

# International Commission on Food Mycology

## 2007 Workshop

*Westin Key West Resort & Marina,  
Key West, Florida USA*

**June 4-6, 2007**





# **International Commission on Food Mycology**

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## Program

**Sunday June 3rd: Welcome dinner (Dock area)**

**Monday June 4th**

0845-0855	Opening remarks, welcome
<b>Session 1: Coffee, cocoa, wine and ochratoxigenic fungi. Chair: Ailsa Hocking</b>	
0855-0920	<b>Overview of the black Aspergilli in food and beverages.</b> Rob Samson, Paramée Noonim, Janos Varga, CBS, The Netherlands
0920-0945	<b>Ochratoxigenic fungi in Vietnamese coffee beans.</b> Su-lin Leong, Swedish University of Agricultural Sciences, Uppsala, Sweden.
0945-1010	<b>Ecology of ochratoxin A producing fungi in Thai coffee beans.</b> Paramée Noonim, Jens Frisvad, Warapa Mahakarnchanakul and Rob Samson, CBS
1015-1045	<b>Coffee / tea break (30 min)</b>
<b>Session 2: Coffee, cocoa, wine and ochratoxigenic fungi (continued). Chair: John Pitt</b>	
1045-1110	<b>Fungal metabolites in coffee: their influence on coffee beverage flavour.</b> Beatriz T. Iamanaka <i>et al.</i> ITAL, São Paulo, Brazil.
1110-1135	<b>Cocoa and cocoa products: fungi and toxins.</b> Marta H. Taniwaki <i>et al.</i> ITAL, São Paulo, Brazil.
1135-1200	<b>Effect of gentian violet on the growth of the N and T genetic types of the <i>Aspergillus niger</i> aggregate: a selective medium.</b> Javier Cabañes, Autonomous University of Barcelona, Spain
1200-1225	<b>Fungi on wine corks.</b> Carmo Basilio*, R. Tenreiro, T. Crespo, and M. V. San Romão. Universidade Nova de Lisboa, Portugal
1230-1330	<b>Lunch break (1 hour)</b>
<b>Session 3: Fungi and mycotoxins in the food chain. Chair: Lloyd Bullerman</b>	
1330-1355	<b>Aflatoxins and the Brazil nut production chain.</b> Monica Olsen, National Food Administration, Sweden.
1355-1420	<b>Mycotoxins in Indonesian food commodities.</b> Endang Sutriswati Rahayu, University of Yogyakarta, Indonesia
1420-1445	<b>Incidence of <i>Aspergillus</i> spp. during production of Grenada nutmegs and testing of essential oils for the control of <i>A. flavus</i> and <i>A. parasiticus</i>.</b> Ludwig Niessen* <i>et al.</i> , Technische Univ. München, Freising, Germany.
1445-1510	<b>Effect of interacting environmental factors, growth and aflatoxin production by <i>A. flavus</i> strains from northern Italy.</b> P. Giorni, A. Pietri, P. Battilani and N. Magan*, Università Cattolica del Sacro Cuore, Piacenza, Italy and Cranfield University, UK
1515-1545	<b>Coffee/tea break (30 min)</b>
<b>Session 4: Fungi and mycotoxins in the food chain (continued). Chair: Naresh Magan</b>	
1545-1610	<b>Mycological problems in maize and maize silage.</b> Ulf Thrane, <i>et al.</i> , Technical University of Denmark
1610-1635	<b>Control methods for fungi and DON in malting barley.</b> Charlene Wolf-Hall, North Dakota State University, USA
1635-1700	<b>Effects of extrusion processing on fumonisins in corn grits.</b> Dojin Ryu*, <i>et al.</i> , Texas Women's University, USA
1700-1725	<b>Optimal laboratory conditions for mycotoxin production and species-mycotoxin connections revisited.</b> Jens C. Frisvad, R.A. Samson. B. Andersen and U. Thrane, Technical University of Denmark & CBS
<b>Evening: Dinner in the gardens of Audubon House</b>	

**NB: Papers are generally 20 min + 5 min discussion**

## Tuesday June 5th

<b>Session 5: Fungi and mycotoxins in the food chain (continued). Chair: Rob Samson</b>	
0830-0855	<b>Co-production of enzymes and mycotoxins by cereal-borne fungi.</b> Birgitte Andersen, <i>et al.</i> , Technical University of Denmark
0855-0920	<b>Effect of interacting environmental factors on growth and temporal mycotoxin production by <i>Alternaria alternata</i> on soybean-extract agar.</b> Maria L. Ramirez, M.S. Oviedo, S.N. Chulze, Universidad Nacional de Rio Cuarto, Argentina
0920-0945	<b>Prevention of postharvest patulin contamination in apples intended for juice production.</b> Vicente Sanchis* <i>et al.</i> , University of Lleida, Spain.
0945-1010	<b>Identification and characterization of moulds isolated from bottled water.</b> Hassan Gourama, et al., Pennsylvania State University
1015-1045	Coffee / tea break
<b>Session 6: Biology and physiology of heat and preservative resistant fungi. Chair: Jens Frisvad</b>	
1045-1110	<b>Occurrence of <i>Paecilomyces variotii</i>/Byssochlamys spectabilis in packaging.</b> Emilia Rico-Muñoz, BCN Research Laboratories, USA.
1110-1135	<b>Ascospore formation in the heat resistant fungus <i>Paecilomyces variotii</i>.</b> Jos Houbraeken and Rob Samson, CBS
1135-1200	<b>The disruption of a cell wall protein in ascospores of <i>Talaromyces macrosporus</i>.</b> Jan Dijksterhuis <i>et al.</i> , CBS and University of Utrecht
1200-1225	<b>Organization of the membrane of germinating spores part 1. Polyene antibiotics and fungal spores.</b> Richard van Leeuwen et al., CBS
1225-1250	<b>Organization of the membrane of germinating spores part 2. The ergosterol cap.</b> Richard van Leeuwen et al., CBS
1250-1345	Lunch break
<b>Session 7: Application of molecular methods to food mycology. Chair: Ulf Thrane</b>	
1345-1410	<b>Molecular methods to follow the fate and activity of <i>Penicillium verrucosum</i> in wheat.</b> Rolf Geisen, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany
1410-1435	<b>Real time PCR detection of emerging toxigenic <i>Fusarium</i> species in Sweden.</b> Lisa Fredlund, National Food Administration, Sweden
1435-1500	<b>Effect of environmental factors on <i>Fusarium</i> toxin gene expression.</b> Naresh Magan, M. Schmidt-Heydt, R. Geisen, M. Jurado and M. T. Gonzalez-Jaén, UK, Germany, Spain
1500-1530	Coffee / tea break
<b>Session 8: discussion session (can go until 1700 if necessary) Moderators: Ailsa Hocking, Monica Olsen, Rob Samson</b>	
1530-1630	<b>Future needs in food mycology research / collaborative studies / other topics?</b>
1700-1800	ICFM Commission meeting – ICFM commissioners only
<b>Evening: Sunset Cruise</b>	



## Wednesday June 6th

<b>Session 9: Ecology and physiology of fungi in foods. Chair: Emilia Rico</b>	
0830-0855	<b>Effect of CO<sub>2</sub> concentrations on growth of fungi at various <math>a_w</math> values and construction of a time to growth model.</b> Ailsa Hocking, M. Begum, D. Miskelly and T. Ross, Food Science Australia
0855-0920	<b>Modelling effect of interacting environmental factors on growth of spoilage moulds.</b> C. C. Tassou, E.Z. Panagou, P. Natskoulis, R. Parra and N. Magan*. Institute of Technology of Agricultural Products, Lycovrissi, Greece, and Cranfield University, UK
0920-0945	<b>The development of FoodMold, a comprehensive, interactive CD guide to foodborne fungi.</b> John Pitt, Food Science Australia.
0945-1005	<b>Mould and yeast problems in the food production chain – report on a survey of the Swedish food industry.</b> Elisabeth Fredlund and Johan Schnürer, National Food Administration, Sweden
1005-1020	<b><i>Dekkera bruxellensis</i> and <i>Lactobacillus vini</i>, a new stable microbial consortium for industrial ethanol production.</b> J. Blomqvist, V. Passoth and J. Schnürer, Swedish University of Agricultural Sciences, Uppsala
1020-1045	Coffee / tea break
<b>Session 10: Ecology and physiology of fungi in foods (continued). Chair: Monica Olsen</b>	
1045-1110	<b><i>F. oxysporum</i> as a spoilage agent of ready-to-drink beverages.</b> Nai Tran-Dinh, Ailsa Hocking* and Nick Charley, Food Science Australia.
1110-1135	<b>Effect of sanitizers on fungi including heat resistant moulds.</b> Shawn Johnson, BCN Research Laboratories, USA.
1135-1200	<b>Enhancement of antifungal activity of propionibacteria.</b> J. Schnürer, H. Lind and A. Broberg, Swedish University of Agricultural Sciences, Uppsala
1200-1225	<b>Antifungal activity of lactic acid bacteria and sourdough bread cultures.</b> Andreia Bianchini* and Lloyd Bullerman, University of Nebraska, Lincoln, Nebraska.
1225-1245	<b>Impact of <i>Rhizopus oligosporus</i> and yeast fermentation on nutritional properties of cereal grains.</b> J. Schnürer, C. Eklund-Jonsson, T. Andlid and M. L. Alminger. Swedish University of Agricultural Sciences
1245-1300	<b>Closing remarks</b>
1300: Lunch	
Afternoon free	

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# **Abstracts**

## **Sessions 1-2**

### **Coffee, cocoa, wine and ochratoxigenic fungi**

# Overview of the black *Aspergilli* in food and beverages

**Robert A. Samson, Paramee Noonim and Janos Varga**

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Black *Aspergilli* (*Aspergillus* section *Nigri*) have a significant impact on modern society and many species cause food spoilage, while others are used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid. They are also candidates for genetic manipulation in biotechnology industries as *A. niger* used under certain industrial conditions has been granted the GRAS (generally regarded as safe) status by the Food and Drug Administration of the US government. Although the main source of black *Aspergilli* is soil, members of this section have been isolated from various other sources. Besides their economical importance, black *Aspergilli* are also important as ochratoxin producing organisms which contaminate several agricultural products including grape derived products, coffee and cocoa.

