1986), Orvehed *et al*. (1988) and Hiltunen and Söderhäll (1992). Stinson and Moreau (1986) showed in their study the SAM dependency of the methyltransferase and that the enzyme is located in the cytosol. It did not show any activity towards structural similar compounds. A partial purification by Hiltunen and Söderhäll (1992) resulted in the determination of the molecular mass of 43 kDa. Consequent kinetic studies revealed a K_m (SAM) of 110 µM at 15 and 30 µM AOH. The inhibition constant K_i for the competitor SAH was 120 µM. The apparent K_m (AOH) was 1.7 µM at 0.8 mM SAM. AME was not found to inhibit the reaction (Hiltunen and Söderhall, 1992).

The aim of the present work was to enhance the knowledge about the AOH-*O*-methyltransferase by identification of putative *O*-methyltransferases in the genome of *A. alternata* and by purification of the AOH-*O*-methyltransferase to throw light in the biosynthesis of AOH and help to understand the important question of regulation of mycotoxin production.

2. Material and Methods

2.1 Strain and Media

Alternaria alternata DSM 12633 was obtained from DSMZ culture collection ("Deutsche Sammlung von Mikroorganismen und Zellkulturen", Braunschweig, Germany). All cultures of *A. alternata* were routinely grown on PDA (Roth, Germany). Conidia were harvested from plates that were incubated 7 days at 28°C with 25 % glycerol and filtered through Miracloth (Calbiochem). Conidia were counted in a Thoma counting chamber and diluted to a concentration of $1*10^6$ condia per ml. Aliquots were stored at -80°C.

For the extraction of DNA, RNA and proteins *A. alternata* was cultured in liquid rice-medium according to Misra and Sinha (1979). 40 g/L of milled rice were dissolved in deionized water and autoclaved. The starch containing supernatant was transferred to a fresh bottle and 30 g/L glucose and 1 g/L yeast extract were added. Deionized water was filled up to the final volume and the medium was autoclaved. 20 ml of the liquid medium was aliquoted in standard Petri dishes and inoculated with $1*10^5$ conidia. Plates were incubated in the dark at 28°C for 3 days (DNA extraction) or 6 days (protein and RNA isolation).

2.2 Molecular biological methods

2.2.1 DNA/RNA extraction

A. alternata was grown for 3 days at 28 °C on liquid rice-medium in surface culture. The mycelium was harvested, washed in deionized water, blotted dry between filter papers and immediately ground to a fine powder in liquid nitrogen. Approximately 100 mg of the powder were transferred to a 1.5 ml reaction tube and 600 µl of lysis puffer (2 % SDS, 10 mM Tris/HCl pH 8.0, 0.1 M NaCl, 2.5 mM EDTA pH 8.0) were added. For cell disruption the suspension was mixed vigorously, frozen in liquid nitrogen for 30 sec and mixed again for 30 sec. The procedure was repeated several times. The sample was thawed at 60 °C. DNA was

isolated by phenol/chloroform extraction and a subsequent isopropanol precipitation over night at 4 °C.

For the RNA isolation *A. alternata* was grown for 6 days at 28 °C on liquid rice-medium in surface culture in the dark. Under these conditions the AOH-*O*-methyltransferase gene is expressed and the corresponding protein is active. As for DNA extraction the mycelium was harvested, washed, blotted dry and ground to a fine powder in liquid nitrogen. The RNA extraction was conducted with the NucleoSpin® RNA Plant kit according to the manufacturer (Macherey & Nagel, Germany).

2.2.2 PCR methods and primer

All PCR experiments were conducted with DreamTaq™ Green DNA Polymerase (Fermentas, Germany) with the supplied $(NH_4)_2SO_4$ buffer containing 2 mM $MgCl_2$ (final concentration) according to the manufacturer. For primer design for the amplification of putative SAM dependent *O*-methyltransferases of *A. alternata* the published genome of the close relative *A. brassicicola* (JGI – DOE Joint Genome Institute, http://genome.jgi-psf.org/Altbr1/Altbr1.home.html) was consulted and conserved domains were identified by protein alignments with the free software BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Table 1 gives the used primer pairs with the obtained fragment length and the optimal annealing temperatures.

Table 1: Primers used for amplification of gene fragments of putative SAM-dependent *O*-methyltransferases in *A. alternata*.

Primer	Sequence (5´-3´)	An. Temp.	Fragment
Mt6for Mt6rev	TTC CTT GTC GAC GTT GGT GGC TCT GG CAT GGC GGG CAC GAT TGC CTT GAG AAT	48 °C	269 bp
Mt5for Mt5rev	GAG ACC TAC TTY GAY TAY ATG RTT CAT CAT YTG CCA RTC CAT	48 °C	524 bp
Mt8for Mt8rev	GAR CAR CCN TGG GAY ACN ATG CAR RTT RTC YTG NGG YTG YTT RTA	50 °C	427 bp
Mt037for Mt037rev	MGN AAY CAY AAR ATG AGC TGG CAT NCC ACT NGT RCA RTT CAT	48 °c	443 bp

Wobble bases: M = A or C; Y = C or T; R = A or G; N= A, T, C or G. An. Temp: annealing temperature.

The full-length genomic sequence of the putative methyltransferase Mt6 was obtained by using 2 step thermal asymmetric interlaced (TAIL)-PCR according to Liu and Whittier (1995) and Arie *et al*. (2000). Two arbitrary degenerate primers (AP1 and AP2) were each combined with a specific primer generated from the obtained gene fragment (Mt6for x Mt6rev). The PCR mixture (25 µl) of the primary TAIL reaction consisted of 1x DreamTaq Green $(NH_4)_2SO_4$ PCR buffer (including 2 mM $MgCl_2$), additional 0.5 mM $MgCl_2$ (final total concentration 2.5 mM), 0.2 mM dNTP, 0.4 µM specific Primer, 5 µM of one AD primer, 20 ng fungal DNA, 1.25 U DreamTaq polymerase. The secondary amplification reactions (50 µl) contained 1x DreamTaq $(NH_4)_2SO_4$ buffer, 2.5 mM $MgCl_2$ (final concentration), 0.2 mM dNTP, 0.2 µM nested specific primer, 4 µM of the same AD primer as in primary reaction and 1 U DreamTaq polymerase. Template was 1 µl of a 1:50 dilution of primary reaction mixture. The cycling conditions for the TAIL-PCR are shown in table 2.

Table 2: Cycling conditions used for TAIL-PCR (according to Liu and Whittier, 1995)

Reaction	Step	Cycle No.	Thermal conditions
Primary	1	1x	95 °C (3 min).
	2	5x	95 °C (15 sec), 63 °C (1 min), 72 °C (2 min).
	3	1x	95 °C (15 sec), 30 °C (3 min), ramping to 72 °C over 3 min, 72 °C (2 min).
	4	10x	95 °C (5 sec), 44 °C (1 min), 72 °C (1 min).
	5	12x	95 °C (5 sec), 63 °C (1 min), 72 °C (2 min), 95 °C (5 sec), 63 °C (1 min), 72 °C (2 min), 95 °C (5 sec), 44 °C (1 min), 72 °C (2 min).
	6	1x	72 °C (5 min).
Secondary	1	1x	95 °C (3 min).
	2	10x	95 °C (5 sec), 63 °C (1 min), 72 °C (2 min), 95 °C (5 sec), 63 °C (1 min), 72 °C (2 min), 95 °C (5 sec), 44 °C (1 min), 72 °C (2 min).
	3	1x	72 °C (5 min).

To confirm the 3´end of the transcript a rapid amplification of cDNA ends (RACE)-PCR was used and finally the complete transcript was amplified with reverse transcription (RT)-PCR. For the generation of cDNA the First Strand cDNA Synthesis Kit (Fermentas, Germany) was used according to the manufacturer. For the 3´-RACE the 3´-RACE adapter Primer was used to synthesize cDNA instead of the supplied oligo-dT Primer of the cDNA synthesis kit. The obtained cDNA with the linked adapter was applied to a PCR reaction with the PCR adapter Primer and the specific primer AaMt6_for2. Subsequently the PCR fragment was diluted 1:50

and applied to a second PCR reaction with the PCR adapter Primer and the specific nested primer Mt6for.

For the amplification of the full-length transcript the cDNA synthesis was conducted with the supplied oligo-dT primer of the kit. The obtained cDNA was applied to a PCR reaction with both specific primers AaMt_start_*Bam*HI x AaMt_end_*Xho*I or AaMt_start_*Xho*I x AaMt_end_*Bam*HI and which had the overlapping restriction sites *Bam*HI and *Xho*I, respectively. All primers used for TAIL-, 3´-RACE- and RT-PCR and subsequent PCRs are given in table 3.

Table 3: Primers used for TAIL-, 3´-RACE-, RT-PCRs and subsequent PCRs.

Primer	Sequence (5´-3´)	Reference
AD1	NGT CGA SWG ANA WGA A	Arie *et al*., 2000
AD2	TGY TGY WSN CAR TTY GG	Arie *et al*., 2000
TAIL1rev	CCT CAT CAT TCC AGT CCT GG	this study
TAIL1revnested	CGC ACC TTT CAC AGG TTG CTC	this study
TAIL2rev	GTG CTC AGA TGT GCG GGT TGC GCC C	this study
TAIL2revnested	CGC GAT GAG CTC CCT ACA CGC GTC C	this study
3´-RACE adapter	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T	Invitrogen
PCR adapter	GGC CAC GCG TCG ACT AGT ACT	Invitrogen
AaMt6_for2	ATG GCG GCC GAT TCY ACT CCG AGC	this study
Mt6for	TTC CTT GTC GAC GTT GGT GGC TCT GG	this study
Oligo-dT	TTT TTT TTT TTT TTT TTT	Fermentas
AaMt_start_*Bam*HI	CGG GAT CCG ATG GCG GCC GAT TCY ACT CCG AGC	this study
AaMt_start_*Xho*I	CTC GAG AAA AGA ATG GCG GCC GAT TCC ACT CCG	this study
AaMt_end_*Bam*HI	GGA TCC CTA CGC AAG CTC AAG CTC GAT C	this study
AaMt_end_*Xho*I	GGC TCG AGC GCA AGC TCA AGC TCG ATC AG	this study

Wobble bases: N: A, G, T or C; S: G or C; W: A or T; R: A or G; Y: C or T.

