CHAPTER 6

Part 1
The Metabolic Degradation of Ochratoxin A by Yeasts

This chapter comprises a collaborative study between the University of the Free State (Bloemfontein) and Potchefstroom University of Christian Higher Education. The aim of the project was to find microorganisms that can degrade OTA to non-toxic compounds.

Contribution made by the candidate

The candidate was assisted by Ms. Tanja van Rooyen (Potchefstoom University) and Prof. Martie Smit (University of the Free State) in the screening for yeasts containing the ability to degrade OTA. The candidate was, however, responsible for the final screening of the yeasts, the quantitation of the hydrolysis and the compilation of the results.

Part 2
The Degradation of Ochratoxin A by Fungi

This study was a collaborative study involving Foodtek, CSIR, Pretoria and the Potchefstroom University of Christian Higher Education.

Contribution made by the candidate

Ms. Annelie Lübben and dr. Gert Marais were responsible for the preliminary experiments. The candidate did the inoculation for the final experiments at Foodtek and was also responsible for the analysis and the compilation of the data.

The Metabolic Degradation of Ochratoxin A

Keywords: Ochratoxin A, metabolism, microorganisms, degradation and prevention

INTRODUCTION

Mycotoxins are toxic secondary metabolites of microorganisms (fungi) that pose a serious threat to the health of both humans and animals (Marquardt and Frohlich, 1992; Steyn and Stander, 1999 and references cited). Ochratoxin A (OTA), a mycotoxin produced by various *Penicillium* and *Aspergillus* species is a common contaminant of various foodstuffs including; spices, coffee, wheat, beer and animal products. Considerable research has been undertaken to detoxify mycotoxins in food especially aflatoxin-containing peanuts and oilseeds; physical, chemical or biological approaches are employed (Scott, 1998). The Food and Agricultural Organisation of the United Nations outlined the following requirements for decontamination processes, the process must:

1. destroy, inactivate or remove the mycotoxin
2. not produce or leave toxic or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed decontaminated feed
3. retain the nutritive value and acceptability of the products
4. not significantly alter important technological properties of the food or feed
5. destroy fungal spores and mycelium in order to avoid the formation of mycotoxins under favorable conditions

Several methods have been tested to detoxify ochratoxin contaminated foods including heat treatment (Josefsson and Moller, 1980; Boudra *et al.*, 1995), ammoniation (Chelkowski *et al.*, 1981), ensiling (Rotter *et al.*, 1989a; 1989b), increasing the dietary concentration of protein (Bailey *et al.,* 1989), cholestyramine (Madhyastha *et al.*, 1992) or phenylalanine (Bailey *et al.*, 1990, Rotter *et al.*, 1989a) or by the use of a non-specific absorbent, like charcoal (Rotter *et al.*, 1989b) but none of these methods showed sufficient results. Fermentation with the yeast *Saccharomyces cerevisiae* degrades patulin during cider manufacture (Doyle *et al.*, 1982; Scott, 1998).

For every biosynthetically formed compound there exists one or more microorganism that have the ability to degrade it either completely or partially; the degradation products can then be utilised by other organisms (Schlegel, 1986). This principle can be explained as follows: Green plants have been synthesising organic material for millions of years, such substances have not accumulated to any marked extent. Only a small fraction of these compounds has been conserved under complete exclusion of air as highly reduced carbon compounds (coal, petroleum and natural gas). This principle is, however, not flawless when it is applied to all compounds and not only biosynthetically produced compounds: no microorganism has yet been discovered that can degrade a number of synthetic compounds including plant-protective agents, detergents and highly polimerised materials (Schlegel, 1986). Microorganisms produce enzymes that catalyses the degradation of compounds. Although all enzymes are initially produced in the cell, some are secreted through the cell wall and function in the cell's environment (intracellular versus extracellular enzymes). Carboxypeptidase A is such an enzyme in mammalian cells that has the ability to hydrolyse OTA to phenylalanine and the non-toxic isocoumarinic acid, ochratoxin α (OTα) [See **Figure 1**, (Doster and Sinnhuber, 1972)]. Micoorganisms have been screened for their ability to degrade OTA, but we are aware of only two that have been identified as possessing this propensity (Wegst and Lingens, 1983; Hwang and Draughon, 1994). *Phenylobacterium immobile*, a Gram-negative soil bacterium was found to degrade OTA: The phenylalanine moiety was mostly attacked by the enzymes of the bacterium to form a phenol- and a dihydrodiol derivative of OTA and finally OTα (Wegst and Lingens, 1983). Hwang and Draughon (1994) screened a number of bacteria, yeasts and moulds for OTA degradation and found *Acinetobacter calcoaceticus* to degrade OTA to OTα.

In an effort to find biological ways to destroy or inactivate OTA in contaminated food, it was decided to screen a number of fungi for ochratoxin degradation. The screening can be done in three possible ways: cells can be removed from a growing culture and resuspended in a non-nutrient solution (resting cells or non-growing but viable cells); or cells in a growing culture; or the enzymes can be extracted from the cells (Pelczar *et al.*, 1986).

Fungi are eucaryotic spore-bearing protists that lack chlorophyll and comprise the moulds and yeasts. Whereas moulds are filamentous and multicellular, yeasts are usually unicellular (Pelczar, *et al.*, 1986).