Black *Aspergilli* are one of the more difficult groups concerning classification and identification. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists. Nuclear and mitochondrial DNA (mtDNA) polymorphisms and PCR-based techniques led to the recognition of at least two species within the *A. niger* species complex (*A. niger*, *A. tubingensis*). Regarding other black *Aspergillus* species, phylogenetic analyses of sequences of the intergenic spacer region and the 5.8 S rRNA gene (ITS region) and the D1-D2 region of the 28 S rRNA gene indicated that, apart from those mentioned earlier, at least five other species belong to section *Nigri*: *A. heteromorphus*, *A. ellipticus*, *A. carbonarius*, *A. japonicus* and *A. aculeatus*. Several other black *Aspergillus* species have recently been described, including *A. vadensis*, *A. costaricensis*, *A. piperis*, *A. lacticoffeatus*, *A. sclerotiumniger* and *A. ibericus*. Taxa are now delimited using a polyphasic taxonomic approach in order to determine the delimitation and variability of the species. The phenotypic analyses, include macro- and micromorphology, secondary metabolite and enzyme profiles. For genotypic studies, partial sequences of the  $\beta$ -tubulin and calmodulin genes and the intergenic transcribed spacer region (ITS region) of the rRNA gene cluster are mostly used. This paper presents an update of the taxonomy of section *Nigri* focussing on the species which are important for food and beverages.

## Ochratoxigenic fungi in Vietnamese coffee beans

Su-lin L. Leong<sup>\*1,4,5</sup>, Lam Thanh Hien<sup>2</sup>, Tran Van An<sup>3</sup>, Nguyen Thu Trang<sup>3</sup>,  
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The European Union has introduced limits for ochratoxin A (OA) of 5 µg/kg in roasted coffee and 10 µg/kg in soluble coffee, to minimise the risk of exposure for the consumer. Limits for OA in green coffee are unlikely to be introduced; nevertheless, infection of green coffee beans by toxigenic *Aspergillus* species in the right conditions will affect the extent of OA contamination in the processed coffee. Vietnam is the second largest producer of coffee in the world, after Brazil, and in 2006, potentially ochratoxigenic *Aspergillus* species were isolated from Robusta (65 samples) and Arabica (11 samples) coffee beans sourced from the primary coffee-growing areas in southern and central Vietnam. *Aspergillus carbonarius*, *A. niger* and yellow *Aspergilli* (*A. ochraceus* and related species in section *Circumdati*) were isolated by direct plating of surface-disinfected beans. Significantly more Robusta than Arabica beans were infected by fungi. *A. niger* was the dominant fungal species, infecting 89% of Robusta beans. *A. carbonarius* and yellow *Aspergilli* were less common, each infecting 12–14% of beans. Other fungi commonly isolated included *Aspergillus flavus* and *A. tamarii*, *Rhizopus* spp. and, less commonly, *A. fumigatus* and *Penicillium citrinum*. These fungi, as well as the yellow *Aspergilli*, typically co-infected beans with the black *Aspergilli*. Black *Aspergilli* of slightly different colony morphologies (*A. carbonarius* + *A. niger*; or two morphologically distinguishable strains of *A. niger*) also often grew from the same bean (95% of samples, average 28% of beans). In particular, *A. carbonarius* was seldom the sole fungus infecting a coffee bean, typically co-infecting with *A. niger*. OA was not produced by *A. niger* (98 isolates) or *A. ochraceus* (77 isolates), but was detected in 110 of 113 isolates of *A. carbonarius*, 10 isolates of *A. westerdijkiae* and one isolate of *A. steynii*. As the majority of *A. carbonarius* isolates are toxigenic, this species appears to be the likely source of OA contamination in Vietnamese coffee beans. Of 30 samples of Robusta beans in which ≥ 20% of beans were infected with either yellow *Aspergilli* or *A. carbonarius*, 10 samples were contaminated with OA at ~0.4–1.8 µg/kg; however, no relationship between extent of infection and OA contamination was observed. Vietnamese green coffee beans were more severely infected with fungi than the levels reported for beans from other parts of the world, yet OA contamination appears to be infrequent.

## Ecology of ochratoxin A-producing fungi in Thai coffee beans

Paramee Noonim<sup>1,3\*</sup>, Jens C. Frisvad<sup>2</sup>, Warapa Mahakarnchanakul<sup>3</sup> and Robert A. Samson<sup>1</sup>

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From our surveys of coffee bean samples harvested throughout the 2006-2007 seasons, a total of 64 Thai coffee bean samples were collected and assessed for the presence of ochratoxin A and potentially ochratoxigenic *Aspergilli*. Samples came from two regions: Arabica coffee (*Coffea arabica*) from Chiangmai Province in the North and Robusta coffee (*Coffea canephora*) from Chumporn province in the South. From the mycological studies, overall percent fungal infection in coffee bean was 98% and reduced to 60% after surface disinfection. There were remarkably differences in ecology of the ochratoxigenic species presented in these two different regions. Arabica coffee bean samples from the North had an average of 77.5% incidence of infection from *Aspergillus* in section *Circumdati* with *Aspergillus westerdijkiae* and *A. melleus* as the predominant species. *A. ochraceus* was not detected in the samples. 75% of the samples were infected with *Aspergillus* section *Nigri* species. One hundred percent of infected Robusta coffee bean samples from the South were infected with *Aspergilli* in section *Nigri* with *A. carbonarius* and *A. niger* the predominant species. *A. westerdijkiae* was only found in one sample. It is possible that the diversity of the fungal population depends on the geographical origin of the coffee, coffee cultivar, and processing method used. Representative isolates of section *Circumdati* (50) and *Nigri* (86) were determined for ochratoxigenic abilities with agar plug method and investigation are in progress. Ochratoxin A levels in coffee bean samples were analyzed using Ridascreen® Ochratoxin A ELISA kits. Of the 64 coffee bean samples analyzed, 98% were contaminated with ochratoxin A at levels varied from 0.12-5.49 ppb for Arabica and 1.3-26.69 ppb for Robusta coffee bean.

## **Fungal metabolites in coffee: their influence on coffee beverage flavour**

**Beatriz T. Iamanaka<sup>1</sup>, E. Vicent <sup>1</sup>, Aldir A. Teixeira<sup>2</sup>, Ana Regina R. Teixeira<sup>2</sup>, Neura Bragagnolo<sup>3</sup> and Marta H. Taniwaki, <sup>1</sup>**

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There is some evidence that the presence of fungi in raw coffee beans can influence the final coffee flavour, often reducing the beverage quality. To investigate this fact, coffee beans from Piraju region (São Paulo State) were collected and the mycobiota was analyzed. The percentage of infection was calculated and each isolate was identified into species or genus. For sensory analyses each sample was roasted and ground and evaluated in three different tasting tests: infusion, diluted espresso and espresso. Parameters such as body, aroma, acidity, bitterness, astringency and sweetness were evaluated. The presence of negative characters such as immature, fermented, stinker, woody, rancid, mouldy, rioy and smoky, and positive aromas such as bread toast, caramel, chocolate, floral were also evaluated. Raw coffee beans were inoculated with the most common species found and sensory analyses were carried out. A total of 443 strains were isolated and the most common genera found were *Penicillium* spp. (45.6%), followed by *Aspergillus* spp. (30.5%) and *Fusarium* spp. (23%). Coffee samples with negative sensorial evaluation and characters such as stinker and mouldy showed contamination by *Aspergillus niger*, exceeding 20%. On the other hand coffee samples which presented a good clean beverage, with distinct aromas such as chocolate, caramel and floral were more infected with *Penicillium* species. The fungi volatile metabolites are also being studied. The solid phase microextraction (SPME) and gas chromatography – olfactometry (GC-O) techniques are being tested to capture the volatiles and to select the most relevant components. These compounds will be identified using gas chromatography – mass spectrometry (GS/MS) technique.