All PCR products were gel purified (MinElute Gel Extraction Kit, Qiagen, Germany), ligated into pDrive vector (Qiagen, Germany) according to the manufacturer´s recommendation and sequenced (Eurofins MWG Operon, Germany) with T7 promoter sequencing primer or SP6 promoter sequencing primer.

2.2.3 Expression of the putative methyltransferase Mt6

The obtained cDNA of the gene Mt6 was cloned via the restriction sites *Bam*HI and *Xho*I into expression vectors for *E. coli* and *Kluveromyces lactis*. For the overexpression in *E. coli* the AaMt_start_*Bam*HI x AaMt_end_*Xho*I fragment was used and cloned into the pET-21b vector (Novagen, Germany). The expression strain was *E. coli* BL21(DE3)pLysS (Stratagene, Germany). For the expression an overnight preculture was cultured in LB broth (10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract) at 37 °C and 200 rpm. The main culture (LB broth) was inoculated 1:100 with the preculture and incubated at 37 °C and 200 rpm to an OD_{600} of 0.6. The protein expression was induced with 1 mM IPTG (final concentration) and the culture was further cultivated at 30 °C and 200 rpm. Samples (10 ml) were taken after 2 h, 3 h, 4 h and 5 h, centrifuged and the pellet was dissolved in 100 mM Tris/HCl pH 8.0. Cells were disrupted via sonification and centrifuged again. Proteins in both supernatant and pellet fractions were separated on a SDS gel and stained with Coomassie Blue R-250 (Imperial Protein Stain, Thermo Scientific, Germany). Protein concentrations were measured photometrically with Bradford reagent (Bio-Rad Protein Assay, Bio-Rad, Germany).

For the overexpression in the yeast *K. lactis* the fragment AaMt_start_*Xho*I x AaMt_end_*Bam*HI was cloned into the vector pKLAC2 (New England Biolabs, Germany). Transformation of yeast cells and protein expression were conducted with *K. lactis* Protein Expression Kit (New England Biolabs, Germany) according to the manufacturer. Since the protein is fused to a secretion signal lysis of yeast cell is not necessary to achieve the overexpressed protein. The secretion signal is removed by Kex protease processing. Culture supernatant samples were analyzed for protein expression on SDS-PAGE.

2.3 Protein biochemical methods

2.3.1 Protein extraction and activity assay

For the protein extraction *A. alternata* was grown for 6 days in liquid rice-medium in surface culture. The mycelium was harvested, washed in deionized water, blotted dry between filter papers and immediately ground to a fine powder in liquid nitrogen. Frozen not-ground mycelium was stored at -80 °C for months without losing protein activity. All following steps were processed at 4 °C or on ice. The powder was transferred to a glass beaker and was solved under gently stirring in the indicated buffer. The obtained viscous solution was centrifuged at 10,000 g for 20 min and the supernatant was again centrifuged at 58,000 g for 1 h. Protein concentration was determined as described in section 2.2.3. The reaction mixture (1 ml) for the activity test contained 50 mM of the respective buffer, 1.25 mM $MgCl_2$, 1 mM SAM, 26 µM AOH and approximately 100 mg protein crude extract. The mixture was incubated at 30 °C overnight or for the indicated time period. The enzyme activity was determined by measuring the conversion of the substrate AOH to AME by HPLC. The reaction was stopped by adding 0.5 ml of ethylacetate. which was also the first extraction step of AOH and AME. After a second addition of 0.5 ml ethylacetate the combined organic phases were evaporated to dryness and the residues were dissolved in 100 µl methanol. The standard HPLC device (Agilent 1100 Series, Agilent, Germany) was equipped with a 25 cm reversed phase column (Luna 5 µm C18(2), Phenomenex, Germany). The analyses were performed at 30°C and a flow rate of 0.7 ml/min. Mobile phase solution was methanol/0.1 M NaH_2PO_4 (2:1), pH 3.2 (according to Shephard *et al*., 1991). Mycotoxins were monitored with a UV detector at 280 nm. For quantification a standard curve with mycotoxin standard solutions was prepared. The standards were purchased from Sigma-Aldrich (Germany) and solved in methanol. Retention times 10.2 ± 0.2 min (AOH) and 23.3 ± 0.1 min (AME).

2.3.2 Protein purification

For the purification of the AOH-*O*-methyltransferase the fast protein liquid chromatography (FPLC) system ÄktaExplorer (GE Healthcare, Germany) was used. For a first screening for appropriate column materials the 1 ml HiTrap Columns (GE Healthcare, Germany) Q Sepharose™ Fast Flow (anion-exchanger), DEAE Sepharose™ Fast Flow (anion-exchanger) and CM Sepharose™ Fast Flow (cation-exchanger) were tested. The ion-exchange columns were equilibrated with the respective buffer which was used for crude extract preparation. Elution was performed with a linear gradient of 0-1 M NaCl in equilibration buffer.

Additionally precipitation experiment with solid $(NH_4)_2SO_4$ were conducted. Samples of crude extract in 100 mM NaPP pH 7.0 were brought to 20%, 40 % and 60 % under gently stirring on ice while keeping the pH to 7.0. After 30 min of precipitation the protein was pelleted by centrifugation at 20,000 g for 30 min. Pellet was dissolved in 100 mM NaPP buffer pH 7.0 and both supernatant and redissolved pellet were tested for activity.

3. Results

3.1 Amplification of putative *O*-methyltransferase gene fragments of *A. alternata*

The genome of *A. brassicicola*, closely related to *A. alternata*, was scanned for SAM dependent *O*-methyltransferases. 7 putative methyltransferases were found: AB06792.1 (AbMt6), AB06186.1 (AbMt5), AB01408.1 (AbMt037), AB07405.1 (AbMt8), AB04168.1 (AbMt3), AB05241.1 (AbMt4) and AB01342.1 (AbMt035). The protein sequences were aligned to determine conserved residues or domains which are shown in figure 1.

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                10         20         30         40         50
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   ---------- ---------- ---------- ---------- ----------
AbMt6   ---------- ---------- ---------- ---------- ----------
AbMt035 ---------- ---------- ---------- --MSTSHSRI AELAAEVVKH
AbMt037 ---------- ---------- ---------- ---------- ----------
AbMt3   MPAFPPTLTS HHIGGCLEAA AGKASYLSAQ AFEFPSSDLE YGKMGSVQQH
AbMt4   ---------- ---------- ----MSFTSI LLAFDKLSRP TERPYIMIAE
AbMt5   ---------- ---------- ---------- ---------- ----------

                60         70         80         90        100
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   ----MSAIPS LPEPSIAAPD EGIVADNVLE ALEALAAGNI TASLR--DNE
AbMt6   ---------- ----MAADAS PSKAALSLLD NIAQLSDGFR KASPG-----
AbMt035 TQQIDEHLNS NGLPYPSFHA EGPVDLGLPP DLEHSRAAVL RASQ---ELN
AbMt037 ---------- ----MLSFQD TIKTLDSIQP SQFSTDAERY EGKEAARRLL
AbMt3   EIATLNSLAA QISELAAKMT KQLEAEKVTP VTLEADSPIK YEKLPG-DVF
AbMt4   HQSTLLELAK EVQQLTTKIV NDLTEKKVPE PSFAIDSDTI PETP---EQI
AbMt5   ---------- ---------- ---------- ---------- ----------

               110        120        130        140        150
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   EKRLRFLEAA RMASVKLEQP WDTMQRLIFC ALPP--NMVQ VGINLGLWRL
AbMt6   -AREGLLDAC RSLIAEVSHP SENMLQLLWA QPAHLSTLWM GVEVKLFHAM
AbMt035 DLLQHPRDLL FNHHVRIRTT RISKLQFFVL ISSQHNALVY LQLISRFEIA
AbMt037 GRLELPFEQA WRLSFETPVL IAGIQTILDL GIWKQWTETD KQNPGASVHL
AbMt3   MTRQLLEDAL KDMWILSQGP SESVFNYVHM AIP---DAAC LNVLNQFDFW
AbMt4   GLRARLNDAA RDLLRLVNGP RNDARTFVCY LY----DLAA WQVACEFNFF
AbMt5   ---------- ---------- ---------- ---------- ----------