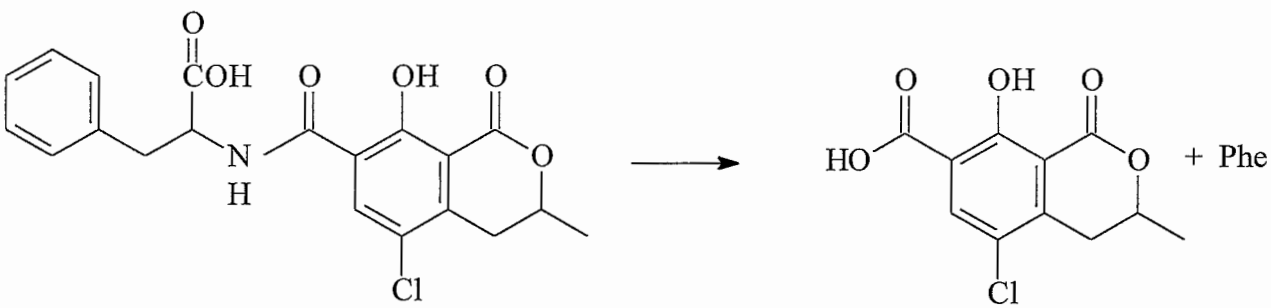

Figure 1: Structures of ochratoxin A (left) and ochratoxin α (right).

Part 1: The Metabolic Degradation of OTA by yeasts

ABSTRACT

Yeasts (323 different strains) were screened as a resting cell suspension for OTA degradation. Seven of these yeasts proved to degrade OTA, of which one, *Trichosporon mucoides* degraded OTA substantially within 48 hours in a growing culture.

Chemicals

OTA was extracted from Durum wheat inoculated with *Aspergillus ochraceus* (Stander *et al.*, 1999) (recrystallised from benzene, m.p. 91 °C, literature 90 °C, van der Merwe *et al.*, 1965). Ochratoxin α was obtained by hydrolysing OTA under reflux in excess 6 M hydrochloric acid for 60 hours. The three hydroxylated ochratoxins which were used as standards: (4*R*)- and (4*S*)-4-hydroxyochratoxin A and 10-hydroxyochratoxin A were supplied by Prof. R. Marquardt, Department of Animal Science, University of Manitoba, Canada. Yeast cultures were kindly provided by Professor Martie Smit University of the Free State, Bloemfontein, South Africa. Peptone, agar, potato dextrose agar, yeast extract and malt extract were obtained from Biolab (Merck). Potassium hydrogen phosphate, potassium phosphate, chloroform, methanol and dichloromethylsilane were obtained from Merck. Vanillin was purchased from Sigma.

Silinating glassware

The glassware was silinated to prevent the adsorption of OTA. Glassware was first treated with chromic acid for 24 hours, then left in water for 24 hours, rinsed three times with water, rinsed three times with distilled water and autoclaved (at 210 °C for 30 minutes). Glassware was placed in a desiccator in the presence of a beaker with dichloromethylsilane (10 ml). A vacuum was applied to the desiccator and it was kept under vacuum for an hour. The glassware was subsequently rinsed with double distilled water and autoclaved (at 210 °C for 30 minutes).

Thin layer chromatography (TLC)

Silica gel 60 aluminium sheets were purchased form Merck. A mobile phase of toluene/acetic acid (4:1) was used. The R_f values of OTA and of OTα under these conditions are: 0.50 and 0.23, respectively. Plates were viewed under UV illumination and non-fluorescing organic compounds were visualised by spraying the plates with vanillin/sulphuric acid and heating at 140 °C.

High Performance Liquid Chromatography (HPLC)

A Hewlett Packard 1090, HPLC system, fitted with a diode array (HP 1090) and fluorescence detector (HP 1100), autosampler and ChemStation software was used. Separations were achieved using a 4.6 mm × 150 mm, 5 µm, C_{18} analytical column (Discovery C_{18}, SUPELCO) fitted with a C_{18} guard cartridge (Spherisorb ODS-2, SUPELCO) and a mobile phase of water/methanol/acetic acid (50:60:2). Injection volume was 20 µl and flow rates of 1 ml/min were used. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 454 nm. Results were quantified relative to the 0 hour values. The percentage recovery of the extraction in **Part 2** was calculated to be: 97.4 % ± RSD 2.79 % (n = 6).

Growth

Optical density was used as an indication of the growth of the yeasts. Samples (20 µl) were taken from the cultures and diluted with physiological salt solution (180 µl). Optical densities (OD_{620}) were read at 620 nm with a Labsystem iEMS reader MF.

SCREENING FOR YEASTS WITH THE ABILITY TO DEGRADE OTA

Procedure

Inoculum liquid medium was prepared by dissolving yeast extract (3 g), maltose (20 g), peptone (10 g) and glucose (15 g) in double distilled water (500 ml). The above liquid medium was subsequently microwaved (5 minutes on high setting) and water (500 ml) added, and autoclaved (at 121 °C for 30 minutes). A selection of frozen cultures (-70°C) of yeasts were plated onto agar plates and kept at 25°C for 48 hours. The yeasts were harvested by adding a needle-eye full of the yeast to the inoculum medium (20 ml in 100 ml Erlenmeyer flasks). The flasks were shaken for 48 hours at 20 °C at 200 rpm. The optical densities (OD) of the cultures in the flasks were measured as described above. Samples (1 ml) of cultures with an OD_{620} of 3.0 and higher were transferred to Eppendorf tubes. The tubes were centrifuged (10 000 rpm, 2 minutes at 10 °C), the supernatant was removed and the pellets were washed by mixing with 50 mM phosphate buffer (1 ml, autoclaved at 121 °C for 30 minutes), centrifuging (10 000 rpm, 2 minutes at 10 °C) and removing the supernatant again. The 50 mM phosphate buffer (1 ml, autoclaved at 121 °C for 30 minutes) was added to the pellets and it was mixed again. The suspended cells were pipetted into silinated vials; OTA (5 µl, 0.2 mg/ml) was added and shaken for 9 days. Samples (200 µl) were taken every three days from the vials and the samples extracted with ethyl acetate (100 µl) by vortexing (1 minute) and centrifuging (1 minute, 10 000 rpm) and subsequent analysis by TLC.