## Cocoa and cocoa products: fungi and toxins

**Marina V. Copetti<sup>1</sup>, Beatriz T. Iamanaka<sup>1</sup>, José L. Pereira<sup>2</sup>, Felipe Nakano, Daniel P. Lemes and Marta H. Taniwaki<sup>1\*</sup>**

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The present study was carried out in order to investigate the toxigenic species and toxins in cocoa and cocoa products. A total of 197 samples was examined during 2005 and 2006 cocoa harvest seasons in Bahia, Brazil. Samples of cocoa were collected at different stages: from trees, during fermentation, drying and storage from the farm. Cocoa products from cocoa companies, such as powder, nibs, cake and shell were also analysed. Cocoa bean samples were surface disinfected with 0.4% chlorine solution for 2 min, then 33 beans from each sample were plated directly onto Dichloran 18% Glycerol agar, and incubated at 25°C for 7 days. For powder and flocculated samples the dilution plate technique was used using the same culture medium and incubation condition. After incubation, plates were examined and all fungal species were first isolated onto Czapek Yeast extract agar (CYA) plates. After growth, other culture media were used for identification. The ability to produce aflatoxin and ochratoxin was tested using the agar plug technique and thin layer chromatography (TLC). The most common fungi isolated were: *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri*, *Eurotium chevalieri*, *Penicillium roqueforti*, *Rhizopus* sp., *Mucor* sp., yeasts and dematiaceous fungi. More than 800 isolates of potentially toxigenic fungi were isolated. All isolates of *Aspergillus parasiticus* and 61% of *A. flavus* tested produced aflatoxins. From *Aspergillus* section *Nigri*, 6.7% of *A. niger* and 100% of *A. carbonarius* produced ochratoxin A (OTA). *Aspergillus westerdijkiae* was isolated from few samples and 100% produced OTA. Ochratoxin A in fresh harvested beans samples was not detected. During fermentation, drying and storage stages the range of OTA contamination was from <0.03 to 5.12, <0.03 to 16.61 and <0.03 to 30.24 µg/kg, respectively. In cocoa powder the range was from 0.41 to 10.78 µg/kg. The results of this work showed that toxigenic fungi and ochratoxin A can be found in cocoa beans and may be of concern for cocoa product consumers. More studies are being carried out in order to evaluate the contamination of aflatoxins in cocoa samples.



## **Effect of gentian violet on the growth of the N and T genetic types of the *Aspergillus niger* aggregate: a selective medium**

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Within the black aspergilli, taxa included in the *A. niger* aggregate are difficult to distinguish by morphological means. Molecular techniques, including RFLP analysis of ribosomal, mitochondrial and chromosomal DNA, RAPD, isoenzymes and sequencing, have been used to clarify the taxonomy of this group, and various authors divided the group into two or more species. In our laboratory, a method that differentiates the *A. niger* aggregate isolates into two ITS-5.8S rDNA RFLP patterns, type N and type T, corresponding to the type species of *A. niger* and *A. tubingensis* respectively was described. A low percentage of isolates belonging to this aggregate are ochratoxin A (OTA) producers. Our investigations show that all the OTA-positive isolates belonging to the *A. niger* aggregate characterised by this method are of type N, whereas type T strains are not able to produce OTA. The ITS-5.8S rDNA and 28S rDNA (D1/D2) sequencing, microsatellite, RAPD and AFLP analyses have been also used to study genetic diversity in the *A. niger* aggregate, and they also confirmed the separation of N and T *A. niger* aggregate strains. In this work, the effect of gentian violet on the growth of *A. niger* aggregate strains has been studied. Gentian violet is a water soluble dye that has multiple uses (e.g. Gram stain, topical antifungal agent). Various authors have tested this dye for its inhibitory effect on the growth of aflatoxigenic strains. More than 100 *A. niger* aggregate strains, including reference cultures of *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* among other species and field isolates have been tested. Most of the N type isolates grew when incubated at 28°C for 3 days at some of the gentian violet concentrations of assayed. All of the T type isolates tested failed to grow at the same conditions.

## Fungi on wine corks

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Natural cork stoppers are an excellent closure material for sealing wine bottles as they enable the proper aging of the wine. Cork is a natural product, therefore recyclable and biodegradable, and cannot be imitated by any synthetic material. Some undesirable smells and some “mouldy” tastes are sometimes present in the bottled wine, this is said to be “cork-tainted”. There are many chemical compounds detected in the cork-tainted wines, but the major source of this defect is considered to be 2,4,6-trichloroanisole (TCA). TCA can contaminate the cork by many processes, but normally results from transformation of the polychlorophenols, used in some wood preservatives and in some pesticides, by the action of some fungi. The study of the natural mycobiota community present in the cork slabs is very important, to provide an indication of the future quality of cork stoppers. Fungal diversity was assessed along the manufacturing process and a comparison was performed between two environmental niches under distinct stress (boiled and non-boiled cork). Fungi were isolated from cork using a direct isolation dilution plating method. The identification of fungi to species level was carried out by morphological and molecular methods. In order to identify the different species present in the whole fungi mixtures, the Denaturing Gel Gradient Electrophoresis (DGGE) technique was applied. Aiming to investigate the possible production of volatile compounds from some of the most frequent species isolated from cork, 4 isolates were inoculated in a cork-based liquid medium. Afterwards, the cultures were analysed using Gas Chromatography (GC) and/or GC-Mass Spectrometry for secondary metabolites evaluation namely TCA. Species diversity was correlated with water activity and some environmental parameters and the occurrence of some volatile compounds in the cork.

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## **Sessions 3-5**

### **Fungi and mycotoxins in the food chain**

# Aflatoxins and the Brazil nut production chain

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The contamination of Brazil nuts with aflatoxins is a well-known phenomenon although the critical points for infection with the aflatoxin producing fungi, *Aspergillus flavus* or *A. parasiticus*, and subsequent toxin formation is not well elucidated. The Brazil nut tree (*Bertholletia excelsa*) is native in the Amazon basin. It grows to great height, up to 50 m (averaging 40 m). The seeds, i.e. the Brazil nuts, are produced within a capsular woody fruit (pod) which is extremely hard. All Brazil nuts available in retail originates from collection in natural stands in the Amazon region of Brazil, Peru and Bolivia. Attempts to grow Brazil nuts in orchards for commercial production have not been successful. It is possible that conidial germination and penetration of *A. flavus/parasiticus* through the floral parts of the young kernels is a source of infection (beside poor drying, processing and storage conditions). Indeed, rotted kernels have been observed inside the hard shell of the brazil nut fruit, suggesting premature invasion and colonization of young nuts. It has also been observed that kernels looking fresh on the outside could sometimes be mould-affected on the inside. *A. flavus/parasiticus* are weak pathogens and would not be able to penetrate an intact hard shell. Furthermore, a study has showed that consumers can, by current behaviour, discriminate aflatoxin contaminated Brazil nuts to a significant extent and that this ability is independent of factors such as sex, age, level of education, ethnic background or knowledge of mycotoxins.

# **Mycotoxins in Indonesian food commodities**

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Fungi and mycotoxin contamination of agricultural commodities is a major problem in Indonesia as well as in other tropical countries. This is due to the high temperature and humidity, which favor the growth of fungi and consequent mycotoxin production. Five mycotoxins have been a major of concern in Indonesia: aflatoxin, deoxynivalenol, fumonisin, ochratoxin, and zearalenone. Priority is given to these five toxins since their producer fungi can grow rapidly and produce their toxins under the tropical conditions. Among these mycotoxins, aflatoxin is the greatest concern and is discussed in this paper. Aflatoxin is the highest priority mycotoxin problem in Indonesian agricultural commodities, particularly corn and peanuts. Current data on aflatoxin contamination in these commodities will be presented.

Samples of corn (139 units) were obtained from farmer (84 units), retailer and wholesaler (55 units) from six production districts, around East and Middle of Java. All corn samples were shelled prior to sub-sampling for further analysis of AFB1 contamination using ELISA method. Data showed that at farmer level, approximately 18% of corn samples were contaminated by more than 20 ppb of AFB1 and 7% even >100 ppb. At retailer and wholesaler levels, 58% of samples contaminated with >20 ppb, and 20% with >100 ppb AFB1. Samples of corn-based food products (96 units) were collected from retailer at the production area and Yogyakarta. Among these samples, 87 were corn snack and 9 were corn grit which is consumed as staple food in certain areas. Approximately 18% of the corn snacks were found to have AFB1 residue higher than 20 ppb and no sample containing more than 100 ppb.

For peanuts, our current data showed that peanuts from farmers in the Central Java region generally contained low levels of AFB1, however, 14% of peanut samples obtained from traders were contaminated by more than 20 ppb AFB1. According to our current data for peanut-snack foods (21 samples) only one contained AFB1 about 21 ppb, while the other samples were <5 ppb. However, 58% among 14 samples of peanut sauce had AFB1 residue >20 ppb, and 14% even >100 ppb, with the maximum level of 120 ppb.

Fumonisin, deoxynivalenol, zearalenone and ochratoxin of corn and feed were also intensively studied. Ochratoxin was also analyzed from coffee and data showed that contamination of this toxin was about 0.3 – 40 ppb, depending on the production area.