               160        170        180        190        200
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   LAKQQGAV-M SVGEMAVELR AEKALLVRVL RWAATQWMVE QVGV---ETY
AbMt6   KHVPDAGAAV HDIAAKCDKN VDPVVVGRML RHLAAMGTVR ETGP---DTF
AbMt035 GKVPVNGE-I TFSRLAAAIG IDEGAMARIL RLGIAYRIFR EPRQ--GVIA
AbMt037 DQLLKWAN-A RAEPNLLHWY ADIMAVGRFL RHIAALYVLE ETDV---DTW
AbMt3   GAVPVDGN-A TFEDIAKYTR LPFEVVSRVI DHAVTMRFFT KPSPTATSVK
AbMt4   EAIPEDGS-A TIQEIAGKVA MDEDRVGRFL RMLSSDRVFE EIEP--DVFK
AbMt5   ---------- ---------- ---------- ---------- ----------
```

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                  210        220        230        240        250
         ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8    RATNITRYLS M--SGFESVI FHVTERNIAL YNAIPKWLAD NAYKQPQDNK
AbMt6    ANTRTSAAFA E--PSYQDSI LYIAENFAPV HQSMKAYFEQ RDWKCPDSGL
AbMt035  HSAASRQIVD D--ARVADWV GASVDDMWPA AQKVVDALTK WP-DAAEPNR
AbMt037  KPTPYSLSLG DTVSHTDQIT QCGTDHTVPT GVNLPYFLKK YNYREPVDLA
AbMt3    HTSRSAALAK D--SGLSALV QMVLDETGPP MFLLPEALRR FSQGKPEISK
AbMt4    HTSRSVLYLK D--KQWRDVM HYQLDEFFRA ASETSESIKE SP-MVTDGQR
AbMt5    ---------- ---------- -------MGP TTQWTAYFAE NGLAEPPRSN

                  260        270        280        290        300
         ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8    NLPFNLSQ-N TDLHFFEWLS --QRPRHQQA FNEYMSFQRV GQKS------
AbMt6    DAPFQHTYNC KGSHYFEYFE --QNPEMGRR FASMMDSWSK GRPR------
AbMt035  TG-FSLAN-D TDLPFHVVLT --QSPQRARR FGGAMSFLTK GDG-------
AbMt037  KLDNYRDMTG GKDFFATCAA --DPVGKGSS FMGLMTALRN HKMS------
AbMt3    NI-KETAF-R LCHSGGEAWG --DHETSWEF IENDGEGEKK GWRQRNFVKF
AbMt4    NA-FVTRH-G VDL--FGYYK --QDPKRAAR FASAMAGVSR RKSSPNQSEA
AbMt5    RSPGGFALGM PDKTAYEIMA --AIPGLATR MNGAMAIDGD IPVTG-----

                  310        320        330        340        350
         ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8    ----WLDVFP LDKYVHASST GSDS--RPLF VDVGGGHGHQ SREVIRRFP-
AbMt6    -----WFSQD YYPVHDRLIS GADKD-APFL VDVGGGSGHD IEGLRQSFQG
AbMt035  --------HS LRYLTDGYPW AATP--PGTV VDLGGSHGDA AFALARRYPN
AbMt037  ----WTDVYD TNRIVDGAEL EAGK---PLF VDIGGAHGLD TSRLLDKHPN
AbMt3    MAYIKDLFHT ENIVLEAIDW KAEG--DITV VDLGGSAGHD DAVLATKFPN
AbMt4    TSLMTIVERH FDNLKESFPW DTIS--GRKV IDVGGGSGHM SVNLARAFPN
AbMt5    -------VYD FSWIATYAAE DAGEEKRELI VDVAGGKGQA LKDILEETPA
                                                  Motif I
                  360        370        380        390        400
         ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8    --WVQGRVVL QDTHAAAIDS --AKSIQGLE VVHHDFTK-- ---AQPTKGA
AbMt6    ---QLPGTLV LQDRPEIVDL --AKLGPGAE AMAHDFMT-- ---EQPVKDA
AbMt035  HHFIVQD--- -LPEVVKNSR --PEYGLNVE FMAHDFFD-- ---EQPVKGA
AbMt037  ---LPANVLI LQDTPEVVAM PIEDLDKRIV KQAYDFFT-- ---PQPQMHA
AbMt3    LKIVVQDLPE VATVFEKEFP --SDLKSRVS FRTHNLFD-- ---PQPVQ-A
AbMt4    LELIVQDSLT MLSSASQNDF --SDLNGRVT FMPHDFFT-- ---KQPVSGA
AbMt5    ---IPAARCV LQDQPHVIAE AVEEHKDSAV LGPVKKIGSS IFGEQPTKGA
                                                        Motif II
                  410        420        430        440        450
         ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8    CVYYLRNILH DWPDQACFNI LSQLKDALAP ---DSVILLD ELILG-----
AbMt6    RAYYLHSIIQ DWNDEVNTAI LKAIVPAMKK G--YSKVLIN DFVVP-----
AbMt035  EVYYYRWTLH NWPDKYCAKT LRALIPALKP ---GAKLLIM DVV---MPSP
AbMt037  RAYFYHAVPH DWPDADCVRM FSQVAAVFKR G--YSKLLIY EVVLP-----
AbMt3    DIYMLKWILH DWPDAESVKI LQALRPALRP ---GARVLFI DYVGKQEPSD
AbMt4    AAYLLRYITH NWSDEDCIRI FRALVPALEK SPAGTPILIN DVV---MPAL
AbMt5    LVYYIRRVLN DWSDHEALQI LKNVRAACAD D---SRVLIA EYLRP-----
                  Motif III
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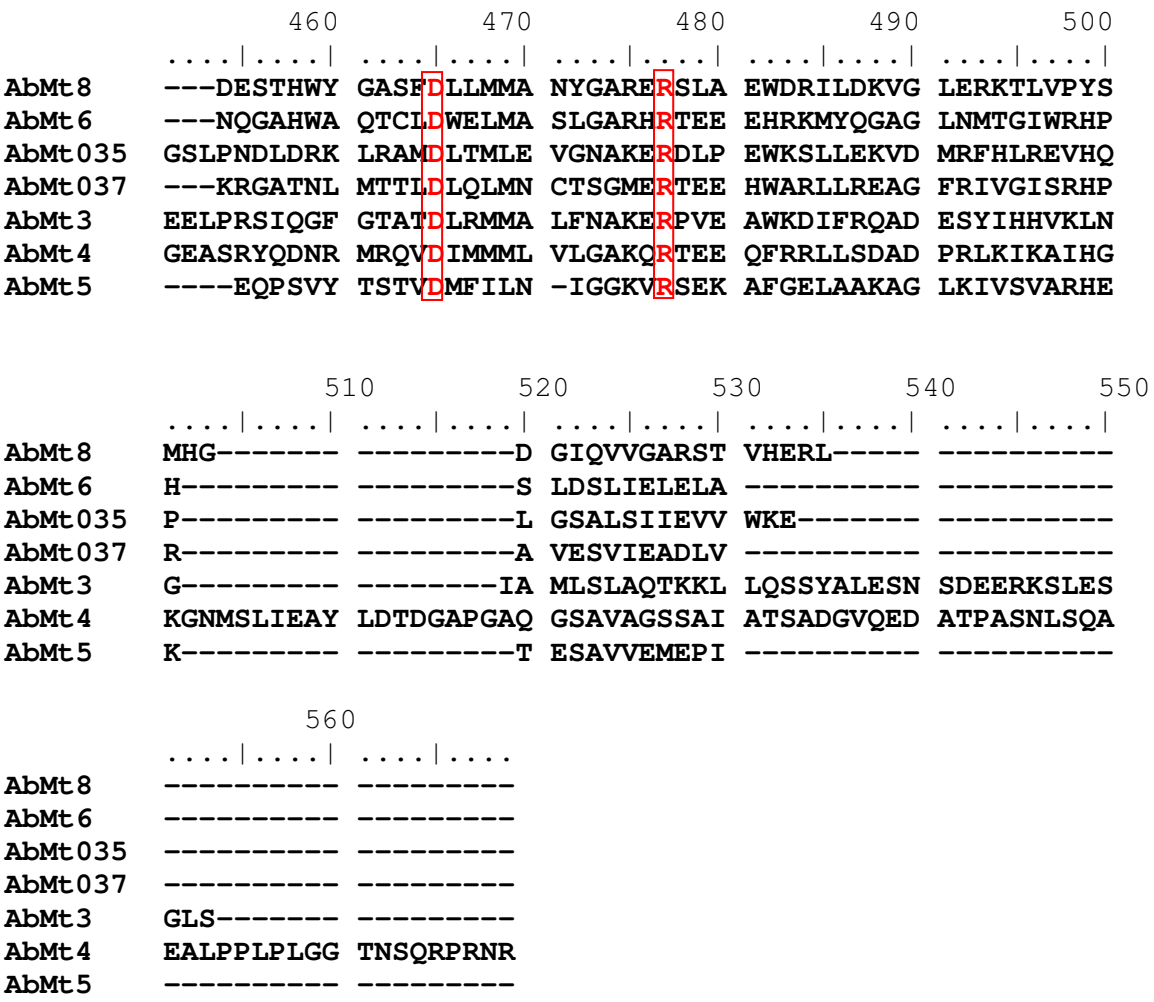

Figure 1: Alignment of putative SAM-dependent O-methyltransferase protein of *A. brassicicola* (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html). Conserved residues or domains were marked with red boxes.

Three conserved motifs were indentified: Motif I consisting of (F/L/V/I) (V/I) D (V/I/L) G G G/S X G, motif II comprising of Q P (V/T) K (G/D) A and motif III containing H D W P D. Additionally the sequences were blasted against the NCBI data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to detect further similarities in homologous genes of other fungi. Based on this results primer were generated (see table 1) and used for the amplification of gene fragments in *A. alternata*. 4 out of 7 approaches were successful and fragments could be amplified. The fragments were 269 bp - 524 bp long and shared high similarity with the homologous genes in *A. brassicicola*. The 4 gene fragments of *A. alternata* were aligned with the respective regions of the homologs of *A. brassicicola* (figure 2).

A

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                 160        170        180        190        200
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   CTGGAGGCTG CCAGGATGGC CTCTGTCAAG CTTGAGCAAC CATGGGACAC
AaMt8   ---------- ---------- --------AG ATTGAGCAGC CATGGGATAC

                 210        220        230        240        250
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   TATGCAGCGG CTGATCTTCT GCGCACTGCC GCCCAACATG GTGCAAGTGG
AaMt8   CATGCAACGA TTGATCTTTT GTGCACTACC GCCCAACATG GTGCAAGTTG
        ---------->
        Mt8for
                 260        270        280        290        300
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   GCATTAATCT GGGGCTCTGG AGGCTACTTG CCAAGCAGCA AGGTGCTGTG
AaMt8   GTATTGATCT TGGGCTGTGG AGGCTACTCA CTAAGCGAGA GGGTGCAGTG

                 310        320        330        340        350
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   ATGAGTGTGG GTGAGATGGC TGTGGAGTTG CGCGCTGAAA AGGCACTACT
AaMt8   ATGAGTGTGA GTGAGATGGC TGTGGAGTTG GGTGCGGAAA AAGCACTGTT

                 360        370        380        390        400
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   AGTGCGAGTG CTGCGGTGGG CCGCGACACA ATGGATGGTG GAGCAGGTTG
AaMt8   AGTGCGAGTG TTGCGGTGGG CTGCGACGCA ATGGATGGTG GAGCAGGTTG

                 410        420        430        440        450
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   GCGTCGAGAC GTACCGGGCG ACAAACATTA CGCGATACCT GTCCATGTCA
AaMt8   GCGTCGAGAC GTACCGTGCA ACAAACATTA CGCGATATCT TTCCATGTCA

                 460        470        480        490        500
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   GGCTTCGAGT CTGTGATTTT CCACGTGTGC GTACTTGCGG CATTTGGTTT
AaMt8   GGGCTAGAGT CTGTCGTATT CCATGTGTGA GTACT--CCG TGTCTGGTCT
                                           ----------

                 510        520        530        540        550
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   TCCAAAGACC ATGACTGACT TGCTTTGACA GGACCGAGAG GAACATTGCA
AaMt8   T--GACAATC ACAGCTGACT CTCTTTG-CA GAACAGAAAG GAATATTGCA
        ----------                    -

                 560        570        580        590        600
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt8   CTGTACAACG CGATACCAAA GTGGCTAGCC GACAATGCCT ACAAGCAACC
AaMt8   CTTTACAACG CGATACCAAA GTGGCTAGCC GACAATTCCT ACAAACAGCC
                                                   <----------
                                                   Mt8rev
                 610        620        630
        ....|....| ....|....| ....|....|
AbMt8   ACAGGACAAC AAAAACCTTC CATTCAATCT
AaMt8   ACAAGACAAC AA-------- ----------
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B

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                710         720         730         740         750
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   CCGACAAGGA TGCACCGTTC CTTGTCGACG TTGGTGGCGG CTCTGGACAC
AaMt6   ---------- --AGATTTTC CTTGTCGACG TTGGTGGC-- -TCTGGGCAC
                                                   Mt6for
                760         770         780         790         800
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   GACATTGAAG GCCTAAGGCA GTCGTTCCAG GGCCAGCTAC CCGGTACGCT
AaMt6   GATATTGAAG GTCTGAGGGA GGCATTCCAT GGCCAGATAC CAGGTACACT

                810         820         830         840         850
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   GGTGCTACAA GATAGACCCG AGATTGTTGA CCTTGCCAAA CTTGGTCCCG
AaMt6   GGTGCTACAG GACAGGCCGG AGATTGTTGA GCTTGCGAAA CTTGGTCCTG

                860         870         880         890         900
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   GCGCCGAGGC CATGGCACAC GACTTCATGA CCGAGCAACC GGTCAAAGAT
AaMt6   GGACGGAGGC GACGGCACAC GACTTCATGA CTGAGCAACC TGTGAAAGGT

                910         920         930         940         950
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   GCACGGGCAT ATTATCTACA TTCCATCATC CAAGATTGGA ACGATGAGGT
AaMt6   GCGCGGGCGT ATTATCTGCA TTCGATTATC CAGGACTGGA ATGATGAGGT

                960         970         980         990         1000
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6   CAATACTGCC ATTCTCAAGG CAATCGTGCC CGCCATGAAG AAGGGATATT
AaMt6   AAACACCGAA ATTCTCAAGG CAATCGTGCC CGCCATGAAT CAC-------
                  Mt6rev
```

