THE MAIZE TRUST

FINAL REPORT

Project no: MTM 11/10
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2. Contact details of the Project Leader *(Refer to application)*

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***Research was coordinated/conducted by Dr H Alberts**

3. Project information *(Refer to application)*

Title	Biological control of mycotoxins in food and feed grain commodities	
Duration of project	Start date: January 2011 End date: December 2013	
Budget year	2011 - 2013	
Total Annual Budget of Project	R 175 000	
Annual amount requested from Maize Trust	R 150 000	
Other sources of funding	Contributor	Amount requested / agreed
MRC	R 25 000	Agreed

4. Co-workers (research team) and other support staff *(Refer to application)*

Name	Qualification	Institution	Role
Prof WCA Gelderblom	PROMEC Unit	Medical Research Council	Research leader
Dr JF Alberts	PROMEC Unit	Medical Research Council	Research coordinator and senior scientist
Prof WH van Zyl	Microbiology Department	Stellenbosch University	Research collaborator
Prof A Botha	Microbiology Department	Stellenbosch University	Research advisor
Ms L du Plessis	Microbiology Department	Stellenbosch University	Researcher
Ms H-M Burger	PROMEC Unit	Medical Research Council	Researcher
Dr HF Vismer	PROMEC Unit	Medical Research Council	Research advisor

5. Summary (Description of the project, capturing the main findings; 250 words)

In this study methods to biologically degrade fumonisins are being developed by expressing bacterial and fungal genes encoding carboxylesterase and aminotransferase enzymes in *Saccharomyces cerevisiae* to achieve detoxification through enzymatic de-esterification and deamination. The aim is to eliminate fumonisin mycotoxin contamination in food/feeds by treatment with food-grade microbial cultures and enzyme preparations. Fungi capable of degrading FB_1 were selected by screening fungal isolates from culture collections and by enrichment procedure from compost-rich soil. Selection was based on

the ability to utilize molecules containing groups related to the terminal end (2-amino-3-hydroxy butyl moiety) of FB_1 as nitrogen source. *A. niger* SU 10864 was able to grow in minimal culture media with FB_1 as the sole nitrogen source. Species identification by microscopic examination, sequencing of the ITS regions and BLAST sequence comparison resulted in 100% homology with *A. niger* CBS 513.88. Recombinant microbial culture- and enzyme preparations were subsequently developed: bacterial and fungal (*A. niger*) genes encoding carboxylesterase and aminotransferase enzymes were targeted and expressed in *S. cerevisiae*, a food-grade yeast with GRAS (generally regarded as safe) status. A selection of fungal and bacterial genes encoding aminotransferase Class III and type B carboxylesterase enzymes acting on substrates with chemical structures related to FB_1 were included in the study. The enzymes were expressed intracellular as well as extracellular in *S. cerevisiae* Y294. The presence of the recombinant proteins in culture fractions was confirmed with SDS-PAGE. Results of fumonisin degradation experiments will conclude this part of the study. The developed treatments will be applied to fumonisin contaminated grain, including maize samples obtained from subsistence farmers in impoverished rural populations as well as for reduction of environmental mycotoxin exposure in animal farming, industrial applications and other post-harvest systems.

6. **Objectives** (Refer to application)

6.1. **Strategic objectives** (Maize Trust objectives)

To support the development of sound mycotoxin risk management practices in the maize supply chain to ensure the delivery of safe products to the consumer.

6.2. **Project objectives** (List main objectives)

Elimination of FB_1 from food intended for human and animal consumption utilising technological approaches:

6.2.1. Degradation of fumonisins by recombinant microbial culture- and enzyme preparations.

6.2.2. Bioprospecting for mycotoxin degrading microbial enzymes.

7. **Work plan** (Refer to application)

7.1. **Work plan as stated in the application** (List main tasks)

- Bioprospecting for mycotoxin degrading (preferably food-grade) microbial enzymes.
- Develop recombinant microbial culture- and enzyme preparations capable of effectively degrading fumonisins [WH van Zyl, SU].
- The effect of the recombinant mycotoxin degrading enzyme- as well as recombinant bacterial, fungal and yeast treatments on the cytotoxic and carcinogenic properties of the fumonisins [WH van Zyl, SU; PROMEC].
- Degradation of mycotoxins in contaminated grain, including samples obtained from subsistence farmers in impoverished rural populations [currently in progress].

7.2. Achieved tasks according to the stated work plan (List measurable units as milestones and provide an indication of progress made)

Milestones	**Achievements**
Screening of fungal isolates from culture collections of SU and the PROMEC Unit for the ability to utilize FB_1 for growth (Table 1 and 2).	Task achieved: A selection of fungal strains was made and cultivated in FB_1 minimal medium as well as Czapek-Dox medium with FB_1 as sole nitrogen source.
Enrichment procedure from compost-rich soil to obtain fungi capable of utilizing 1,4 diaminobutane as nitrogen source. Enrichment procedure from compost-rich soil to obtain fungi capable of utilizing FB_1 as nitrogen source.	Task achieved: One fungal isolate, capable of utilizing 1,4 diaminobutane as nitrogen source, was isolated from compost-rich soil. Task achieved: One fungal isolate, capable of utilizing FB_1 as nitrogen source, was isolated from compost-rich soil.
Identification of fungal isolates capable of utilizing 1,4 diaminobutane and FB_1 as nitrogen source.	Task achieved: Species identification of the positive 1,4 diaminobutane isolate resulted in 100% homology with *Fusarium solani*. Task achieved: Species identification of a positive FB_1 isolate of the SU culture collection resulted in 100% homology with *Aspergillus niger* CBS 513.88. Task achieved: Identification of positive isolates from the culture collections. Induction of possible aminotransferase enzymes for FB_1 degradation.
Targeting of aminotransferase enzymes produced by positive fungal strains obtained from preliminary experiments.	Task achieved: Aminotransferase enzymes: Gene sequences encoding an acetylornithine aminotransferase (CAK42339.1) and alanine aminotransferase (CAK40604.1) of *A. niger* CBS 513.88; aminotransferase of Bacterium ATCC 55552 (ADO15008.1); and aminotransferase (ACS27061.1) of *Sphingopyxis macrogoltabida*. Carboxylesterase enzymes (*A. niger* CBS 513.88): CAK38021; CAK38143; CAK41615; CAK41773; CAK47056.