Based on the current data on mycotoxins, particularly aflatoxin level at agricultural products, the Department of Food and Agricultural Product Technology, Gadjah Mada University has developed several strategies for prevention and control of this mycotoxin, particularly in corn and peanuts, in collaboration with other universities, research institutes, government, farmer, traders, and industries. Several performance indicators are targeted such as increasing the productivity, quality, safety (low aflatoxin level) as well as market share of these commodities, increasing awareness of the aflatoxin problem and income of farmers.

## **Incidence of *Aspergillus* spp. during production of Grenada nutmegs and testing of essential oils for the control of *A. flavus* and *A. parasiticus***

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Grenada is the southernmost of the Caribbean Windward Islands, north of Trinidad and west of Barbados. Nutmeg production and export is one of the main pillars of Grenada's economy and contamination of nuts with aflatoxins causes serious trade restrictions. In order to increase process hygiene, fungal contamination of nutmegs was monitored during the production process at Grenada. Among other *Aspergillus* spp., *A. flavus* and *A. parasiticus* were frequently isolated. from windfallen nuts, from washing water, and from nuts and dust taken from drying and storage facilities. In order to test nutmeg fungal isolates for their aflatoxin producing capabilities, Palm fat agar (PFA) was developed on the basis of emulsified palm fat (Palmin®). The medium proved to be an interesting alternative to the use of coconut cream agar. All *A. parasiticus* and 50 % of *A. flavus* isolates produced aflatoxins on PFA as indicated by bright blue fluorescence and TLC analysis. Aimed at inhibiting growth of aflatoxin producers during drying and storage of nutmegs, effects of eight different essential oils on growth and aflatoxin production by *A. flavus* and *A. parasiticus* isolates were investigated. Oils from *Cinnamomum zeylanicum* (Ceylon cinnamon) and *C. cassia* (Chinese cinnamon) had the most pronounced effect among the oils tested. Essential oils produced from nutmeg and mace had no inhibitory effect on fungal growth. Quantitative analysis of aflatoxin production of *A. parasiticus* grown in liquid PFA in the presence of the *C. cassia* oil revealed inhibition of growth and aflatoxin production at higher doses and a marked increase of toxin production at sublethal doses.



## Effect of interacting environmental factors, growth and aflatoxin production by *A. flavus* strains from northern Italy

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Maize is widely grown in northern Italy where the main concern is *Fusarium* section *Liseola* species and fumonisins with a high incidence in most years. DON is detected occasionally determined primarily by weather conditions. In 2003/2004 for the first time significant problems arose due to aflatoxin (AFA) contamination of maize. This was diagnosed because of the very high AFM<sub>1</sub> found in milk and derived products. There was thus a need for more detailed information on the extent of this problem, the susceptible regions and the ecological reasons why *A. flavus* and AFA had suddenly become important. The relative diversity of *Aspergillus* section *Flavi* in the main 6 regions were determined and the relative population structure with regard to *A. flavus*, *A. parasiticus* and production of AFA were determined. The ecological parameters (water availability, temperature and gas composition) on growth and AFA production were identified and compared with other data from other parts of the world. This information will be use to develop a systems-based plant pathogen model to predict the high risk regions in northern Italy where most of the maize is destined for animal feed for the economically important dairy-based industries.

## **Mycological problems in maize and maize silage**

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Mycotoxin contamination of cattle feed has been suggested as a contributing factor in diseases among animals with very different symptoms ranging from disorders in the digestive system, diarrhea, growth retardation, poor lactation, pulmonary edema, deterioration of liver or kidney function, circulatory shock and death. In collaboration with agricultural scientists our aim is to determine if fungi and their mycotoxins are responsible for diseases and poor performance in cattle. Further concern is if mycotoxins in the feed can be carried-over into blood and milk. Surveys of Danish maize and maize silage have shown that the most common and potent mycotoxin producers in maize in the field are members of the genera *Fusarium*, *Epicoccum*, *Phoma* and *Alternaria*. In silage *Penicillium roqueforti* is the predominant species. The related *P. paneum* often co-occurs with *P. roqueforti* and the two toxigenic species are difficult to isolate from each other. Even healthy looking silage samples contain these two species in up to  $3 \times 10^3$  colony forming units/gram. Other mycotoxin producing fungi found in silage were *Monascus ruber* and *Byssoschlamys nivea*, whereas *Aspergillus fumigatus* seems to be rare. There is a focus on *Fusarium* mycotoxins in maize silage; even though growth of *Fusarium* is considerably inhibited during the fermentation due to the low pH, the high CO<sub>2</sub> and the competition with the lactic acid bacteria present in the silage. In contrast, *Penicillium* and *Monascus* species are much more competitive under these growth conditions. During surveys of maize silage *Fusarium* toxins - trichothecenes, zearalenone and fumonisins - are detected in varying amounts, but so far other mycotoxins are not included in the analyses. On-going surveys will determine the ecological conditions in silage silos and the presence of the potential mycotoxins, including other than *Fusarium* mycotoxins, will be determined by bio-assay guided chemical analyses.

## **Control methods for fungi and DON in malting barley**

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Fungal infections in grains can lead to quality and safety concerns in malt and beer. While some amount of water soluble mycotoxins, such as DON, may be lost during barley steeping, fungi are still capable of growth and mycotoxin production during steeping, germination and kilning. Therefore, detoxification of grain before malting may not be practical unless further growth of the mold is also prevented. Methods to reduce the amount of mold growth during malting are needed. Physical, chemical and biological methods are reviewed. Irradiation looks very promising as a means to prevent *Fusarium* growth during malting, but the effect on the surviving microflora to produce mycotoxins and the effect on malt quality needs further study. Chemical treatments such as ozonation, which would not leave residual chemical in the beer, also appear to be promising. Although biological control methods may be desirable, due to the use of natural inhibition, the effects of these inhibitors on malt and beer quality requires further investigation. It may also be possible to incorporate detoxifying genes into fermentation yeasts, which would result in detoxification of the wort when mold growth is no longer a problem. Development of these types of technological interventions should help improve the safety and quality of products, such as beer, made from fungal infected grain.

## Effects of extrusion processing on fumonisins in corn grits

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Fungi in the genus *Fusarium* frequently infect corn and may produce a variety of toxic metabolites in the field and during storage. Fumonisins, particularly fumonisin B<sub>1</sub> (FB<sub>1</sub>), in corn has been a major concern for farmers, the food industry, and regulatory agencies due to its prevalence and toxicity. While FB<sub>1</sub> is known to be fairly heat stable, extrusion processing has been shown to apparently reduce concentrations of FB<sub>1</sub> significantly. Extrusion processing is one of the most versatile technologies available to the food industry, and is used extensively in the production of breakfast cereals, snack foods and pet foods. As the food matrix passes through the barrel of an extruder, very high temperatures and pressures can be reached simultaneously with severe mechanical shear forces that may result in greater reductions of toxins than conventional food processes. The effects of extrusion processing on fumonisins were investigated using a laboratory scale co-rotating twin screw extruder at various temperatures, moisture contents, and screw speeds. Corn grits artificially contaminated with FB<sub>1</sub> at 5 µg/g lost from 46 to 76% of the FB<sub>1</sub> during extrusion cooking of the grits at the different extrusion parameters used in this study. Addition of sugars especially glucose at 10%, further reduced the FB<sub>1</sub> concentrations by up to 93%. When tested in a rat feeding bioassay it was found that the toxicity of FB<sub>1</sub> may also be reduced by extrusion processing in addition to the significant reduction in the measurable amount of FB<sub>1</sub>.

## Optimal laboratory conditions for mycotoxin production and species-mycotoxin connections revisited

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Production of mycotoxins and other secondary metabolites depends heavily on the growth substrate and environmental factors. For many years wheat, rice or corn were used for mycotoxin screening, but in the last 25 years agar media have been used extensively for rapid screening. The most widely used medium for mycotoxin and other secondary metabolite production is yeast extract sucrose (YES) agar, which originated in yeast extract sucrose broth used for aflatoxin production in the sixties. The combination of the agar plug method and YES agar proved to be a very effective method to enhance the expressions of most interesting secondary metabolites. YES agar has been used for *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, *Trichoderma* and many other common mycotoxigenic genera. Problems with differences in brands of yeast extract were solved by adding magnesium sulphate and traces of copper sulphate and zinc sulphate to the medium. In some cases YES or the slightly better medium DRYES are not sufficient for production of certain secondary metabolites. For *Penicillium* and *Aspergillus* the diagnostic medium CYA is a good addition to the medium regimen, especially when YES agar is giving a poor sporulation. A further addition of a malt extract based medium may give some extra anthraquinones and other pigments. Finally a medium without a pure carbon source may be optimal for production of certain metabolites such as penicillin and gliotoxin. One such medium is YE (YES agar without sucrose). For *Fusarium*, YES is often supplemented with PDA (potato dextrose agar). For *Alternaria* species the medium DRYES has been very effective. We have recently compared these media to other suggested secondary metabolite media. One of them is YES agar with bee pollen, claimed to enhance ochratoxin A (OTA) production. Results from these studies will be reported. By using different optimal conditions for ochratoxin A production we suggest that recent reports on OTA being produced by *Aspergillus tubingensis*, *A. fumigatus*, *A. versicolor* and certain *Penicillia* are not correct. This is a matter of using 1) a series of media, 2) a series of chemical confirmatory steps and 3) correct species identification. Most reports had the fungal identification correct, but the chemical confirmation was not convincing.