C

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                710         720         730         740         750
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037 GGGCAAGGGA AGCAGTTTCA TGGGTCTGAT GACTGCGTTG CGGAACCATA
AaMt037 ---------- ---------- ---------- ---------- -GGNACCATA

                760         770         780         790         800
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037 AGATGAGCTG GACAGACGTA TATGACACGA ACCGCATCGT CGATGGTGCA
AaMt037 AGATGAGCTG GACAGATGTG TACGATACGA ACCGCATCGT CGATGGTGCT
        Mt037for
                810         820         830         840         850
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037 GAGCTAGAGG CTGGGAAACC ATTGTTCGTA GACATCGGTG GCGCGCACGG
AaMt037 GATGTCCAGA CTGGAAAACC TTTGTTTGTG GACATTGGCG GCGCGCACGG

                860         870         880         890         900
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037 TCTCGATACT TCGCGTCTTC TGGATAAACA TCCCAATTTG CCCGCCAACG
AaMt037 TCTCGATACT TCGCGTCTCC TCGACAAGCA TCCAAACCTG CCTGCCAACG
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                910        920        930        940        950
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  TCCTGATTCT GCAAGATACC CCGGAAGTCG TCGCCATGCC GATAGAGGAT
AaMt037  TTCTGGTCCT GCAAGATACT CCCGAAGTCG TCGCAATGCC CATTGAAGAC

                960        970        980        990        1000
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  CTGGACAAGA GGATCGTGAA ACAAGCCTAC GACTTCTTCA CACCTCAGCC
AaMt037  TTGGATAAGA GGATAGTGAA ACAAGCCTAC GACTTCTTCA CGCCTCAACC

                1010       1020       1030       1040       1050
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  CCAAATGCAC GCACGCGCTT ATTTCTACCA TGCCGTGCCA CACGACTGGC
AaMt037  CCAAAAGCAC GCGCGCGCTT ACTTCTTCCA TGCCGTACCC CACGACTGGC

                1060       1070       1080       1090       1100
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  CTGACGCGGA CTGCGTACGC ATGTTTTCGC AAGTAGCCGC TGTATTCAAA
AaMt037  CTGACGCGGA CTGCGTTCGC ATGTTCTCGC AAGTGGCCGC TGTGTCCAAG

                1110       1120       1130       1140       1150
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  CGAGGATACT CCAAGCTTTT GATCTATGAG GTCGTGCTAC CGAAGAGGGG
AaMt037  CGCGGATACT CCAAGCTCCT GATATACGAG GTTGTGTTGC CGAAGAAGGG