Table 1: A selection of yeasts screened for their ability to degrade OTA

Genus	**species**	**Culture collection number**		
Aeromonium	*strictum*		TVN 306	
Aureobasidium	*pullulans*			NRRL Y-02311-1
Babjevia	*anomalus*	CBS 6740T		
Brettanomyces	*anomalus*			CSIR Y-0505
Bullera	*dendrophila*	CBS 6074 T		CSIR Y-0499T
Bullera	*singularis/dendrophila*	CBS 6460		
Candida	*albicans*			CSIR Y-0132
Candida	*albicans*	CBS 0562		UOFS Y-0198
Candida	*albicans*	CBS 5736		
Candida	*albicans*	CBS 562		
Candida	*catehulata*	CBS 0564		
Candida	*cylindracea*	CBS 6330		
Candida	*diddensiae*			CSIR Y-0060
Candida	*edax*			CSIR Y-636T

Candida	*edax*	CBS 5657		CSIR Y-0636
Candida	*glabrata*		CSIR Y-0007	
Candida	*glaebosa*	CBS 5691T		CSIR Y-0581
Candida	*haemulonii*	CBS 5149 T		
Candida	*humilis*	CBS 5658T		
Candida	*inconspicua*	CBS 2833		
Candida	*inconspicua*	CBS 0990		
Candida	*inconspicua*	CBS 0180 T		
Candida	*inconspicua*	CBS 4258		UOFS Y-0392
Candida	*insectamans*	CBS 6033T		
Candida	*intermedia var. intermedia*	CBS 7153		
Candida	*magnoliae*		CSIR Y-0118	
Candida	*maltosa*	CBS 5611 T		
Candida	*mogii*	CBS 2032 T		
Candida	*parapsilosis*	CBS 0604	CSIR Y-0685	
Candida	*peltata*	CBS 5564		
Candida	*quercuum*	CBS 6422		
Candida	*rugosa*		CSIR Y-0295	
Candida	*rugosa*			CSIR Y-0299
Candida	*salmanticensis*	CBS 5121		
Candida	*shehatae*			Y 0492
Candida	*silvicultrix*	CBS 6269	CSIR Y-0481	
Candida	*sp. "saitoana"*			Y 756
Candida	*tenuis*	CBS 2885	CSIR Y-0597	
Candida	*tropicalis*			OC 0003
Candida	*tropicalis*	CBS 0094 T		
Candida	*vartiovaarai*	CBS 4289		
Candida	*wickerhamii*		CSIR Y-0926	
Candida	*wickerhamii*		CSIR Y-0799	
Candida	*wickerhamii*			IGC3244
Candida	*wickerhamii*	CBS 6395		CSIR Y-0038
Chrysemonas	*luteola*		TVN 293	
Cryptococcus	*albidus var. aerius*	CBS 0155 T		
Cryptococcus	*lamrentii*		TVN 129	W 36
Cryptococcus	*laurentii*		TVN 329	
Cryptococcus	*laurentii*		TVN 300	UOFS Y-0217
Cryptococcus	*terreus*	CBS 1895T		
Cryptococcus	*terreus*	CBS 1895 T		
Debaryomyces	*anomala*	CBS 0076	CSIR Y-0504	
Debaryomyces	*hansenii*		TVN 163	G 210
Debaryomyces	*hansenii*			CSIR Y-0016
Debaryomyces	*melissophilus*	CBS 7060		
Debaryomyces	*yamadae*	CBS 7035		CSIR Y-0887
Dekkera	*bruxellensis*			Y 0562
Dipodascopsis	*tothii*			UOFS Y 0811T
Dipodascopsis	*uninudeata var. wickerhamii*	CBS 741.74		
Dipodascus	*ingens*			UOFS Y-0104
Dipodascus	*tetrasperma*	CBS 765.70 T		
Exophiala	*dermatitidis*			UOFS Y-2048