## Co-production of enzymes and mycotoxins by cereal-borne fungi

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The ability of a fungus to establish itself as a pathogen on plants or as a spoiler of food raw materials is likely to depend on its production of extracellular enzymes as well as secondary metabolites including toxins. Extracellular enzymes should be secreted into the medium in order to degrade the material. It is our hypothesis that secondary metabolites have to be secreted into the environment to protect the nutrient source, and inhibit plant or animal defence. Secondary metabolites that stay in the conidial cell wall probably protect the conidia from being eaten by insects and other animals. Co-production of extracellular enzymes and secondary metabolites has rarely been examined. We examined the meat-borne *Penicillium nordicum* and compared it with cereal-borne *Penicillia* such as *P. verrucosum*, *P. polonicum*, *P. cyclopium* and *P. freii*. These fungi were grown on Czapek based media with and without sucrose and xylan. Samples from exudate droplets, spores, mycelium and agar were analyzed for their content of enzymes and secondary metabolites using AZCL assays and HPLC-DAD, respectively. The data were analyzed using principal component analysis and showed that there are strong correlations between enzyme production and secondary metabolite production. Furthermore some secondary metabolites are clearly only present in the conidia, while others are secreted into the growth medium or the exudate droplets. In general, exudate droplets contained very large amounts of both extracellular enzymes and secondary metabolites, and thus play a major role in mycotoxin contamination of foods and for the aggressiveness of the fungus.



## **Effect of interacting environmental factors on growth and temporal mycotoxin production by *Alternaria alternata* on soybean-extract agar**

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The objective of this study was to determine the effects of water activity ( $a_w$ ; 0.995-0.90), temperature (18 and 25°C), time of incubation (7-35 days) and their interactions on mycelial growth and mycotoxin production (alternariol (AOH) alternariol monomethyl ether (AME) and tenuazonic acid (TeA)) on 2% soybean extract agar by two strains of *A. alternata* isolated from soybeans in Argentina. Optimal  $a_w$  levels for growth were in the range 0.995-0.94 with an optimum temperature of 25°C. Maximum growth rates were obtained at the highest  $a_w$  (0.995) and 25°C with growth decreasing as the water availability of the medium was reduced. Both strains were able to growth at the lowest  $a_w$  assayed (0.90). Greatest production of all three mycotoxins occurred at 0.98  $a_w$  and 25°C. The three mycotoxins were produced most rapidly after 7 days only at 25°C and 0.995  $a_w$ . All other conditions required 7-14 days before the toxins were produced. Maximum amounts of all toxins were produced at the 0.98  $a_w$  treatment after three weeks at 25°C for both strains. Production of AOH, AME and TeA occurred over a narrower range of  $a_w$  (0.995-0.94) than that for growth (0.995-0.90). Two dimensional contour maps of  $a_w$  x temperature profiles were developed from these data to identify areas where conditions indicate a significant risk of accumulation of these three mycotoxins.

# **Prevention of postharvest patulin contamination in apples intended for juice production**

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Accumulation of patulin in apples and pears and subsequently in their by products is caused mostly by *Penicillium expansum* which causes blue mould in these fruits. Patulin can damage organs and tissues in animals and some studies revealed carcinogenic and teratogenic effects. There is a significant risk of patulin contamination in apple by-products as almost 100% of *P. expansum* isolates are patulin producers. To prevent the patulin contamination in apples intended for juice production, the influence of cold storage and further ambient/deck storage steps prior to processing were studied.

Duration of cold storage influenced patulin accumulation. However, when lesions were smaller than 1 cm, patulin accumulation was not detected. Modified atmospheres prevented patulin accumulation during longer storage. Ripeness degree of apples at harvest affected fungicide efficiency, but fruit spoilage could not be completely prevented. The biocontrol agent *Candida sake* seemed to be an alternative to fungicides. Postharvest treatments assayed in these studies lost their efficiency during a further storage at 20 °C in which a significant increase in patulin levels was observed after 48 h.

Both cold and deck storage are critical in patulin control. Duration of cold storage should be calculated depending on postharvest treatments and quality of apples when harvested. Further deck storage should be avoided. When not possible, quality of apples at the end of cold storage should be assessed in order to state a critical limit for the duration of such storage. The implementation of HACCP principles at these two steps may reduce significantly the risk of patulin contamination.

## Identification and characterization of molds isolated from bottled water

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A study was conducted to isolate, identify and characterize molds and bacteria in bottled water. One hundred and five bottled water samples were purchased in supermarkets in the southeastern Pennsylvania area. These samples were examined for molds, aerobic heterotrophic colony count, and coliforms using the membrane filter method. The samples were analyzed immediately after purchase and after storage at room temperature for six months. Bacterial isolates were identified using the fatty acid profiling technique. Mold isolates were identified using macroscopic and microscopic examinations, and fatty acid profile analysis. The most predominant mold species that were isolated before storage included *Penicillium glabrum*, *P. citrinum*, *P. chrysogenum*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Fusarium* sp. *Acremonium* sp. and *Aspergillus fumigatus*. The less predominant mold isolates included *Alternaria*, *Paecilomyces*, *Trichoderma*, *Rhizopus* and *Phoma*. Generally the same mold species and genera were isolated after six months of storage, although at lower levels. A comparison between bacterial counts and mold counts revealed that the samples that contained high levels of bacteria had lower mold counts. None of the samples had visible fungal mycelia before storage; however after storage three samples contained visible mold filaments. Production of mycotoxins by selected mold isolates was also investigated in vitro using laboratory media. Some of the *Penicillium* isolates produced citrinin, penicillic acid, and patulin.

## **Session 6**

### **Biology and physiology of heat and preservative resistant fungi**

## **Occurrence of *Paecilomyces variotii*/*Byssoschlamys spectabilis* in packaging (empty PET bottles) in the U.S.**

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*Paecilomyces variotii* is a thermophilic, heat-resistant, preservative-resistant fungus that can grow at low oxygen pressure. It is now known that *P. variotii* is the anamorph state of *Byssoschlamys spectabilis* and that the ascospores of this species are one of the most heat-resistant fungal ascospores. All these characteristics make *P. variotii*/*B. spectabilis* the ideal beverage spoilage fungi that cost the beverage industry large economic losses. The objectives of this study were: (1) to evaluate the microbial quality and occurrence of *P. variotii*/*B. spectabilis* in empty PET bottles used in the beverage industry in the U.S, and (2) to study the filling temperature survival and spoilage potential of the *Paecilomyces variotii* isolates found in empty PET bottles. Empty PET bottles were collected from two locations: in one of the plants the bottles were manufactured in the building next to the plant (on-site) and in the other, the bottles were brought into the plant from an outside manufacturing plant (off-site). The total number of bottles collected was almost 1,400. Half of these were used to determine their microbial quality (by the plating method) and another half were used to determine the occurrence of *Byssoschlamys spectabilis* (by the heat-shocking method). The number of yeasts, mold, *P. variotii* (PHRM) and *B. spectabilis* colonies (HRM) was recorded. For the filling temperature survival study, four sets of five empty bottles per each product were inoculated with isolates of *Paecilomyces variotii* found in empty bottles at two different inoculum levels, a low (100 spores, approximately) and a high (100,000 spores, approximately). Inoculated bottles were filled with either a lemon-lime flavored sport drink or sweetened tea at 180°F (82°C) and capped. After 15 sec, bottles were inverted for 7 sec to simulate processing procedures. After 60 sec, bottles were cooled down in an ice-water bath with agitation. Bottles were carefully placed in a 30°C incubator for up to two months. By the plating method, most bottles were not a significant source of yeasts. Mold content was higher for the off-site manufactured bottles. In the case of the presumptive heat-resistant molds (PHRM) or *P. variotii* count, the bottles manufactured in-site had a higher contamination than the ones manufactured off-site. However, when the heat-shock method for the detection of HRM was used, the opposite was true. This again emphasizes the importance of using the right methodology. Only one bottle out of 537 of the on-site bottles contained one ascospore of *Byssoschlamys spectabilis*, while four bottles out of 166 of the off-site bottles contained at least one ascospore. One bottle contained as many as 6 ascospores. None of the bottles from the filling temperature survival study showed growth after two months of incubation. The filling temperature of 180°F (82°C) seemed to be effective at inactivating spores (conidia) of *Paecilomyces variotii* present in the empty bottles at both of the inocula levels studied. More research is being conducted with different products and processing variables as well as with ascospores. In conclusion, *Paecilomyces variotii*/*Byssoschlamys spectabilis* was found in empty PET bottles after heat shock at a rate of up to 2.5% in some cases. The filling temperature of 180°F (82°C) inactivated conidia of isolates of *Paecilomyces variotii* inoculated in empty bottles and the inoculum size did not influence the deactivation.