                1160       1170       1180       1190       1200
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  TGCCACGAAC TTGATGACTA CGCTGGACCT GCAGCTTATG AACTGTACAA
AaMt037  AGCCACGAAC TTGATGACGA CGTTGGATTT GCAGCTCATG AACTGCACGA
                                                   <-- Mt037rev
                1210       1220       1230       1240       1250
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt037  GTGGAATGGA GAGGACGGAG GAGCACTGGG CGAGGTTGTT GAGGGAAGCT
AaMt037  GTGGCATGAA TCTGA----- ---------- ---------- ---------
```

D

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                860        870        880        890        900
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt5    GCGCGACAGC CCTTTTGTCA ACGCCTATCA GTGCAAAGGC GAGACCTACT
AaMt5    ---------- ---------- ---------- ---------- GAGACCTACT

                910        920        930        940        950
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt5    TCGACTACAT GAACAAGCCC GAAAACGTGC GCATGTTTGA TGCATTCAAT
AaMt5    TCGATTATAT GAACAAGCCC GAGAACGCAC GCATGTTCGA TGCATTCAAT
          --> Mt5for
                960        970        980        990        1000
        ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt5    GAGACGATGA CGCTGCGGAA GCCAGGCGAG CACGACACAT TTGTGCAAGC
AaMt5    GGGACGATGA CACTCCGGAA GCCAGGCGAG CACGACACGT TCGTTGCAGC
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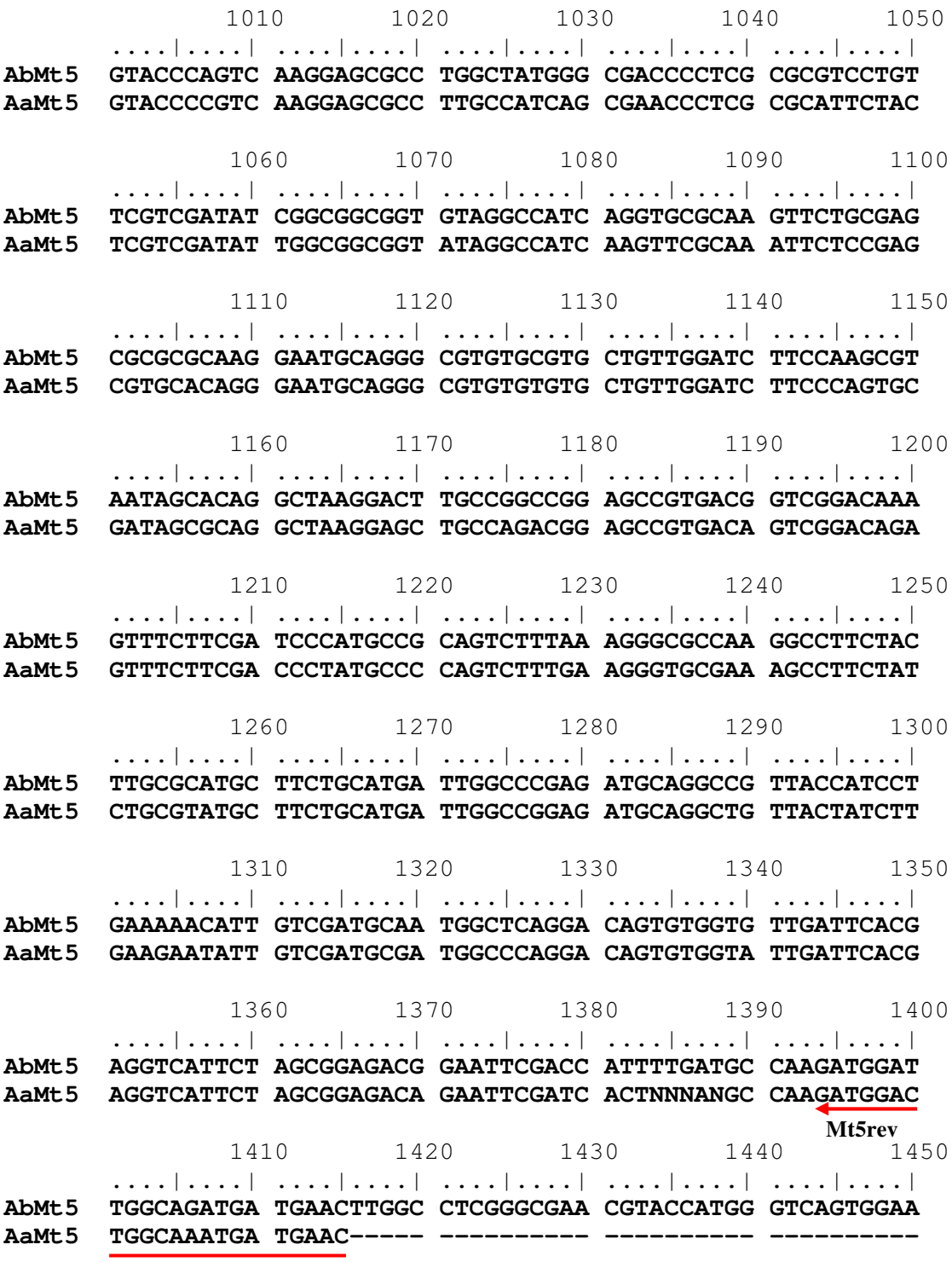

Figure 2: Alignment of putative SAM-dependent *O*-methyltransferase gene fragments of *A. alternata* (Aa) with fragments of *A. brassicicola* (Ab). **A.** Alignment for methyltransferase 8 (Mt8). **B.** Alignment for methyltransferase 6 (Mt6). **C.** Alignment for methyltransferase 037 (Mt037). **D.** Alignment for methyltransferase 5 (Mt5).
Primer position: red arrow; putative intron position: dashed black line.

3.2 Identification of the whole genomic and cDNA sequence and expression of Mt6 in *A. alternata*

Using TAIL-PCR the 5´-end of the Mt6 gene could be identified while the 3´-end was determined by 3´RACE-PCR. The genomic fragment consisted of 1259 bp. The cDNA sequence was finally obtained by RT-PCR and cloned into pDrive vector. The cDNA fragment has a length of 1212 bp. When compared with the genomic sequence the location of a single intron of 47 bp was detected. The intron position in the gene of *A. alternata* matches well with the one in *A. brassicicola*. For further cloning into expression vectors the PCR product was tagged with *Xho*I and *Bam*HI restrictions sites. Both the genomic and cDNA sequence were aligned with the corresponding gene of *A. brassicicola* as shown in figure 3. All employed primers were marked within the sequence and the putative SAM binding domain was highlighted.

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                    10         20         30         40         50
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     ATGGCCGCCG ATGCAAGCCC GAGCAAGGCT GCCCTCTCCC TCCTCGACAA
AaMt6 gDNA     ATGGCGGCCG ATTCCACTCC GAGCAAGGCT GCTCTTTCTC TCCTCGACAA
AaMt6 cDNA     ATGGCGGCCG ATTCCACTCC GAGCAAGGCT GCTCTTTCTC TCCTCGACAA
               AaMt6_for2 / AaMt_start_BamHI /_XhoI
                    60         70         80         90        100
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     CATTGCGCAG CTCAGCGATG GCTTCAGGAA GGCCAGTCCC GGCGCCCGCG
AaMt6 gDNA     CATTGCGCAA CTCAGCGATG GCTTCAAGAA TGGGAGTCCT GGCGCGAGAG
AaMt6 cDNA     CATCGCGCAA CTCAGCGATG GCTTCAAGAA TGGGAGTCCT GGCGCGAGAG

                   110        120        130        140        150
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     AAGGGCTGCT GGACGCGTGC AGGAGCCTCA TCGCCGAAGT CAGCCATCCA
AaMt6 gDNA     AAGGGTTGCT GGACGCGTGT AGGGAGCTCA TCGCGGAAGT CAGCAATCCA
AaMt6 cDNA     AAGGGTTGCT GGACGCGTGT AGGGAGCTCA TCGCGGAAGT CAGCAATCCA
                          TAIL2revnested
                   160        170        180        190        200
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     TCCGAGAACA TGCTGCAGCT GTTATGGGCG CAGCCCGCCC ATCTGAGCAC
AaMt6 gDNA     TCTGAGAATA TGCTGCAATT GTTATGGGCG CAACCCGCAC ATCTGAGCAC
AaMt6 cDNA     TCTGAGAATA TGCTGCAATT GTTATGGGCG CAACCCGCAC ATCTGAGCAC
                                     TAIL2rev
                   210        220        230        240        250
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     ACTCTGGATG GGCGTCGAAG TCAAGCTCTT CCACGCCATG AAGCATGTCC
AaMt6 gDNA     ACTTTGGATG GGCGCGGAAG TGAAGCTGTT TCAGGCCATG AAAGACGTCC
AaMt6 cDNA     ACTTTGGATG GGCGCGGAAG TGAAGCTGTT TCAGGCCATG AAAGACGTCC

                   260        270        280        290        300
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA     CGGACGCGGG TGCTGCCGTC CACGACATCG CGGCCAAGTG CGACAAGAAC
AaMt6 gDNA     CGGAATCTGG TGCTACTGTT GGAGACATCG CGAAGAAGTG CGAAAAGAAT
AaMt6 cDNA     CGGAATCTGG TGCTACTGTT GGAGACATCG CGAAGAAGTG CGAAAAGAAT
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                  310         320         330         340         350
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GTTGACCCAG TCGTCGTCGG GCGCATGCTT CGTCATCTCG CTGCCATGGG
AaMt6 gDNA   GTTGATCCGA TTATCGTTGG ACGCATGCTT CGACATCTTG CTGCTATGGG
AaMt6 cDNA   GTTGATCCGA TTATCGTTGG ACGCATGCTT CGACATCTTG CTGCTGTGGG

                  360         370         380         390         400
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CACGGTGCGC GAGACTGGCC CGGATACTTT TGCAAACACA CGCACCTCAG
AaMt6 gDNA   CACAGTGCGC GAGACTGGTC CCGGTACCTT TGCAAACACT CCTACTTCTT
AaMt6 cDNA   CACAGTGCGC GAGACTGGTC CCGGTACCTT TGCAAACACT CCTACTTCCT

                  410         420         430         440         450
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CTGCCTTTGC TGAACCATCC TACCAAGACT CGATCCTGTA CATTGCCGAA
AaMt6 gDNA   CCGCATTCGC CGAACAATCG TATCAAGACT CAATAAAATA TATCGCAGAG
AaMt6 cDNA   CCGCATTCGC CGAACAATCG TATCAAGACT CAATAAAATA TATCGCAGAG

                  460         470         480         490         500
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   AACTTTGCGC CGGTCCATCA GTCCATGAAG GCGTACTTTG AGCAGCGTGA
AaMt6 gDNA   AACTTTGCGC CGGCGCATCA ATCCATGAAG TCGTACTTTG ATCAGCGTGA
AaMt6 cDNA   AACTTTGCGC CGGCGCATCA ATCCATGAAG TCGTACTTTG ATCAGCGTGA

                  510         520         530         540         550
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CTGGAAATGT CCCGATTCCG GCCTCGATGC GCCTTTTCAG CACACCTACA
AaMt6 gDNA   CTGGAAATGC CCCGACTCTA GTCTCGATGC GCCATTCCAG CATGCCTACA
AaMt6 cDNA   CTGGAAATGC CCCGACTCTA GTCTCGATGC GCCATTCCAG CATGCCTACA

                  560         570         580         590         600
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   ACTGCAAAGG GAGCCATTAC TTTGAGTACT TTGAGCAAAA CCCCGAAATG
AaMt6 gDNA   ACTGCAAGGG AACCCACTAC TTTGAATTCT TCCAGAAAAA CCCTGAAGTA
AaMt6 cDNA   ACTGCAAGGG AACCCACTAC TTTGAATTCT TCCAGAAAAA CCCTGAAGTA

                  610         620         630         640         650
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GGACGCCGCT TCGCTAGCAT GATGGACTCG TGGAGCAAGG GACGGCCGCG
AaMt6 gDNA   GGACGGCGCT TCGCTAGTAT GATGGACTCT TGGAGCAAGG GACGGCCGCG
AaMt6 cDNA   GGACGGCGCT TCGCTAGTAT GATGGACTCT TGGAGCAAGG GACGGCCGCG

                  660         670         680         690         700
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GTGGTTCTCC CAGGATTACT ACCCGGTCCA CGATCGCTTG ATCAGCGGTG
AaMt6 gDNA   GTGGTTCTCC AAGGATTACT ACCCTGTCGA AGATCGGCTG ATCAGCGGTG
AaMt6 cDNA   GTGGTTCTCC AAGGATTACT ACCCTGTCGA AGATCGGCTG ATCAGCGGTG

                  710         720         730         740         750
             ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CCGACAAGGA TGCACCGTTC CTTGTCGACG TTGGTGGCGG CTCTGGACAC
AaMt6 gDNA   CCGAGAAGGA TGTACCGTTT CTCGTCGACG TAGGCGGCGG CTCTGGGCAC
AaMt6 cDNA   CCGAGAAGGA TGTACCGTTT CTCGTCGACG TAGGCGGCGG CTCTGGGCAC
                                    V   D   V   G   G   G   Mt6for
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```
                    760         770         780         790         800
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GACATTGAAG GCCTAAGGCA GTCGTTCCAG GGCCAGCTAC CCGGTACGCT
AaMt6 gDNA   GATATTGAAG GTCTGAGGGA GGCATTCCAT GGCCAGATAC CAGGTACACT
AaMt6 cDNA   GATATTGAAG GTCTGAGGGA GGCATTCCAT GGCCAGATAC CAGGTACACT

                    810         820         830         840         850
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GGTGCTACAA GATAGACCCG AGATTGTTGA CCTTGCCAAA CTTGGTCCCG
AaMt6 gDNA   GGTGCTACAG GACAGGCCGG AGATTGTTGA GCTTGCGAAA CTTGGTCCTG
AaMt6 cDNA   GGTGCTACAG GACAGGCCGG AGATTGTTGA GCTTGCGAAA CTTGGTCCTG

                    860         870         880         890         900
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GCGCCGAGGC CATGGCACAC GACTTCATGA CCGAGCAACC GGTCAAAGAT
AaMt6 gDNA   GGACGGAGGC GATGGCACAC GATTTCATGA CTGAGCAACC TGTGAAAGGT
AaMt6 cDNA   GGACGGAGGC GATGGCACAC GATTTCATGA CTGAGCAACC TGTGAAAGGT
                                                 <-- TAIL1revnested
                    910         920         930         940         950
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GCACGGGCAT ATTATCTACA TTCCATCATC CAAGATTGGA ACGATGAGGT
AaMt6 gDNA   GCGCGGGCGT ATTATCTGCA TTCGATTATC CAGGACTGGA ATGATGAGGT
AaMt6 cDNA   GCGCGGGCGT ATTATCTGCA TTCGATTATC CAGGACTGGA ATGATGAGGT
                                              <-- TAIL1rev
                    960         970         980         990        1000
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CAATACTGCC ATTCTCAAGG CAATCGTGCC CGCCATGAAG AAGGGATATT
AaMt6 gDNA   AAACACCGAA ATCCTCAAGG CAATAGTGCC AGCCATGAAG AAGGGGTATT
AaMt6 cDNA   AAACACCGAA ATCCTCAAGG CAATAGTGCC AGCCATGAAG AAGGGGTATT
                       <-- Mt6rev
                   1010        1020        1030        1040        1050
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   CAAAGGTCCT CATCAACGAT TTTGTGGTTC CAAATCAGGG CGCGCATTGG
AaMt6 gDNA   CCAAGGTCCT TGTGAGCGAC TTTGTAGTCC CGAACCAAGG CGCGCATTGG
AaMt6 cDNA   CCAAGGTCCT TGTGAACGAC TTTGTAGTCC CGAACCAAGG CGCGCATTGG

                   1060        1070        1080        1090        1100
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GCGCAGAGTA GGCTGTCCTC GTCTCTTGAT GCCAGCACCT GCTAACGTAA
AaMt6 gDNA   GCGCAGAGTA AG-TCTTCTG GTTTAATGAT ATGACATGCG ACTAACGAAC
AaMt6 cDNA   GCGCAGA--- ---------- ---------- ---------- ----------
                      - - - - - - - - - - - - - - - - - - - - - - - -
                   1110        1120        1130        1140        1150
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   AATAGCATGC CTTGACTGGG AGCTCATGGC CAGTCTTGGA GCCCGACACC
AaMt6 gDNA   GGTAGCTTGT CTCGACTGGG AGCTCATGGC CAGTCTTGGA GCGCGACACC
AaMt6 cDNA   -----CTTGT CTCGACTGGG AGCTCATGGC CAGTCTTGGA GCGCGACACC
             - - - -
                   1160        1170        1180        1190        1200
               ....|....| ....|....| ....|....| ....|....| ....|....|
AbMt6 gDNA   GAACCGAAGA GGAGCACCGG AAAATGTACC AAGGTGCAGG TTTGAACATG
AaMt6 gDNA   GAACGGAAGA GGAACACCGA AAGATGTACG AAGGGGCAGG CTTGAAAATG
AaMt6 cDNA   GAACGGAAGA GGAACACCGA AAGATGTACG AAGGGGCAGG CTTGAAAATG
```