Exophiala	*sp.*		VDW 0067	
Fellomyces	*penicillatus*		TVN 314	
Galactomyces	*geotrichum*	CBS 0772.71 T		
Galactomyces	*geotrichum*			UOFS Y-0079
Galactomyces	*geotrichum group a alt*			UOFS Y-0078
Galactomyces	*geotrichum group a alt*			UOFS Y-0077
Galactomyces	*sp.*		VDW 24	
Geotrichum	*candidum*	CBS 0193.34		
Geotrichum	*candidum*	CBS 0774.71		CSIR Y-1004
Geotrichum	*candidum*			UOFS Y-0073
Geotrichum	*candidum*			UOFS Y-0094
Geotrichum	*candidum*			UOFS Y-0092
Geotrichum	*candidum*			UOFS Y-0088
Geotrichum	*candidum*			UOFS Y-0081
Geotrichum	*candidum*			UOFS Y-0095
Geotrichum	*capitatum*	CBS 0572.82		
Geotrichum	*eriensis*	CBS 694.83		
Geotrichum	*fermentans*	CBS 0439.83		
Geotrichum	*fermentans*	CBS 409.34		
Geotrichum	*fragrans*	CBS 0164.32		
Geotrichum	*fragrans*	CBS 0127.76		
Geotrichum	*fragrans*			UOFS Y 0107
Geotrichum	*ingens*			UOFS Y-0093
Geotrichum	*ingens*			UOFS Y-0099
Geotrichum	*ingens*			UOFS Y-0100
Geotrichum	*klebahnii*	CBS 0625.85		
Geotrichum	*sericeum*	CBS 0192.55 T		
Geotrichum	*sericeum*	CBS 0192.55 T		
Geotrichum	*sericeum*	CBS 750.85		
Geotrichum	*sp.*			UOFS Y-0074
Geotrichum	*sp.*		VDW 0153	
Geotrichum	*sp.*		VDW 0152	
Geotrichum	*sp.*		VDW 142	
Geotrichum	*sp.*		VDW 143	
Geotrichum	*sp.*		VDW 149	
Geotrichum	*sp.*			UOFS Y-0111
Geotrichum	*sp.*		VDW 0145	
Geotrichum	*sp.*		VDW 146	
Geotrichum	*sp.*		VDW 150	
Geotrichum	*sp.*		VDW 144	
Geotrichum	*sp.*		VDW 151	
Geotrichum	*sp. fragrans*			UOFS Y-0101
Geotrichum	*spicifer*	CBS 0758.85		
Geotrichum	*terrestre*	CBS 0278.86 T		
GM 108			TVN 294	
GM 116			TVN 302	
GM 127			TVN 313	
GM 134			TVN 320	
GM 141			TVN 327	

GM 144			TVN 330	
GM 145			TVN 331	
GM 146			TVN 332	
GM 147			TVN 333	
Hanseniaspora	*guilliermondii*	CBS 0465 T		
Hanseniasporum	*guillermondi*	CBS 0465T		
Hasegawaea	*japonica var. versatilis*	CBS 0099		
Kluyveromyces	*delphensis*	CBS 2170 T		
Kluyveromyces	*lactis var. lactis*	CBS 2103		
Kluyveromyces	*lodderae*	CBS 2757T		
Kluyveromyces	*marxianus*	CBS 4857		
Kluyveromyces	*polysporus*	CBS 2163 T		
Lipomyces	*tetrasporus*			UOFS Y 0158
Lipomyces	*kockii*			UOFS y O168
Lipomyces	*kononenkoae*	CBS 2514T		
Lipomyces	*sp.*			UOFS Y-0171
Lipomyces	*sp.*		TVN 272	
Lipomyces	*tetrasporus*			UOFS Y 2034
Lipomyces	*tetrasporus*			UOFS Y0179
Lipomyces	*tetrasporus*			UOFS Y-2032
Lipomyces	*tetrasporus*			UOFS Y-0190
Lipomyces	*tetrasporus*			UOFS Y-0188
Lipomyces	*tetrasporus*			UOFS Y-0192
Lipomyces	*tetrasporus*			UOFS Y-0189
Lipomyces	*tetrasporus*			UOFS Y-2082
Lipomyces	*tetrasporus*			UOFS Y-0193
Lipomyces	*tetrasporus*			UOFS Y-0180
Lipomyces	*tetrasporus*			UOFS Y 0191
LM10			VDW 30	
LM2			VDW 29	
M 183(38)			TVN 50	
M 190			TVN 164	
Candida	*silvae*		TVN 168	UOFS-Y0491
M 252			TVN 52	
M 292			TVN 170	
M 295(12)			TVN 54	
M 356(12)			TVN 58	
Pichia	*pini*		TVN 19	UOFS-Y0490
M 459			TVN 016	
M 464			TVN 25	
M 465			TVN 21	
M 468			TVN 23	
M 544			TVN 17	
M 568			TVN 199	
M 884(32)			TVN 87	
M 885			TVN 89	
M 888			TVN 202	
M>	*pullchirina*		TVN 138	G 300
Malay			TVN 310	GM 124