## **Ascospore formation in the heat resistant fungus *Paecilomyces variotii***

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*Paecilomyces variotii* is a commonly occurring world-wide species and is an important food spoilage organism. This species is often associated with spoilage of heat treated products and it is suggested that this species is able to form extremely heat resistant ascospores. However, induction of these structures on agar media is difficult and poorly understood. Although no teleomorph is described for *Paecilomyces variotii*, the genus *Byssoschlamys* has been linked to it. In this study we elucidate the phylogenetic relationships of *P. variotii* with *Byssoschlamys* by analyses of the nuclear ribosomal internal transcribed spacers (ITS1, ITS2) and the 5.8S rRNA gene, as well as partial tubulin, and calmodulin gene. Besides sequence analyses, also mating experiments were performed to induce the teleomorph formation. Experiments on the presence of MAT1-1 and MAT1-2 genes in selected isolates were initiated.

From the sequence and other data it could be concluded that *P. variotii* is the anamorph of *Byssoschlamys* (*Talaromyces*) *spectabilis*. Mating experiments with several *P. variotii* strains, isolated by a heat shock method or originating from heat treated products, showed that this species has a heterothallic reproduction mode. Ascospore formation can be induced by crossing strains of different mating types with each other. Potato Dextrose Agar proved to be more suitable for this purpose than Corn Meal agar or Malt Extract agar. The type strain of *P. variotii*, CBS 102.74, did not produce a teleomorph state with any of the tested strains. However, the type culture of *B. spectabilis* CBS 101075, was able to mate with the test strains.



## **The disruption of a cell wall protein in ascospores of *Talaromyces macrosporus***

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Ascospores of *Talaromyces macrosporus* are very stress-resistant eukaryotic structures that survive extreme temperatures, drought and high pressure. They exhibit constitutive dormancy and germinate only after an extreme trigger as, for instance, a heat treatment at 85°C for 10 min. Activation is correlated with changes in the cell wall as is observed with different techniques. Fluorescence microscopy indicates a change in the permeability of the cell wall and X-ray diffraction reveals a recrystallisation event during activation. Heat activation does not occur at 65 °C, but strongly increases at 70 °C. At this temperature large amounts of a protein are released from the spores. The sequence of the protein is obtained via N-terminal sequencing and degenerative primers were constructed. These were used to generate a fragment from isolated mRNA by PCR but only a partial sequence of the protein was found. By means of RACE-PCR a complete cDNA from a mRNA was obtained and new primers were designed for the isolation of the genomic DNA sequence. This revealed the presence of one intron of 69 bp in the gene. The genome of *Talaromyces macrosporus* contains one copy of this gene and its expression is strictly related to the formation of fruit bodies (that contain ascospores). The total sequence of the protein was used to produce a construct to generate a disruption mutant via homologous recombination.

## **Organization of the membrane of germinating spores**

### **Part 1: Polyene antibiotics and their effect on the fungal membrane.**

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Formation of (carcinogenic) mycotoxins by food-spoilage fungi and the developing resistance of fungi against the preservatives benzoate and sorbate, (new) antifungals are an ongoing concern for food industry. Macrolide polyene antibiotics were first discovered during the late forties. They are almost exclusively produced by the soil actinomycete *Streptomyces* sp. and are effective against a wide range of fungal species. Many different polyene antibiotics are nowadays discovered and characterized. Polyene antibiotics have a ring structure in which a series of conjugated double bonds is located opposite to a number of hydroxyl functions. A number of polyenes possess a mycosamine group, rendering the molecule amphoteric. In the seventies and eighties compelling evidence has been presented that this class of antibiotics targets ergosterol, a main sterol of fungal membranes. Subsequent research documented that different types of polyene antibiotics may display different modes of action despite that they share a common target. Larger polyenes like amphotericin and nystatin can form complexes with ergosterol that become pores in the plasma membrane. This results in the collapse of vital ion gradients and loss of cell components resulting in death of the cells. The smaller uncharged filipin cannot form pores but may act by a different mechanism. It is supposed that filipin forms large complexes with sterols that are located between the leaflets of the lipid bilayer, resulting in disruption of the membrane. Natamycin like the other polyene-antibiotics specifically binds to ergosterol in the membrane, but there is evidence that its mechanism is different from the other compounds. Novel insights show that sterols may have different functions in the cell with respect to protein sorting and membrane trafficking. It is tantalizing to study the role of natamycin in this respect

## Organization of the membrane of germinating spores

### Part 2: The ergosterol cap

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The polyene antibiotic filipin is occasionally used as a fluorescent marker for sterol distribution in fungal cells, but it has never been evaluated if the compound realistically reports ergosterol location and causes artefacts. For instance, the excessive formation of filipin-sterol aggregates has to be avoided; this is best achieved by short filipin incubation and low concentrations and low temperatures after staining. Further, the fungal cell wall might display variable permeability for filipin and as a result might suggest certain filipin “hot spots” at, for instance, growing apices of fungal hyphae. We confirmed that membrane staining was evenly distributed as judged by FM4-64 staining of conidia. The reliability of filipin as a fluorescent sterol marker was further tested by attenuation of the fluorescent signal of filipin with natamycin that also binds more specifically to ergosterol. This was done on germinated conidia and mutant *S. cerevisiae* strains. These so-called *ergΔ* mutants contain an altered set of sterols and exhibited ergosterol dependent fluorescence. With our new filipin staining method, conidia of *Penicillium discolor* revealed a homogeneous distribution of ergosterol during swelling, followed by an increase of fluorescence at the presumptive site of germtube emergence and the apex of the germtube itself. Filipin was also enriched at septation sites in hyphae. Actin appeared to play a role in stabilizing and localization of ergosterol in that both were reversibly affected by latrunculin A. Furthermore inhibition of the sphingolipid biosynthesis with myriocin resulted in a small decrease of fluorescent intensity at the site of ergosterol polarization. These results suggest that careful staining with filipin under controlled conditions gives reliable data and that a polarized ergosterol cap exists at conidia during polarisation establishment and maintenance.

## **Session 7**

### **Application of molecular methods to food mycology**

## **Molecular methods to follow the fate and activity of *Penicillium verrucosum* in wheat**

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The main habitat of *P. verrucosum* is cereals and especially wheat. *P. verrucosum* is responsible for the contamination of cereals with ochratoxin A. It has been shown that the main intake of ochratoxin A by the consumer is via the consumption of cereals and cereal products, demonstrating the food safety importance of this fungal species. It is also known that a certain level of contamination by *P. verrucosum* indicates the presence of ochratoxin A in the cereal sample. *P. verrucosum* is a slow growing species and several days are needed to identify and quantify this fungus from cereal samples by conventional methods. In addition no information can be gained by these techniques about the physiological status of the fungus, in particular its ability to produce ochratoxin A under the current growth conditions. To be able to rapidly detect, quantify and monitor the capacity of ochratoxin A biosynthesis by this fungus, a set of molecular methods has been developed, which allows the early assessment of the risk caused by the presence and growth of *P. verrucosum*. These methods include a specific PCR, a Real Time PCR, a reverse transcriptase Real Time PCR and a microarray approach to detect and quantify *P. verrucosum*, as well as to analyse the activity of ochratoxin A biosynthesis genes. To develop the single gene based methods the ochratoxin A polyketide synthase gene of *P. verrucosum* (*otapksPV*) has been isolated by heterologous cloning. The microarray carries oligonucleotides specific for ochratoxin A producing *Penicillia*. It has been shown in model experiments and under practical storage conditions, that these molecular methods can detect *P. verrucosum* specifically in wheat and can very early identify the activation of the ochratoxin A biosynthesis genes, in fact before reasonable amounts of ochratoxin A are produced. The expression of the biosynthesis genes is very well correlated to the biosynthesis of ochratoxin A. Several general features of ochratoxin A biosynthesis gene activation in *P. verrucosum* could be worked out by using these methods. In addition to these instrumental methods, the basis for a reporter gene approach in *P. verrucosum* have been established. The rationale behind this approach is the usage of promoter signals from the ochratoxin A biosynthesis genes to drive the transcription of reporter genes such as the genes for the LacZ or the GFP proteins and to study the activity of these genes under food relevant conditions. A prerequisite for this approach is a functional transformation system. For *P. verrucosum* an *Agrobacterium tumefaciens* mediated transformation system (ATMT) has been established and heterologous gene expression has been demonstrated for the first time for this fungal species.

## Real time PCR detection of emerging toxigenic *Fusarium* species in Sweden

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*Fusarium graminearum* is an emerging plant pathogen in the Nordic countries and is increasingly found in Swedish cereal grain. The main toxins produced by *F. graminearum* are deoxynivalenol (DON) and zearalenone (ZEA), both toxic to humans and animals. Maximum levels of these toxins are set to 1250 ppb for DON and 100 ppb for ZEA within the European Union (EC No. 1881/2006). Identification of *Fusarium* species by traditional methods requires specific skill and experience and there is an increased interest for new molecular methods for identification and quantification of *Fusarium* from environmental samples. Real-time PCR with probe technology (Taqman) can be used for the identification and quantification of several species of *Fusarium* from cereal grain samples. There are several critical steps that need to be considered when establishing a Real Time PCR method for DNA quantification. In our study, several DNA extraction methods were evaluated, including the extraction robot EZ1 (Qiagen), Fast DNA spin kit for soil (Qibiogene), DNeasy Plant kit (Qiagen) and a CTAB-based method. Parameters such as DNA purity and stability, PCR inhibitors, and PCR efficiency were investigated. Our results showed that the DNeasy Plant kit (Qiagen) in combination with sonication gave the best results with respect to yield whereas several other methods gave good PCR efficiency (above 90%) and DNA stability. To avoid effects of PCR inhibitors the DNA samples had to be diluted 5-10 times before PCR analysis. The DNeasy Plant kit (Qiagen) was used to analyse 30 wheat samples for the presence of *F. graminearum* and *F. culmorum*. The DNA level of *F. graminearum* could be correlated to the level of DON ( $r^2=0.9$ ) whereas no correlation was found between *F. culmorum* and DON. This indicates that *F. graminearum*, and not *F. culmorum*, is the main producer of DON in Swedish wheat.