	Gene sequences were codon-optimized for expression in *S. cerevisiae* and synthesized (Geneart). PacI and AscI were included as restriction sites for cloning into expression vector pRDH122 under control of the ENO1 promoter and terminator. A *zyn* secretion signal was included for extra-cellular production of the enzyme.
Cloning of genes and expression in *S. cerevisiae* strain Y294.	Task achieved: Cloning and expression of aminotransferase enzymes in *S. cerevisiae* (yeast integrated ligation). Task finalizing: Cloning and expression of carboxylesterase enzymes in *S. cerevisiae.*
Protein profile analyses (SDS-PAGE).	Task achieved: extra-cellular detection of aminotransferase enzymes. Task in progress: extra-cellular detection of carboxylesterase enzymes.
Fumonisin degradation experiments.	Task in progress: FB_1, HFB_1 and HFB_2 treated with enzyme fractions; determination of concentrations with LC/MS.

8. Detailed report covering the research conducted during the full grant period (Introduction, methods, results, discussion, tables, figures, etc.)

Introduction

The fumonisins are a group of mycotoxins mainly produced by the filamentous fungi *Fusarium verticillioides* and *F. proliferatum* that frequently contaminate maize (Wild and Gong, 2010). The fumonisin mycotoxins are important environmental and carcinogenic agents, causing toxic responses when ingested by mammals. Fumonisin B_1 (FB_1), fumonisin B_2 and fumonisin B_3 are the most abundant naturally occurring fumonisins, while FB_1 is the most prevalent fumonisin mycotoxin detected in maize intended for human consumption.

Mycotoxin contamination poses an enormous threat to the international trade of foods and feeds, because of the worldwide distribution of toxigenic fungi in agricultural products (Wild and Hall, 2000). Approximately 60% of Africa's grain supplies are at risk owing to fungal contamination and mycotoxin formation, thereby contributing to food insecurity in Africa. Maize is the staple food commodity in southern Africa, and known to be frequently contaminated with unacceptable levels of fumonisins, especially in areas where regulations are either lacking or not enforced. Although commercial maize is contaminated

with low levels, the high intake there-of could also be a risk factor for disease development in impoverished communities.

Several methods are employed to reduce fumonisin mycotoxins in food sources (Wild and Gong, 2010). Methods to control fumonisins in crops are mainly applied pre-harvest or during harvesting and processing, since *Fusarium* spp. infect maize in the field, not during storage, while the highest levels of fumonisins are present at harvest. There are different approaches in controlling fumonisins in maize. Replacing maize by another staple food in the diet could be an option, but the lack of alternative sources in certain populations makes it almost impossible (Shephard et al., 2008). Several pre-harvest practices are employed to reduce crop stress including improved agricultural practices with regards to sowing, plant density, fertilization, application of chemical agents and resistant maize cultivars (Wild and Hall, 2000). These approaches are expensive and have limited applicability. Sorting and washing of kernels and processing at high temperatures could reduce fumonisin levels (Wild and Gong, 2010). However, cooking and processing methods do not destroy the heat stable fumonisins and therefore retain the toxicity properties. While detoxification by physical and chemical methods are not always effective or economically feasible, concern regarding the negative environmental impact of pesticides caused an increasing interest in biological approaches to control mycotoxins in crops.

Practical, affordable and environmentally sound methods to biologically detoxify fumonisins are the focus of this research initiative. Biological detoxification is likely to offer an attractive alternative for eliminating toxins as well as safe-guarding the desired quality of food and feed (Alberts et al., 2009; Heinl et al, 2009; Wu et al., 2009). In this study methods to biologically degrade fumonisins are developed by expressing fungal genes encoding carboxylesterase and aminotransferase enzymes in *S. cerevisiae* to achieve detoxification through enzymatic de-esterification and deamination. Food-grade microbial strains expressing mycotoxin-degrading enzymes could support the development of commercial enzymatic additives capable of degrading these toxins in food.

Materials and Methods

Fungal strains

The fungal strains employed in experiments are listed in Table 1. The project focuses on food-grade fungi, including strains of *A. niger*, *Pleurotus* spp. as well as other saprophytic, edible mushrooms and wild yeast. The strains are part of fungal culture collections belonging to the Microbiology Department of SU and the PROMEC Unit of the MRC. Cultures were maintained on malt extract agar slants.