Myxozyma	*lipomycoides*	CBS 7038 T		
Oranges	*sa*		TVN 171	M 283
Oranges	*sa*		TVN 176	M 256
Oranges	*vw*		TVN 220	M 707
Pachitrichosporon	*transvaalensis*		TVN 132	
Pichia	*membranaefaciens*		TVN 154	G 104
Pichia	*onychis*	CBS 5587 T		
Pichia	*cactophila*	CBS 6926 T		
Pichia	*etchellsii*			Y 0858
Pichia	*guilliermondii*	CBS 2030 T		NRRL Y-2075 T
Pichia	*haplophila*	CBS 2028 T		CSIR Y-0225 T
Pichia	*haplophilia*	CBS 4329		
Pichia	*holstii*		TVN 33	
Pichia	*jadinii*	CBS 4885		CSIR Y-0227
Pichia	*membranaefaciens*	CBS 0107		CSIR Y-0035
Pichia	*mississipiensis*			Y 0306
Pichia	*pini*	CBS 0744 T		
Pichia	*quercuum*	CBS 2283T		
Pichia	*quercuum*	CBS 2283 T		
Pichia	*scolyti*	CBS 4803		CSIR Y-0445
Pichia	*spartinae*	CBS 6059 T		
Pichia	*subpelliculosa*		TVN 334	
Pine			TVN 338	GM 152
Rhodotorula	*minuta*		TVN 341	UOFS Y-0138
Rhodosporidium	*lusitaniae*	CBS 7604 T		
Rhodosporidium	*sphaerocarpum*	CBS 6985		
Rhodosporidium	*toruloiides*	CBS 0014		
Rhodoturula	*acuta*	CBS 7053		
Rhodoturula	*araucariae*	CBS 6031 T		
Rhodoturula	*aurentiaca*		TVN307	UOFS Y-2049
Rhodoturula	*foliorum*	CBS 5234	CSIR Y-0017 T	
Rhodoturula	*glutinis*		TVN 149	G 318
Rhodoturula	*glutinis*		TVN 311	UOFS Y-0123
Rhodoturula	*lactosa*	CBS 5827		
Rhodoturula	*minuta*		TVN 350	
Rhodoturula	*minuta*		TVN 301	
Rhoduturula	*minuta*		TVN 286	
Rhodotorula	*mucilaginosa*	CBS 5951		
Rhodoturula	*mucilaginosa*		TVN 321	
Rhodoturula	*philyta*		TVN 326	
Rhodoturula	*sp.*	CBS 5143		
Rhodoturula	*sp.*		TVN 309	
Rhodoturula	*sp.*		TVN 298	
Saccharomyces	*buyanus*	CBS 1505		
Saccharomyces	*castellii*	CBS Y309T		
Saccharomyces	*cerevisiae*	CBS 2814		
Saccharomyces	*cerevisiae*	CBS 2354		
Saccharomyces	*cerevisiae*	CBS 4903		
Saccharomyces	*cerevisiae*	CBS 1171 NT		

Saccharomyces	*dairensis*	CBS 633		
Saccharomyces	*dairensis*	CBS 6904		
Saccharomyces	*dairensis*			CSIR Y-471
Saccharomyces	*dairensis*	CBS Y21T		CSIR Y-1392
Saccharomyces	*exiguus*	CBS 0835		CSIR Y-0441
Saccharomyces	*exiguus*			CSIR Y-0572
Saccharomyces	*exiguus*	CBS 379T		
Saccharomyces	*exiguus*	CBS 6440		
Saccharomyces	*exiguus*			CSIR Y-0348
Saccharomyces	*kluyverii*	CBS 6545		
Saccharomyces	*kluyverii*	CBS S. 4104		
Saccharomyces	*kluyverii*	CBS 2861		
Saccharomyces	*kluyverii*	CBS 3082T		
Saccharomyces	*paradoxus*	CBS 7400		
Saccharomyces	*pastorianus*	CBS 1503		
Saccharomyces	*pastorianus*	CBS 1513		
Saccharomyces	*pastorianus*	CBS 1538		
Saccharomyces	*sewazzii*	CBS 7721		
Saccharomyces	*unisporus*	CBS 3987		
Saccharomyces	*unisporus*	CBS 399		
Saccharomyces	*unisporus*	CBS 3004		
Saccharomyces	*unisporus*	CBS 398T		
Saccharomycodes	*ludwigii*	CBS 5929		
Saccharomycodes	*ludwigii*			Y 0022
Saccharomycodes	*sinensis*	CBS 7075 T		
Saccharomycopsis	*capsularis*	CBS 5638		CSIR Y-0447
Schizosaccharomyces	*japonicus var. japonicus*	CBS 7116		
Schizosaccharomyces	*octosporus var. octosporus*	CBS 1804		CSIR Y-0934
Schizosaccharomyces	*octosporus var. octosporus*	CBS 6206		
Schizosaccharomyces	*pombe*	CBS 1058		
Schizosaccharomyces	*pombe*	CBS 1059		
Schwanniomyces	*occidentalis*	CBS 4668		CSIR Y-0828
Sporidiobolus	*microsporus/johnsonii*	CBS 5937		CSIR Y-0144
Sporidiobolus	*pararoseus/johnsonii*			CSIR Y-0011
Sporobolomyces	*tsugae*	CBS 7096		
Sporobolomyces	*roseus*	IGC 4222		
Sporopachydermia	*quercuum*	CBS 8070		CSIR Y-0606
T102			VDW 0084	
T16			VDW 0070	
T17			VDW 0068	
T21			VDW 0071	
T63			VDW 0072	
T64			VDW 0065	
T69			VDW 0075	
T74			VDW 0074	
T75			VDW 0069	
T82			VDW 0076	
T88			VDW 0078	
T89			VDW 0079	