## Effect of environmental factors on *Fusarium* toxin gene expression

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The production of secondary metabolites such as trichothecenes and fumonisins are strongly dependent on nutritional matrices, and environmental factors such as temperature, pH and water availability. While there is significant body of information on the effect of such fluctuating conditions on phenotypic production of the mycotoxins, less information is available on how these factors affect triggering of gene expression involved in mycotoxin production. We have thus examined for the first time the impact of interacting environmental factors on trichothecene and fumonisin biosynthetic gene clusters using microarrays and quantitative PCR. This has been related to phenotypic mycotoxin expression. For example, when the monitored parameters favoured high tri gene expression in *F. culmorum* and *F. graminearum* then high production of DON was observed and vice versa, depending on the genes. The *FUM1* gene expression in *F. verticillioides* was influenced by interactions between osmotic or matric stress and temperature. These results suggest that early stimulation of mycotoxin gene expression can occur, especially under environmental stress. This will enable a better understanding to be made of the influence of these interacting factors on regulation of important biosynthetic toxin gene clusters and could be an excellent tool for examining in detail potential control strategies, both pre- and post-harvest in important staple foods.

## **Sessions 9-10**

### **Ecology and physiology of fungi in foods**



## Effect of CO<sub>2</sub> concentrations on growth of fungi at various $a_w$ values and construction of a time to growth model

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We studied the effect of modified atmospheres on growth of some common xerophilic food spoilage fungi (*Eurotium rubrum*, *E. herbariorum*, *Aspergillus penicillioides*, *A. niger*, *A. flavus* and *Penicillium roqueforti*) using low levels of oxygen (5, 1 and 0.5%) in combination with elevated carbon dioxide (20, 35, 50 and 65%) over a range of water activity (0.90 to 0.77  $a_w$ ). Agar plates inoculated with fungal spores were packaged in pouches made from high barrier film filled with respective gas mixtures and incubated at 25°C. Water activity was measured using an Aqua Lab CX-3 instrument and head space gas composition in each pouch was analysed by Gas Pace analyser. Results indicated that *E. rubrum* was the most tolerant species at low oxygen and elevated carbon dioxide concentrations at high water activity, growing within 14.5 days in 65% CO<sub>2</sub> + 1% O<sub>2</sub> at 0.90  $a_w$ . However, at 0.78  $a_w$ , the sensitivity of *E. rubrum* increased markedly, and at 35% CO<sub>2</sub> + 1% O<sub>2</sub> no growth was observed at 100 days. *E. herbariorum* was very sensitive to 0.5% O<sub>2</sub> + 35% CO<sub>2</sub> at  $a_w$  0.81, with no growth in CO<sub>2</sub> concentrations higher than 35% at  $a_w$  0.78 and 20% CO<sub>2</sub> at 0.77  $a_w$ . *Aspergillus penicillioides* was also sensitive at 35% CO<sub>2</sub> + 1% O<sub>2</sub> at 0.78  $a_w$ . Analysis of the data showed that oxygen concentration was not a significant factor in growth inhibition at levels of 1% or greater, and that growth inhibition relied on the interaction of CO<sub>2</sub> concentrations with  $a_w$ . However, oxygen levels below 0.5% were inhibitory to growth. We used the combined datasets from all species to construct a predictive model for time to visible growth at various  $a_w$  and CO<sub>2</sub> concentrations.

# Modelling effect of interacting environmental factors on growth of spoilage moulds

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Mathematical modelling has proved to be a valuable tool in the food industry to predict microbial growth as a function of environmental factors such as pH, temperature and water availability. However, less attention has been paid to filamentous fungi because of their mycelial growth habitat. Many studies have also only considered single factors in modelling fungal growth whether using growth/no growth thresholds, or empirical mechanistic approaches. The use of secondary polynomial model development using surface response contour plots has received particular attention for mycotoxigenic moulds. Using *Aspergillus niger*, and *A. carbonarius* as examples we have examined the development of models which take into account interacting environmental factors of  $a_w$  and temperature using polynomial functions and validated these against different models for microbial growth available in the literature (e.g. Miles, Davey and Rosso). Attention has been on modelling the impact of interacting conditions because of the potential synergistic efficacy in terms of extending shelf-life of food products. However, the Gamma hypothesis suggests that multiple inhibitory factors combine independently and this is used effectively as part of the Hurdle concept in food manufacture. Recent studies with bacteria suggest that when examining time to detection (TTD) then factors such as pH, salt and organic acids may have independent effects on this parameter (TTD). If there is no synergistic effect of such parameters for a wide range of food spoilage bacteria it may be worthwhile giving consideration to such effects on food spoilage moulds by testing the validity of the Gamma hypothesis.

# **The development of FoodMold, a comprehensive interactive CD guide to foodborne fungi**

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In about 1992, when modern computer technology was still in its infancy, I decided that it would be nice to produce a computer-based key to foodborne fungi, based on the 1985 "Fungi and Food Spoilage" by Pitt and Hocking. This would allow the viewing of pictures in quite faithful colours, and that would greatly assist identifications in some genera. Soon after that, I came across a new computer program called "ToolBook", produced in the United States by a company called Asymetrix. I purchased a copy of Version 1.5, and set to work to learn how to use it. ToolBook is a very distinctive program with its own computer language. It is designed to provide information as a set of pages – computer screens – just made for single species and readily addressed by hyperlinks such as keys or indexes. ToolBook seemed the ideal system for what I wished to achieve. I started work and christened my program "FoodMold".

A couple of years later, ToolBook Version 3.5 appeared, with some greatly improved interfaces. I presented a partly completed work to Chapman and Hall in London, who were publishing the second edition of "Fungi and Food Spoilage" and they expressed interest in marketing FoodMold. Chapman and Hall were almost immediately sold off and closed down, and FoodMold languished from 1998 until about 2004. In the meantime computer technology had made enormous strides. In particular, the advent of the CD made the handling of large files – which FoodMold had become – simple.

When we began work on the third edition of Fungi and Food Spoilage with new publishers, interest again increased. After a lot of trouble, I was able to locate ToolBook again – now Version 8.5 and owned by "Click2Learn", soon to become "SumTotal". This version has vast improvements for the user, especially in set up on a hard drive. At about this time, BCN Research Laboratories became interested in a cooperative venture, and here today, at last, 15 years later, FoodMold is nearing completion. This presentation will go through some of the developmental steps and provide an overview of the capabilities of this program.

# **Mould and yeast problems in the food production chain – report on a survey of the Swedish food industry**

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Food and feed mycologists tend to believe that growth of mould and yeast is an important problem in the food production chain and that this may pose a risk to the consumer, as well as economical losses for the producer. The food producing companies turn to food mycology laboratories for assistance when problems have arisen and mould isolates need identification, making it easy to hold on to that idea. To learn more of the perceived extent of these problems we systematically contacted Swedish food producing companies with questions related to microbial growth in the production chain, sources of contamination, analytical requirements, level of education of the staff, internal quality control programs, and strategies for reducing mould and yeast in the production. The companies that participated in the survey represented five product groups: <sup>1</sup>bread and cereal products, <sup>2</sup>cheese and dairy products, <sup>3</sup>juice and other liquid products based on fruit, <sup>4</sup>candy and sugar, and <sup>5</sup>cereal grain for feed. The survey results showed that the three products most sensitive to mycological spoilage were cheese, fruit juice, and cereal grain. Hard cheese was susceptible to *Penicillium* contamination and growth during process and storage. One company systematically restored the cheese by cutting off the mouldy parts, others used them for making soft “melting-cheese” through a heating process. Yeast, which was the major problem in fruit-juice, entered the production chain with the fruits, grew during storage and reduced the shelf life of the product, especially in low- or non-pasteurized products. Mycotoxins were not considered as risks in these two types of products in the internal quality control system (HACCP) of these companies. In cereal-based products, mycotoxin analysis was a part of the regular quality control systems and the mould contamination of the process often originated from the raw material. Generally, losses due to fungal spoilage are not regarded as a major problem by the food industry.

## ***Dekkera bruxellensis* and *Lactobacillus vini*, a new stable consortium for industrial ethanol production**

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The yeast and lactic acid bacteria populations in an industrial ethanol fermentation process were investigated using PCR-fingerprinting and rDNA sequencing. This process had originally been inoculated with *Saccharomyces cerevisiae*. However, in the stably running process, the dominating yeast was instead *Dekkera bruxellensis*. There was also a rather high number of lactic acid bacteria, which almost exclusively belonged to *Lactobacillus vini*. *D. bruxellensis* is a common contaminant in the wine industry, but is also a production organism in the brewing of Lambic beer and in the generation of sour dough. Although lactic acid bacteria usually are unwanted in ethanol fermentations, they did not seem to affect the process in a negative way. Accordingly, the number of yeast cells was highest when the number of lactic acid bacteria was highest, only low levels of non-desired side-products were detected and the ethanol productivity was in the normal range. Thus, *D. bruxellensis*, together with *L. vini*, can be regarded as ethanol production organisms.