Culture media

FB_1 minimal medium. Yeast carbon base (1.17% w/v), FB_1 (0.75 mM in PBS pH 7.4, filter sterilized). *Czapek-Dox broth.* Glucose (3% w/v), $NaNO_3$ (0.3% w/v), K_2HPO_4 (0.1% w/v), $MgSO_4$ (0.05% w/v), KCl (0.05% w/v), $FeSO_4$ (0.001% w/v), *Modified Czapek-Dox broth (MCD).* Glucose (3% w/v), K_2HPO_4 (0.1% w/v), $MgSO_4$ (0.05% w/v), KCl (0.05% w/v), $FeSO_4$ (0.001% w/v), FB_1 (0.75 mM in PBS pH 7.4, filter sterilized), 2 mM pyruvate (filter sterilized). *Diaminobutane medium.* Yeast carbon base (1.17% w/v), 1,4 diaminobutane (2.34% w/v), chloramphenicol (0.5% w/v), filter sterilized. *SC-URA selective medium.* Succinic acid (2%), NaOH (1.2%), Yeast nitrogen base without amino acids and ammonium sulphate (0.34%), amino acid pool (-URA) (0.14%), $(NH_4)_2SO_4$ (1%).

Screening of fungal isolates from culture collections of SU and the PROMEC Unit for the ability to utilize FB_1 for growth

FB_1 minimal medium that supports growth of fungi capable of utilizing amino groups from FB_1 was employed. Fungal isolates (Table 1) were cultivated in FB_1 minimal medium for six months at 26°C while sub-culturing monthly to minimize the possibility of oligotrophic growth. In subsequent experiments positive isolates were sub-cultured in modified Czapek-Dox broth with AFB_1 as sole nitrogen source for four months, sub-culturing monthly.

Enrichment from compost-rich soil to select fungi capable of utilizing 1,4 diaminobutane as nitrogen source

Compost-rich soil samples were collected from the bottom, middle and top of a compost heap. Samples (1 g) were suspended in 10 ml distilled water, stirred for 30 min. and allow to settle for 15 min. An aliquot (5 ml) of each sample supernatant was pooled. An aliquot (0.1 ml) of the pooled and separate supernatants were inoculated in Diaminobutane medium (50 ml) respectively. Cultures were incubated at 26°C for four weeks on a rotary shaker, where after cultures were sub-cultured in FB_1 minimal medium (1 ml) and cultivated for four months, sub-culturing monthly.

Table 1. Fungal strains from culture collections of the PROMEC Unit and SU included in the study.

Fungal isolate	Strain no.	Substrate	Location
Aspergillus terreus	MRC 424	Maize	Mpumalanga, RSA
A. terreus	MRC 458	Unknown	RSA
Aspergillus fischeri	MRC 723	Cassava	Mozambique
Aspergillus niger gr.	MRC 741	Peanuts	Mozambique
A. niger gr.	MRC 742	Rice	Mozambique
A. niger gr.	MRC 743	Bean leaves	Mozambique
A. niger gr.	MRC 744	Maize	Mozambique
A. niger	MRC 898	Pistachio nuts	Iran
A. fischeri	MRC 920	Cassava	Mozambique
A. fischeri	MRC 1148	CBS 681.77	CBS
A. niger	MRC 1315	Maize malt	King Williamstown, RSA
Aspergillus oryzae	MRC 2837	NRRL 3485	CSIR
A. terreus	MRC 3947	Maize	Lydenburg, RSA
A. niger	MRC 5384	House	Stellenbosch, RSA
A. terreus	MRC 5385	House	Stellenbosch, RSA
A. niger	MRC 6977	Soil	Kruger National Park, RSA
A. terreus	MRC 6980	Soil	Kruger National Park, RSA
A. terreus	MRC 6981	Soil	Kruger National Park, RSA
A. niger	MRC 7419	Betel nuts	Durban, RSA
A. niger	MRC 8975	Maize kernel	Simdlangentsha, KZN, RSA
A. niger	MRC 8985	Maize kernel	Centane, RSA

A. niger	MRC 8986	Maize kernel	Centane, RSA
A. niger	MRC 8987	Maize kernel	Bizana, RSA
A. niger	MRC 8989	Maize kernel	Hlabisa, KZN, RSA
A. niger	MRC 8990	Onion	ARC, Stellenbosch, RSA
A. niger	MRC 8991	Onion	ARC, Stellenbosch, RSA
A. niger	MRC 8992	Onion	ARC, Stellenbosch, RSA
A. niger	MRC 8993	Onion	ARC, Stellenbosch, RSA
A. niger	MRC 8994	Onion	ARC, Stellenbosch, RSA
A. niger	MRC 9007	Beer	Unknown
A. niger	MRC 9008	Beer	Unknown
A. niger	MRC 9009	Beer	Unknown
A. niger	MRC 9012	Beer	Unknown
A. niger	MRC 9013	Beer	Unknown
A. niger	MRC 9014	Beer	Unknown
A. niger	MRC 9015	Beer	Unknown
A. niger	SU 10864	-	-
Pleurotus ostreatus	SU, St3-3	-	-
Pleurotus sanguineus	SU, SCC108	-	-
Saccharomyces cerevisiae	SU, Vin 13	-	-
Pleurotus florida	SU, 282	-	-

Enrichment procedure from compost-rich soil to select fungi capable of utilizing FB_1 as nitrogen source

Compost-rich soil samples were collected and prepared as described above and cultivated in FB_1 minimal medium for four months while sub-culturing monthly. Positive cultures were sub-cultured in MCD medium with FB_1 as sole nitrogen source for 4 months, sub-culturing monthly.