T92			VDW 0080	
Torulaspora	*glubosa*			Y 0574
Tremella	*fuciformis*	CBS 6970		
Trichosporon	*lodderae*	CBS 1924		
Trichosporon	*beigelii*			UOFS Y-0063
Trichosporon	*beigelii*			UOFS Y-0113
Trichosporon	*beigelii*		VDW 0091	
Trichosporon	*cutaneum*			UOFS Y-0264
Trichosporon	*cutaneum var. cutaneum*	CBS 2466NT		
Trichosporon	*ovoides*			UOFS Y-0106
Trichosporon	*pullulans*	CBS 2535		
Trichosporon	*sp.*		VDW 109	UOFS Y-0118
Trichosporon	*sp.*		VDW 114	
Trichosporon	*mucoides*		VDW 0061	UOFS Y-2041
Trichosporon	*sp.*		VDW 0115	
Trichosporon	*sp.*		VDW 113	
Trichosporon	*sp.*		VDW 0111LN	
Trichosporon	*sp.*			Y 0561
Trichosporon	*sp.*		VDW 0108LN	
Trichosporon	*sp.*		VDW 0110	
Trichosporon	*mucoides*		VDW 61	UOFS Y-2041
Wingea	*robertsiae*	CBS 2934 T		CSIR Y-0208 T
Yarrowia	*lipolytica*			Y 0513
Yarrowia	*lipolytica*	CBS 5699		
Yarrowia	*lipolityca*	CBS 6114		
Yarrowia	*lipolytica*	CBS 0599 T		
Yarrowia	*lipolytica*			CSIR Y-0088
Yarrowia	*lipolytica*	CBS 6124.2		
Yarrowia	*lipolytica*		TVN 47	M663/5
Yarrowia	*lipolytica*	CBS 2073		
Zygozyma	*sp.*			UOFS Y-0182
			TVN 22	
			TVN 12	
			TVN 318	
			TVN 312	
			TVN 287	GM 101
			TVN 353	
				UOFS Y-0230
				CSIR Y-0636 T
				UOFS Y-0225
			TVN 291	
			TVN 336	
			TVN 316	
			TVN 325	
			TVN 299	
				CSIR Y-0433T
			TVN 288	
				CSIR Y-0447
			TVN 297	

				ZZ6
				ZZ7
				ZZ3
				ZZ4
				ZZ2
				ZZ8
			TVN 166	
				ZZ9
		CBS 109/104		
			TVN 056	
				CSIR Y 1118

RESULTS

The yeasts reported in **Table 2** showed the ability to degrade OTA, as indicated by TLC. It was decided to substantiate the observations and to investigate the reaction kinetics of the degradation of OTA by these yeasts by quantifying the reaction(s) on HPLC. The presence of certain substrates can induce the production of enzymes that can catalyse the degradation of the substrate (Schlegel, 1986), it was therefore decided to investigate the hydrolysis of OTA by the yeasts that tested positive for degradation as growing cells.

Table 2: Yeasts that screened positive for OTA degradation

Genus	**Species**	**Culture collection number**		
Rhodotorula	*glutinis*		TVN 311	UOFS Y-0123
Unidentified fungus			TVN 318	UOFS Y-0132
Rhodotorula	*aurantiaca*		TVN 307	UOFS Y-2049
Trichosporon	*mucoides*		VDW 61	UOFS Y-2041
Rhodotorula	*minuta*		TVN 341	UOFS Y-0138
Cryptococcus	*laurentii*		TVN 300	UOFS Y-0217
Pichia	*guilliermondii*	CBS 2030 T		NRRL Y-2075 T
Pichia	*scolyti*	CBS 4803		CSIR Y-0445
Aureobasidium	*pullulans*			NRRL Y-02311-1

EXPERIMENTS TO SUBSTANTIATE THE ABILITY OF THE YEASTS IN TABLE 2 TO METABOLISE OTA

The liquid medium for inoculation was prepared in a similar manner as in the screening for the yeasts. The frozen cultures (-70°C) of the 7 yeasts that tested positively for OTA degradation were plated onto agar plates and kept at 25°C for 48 hours. The yeasts were harvested by adding a needle-eye full of the yeast to inoculum medium (20 ml in falcon tubes) in duplicate. The flasks including standards without yeasts were shaken for 48 hours at 25 °C at 200 rpm. OTA (100 µl of a solution of 20 mg/ml in 0.1M sodium bicarbonate) was added to the flasks. The flasks were returned to the shaker and samples (500 µl) were obtained at 0, 12, 24 and 48 hours after the addition of the OTA. The samples were extracted with ethyl acetate (1 ml), centrifuged at 1400 G for 10 minutes and 500 µl of the ethyl acetate layer was dried under a stream of nitrogen, resuspended in methanol/water (1:1) and 20 µl injected onto the HPLC.

RESULTS AND DISCUSSION

Only four of the yeasts reported in **Table 2** viz. *Trichosporon mucoides*, *Areobasidium pullulans*, *Rhodotorula glutinis* and *Pichia guilliermondii* showed a significant degradation of OTA (**Figure 2**). It was evident from **Figure 2** that one of the yeasts (*Trichosporon mucoides*) led to enhanced hydrolysis of OTA. The experiment was therefore, repeated with this yeast, at 3 concentrations of 2 mg, 4 mg and 8 mg of OTA added to 30 mg of yeast culture suspension. Samples were taken at 0, 6, 12, 24, 48, 60 and 84 hours after addition of OTA (**Figure 3**). In all four instances only hydrolysis of OTA (the formation of ochratoxin α) was observed; none of the known hydroxy-ochratoxins was observed (**Figure 4**). **Figure 3** illustrates the decrease in OTA concentration and the commensurate increase in OTα concentration as the OTA is hydrolysed over time by the yeast.