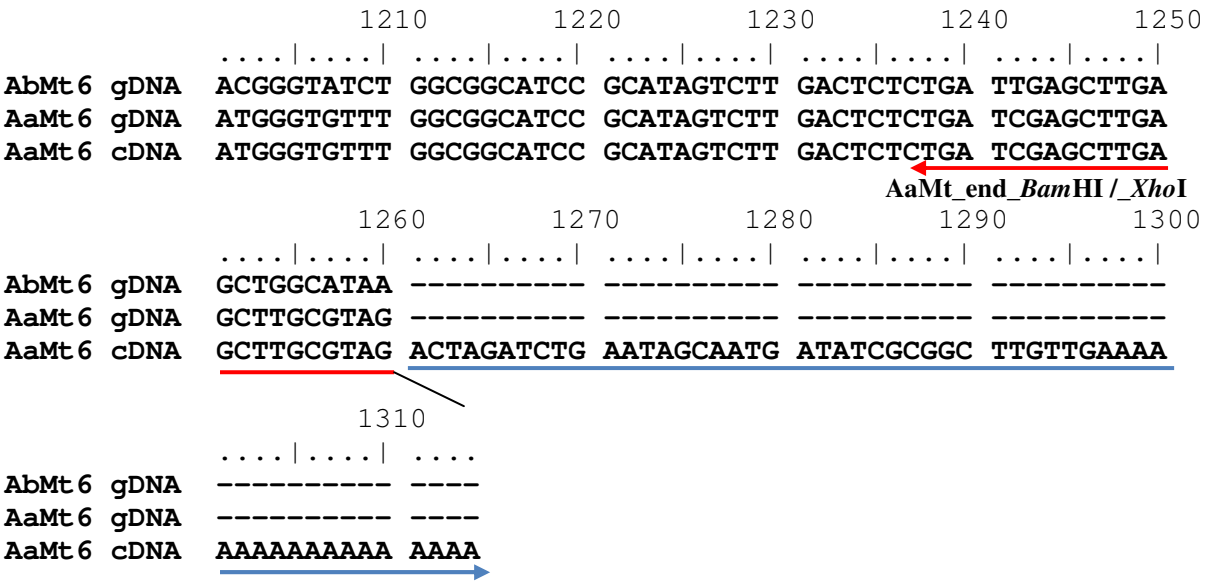

Figure 3: Alignment of the genomic and cDNA sequence of the putative SAM-dependent *O*-methyltransferase gene Mt6 of *A. alternata* (Aa) with the corresponding gene of *A. brassicicola* (Ab). Primers used for amplification: red arrow; intron sequence: black dashed line; 3´UTR: blue arrow; SAM binding domain: red box, the translated amino acid sequence is indicated below.

For the expression of the gene in *E. coli* the 5´-end was tagged with the restriction site *Xho*I and the 3´end with the restriction site *Bam*HI. The tagged fragment was cloned into the expression vector pET-21b. In a first overexpression experiment several clones were screened for proper gene expression after 2 h of induction and one clone was chosen for detailed experiments. These experiments revealed the presence of the protein as insoluble inclusion bodies in the pellet fraction while the protein could not be detected in the soluble supernatant fraction (figure 4).

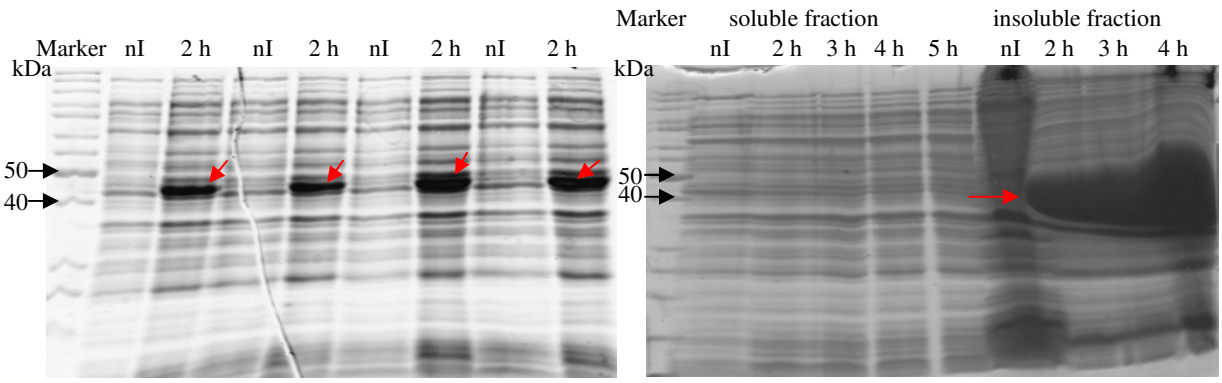

Figure 4: SDS PAGEs of overexpression experiments of *A. alternata* methyltransferase 6 protein in *E. coli*. Left: An initial screening of several clones. A separation of pellet and supernatant fraction was not conducted. Right: A more detailed experiment with one clone. Supernatant (soluble fraction) and pellet (insoluble fraction) were applied separately. The expressed protein has a molecular weight of approx. 45 kDa and is indicated by a red arrow. nI: not induced; 2h, 3h, etc.: time point of sampling after induction with 1 mM IPTG.