Identificaton of fungal isolates capable of utilizing 1,4 diaminobutane and FB_1 as nitrogen source

Pure cultures of fungal isolates capable of maintaining growth in the selective media throughout the incubation periods, were prepared on malt extract agar and identified utilizing traditional keys and descriptions in literature as well as molecular techniques. Genomic DNA was isolated and identification to species level conducted with analyses of the internal transcribed spacer- (ITS) of ribosomal DNA. The ITS regions were be amplified by the polymerase chain reaction (PCR) using selected universal oligonucleotide primers. The PCR products were purified by column chromatography (Nucleospin^R Extract II, Separations) and sequenced using a Perkin Elmer ABI PRISM model 3100 genetic sequencer. Data from the forward and reverse sequences was compared and aligned by using DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The fungal isolates were identified by comparing known sequences using the BLAST program of the National Centre for Biotechnology Information (NCBI).

Induction of possible fumonisin degrading enzymes

The most promising strains from the culture collection experiments (MRC 8975, 8990, 8993, 742, 8992, 9013, 7419, 6977) were cultivated in a modified Czapek-Dox medium (50 ml; without sodium nitrate) supplemented with 1,4 diaminobutane (7,5 mg/ml), pyruvate (3 mM) and pyridoxal 5'-phosphate (PLP; 10 µM). 1,4 Diaminobutane was present as the sole nitrogen source, PLP and pyruvate as co-factor and co-substrate respectively, to enhance aminotransferase activity. Cultures were incubated on a shaker incubator at 30°C for 8 days. Cell densities were monitored spectrophotometrically at 600 nm.

Induction experiments: fumonisin degradation and chromatographic analyses

Following incubation, 1,4 diaminobutane cultures were centrifuged (10 000 rpm/10 min) and the extra-cellular fractions aliquoted (500 µl) to 2 ml screw cap Eppendorf tubes, supplemented with FB_1 (final concentration 0.79 mM) and incubated at 30°C for 72 h. For chromatographic analyses extra-cellular culture fractions were 100x diluted with acetonitrile:H_2O (1:1) and derivatized with o-phthaldialdehyde (OPA) as described by Sydenham et al. (1996). FB_1 and 1,4 diaminobutane were quantified by isocratic HPLC analysis (Waters liquid chromatograph) utilising a Phenomenex Luna C18 (5 µm) column with methanol:0.1 M NaH_2PO_4 (82:16; pH 3.35) as mobile phase (1 ml/min). Compounds were measured with fluorescence detection (Waters 470 Scanning fluorescence detector; excitation 335 nm; emission 440 nm).

Selection of gene sequences: aminotransferase and carboxylesterase enzymes for expression in *S. cerevisiae*

Selection of genes focused firstly on aminotransferase Class III enzymes which are PLP dependent with pyruvate as co-substrate. The selection included EC 2 enzymes acting on substrates with chemical structures related to FB_1. Type B carboxylesterase enzymes of *A. niger* were also included in the study. Suitable aminotransferase and carboxylesterase enzymes produced by fungi isolated through FB_1 enrichment procedures were identified. *S. cerevisiae*, a food-grade yeast strain with GRAS status was employed as host strain. Aminotransferase gene sequences: Acetylornithine aminotransferase (*A. niger* CBS 513.88; CAK42339.1); aminotransferase (Bacterium ATCC 55552; ADO15008.1); aminotransferase (*Sphingopyxis macrogoltabida;* ACS27061.1); alanine aminotransferase (*A. niger* CBS 513.88; CAK40604.1). Carboxylesterase gene sequences: Carboxylesterase CAK38021; CAK38143; CAK41615; CAK41773; CAK47056. Gene sequences were optimized for expression in *S. cerevisiae*, relevant restriction sites (PacI and Asc) and secretion signals (xyn2) included, and synthesized (Geneart).

Cloning and expression in *S. cerevisiae* Y294: aminotransferase and carboxylesterase enzymes

Commercial plasmids harbouring the genes were digested with PacI and AscI restriction enzymes, where after the resulting DNA fragments were cloned into expression vector pRDH122 under control of the ENO1 promoter and terminator and transformed into *E. coli.* Positive transformants were identified by mini-prep plasmid isolation and subsequent restriction enzyme digest evaluations. For intracellular expression of the genes, plasmids were directly sub-cloned into *S. cerevisiae* Y294 through electroporation.

Extracellular expression of the enzymes was accomplished by Yeast Integrated Ligation (YIL). Forward primers were designed to partly anneal to the xyn2 secretion signal and partly anneal to the end of promoter sequence of the vector. Reverse primers were designed to partly anneal to the end of the xyn2 sequence and the start of gene sequence. A PCR reaction was performed to amplify xyn2 and the DNA sequences homologous to

the promoter and start of the gene. The plasmid containing the gene was digested with PacI between the promoter and gene. The digested plasmid and fragment was transformed directly into *S. cerevisiae* by electroporation. Selection was accomplished by cultivating the transformants on SC-URA selective agar. The presence of the genes in positive transformants was confirmed by amplification of the gene fragments by PCR whilst employing primers specific to the genes.

Confirmation of recombinant enzyme production: SDS-PAGE

Positive transformants were cultivated in SC-URA selective liquid medium (10 ml) for 3 days at 30°C on a shaker. The presence of constitutively produced recombinant aminotransferase and carboxylesterase enzymes in *S. cerevisiae* Y294 culture extracts were confirmed through fractionation of the extra- and intracellular proteins by SDS-PAGE (Sambrook et al., 1989). A 0.1% SDS-10% PAGE analysis was performed using Coomassie Blue R-250 (intracellular fractions) and silver (extracellular fractions) staining techniques, where after the protein profiles were compared. Yeast transformants, harbouring the plasmid without the genes, were included as controls.