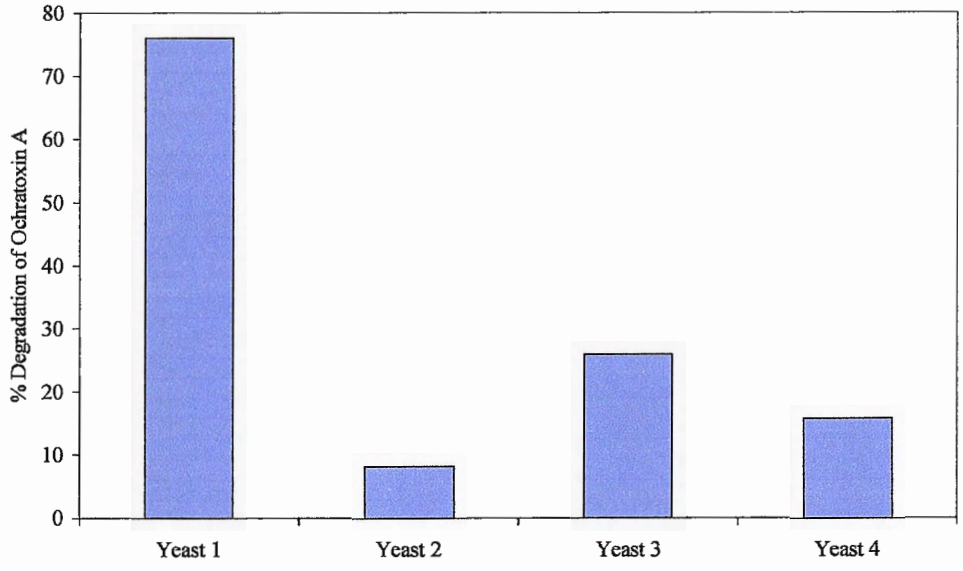

Figure 2: The degradation of OTA over a period of 48 hours by the four best yeasts: *Trichosporon mucoides* (1), *Areobasidium pullulans* (2), *Rhodotorula glutinis* (3) and *Pichia guilliermondii* (4).

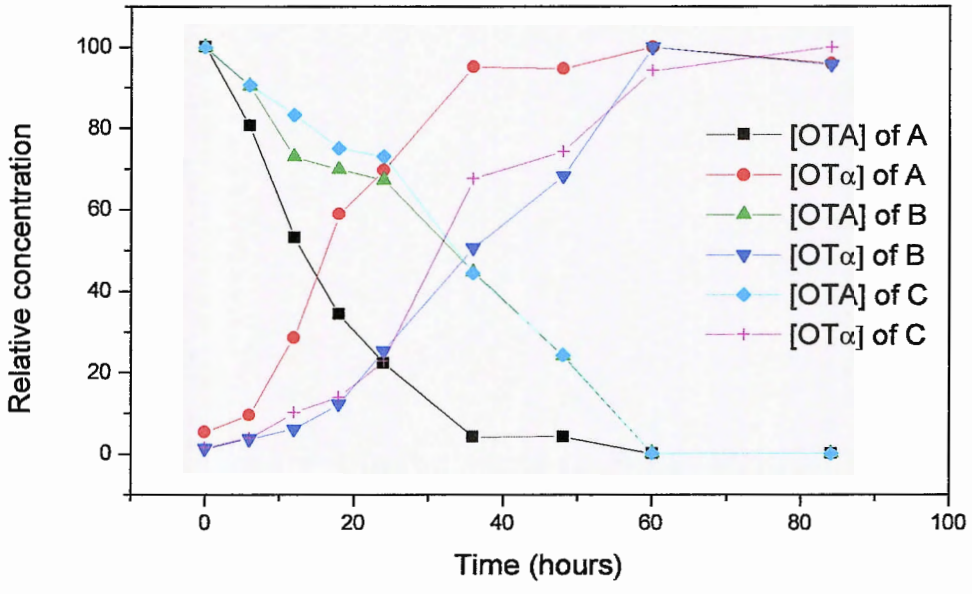

Figure 3: The degradation of OTA and the formation of OTα in A: addition of 2 mg of OTA, B: addition of 4 mg of OTA and C: addition of 8 mg of OTA to media inoculated by *Trichosporon mucoides*.

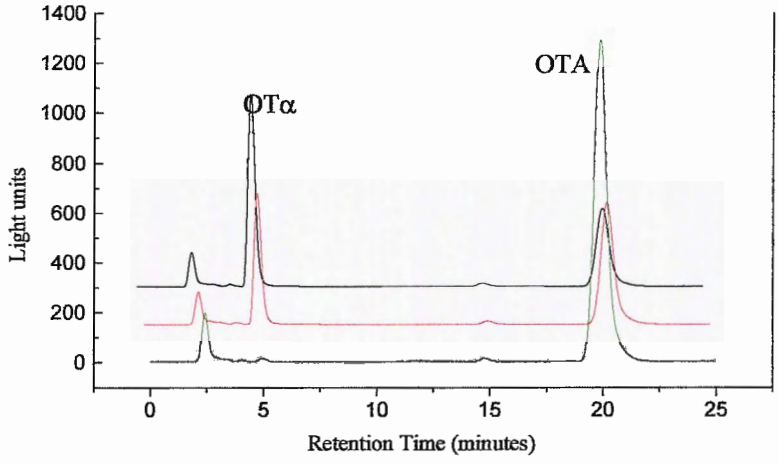

Figure 4: HPLC chromatograms depicting the degradation of OTA and the formation of OTα: After 0 hrs (green line), after 24 hrs (red line) and after 48 hrs (black line) upon addition of OTA to media inoculated with *Trichosporon mucoides.*

From the foregoing it is evident that *Trichosporon mucoides* contains the propensity to effectively degrade OTA to OTα (**Figure 4**).