## ***Fusarium oxysporum* as a spoilage agent of ready-to-drink beverages**

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Spoilage of ready-to-drink fruit and dairy beverages by micro-organisms results in fermentation, pack swelling and the production of off-flavours. Yeast and lactic acid bacteria are the most common cause of spoilage in low pH beverages such as fruit juices. However, filamentous fungi are increasingly causing problems in a number of beverage types, from bottled water, to juice products and dairy-based drinks. As shelf stable beverage products usually undergo some heat processing (pasteurisation or ultra-high temperature, UHT) the filamentous fungi causing spoilage are often heat resistant. Recently, the filamentous fungus *Fusarium oxysporum*, which is considered as a serious plant wilt pathogen, but not as a common spoilage micro-organism, has been isolated from UHT juices and dairy drinks in Australia and overseas. *F. oxysporum* is able to grow at low O<sub>2</sub> tension, but is not reported to be heat resistant and the mechanisms that enable it to contaminate a heat processed product that is aseptically filled into its final container are not understood. *F. oxysporum* also poses a food safety issue as it has been reported to produce a number of mycotoxins including fumonisin, moniliformin, and zearalenone. This study examines *F. oxysporum* spoilage in fruit juice products, the moist and dry heat resistance characteristics of *F. oxysporum*, and the potential of spoilage strains to produce mycotoxins.

## **Efficacy of commonly used sanitizers against foodborne fungi**

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Most research on the effect of sanitizers on spores has been conducted on bacteria, mainly on foodborne pathogens as well as sporeformer bacteria (*Clostridium* sp., *Bacillus* sp., *Alicyclobacillus acidoterrestris*, etc.). This study was undertaken to study the efficacy of several commonly used sanitizers at the no-rinse concentration against a number of common foodborne fungi. A modified AOAC Germicidal and Detergent Sanitizers Method was used. Disinfectants were obtained from commercial sources. The most commonly used sanitizers in the food and beverage industry were selected. Each sanitizer was prepared daily using sterile distilled water according to the label instructions. Concentrations were checked using the appropriate test kit. The exposure times were 5 and 15 min at room temperature. All test solutions were at room temperature. The best performing sanitizer was chlorine dioxide (acidified sodium chlorite) at 200 ppm. After 15 min, there were no survivors. The second best performing sanitizer was iodine (iodophor) at 25 ppm. After 15 min of exposure, most of the fungi were significantly reduced or killed. In many cases, there were no survivors. Sodium hypochlorite at 200 ppm did not perform as well as expected. To inhibit fungi, the concentration should be increased or a longer exposure time should be chosen. Quaternary ammonium compounds (QUAT) and acid QUATS performed better than expected at 200 ppm. Some fungi were more sensitive to these compounds than others. The acid sanitizer studied performed better than the hypochlorite, acid Quat and the two peroxyacetic acids but was still not good enough to decrease counts to an acceptable level which ideally is <1 cfu/area swabbed. Two peroxyacetic compounds were studied at the recommended concentrations of 98 and 128 ppm. Neither was effective against all the fungi studied even after 15 min of exposure. More work needs to be done on finding the kill time for each of the sanitizers, the effect on surfaces such as stainless steel carriers, and the effect on biofilms. More research is being performed on the effect of these sanitizers on ascospores, the heat-resistant spores of heat-resistant molds (HRM). Even though chlorine dioxide has shown the highest efficacy against fungi, it is not easy to use and requires training of the employees. Due to the efficacy against fungal spores, the lower cost of ready-to-use sanitizer solution and ease of use, iodophors are the sanitizer of choice for fungal control.

## Enhancement of antifungal activity of propionibacteria

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Dairy propionibacteria are widely used in starter cultures for Swiss type cheese. These bacteria can ferment glucose, lactic acid and glycerol to propionic acid, acetic acid and carbon dioxide. Glycerol is in some lactic acid bacteria converted to 3-hydroxypropionaldehyde (3-HPA) also known as reuterin, a well-known antimicrobial. We examined the antifungal effect of dairy propionibacteria in combination with glycerol. Five type strains of propionibacteria were tested against the yeast *Rhodotorula mucilaginosa* and the molds *Penicillium commune* and *Penicillium roqueforti*. A dual culture assay with different concentrations of glycerol in the top layer, as well as a microtitre plate assay with broth cultures from media with glycerol or lactic acid as carbon source was used. The conversion of <sup>13</sup>C glycerol by *P. jensenii* was followed with NMR. In the dual culture assay, the degree of inhibition of the molds was strongly enhanced by increasing glycerol concentrations, while the yeast was less affected. The two *Propionibacterium freudenreichii* strains showed the strongest glycerol enhancement of inhibition. In the broth cultures, decreased pH in glycerol medium was probably responsible for the complete inhibition of the yeast and mold by three of five propionibacteria. NMR spectra of the glycerol conversion showed that propionic acid was the dominant metabolite, while 3-HPA was not detected. We conclude that the increased antifungal effect seen by glycerol addition to propionibacteria is due to production of propionic acid, with ensuing pH reduction. This finding should aid fungal spoilage control in food and feed industry since dairy propionibacteria, glycerol and propionate already have regulatory approval and are used in different production processes.



## **Antifungal activity of lactic acid bacteria and sourdough bread cultures**

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Increased public concern over chemical food additives has prompted searches for more natural antifungal agents. One source for these agents are the lactic acid bacteria (LAB), which have been used to ferment foods for years and are known to positively influence the gastrointestinal tract of humans and animals. Studies have suggested that LAB may have different mechanisms of antimicrobial activity, including competitive growth, metabolites (lactic, phenyllactic, propionic and acetic acids), pH, or a combination of these factors. This presentation will review some of these studies and present data on the antifungal activity of LAB from studies done in our laboratory on the effect of sourdough bread cultures and food grade LAB isolates on growth of spoilage and mycotoxigenic molds, both with intact cultures and culture filtrates. Studies were also done to evaluate the effect of pH on the antifungal activity of sourdough bread cultures. Results have shown that different sourdough bread cultures have remarkably different antifungal activities that may be lost depending upon the pH of the culture filtrate. Among those LAB isolates obtained, the ones from fermented plant foods proved to be stronger inhibitors of mold growth than those from dairy products, and the most inhibitory isolates were obtained from kimchi. The inhibitory effect of the isolates was greatly reduced when only the filtrate was tested, compared with the whole culture grown as a co-culture with the mold. Work is in progress to develop food grade growth media for LAB which would improve the antifungal activity of these culture filtrates. As future research, studies with stabilized fermentates (whole culture material with inactive bacteria cells) are needed to compare activities of fermentates and filtrates.

## **Impact of *Rhizopus oligosporus* and yeast fermentation on nutritional properties of cereal grains**

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Soybean tempeh has a long tradition of use in Indonesia as a protein rich food component. During fermentation with *R. oligosporus* the soybeans are bound by the white mycelium to a cake, and enzymes released by the fungus make the protein-rich product more digestible. We developed a patented process where intact cereal grain kernels can be fermented with *R. oligosporus* to barley tempeh. The resulting product has a firm texture, a neutral taste and can be used in many food dishes. During the original soybean process a multitude of microorganisms develop together with *R. oligosporus*, whereas the grain fermentation process originally was developed as an axenic system. The *R. oligosporus* fermentation to barley tempeh increases provitamin D (ergosterol) 100-fold and also reduces the mineral binding phytate (depending on process). Fungal fermentation of the cereals also gives products with high amounts of dietary fibre and a low glycaemic index, *i.e.* with slow carbohydrates. The effects of different pretreatments such as pearling, rolling, moistening, autoclaving and soaking before fermentation on phytate and mineral contents were investigated in whole grain barley and oat tempeh fermented with *R. oligosporus*. Pearling was the most effective pretreatment for reduction of phytate content for both oats and barley. Nevertheless, mineral contents were reduced, and most likely also cell wall rich fractions were reduced by this process. Initially, the phytate content in the oats and barley samples were reduced by 74% (3.3  $\mu\text{mol/g}$ , d.m.) and 89% (1.4  $\mu\text{mol/g}$ , d.m.), respectively. However, to improve iron absorption the phytate levels should not exceed 0.5  $\mu\text{mol/g}$ , and further phytate degradation was necessary. Therefore, in the final experiments barley samples were exposed to an optimised process with prolonged soaking at a higher temperature and the pearling residues were returned before fermentation. When the outer layers of the barley kernels were returned before fermentation the phytate content was successfully reduced by 97% to 0.4  $\mu\text{mol/g}$  (d.m.) and Fe and Zn levels were well preserved. We also developed barley tempeh co-cultivation systems by introducing different lactic acid bacteria and yeast species. Additional data on co-inoculation effects on contents of the vitamin folate will also be included in this presentation