This result could not be enhanced by lowering expression temperature or decreased IPTG concentrations. Nevertheless the molecular mass of the protein was determined to be approx. 45 kDa. Activity tests of the pellet fraction were negative, i.e. AME production was not observed.

To overcome the problem of protein insolubility a eukaryotic expression system was tested. For the expression in the yeast *K. lactis* the gene fragment was amplified using the primer combination AaMt_start_*Bam*HI and AaMt_end_*Xho*I und cloned into the vector pKLAC2. The SDS-PAGE revealed no visible overexpression or rather secretion of an overexpressed protein in the culture broth (result not shown). The activity test with culture broth was also negative. The Blast search in the genome of *A. brassicicola* (see section 3.1) revealed the existence of min. 7 putative methyltransferases whereof 4 were also found in *A. alternata*. Due to the expression experiments it cannot be fully excluded that methyltransferase Mt6 exhibits AOH-*O*-methyltransferase activity, but it is also possible that one of the other genes encodes for the desired protein.

Therefore, further expression experiments were not conducted. A protein purification strategy was pursued instead.

3.3 Characterization of the AOH-*O*-methyltransferase of *A. alternata*

Prior to purification experiments pH- and salt stability of the protein was tested. 100 mM Tris and NaPP buffer in the range of pH 6 to pH 8 were prepared. Protein crude extract was prepared in the respective buffer and applied to the activity test. As shown in figure 5 A the protein was more active in NaPP buffer as in Tris buffer of the same pH value. The pH optimum for both buffers was around pH 7 but the pH tolerance was in range of pH 6.5 to pH 8. Salt tolerance tests were performed in 100 mM NaPP buffer pH 7. The tolerance towards 1 M NaCl, 1 M KCl and 1 M $(NH_4)_2SO_4$ was tested (see figure 5 B).

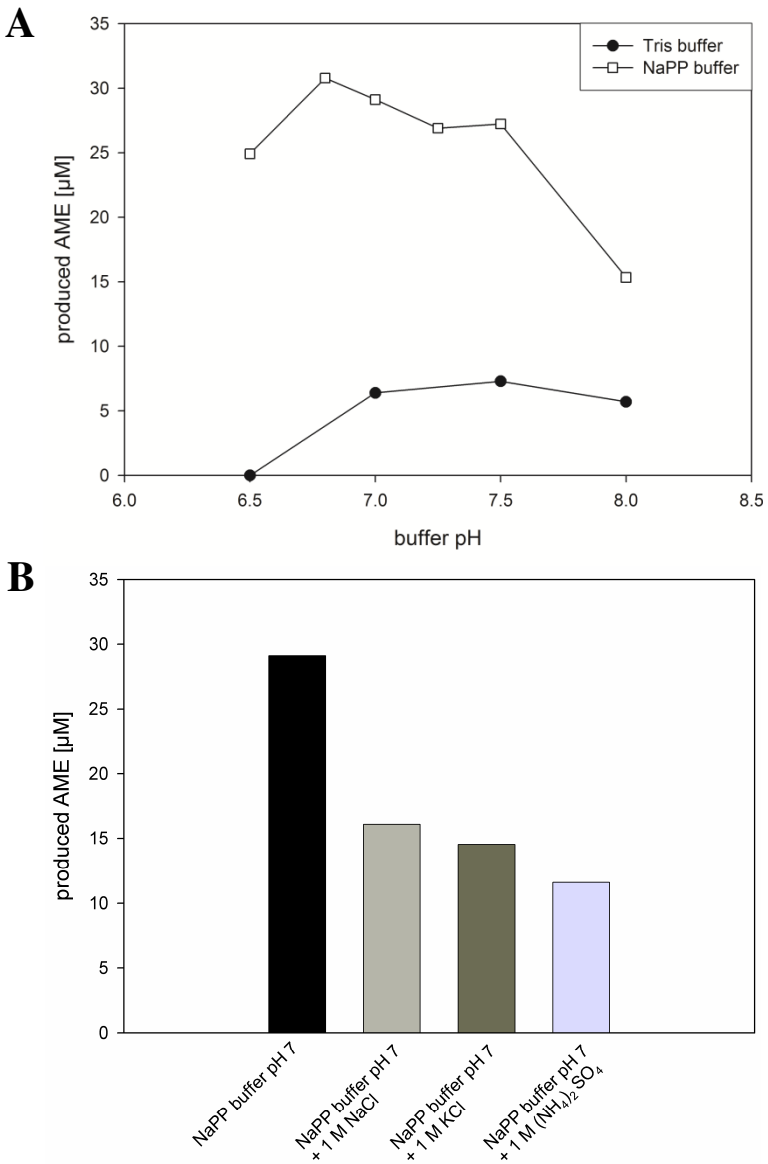

Figure 5: Salt and pH tolerance of *A. alternata* AOH-*O*-methyltransferase in protein crude extract. **A**. pH tolerance of the protein in 100 mM Tris and NaPP buffer. Protein crude extract was prepared in the respective buffer of the indicated pH and applied to the activity test. Activity was determined by detecting produced AME after 16 h. **B**. Salt tolerance of the protein. Protein crude extract was prepared in 100 mM NaPP buffer pH 7. 1 M of the indicated salt was added additionally to the activity test.

The protein activity was survived in the presence of the salts but was reduced to 55.25 % (NaCl), 49.93 % (KCl) and 39.8 % ($(NH_4)_2SO_4$).

3.4 Partial purification of the *A. alternata* AOH-*O*-methyltransferase

In a first step purification by ion exchange chromatography was tested. Protein crude extract was prepared in 50 mM MES buffer pH 6.5 for cation exchange chromatography and in 20 mM Tris/Cl buffer pH 8 for anion exchange chromatography. While the methyltransferase was not bound on CM Sepharose (cation exchanger) and the protein activity was detected in the flow-through it was bound on DEAE Sepharose (anion exchanger) and AOH-O-methyltransferase activity was eluted as a single peak with approx. 0.3 M NaCl. These results may be helpful for further experiments and a subsequent application to a second weaker anion exchanger appears to be reasonable.

A second strategy comprises a precipitation of protein and a subsequent application to hydrophobic interaction chromatography (HIC). Therefore $(NH_4)_2SO_4$ precipitation tests were performed and protein activity was determined in pellet and supernatant fraction after precipitation with 20 %, 40 % and 60 % $(NH_4)_2SO_4$. Approx. 70 % of protein activity was detected in the pellet fraction of 40 % $(NH_4)_2SO_4$ precipitation (table 4).

Table 4: Fractional precipitation of AOH-*O*-methyltransferase with solid $(NH_4)_2SO_4$. Shown are the measured protein activities expressed in percent in supernatant and pellet fractions.

Salt concentration	Activity supernatant	Activity pellet
0 %	100 %	---
20 %	70 %	10 %
40 %	15 %	65 %
60 %	0	80 %

First experiments using HIC resulted in a tight binding of AOH-*O*-methyltransferase on the column material independent which type of HIC was used, e.g. Phenyl-Sepharose, Butyl-Sepharose. An elution of protein was not possible. Further elution strategies are currently under investigation.

4. Discussion

Contamination of food and feeds with *Alternaria* toxins is an unpredictable problem concerning health risk of humans and livestock since risk assessment data are not available. For a better understanding of the regulation of *Alternaria* toxins the knowledge of the biosynthetic gene clusters is crucial. This work focuses on one gene involved in the biosynthesis of the most studied *Alternaria* toxins AOH and AME which product carries out the methylation of AOH to AME. The biosynthetic gene of many secondary metabolites and therefore mycotoxins are organized in clusters: aflatoxin (Yu *et al*., 2004), trichothecene (Kimura *et al*., 2007), fumonisin (Procter *et al*., 2003) and zearalenone (Kim *et al*., 2005). As shown for *O*-methyltransferase I gene (dmtA) from *Aspergillus parasiticus* cloning of this gene enables identification of neighbor genes by genome walking (Motomura *et al*., 1999). For the identification of gene sequences two strategies can be pursued: a genetic strategy which involves knowledge about conserved regions of the respective gene/enzyme class and a biochemical strategy which means a purification of the protein. For both strategies a reliable and fast activity test is necessary which was already published for the AOH-*O*-methyltransferase by Stinson and Moreau (1986) and Orvehed *et al*. (1988) and was adapted in this work.