Results

Screening of fungal isolates from culture collections of SU and the PROMEC Unit for the ability to utilize FB_1 for growth

Growth of fungal strains in FB_1 minimal medium and MCD medium is summarized in Table 2. The composition of the culture media was selected to support minimal and selective growth of fungi. Although growing slowly, several fungal strains could initially maintain growth in FB_1 minimal medium, possibly due to oligotrophic growth. Growth was generally spherical and hazy. However, after several times sub-culturing in FB_1 minimal medium and eventually in MCD medium, only a few strains continue to grow. Strains capable of growing in the selective media include *A. niger* SU 10864, *P. sanguineus* SCC108, *A. fischeri* MRC 1148. *S. cerevisiae* SU Vin13, *P. ostreatus* Su St3-3, *P. florida* SU 282 and several *A. niger* strains could not maintain growth in the selective media throughout the experiments. An *A. niger* strain SU 10864 was able to exist in the FB_1 minimal medium for five months as well as in MCD medium. Traditional microscopic examination, sequencing of the ITS regions and BLAST sequence comparison resulted in 100% homology with *A. niger* CBS 513.88.

Table 2. Growth in selective media with FB_1 as nitrogen source: diaminobutane fungal isolate and fungal strains from culture collections.

Fungal strain no.	Cultivated in FB_1 minimal medium	Comments	Sub-cultured
Diaminobutane isolate from compost-rich soil	16032011	Growth	1. 28032011: FB_1 medium Growth 2. 28042011 Growth 3. 05052011 Growth 4. 07062011 Growth 5. 05072011 6. 29072011: MCD medium 23082011: No growth
S. cerevisiae SU Vin13	16032011	No growth	1. FB_1 medium 2. 04082011: MCD medium 10082011: No growth 23082011: No growth
P. ostreatus SU St3-3	16032011	Growth	1. 16032011:FB_1 medium Growth, spherical, hazy 2. 28042011 Growth, spherical 3. 05052011 Growth, spherical 4. 07062011 Growth, spherical 5. 05072011 Growth, spherical 6. 04082011: MCD medium 23082011: No growth
A. niger SU 10864 (100% homology with *A. niger* CBS 513.88)	16032011	Growth	1. 16032011: FB_1 medium Growth, large, spherical 2. 12042011 Growth 3. 05052011 Growth, large, spherical 4. 07062011 Growth, hazy, spherical 5. 05072011 Growth, spherical, hazy 6. 28072011: MCD medium 10082011: Growth 23082011: Growth
P. florida SU 282	13042011	19052011: Growth	1. 31052011: FB_1 medium 23062011: Small, spherical growth 2. 04072011 11072011: Small, spherical growth

			3. 04082011 10082011: No growth 4. 04082011: MCD medium 10082011: No growth 23082011: No growth
P. sanguineus SU SCC108	13042011	23062011: Growth	1. 04072011: FB_1 medium 06072011: Growth 2. 04082011: MCD medium 10082011: Growth 23082011: Growth
MRC 1148	13042011	26052011: Growth	1. 31052011: FB_1 medium 17062011: Growth, hazy 27062011: Good growth 2. 01072011 04072011: Growth, hazy, spherical 14072011: Large, white, hazy spherical 3. 04082011: MCD medium 10082011: Hazy 23082011: Growth
MRC 1315	13042011	03052011: Growth	1. 31052011: FB_1 medium 08062011: Growth 2. 01072011 04072011: Growth, hazy, spherical 14072011: Large, hazy, spherical 3. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 2837	13042011	19052011: Growth	1. 31052011: FB_1 medium 08062011: Growth 2. 04072011 08072011: Growth, hazy 3. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 8985	13042011	03052011: Growth	1. 31052011: FB_1 medium 23072011: Growth 2. 04072011 08072011: Growth, hazy 3. 04082011: MCD medium 10082011: No growth 23082011: Growth
MRC 8987	13042011	27062011: No growth, terminated	1. 08082011: MCD medium Freshly inoculated 23082011: Growth
MRC 8989	13042011	03052011: Growth, spherical	1. 31052011: FB_1 medium 17062011: Growth, small, spherical 2. 04072011 06072011: Growth, hazy 3. 04082011: MCD medium

			10082011: Growth, hazy 23082011: Growth
MRC 424	06052011	23072011: No growth, terminated	1. 08082011: MCD medium Freshly inoculated 23082011: Growth
MRC 458	09052011	14072011: Slight growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 723	09052011	27062011: No growth, terminated	1. 08082011: MCD medium Freshly inoculated 23082011: No growth
MRC 741	09052011	03052011: Growth	1. 31052011: FB_1 medium 08062011: Growth 27062011: Good growth 2. 01072011 04072011: Growth, large, spherical, hazy 3. 04082011: MCD medium 10082011: Growth 23082011: Growth
MRC 742	09052011	03052011: Growth	1. 31052011: FB_1 medium 08062011: Growth 2. 01072011 04072011: Growth, hazy 3. 04082011: MCD medium 10082011: Growth, hazy 23082011: Growth
MRC 743	09052011	03052011: Growth	1. 31052011: FB_1 medium 08062011: Growth 2. 01072011 04072011: Growth, hazy, spherical 3. 04082011: MCD medium 10082011: Hazy 23082011: Growth
MRC 744	23052011	23072011: Growth	1. 04072011: FB_1 medium 08072011: Growth, small, hazy, spherical, black 2. 04082011: MCD medium 10082011: Hazy 23082011: No growth
MRC 898	23052011	27062011: Growth 14072011: Large growth, spherical, black	1. 04082011: MCD medium 10082011: Growth 23082011: Growth
MRC 920	23052011	04072011: Growth, small, hazy, spherical	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 3947	23052011	31052011: No 14072011: No, terminated	1. 08082011: MCD medium Freshly inoculated 23082011: Growth
MRC 5384	23052011	31052011: No, terminated	1. 08082011: MCD medium Freshly inoculated 23082011: Slight growth