Part 2: The ability of fungi to metabolise OTA

The success achieved with the studies involving yeasts, particularly *Trichosporon mucoides* stimulated us to extend the study to a few other fungi, *Cochliobolus sativus* (MRC 10870), *Penicillium islandicum* (MRC 1583) and *Metarhizium anispoliae* (MRC 11853) [obtained from the culture collection of the CSIR, Pretoria]. *Metarhizium anispoliae* (Hyphomycetes) is available as a commercial mycoinsecticide under the name Metaquino and has been used for example to control the rhinoceros beetle (Oryctes) in the islands of Western Samoa and Tongatapu (Kendrick, 1992). *Penicillium islandicum* is often found on rice and *Cochliobolus sativus* is a parasite on wheat (Kendrick, 1992, Marasas, 1999).

Materials

See Part 1 for the chemicals used. NH_2-LC (aminopropyl, 3 ml sample capacity, 500 mg packing material) solid phase extraction columns were purchased from SUPELCO.

High Performance liquid chromatography (HPLC)

Similar HPLC conditions as in **Part 1** were used. The percentage recovery of the extraction in **Part 2** was calculated to be: 97.4 % ± RSD 2.79 % (n = 6).

PROCEDURE

Lyophilised frozen cultures of *Cochliobolus sativus* (MRC 10870), *Penicillium islandicum* (MRC 1583) and *Metarhizium anispoliae* (MRC 11853) were plated onto potato dextrose agar plates, and the petri dishes incubated at 27 °C for 12 days in the dark. Three different concentrations of OTA (0 mg, 50 mg and 150 mg) were used in duplicate for each of the 3 microorganisms grown in Erlenmeyer flasks (500 ml) containing malt extract (5 g), 0.1 M sodium bicarbonate (12.5 ml) and distilled water (250 ml). The above liquid media were inoculated with a spore suspension of the microorganisms (2 ml); and the flasks placed on a rotary shaker for 16 days.

EXTRACTION

Chloroform (100 ml) was added to the Erlenmeyer flasks containing the fermented materials. The content of the flasks were filtered through fluted filter paper and the two phases were separated. The chloroform layer was kept. Amino solid phase extraction columns were conditioned with chloroform (2.5 ml), whereafter the chloroform extracts (2 ml) were transferred to the columns. The columns were washed with chloroform (2.7 ml) and the samples were eluted with methanol/acetic acid (4:1, 2.5 ml).

ANALYSIS

N-(5-chloro-2-hydroxybenzoyl)-phenylalanine (100 µl, 1.16 µg/ml) was added to the OTA containing extracts to act as internal standard and it was subsequently injected onto the HPLC (50 µl) under similar conditions as described in **Part 1**.

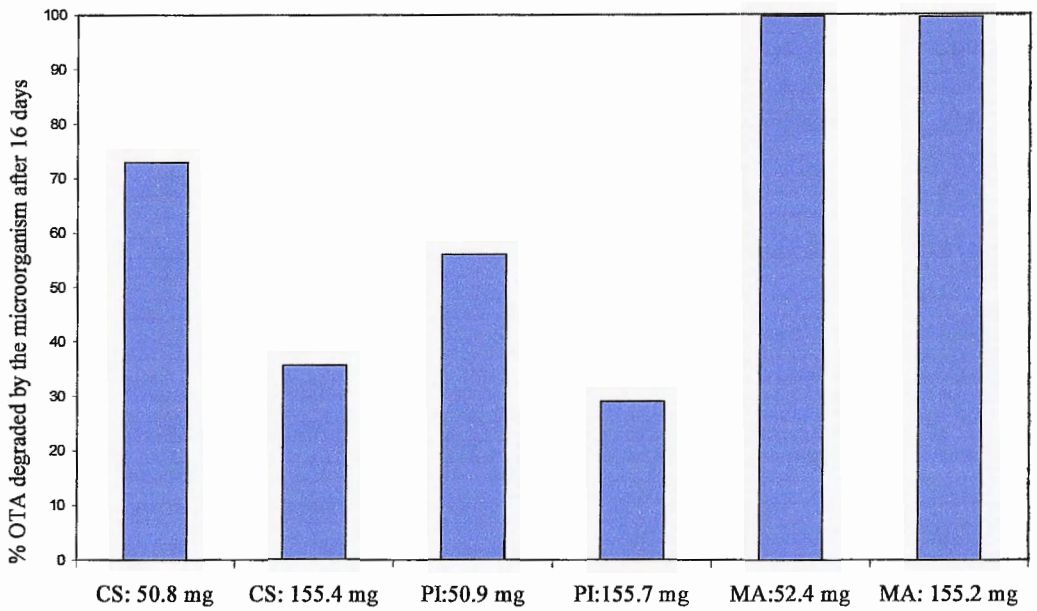

Figure 5: The degradation of OTA (amounts indicated) over a period of 16 days by *Cochliobolus sativus* (CS), *Penicillium islandicum* (PI) and *Metarhizium anispoliae* (MA).

RESULTS

All three of the fungi showed a promising ability to degrade OTA although the time required is very long (**Figure 5**). The concentration of OTA that was used for this study is much higher than the level of contamination in foods. *Cochliobolus sativus* (MRC 10870) and *Penicillium islandicum* (MRC 1583) showed a higher efficiency in hydrolysing the OTA at the lower concentration than at the higher OTA concentration. More work needs to be done into the ability of these fungi to degrade lower concentrations of OTA over shorter periods of time.

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