SAM-dependent O-methyltransferases share little sequence identity but the structural fold is highly conserved (Martin and McMillan, 2002). As stated by Fauman *et al*. (1999) a comparison of several SAM-dependent methyltransferases revealed a 13 % protein sequence identity at best. Among the non-DNA methyltransferases only three conserved motifs can be found (Kagan and Clarke, 1994). The comparison of 7 putative SAM-dependent *O*-methyltransferases from *A. brassicicola* (figure 1) revealed three conserved motifs and five conserved residues within the alignment. Kagan and Clarke (1994) compared in their work a wide variety of different methyltransferases (84 in all) and named consensus sequences for the three found motifs. Motif I in their work corresponds to motif I in this work and comprises of the consensus sequence (V/I/L) (L/V) (D/E) (I/V) G (G/C) G (T/P) G. Motif I in the present study consist of the consensus sequence (F/L/V/I) (V/I) D (V/I/L) G G G/S X G and matches therefore quite well. The differences of both consensus sequences may be explained when sequence selection of the Kagan and Clarke (1994) study is taken into account: only one fungal methyltransferase was included in their comparison of 84 methyltransferases. In previous studies this motif was identified to be part of the SAM binding pocket (Cheng *et al*., 1993). The more general consensus sequenced was determined to be h h (D/E) h G X G X G whereas h stands for an hydrophobic residue (Wu *et al*., 1992) and matches very well with

motif I of the present study. The hydrophobic residues form a hydrophobic platform on one side of the purine and ribose rings of SAM and the glycine residues are involved in proper positioning of the SAM in the binding pocket by forming a tight loop (Cheng *et al*., 1993; Kagan and Clarke, 1994). The second motif named by Kagan and Clarke (1994) can only be found in parts in the alignment of *A. brassicicola* methyltransferases: Q P (V/T) K (G/D) A in the present study compared to (P/G) (Q/T) (F/Y/A) D A (I/V/Y) (F/I) (C/V/L). The function as well as the significance of motif II and III are not clear since they are not always found in methyltransferases (Wu *et al*., 1994). Although motif III of this study H D W P D is very well conserved it differs completely from the described motif III L L (R/K) P G G (R/I/L) (L/I) (L/I/V/F) (I/L) in the Kagan and Clarke (1994) study. While common sequence motifs excepting motif I were not found the identified domains will help to enhance the scarce knowledge about fungal methyltransferases and enabled the amplification of at least four methyltransferases in *A. alternata*. The universal validity of the motifs which were revealed in the present work will be further investigated in related fungi.

Based on the gene fragment the complete sequence of *A. alternata* gene Mt6 was elucidated. The translated protein sequence contained a well conserved motif I sequence L V D V G G G S G and shares high similarity to other fungal *O*-methyltrasferases. Applied to the NCBI blast search against the non-redundant database the translated protein sequence of Mt6 showed the highest identitiy of 85 % to an *O*-methyltransferase of *Pyrenophora tritici-repentis* (accession number XP_001937233.1) with unknown function. The sequence similarity and the presence of motif I suggests the protein to be an SAM-dependent *O*-methyltransferase. Furthermore the gene is expressed in AOH producing mycelium which promotes it to a promising candidate for AOH-*O*-methyltransferase. However, all attempts to get the soluble form of the protein failed and the proof is still required. Expression of Mt6 protein in *E. coli* was successful as shown in figure 4 but resulted in the formation of inclusion bodies. Inclusion bodies are formed when the heterologous protein failed to reach its native conformation due to the lack of post-translational modifications or high product yields obtained by strong promoters and high inducer concentrations (Baneyx and Mujacic, 2004). Additionally, highly hydrophobic proteins are more susceptible to accumulate and form inclusion bodies (Singh and Panda, 2005). AOH-*O*-methyltransferase proved to be highly hydrophobic during protein purification. It bound tightly to Phenyl Sepharose column material and could not be eluted by decreasing ionic strength. As reported by Hiltunen and Söderhäll (1992) the protein can be eluted by using 40 % ethylene glycol. A third reason for insoluble expression in *E. coli* may be the C-terminal His-tag linked to the protein due to protein purification considerations

which may interfere protein folding. Nevertheless, reducing expression temperature or decreasing inducer concentration did not result in production of soluble protein and changing the expression system did not result in visible protein expression at all. Although the activity test was negative, it cannot be excluded completely that Mt6 protein exhibits AOH-*O*-methyltransferase activity because both expression strategies failed. Nevertheless, it is also possible that another putative *O*-methyltransferase gene encodes for the protein.

Therefore, the biochemical strategy appears to be promising and will be continued. Determined characteristics of unpurified enzyme are in agreement with previous studies of Stinson and Morau (1986) and Hiltunen and Söderhäll (1992). Tris buffer inhibits strongly enzyme activity. At pH 7.0 the activity was reduced to 22 % in Tris buffer compared to phosphate buffer which matches with reported 19.1 % by Stinson and Moreau (1986). Additionally pH optimum was described between pH 7 and 8 (Stinson and Moreau, 1986) which was confirmed in this study. High salt tolerance enables different purification strategies. Anion exchange chromatography showed to be very encouraging and will be the basis for further purification attempts.

The final aim of this study is the identification of the whole gene cluster responsible for AOH and AOH-derivative production. To achieve this aim only the sequence of one gene is theoretically necessary because further sequences of the cluster can be obtained by PCR based methods. Nevertheless, the public accessibility of the *A. alternata* genome would simplify the cluster identification and would offer the possibility to elucidate the function of other polyketide cluster as a previous study revealed the existence of 11 putative polyketide synthases in *A. alternata* which are organized in clusters (Fetzner et al., 2011).

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V. Concluding Remarks

In the present work a reproducible process was developed. As shown in chapter 4.2 this process enables variation of all process parameters and research of their effects on mycotoxin production. Due to medium optimization mycotoxin concentrations of 7.75 mg/L (30 µM) AOH, 4.81 mg/L (17.7 µM) AME and 36.54 mg/L (185.3 µM) TA were obtained by fermentation with glucose and aspartic acid. This process offers the possibility for a scale-up to larger volumes. For fungal fermentations other fermentation systems may be more favorable, e.g. solid-state fermentation or airlift reactor systems, and complex media can enhance the production yield. Furthermore, the knowledge of the biosynthetic genes would allow genetic manipulation of the production strain for further increase of production yields and to ensure the production of a single toxin by knockout of other mycotoxin production genes. Therefore, this work provides a good basis and offers a high potential for further process optimization. However, as shown by previous toxicological studies, significant effects of *Alternaria* toxins appeared in the µM-range which can be provided by the presented process. The mycotoxins can be purified easily by extraction with different solvents and a subsequent one-step chromatography. Protocols were published for the extraction and purification in gram-quantities (Chu and Bennett, 1981).

Alternaria toxins can be produced both biotechnologically and chemically. The biochemically produced mycotoxins AOH, AME and TA can be purchased from Sigma-Aldrich. At the time of this writing the costs for the mycotoxins are as follows (excluding value added tax): 5 mg AOH 411 €, 5 mg AME 187.50 €, 10 mg TA copper salt 201.50 €. The prices varied considerably during this thesis; in the beginning costs for AOH and AME were considerably higher (around 600 € per 5 mg) and ALT was still offered. Details about production process or purification strategies were not provided by Sigma-Aldrich. The costs of the presented process in this work are considerably lower due to the simultaneous production of all three mycotoxins. Alternatively, total chemical syntheses of AOH/AME (Koch et al., 2005), ALT/iso-ALT (Altemöller et al., 2006) and TA (Schobert et al., 2004) were developed. In case of AOH and AME the palladium-catalyzed synthesis comprised seven steps and achieved an overall yield of 22.2 %. The synthesis required approximately 14 days. The chemical synthesis is time-consuming and expensive; in a small scale, i.e. few milligrams, biotechnological produced mycotoxin is therefore more cost-effective (J. Podlech, personal communication). Consequently, a reproducible process was established which was primarily intended for the study of mycotoxin production, but which is also competitive to chemical synthesis and can provide sufficient amounts of mycotoxins for toxicological studies.

VI. References

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Publications

Conference contributions – Oral presentations

Screening of *Alternaria alternata* strains and media for alternariol production in fluid submerse fermentation. K. Brzonkalik, A. Neumann, C. Syldatk (2009). Talk at the 27. DECHEMA Jahrestagung der Biotechnologen, Mannheim, Germany.

Process optimization of alternariol production with the filamentous fungus *Alternaria alternata*. K. Brzonkalik, T. Herrling, A. Neumann, C. Syldatk (2010). Talk at the 28. DECHEMA Jahrestagung der Biotechnologen, Aachen, Germany.

Conference contributions – Poster presentations

Strain-improvement und media optimization for the production of the mycotoxin alternariol from *Alternaria alternata*. K. Brzonkalik, A. Neumann, C. Syldatk (2009). Poster at the VAAM annual meeting 2009, Bochum, Germany.

Phylogenetic evidence for the neofunctionalization of an enzyme originally involved in the primary metabolism of basal fungi. K. Hoffmann, R. Winkler, K. Brzonkalik, K. Voigt (2009). Poster at Botany & Mycology 2009, Snowbird, Utah, USA.

Screening of Alternaria alternata strains and media for alternariol production in fluid submerse fermentation. K. Brzonkalik, A. Neumann, C. Syldatk (2009). Poster at the 3rd Congress of European Microbiologists (FEMS), Gothenburg, Sweden.

Screening of different carbon sources and gas flow rates for alternariol production with *A. alternata* in fluid submerse fermentation. T. Herrling, K. Brzonkalik, A. Neumann, C. Syldatk (2010). Poster at the DECHEMA Conference „Bioprozessorientiertes Anlagendesign“, Nürnberg, Germany.

Identification and isolation of the alternariol-*O*-methyltransferase in *Alternaria alternata*. K. Brzonkalik, A. Stoppa, A. Neumann, C. Syldatk (2010). Poster at the VAAM annual meeting 2010, Hannover, Germany.

Process optimization of alternariol production with the filamentous fungus Alternaria alternata. K. Brzonkalik, T.Herrling, C. Syldatk, A. Neumann (2011). Poster at the VAAM annual meeting 2011, Karlsruhe, Germany.

Publications

The influence of different carbon and nitrogen sources on mycotoxin production in *Alternaria alternata*. K. Brzonkalik, T. Herrling, C. Syldatk, A. Neumann (2011). International Journal of Food Microbiology 147: 120-126.

Process development for the elucidation of mycotoxin formation in *Alternaria alternata*. K. Brzonkalik, T. Herrling, C. Syldatk, A. Neumann. Under review in Applied Microbiology and Biotechnology.