MRC 5385	01062011	08072011: Growth	1. 08082011: MCD medium Freshly inoculated 23082011: No growth
MRC 6977	01062011	08062011: Growth	1. 01072011: FB_1 medium 04072011: Growth, hazy, spherical 2. 04082011: MCD medium 10082011: Hazy 23082011: Growth
MRC 6980	01062011	08062011: Growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 6981	01062011	08062011: No growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 7419	01062011	08062011: Growth	1. 01072011: FB_1 medium 04072011: Growth, hazy 2. 04082011: MCD medium 10082011: Growth 23082011: Growth
MRC 8975	01062011	08062011: Growth 27062011: Good growth, spherical, black	1. 01072011: FB_1 medium 04072011: Growth, hazy 11072011: Large growth, hazy 2. 04082011: MCD medium 10082011: Hazy 23082011: Growth
MRC 8986	01062011	17062011: Growth	1. 04072011: FB_1 medium 14072011: Slight growth 2. 04082011: MCD medium 10082011: Growth 23082011: Growth
MRC 8990	01062011	08062011: Growth	1. 01072011: FB_1 medium 04072011: Growth 2. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 8991	01062011	08062011: Growth	1. 04072011: FB_1 medium 08072011: Growth, hazy 2. 04082011: MCD medium 10082011: Hazy 23082011: No growth
MRC 8992	01062011	08062011: Growth	1. 01072011: FB_1 medium 04072011: Growth, hazy 2. 04082011: MCD medium 10082011: Hazy 23082011: Slight growth
MRC 8993	29062011	04072011: Growth	1. 04082011: MCD medium 10082011: Growth 23082011: No growth
MRC 8994	29062011	06072011: Growth	1. 04082011: MCD medium 10082011: Hazy 23082011: Growth
MRC 9007	29062011	04072011: No growth	1. 08082011: MCD medium Freshly inoculated

			23082011: No growth
MRC 9008	29062011	04072011: No growth	1. 08082011: MCD medium Freshly inoculated 23082011: No growth
MRC 9009	29062011	04072011: Growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 9010	29062011	04072011: Growth 11072011: Good growth	1. 04082011: MCD medium 10082011: No growth 23082011: Slight growth
MRC 9012	29062011	04072011: Growth	1. 04082011: MCD medium 10082011: No growth 23082011: Slight growth
MRC 9013	29062011	04072011: Growth 06072011: Good growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 9014	29062011	04072011: Growth 11072011: Good growth	1. 04082011: MCD medium 10082011: No growth 23082011: No growth
MRC 9015	29062011	04072011: Growth 08072011: Good growth	1. 04082011: MCD medium 10082011: No growth 23082011: Growth

Enrichment from compost-rich soil to select fungi capable of utilizing 1,4 diaminobutane as nitrogen source

The enrichment procedure from compost-rich soil produced one fungal isolate capable of growing in a medium with 1,4 diaminobutane as nitrogen source. The isolate was also capable to exist in FB_1 minimal medium for five months (Table 2). Traditional microscopic examination, sequencing of the ITS regions and BLAST sequence comparison resulted in 100% homology of the 1,4 diaminobutane isolate with *F. solani*.

Enrichment from compost-rich soil to select fungi capable of utilizing FB_1 as nitrogen source

The enrichment procedure from compost-rich soil with FB_1 as nitrogen source produced one fungal isolate capable of utilizing FB_1 as nitrogen source (Table 3). The isolate was capable of existing in the FB_1 minimal medium for four months as well as in MCD medium with FB_1 as sole nitrogen source. After sub-culturing to minimize oligotrophic growth, the isolate will be identified by traditional and molecular techniques as described above.

Detection and selection of *A. niger* aminotransferase enzymes for degradation of FB_1 was approached in two ways. Firstly, selected fungal strains from previous experiments were cultivated in a liquid medium with 1,4 diaminobutane as the sole nitrogen source for growth. In this way, the ability to grow indicates the utilization of the amino groups of 1,4 diaminobutane and therefore the possible production of aminotransferase enzymes by the fungus. Medium supernatant could serve as a source of enzymes to fractionate and detect the relevant enzyme activity. Secondly, characterized genes encoding aminotransferase enzymes of *A. niger* were selected for molecular expression in laboratory and industrial *S. cerevisiae* strains.

Table 3. Enrichment from compost-rich soil to select fungi capable of utilizing FB_1 as nitrogen source.

Sample	Description	Cultivated in FB_1 minimal medium	Sub-cultured
A	Top, middle and bottom samples mixed; starved	18032011 Top, middle and bottom: Growth	1. 06052011: FB_1 medium No growth
B	Top, middle and bottom samples kept separate; starved	24032011 Top: No growth Middle and bottom: Growth	1. 06052011: FB_1 medium No growth
C	Top, middle and bottom samples kept separate; not starved	13042011 Top, middle and bottom: Growth	1. 06052011: FB_1 medium Growth, hazy, spherical 2. 01062011 Growth, hazy, spherical 3. 01072011 Growth, hazy, spherical 4. 04082011: MCD medium 10082011: Top, middle and bottom: Growth 18082011: Top, middle and bottom: Growth

Table 4. Cell densities (A_{600}) of *A. niger* strains in a minimal medium with 1,4 diaminobutane as sole nitrogen source.

A. niger strain	Day 0	Day 8
MRC 742	0.047	0.571
MRC 7419	0.083	0.275
MRC 8992	0.062	0.366
MRC 8975	0.045	0.306
MRC 8990	0.068	0.221
MRC 8993	0.053	0.320
MRC 9013	0.063	0.202
MRC 6977	-	0.390

Induction of possible fumonisin degrading enzymes

All *A. niger* strains evaluated could grow in the medium with 1,4 diaminobutane as sole nitrogen source, indicating utilization of the amino groups of 1,4 diaminobutane and therefore the production of possible aminotransferase enzymes (Table 4). No growth was recorded in the control medium without 1,4 diaminobutane but supplemented with PLP and pyruvate. However, growth recorded in cultures with 1,4 diaminobutane as nitrogen source was slow and cell densities remained relatively low.

Fumonisin degradation and chromatographic analyses

The HPLC retention times of OPA derivatized FB_1 and 1,4 diaminobutane were very similar. To obtain optimal separation of the two compounds different mobile phases were evaluated in an isocratic system and methanol: 0.1 M NaH_2PO_4 (82:16; pH 3.35) selected as the most suitable mobile phase. Results indicating utilization of 1,4 diaminobutane as a nitrogen source were supported by a substantial decrease (>99%) in 1,4 diaminobutane concentration exhibited by all the fungal isolates during growth (Table 5). Concomitantly, treatment of FB_1 with fungal extra-cellular fractions, harbouring possible aminotransferase enzymes, resulted in a decrease in FB_1 concentration (7,43 – 22,13%).

Fig. 1. HPLC chromatogram indicating FB_1 and 1,4 diaminobutane eluting at 3.731 and 4.359 min. respectively following derivatization with OPA.

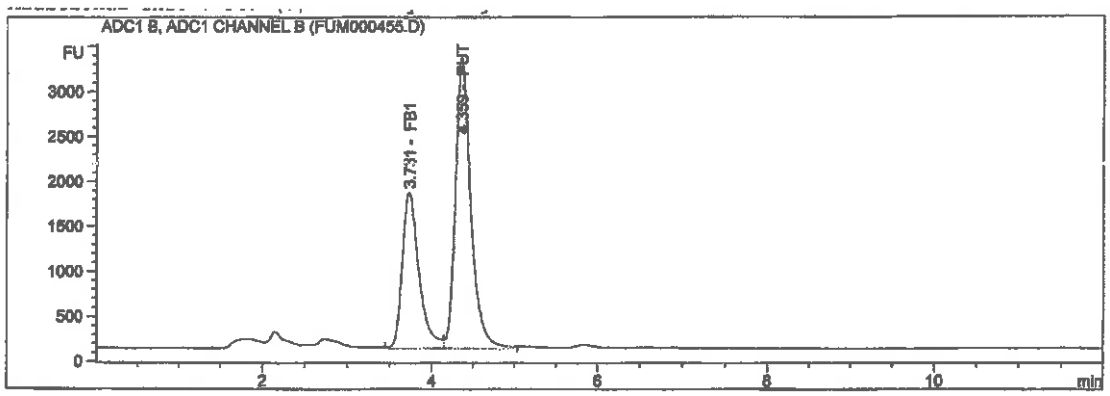


Table 5. FB_1 and 1,4 diaminobutane concentrations following treatment with *A. niger* extracellular fractions.

A. niger strain	FB_1 (µg/ml)	% FB_1 reduction	1,4 diaminobutane (µg/ml)	% 1,4 diaminobutane reduction
Control FB_1 medium	570.00	-	-	-
Control 1,4 diaminobutane medium	-	-	7500.00	-
MRC 742	509.45±147.66	10.62	17.43±1.05	99.77
MRC 6977	527.66±41.02	7.43	17.37±0.15	99.77
MRC 7419	443.85±117.23	22.13	13.87±4.00	99.82
MRC 8990	468.18±12.61	16.10	14.14±0.80	99.81
MRC 8992	451.24±17.03	20.83	14.27±0.63	99.81
MRC 8993	544.04±80.97	4.56	14.88±0.54	99.80
MRC 8975	493.19±20.47	13.48	13.14±0.39	99.82
MRC 9013	470.49±25.32	17.46	12.66±0.47	99.83

Cloning and expression of aminotransferase and carboxylesterase genes: confirmation of positive *E. coli* transformants

Restriction enzyme digests: DNA fragments encoding aminotransferase and carboxylesterase enzymes were separately cloned into expression vector pRDH122 and transformed into *E. coli*. Positive transformants were identified by mini-prep plasmid isolation and subsequent restriction enzyme digest evaluations where after it was sub-cloned into *S. cerevisiae* Y294 (Fig. 2).

Amplification of gene fragments: The presence of the genes in positive transformants was confirmed by amplification of the gene fragments by PCR whilst employing primers specific to the gene sequences (Fig. 3).

Recombinant enzyme production: SDS-PAGE

Extracellular expression of enzymes: SDS-PAGE analysis (10%) of the extracellular yeast culture fractions exhibited clear presence of all aminotransferase proteins included in the study at 40-60 kDa (Fig. 4).

Intracellular expression of enzymes: The proteins could not be clearly distinguished in the intracellular fractions, possibly due to the vast presence of background proteins (data not shown).

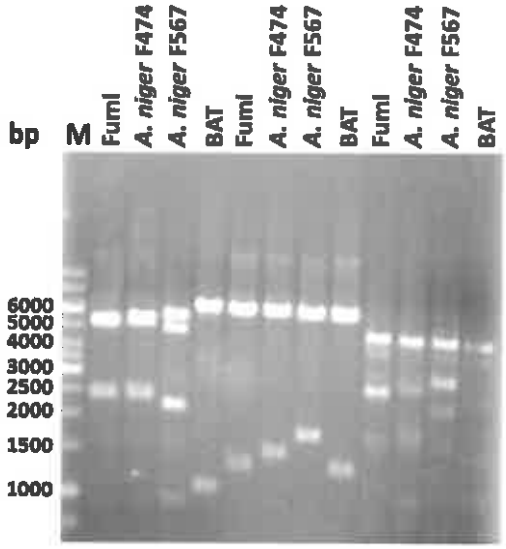
Description of codes:

FUMI: Aminotransferase of *Sphingopyxis macrogoltabida*

ANF474: Acetylornithine aminotransferase of *A. niger* CBS 513.88

ANF567: Alanine aminotransferase of *A. niger* CBS 513.88

BAT: Aminotransferase of Bacterium ATCC 55552



Restriction enzyme digests:

Lane 1: **RDH122-FumI** cut with NcoI (2401,5205)
Lane 2: **RDH122-AnF474** cut with NcoI (2411, 5351)
Lane 3: **RDH122-AnF567** cut with PvuII (2212, 5831)
Lane 4: **RDH122-BAT** cut with PvuII (1079, 6530)

Lane 5: **RDH122-FumI** cut with PacI and AscI (1289, 6317)
Lane 6: **RDH122-AnF474** cut with PacI and AscI (1445, 6317)
Lane 7: **RDH122-AnF567** cut with PacI and AscI (1724, 6317)
Lane 8: **RDH122-BAT** cut with PacI and AscI (1292, 6317)

Lane 9: **RDH122-FumI** cut with BamHI and BglII (2737, 4869)
Lane 10: **RDH122-AnF474** cut with BamHI and BglII (2893, 4869)
Lane 11: **RDH122-AnF567** cut with BamHI and BglII (3172, 4869)
Lane 12: **RDH122-BAT** cut with BamHI and BglII (2740, 4869)

Fig. 2. Restriction enzyme digests to confirm the identity of positive *E. coli* transformants harbouring aminotransferase genes.

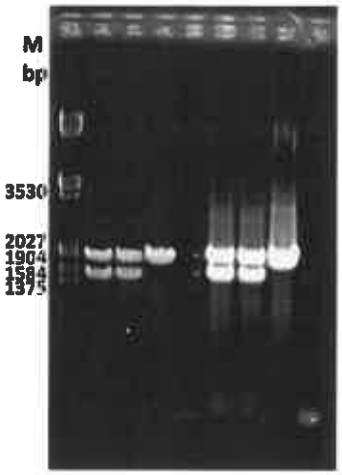
A. FumI and BAT:

Lane 1: Lambda marker
Lane 2: RDH-Fum (1) 10 ul
Lane 3: RDH-Fum (2) 10 ul
Lane 4: positive control (plasmid DNA) 10 ul
Lane 5: negative control (wild type *S. cerevisiae* DNA) 10 ul
Lane 6: RDH-BAT (2) 10 ul
Lane 7: RDH-BAT (4) 10 ul
Lane 8: positive control (plasmid DNA) 10 ul

Fig. 3a. PCR amplification of aminotransferase gene sequences to confirm the identity of positive *S. cerevisiae* transformants (FumI and BAT).

B: A. niger F567:

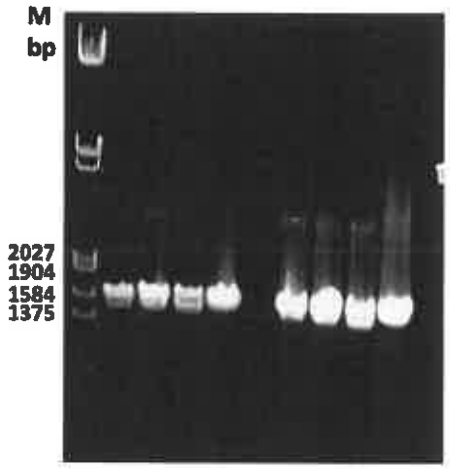
- Extracted DNA from *S. cerevisiae* Y294 transformants
- PCR with primers annealing to ENO1 promoter and ENO1 terminator
- Expected size approx 1900bp
- Primers also amplify enolase 1 gene in *S. cerevisiae* genomic DNA, which is approx 1500 bp
- Still positive result



Lane 1: Lambda marker
Lane 2: RDH-567 (1) 10 ul
Lane 3: RDH-567 (2) 10 ul
Lane 4: positive control (plasmid DNA) 10 ul
Lane 7: negative control (Wild type *S. cerevisiae* DNA) 10 ul
Lanes 8 – 11: Same as above but 20 ul loaded

C: *A. niger* F474

- PCR with primers annealing to ENO1 promoter and ENO1 terminator
- Expected size approx 1650bp
- Positive result, but primers also amplify enolase 1 gene in *S. cerevisiae* genomic DNA, which is approx 1500 bp



Lane 1: Lambda marker
Lane 2: RDH-474 (1) 10 ul
Lane 3: RDH-474 (3) 10 ul
Lane 4: RDH-474(4) 10 ul
Lane 5: positive control (plasmid DNA) 10 ul
Lane 6: negative control
(wild type *S. cerevisiae* DNA) 10 ul
Lanes 7 – 11: Same as above but 20 ul loaded

Fig. 3b and 3c. PCR amplification of aminotransferase gene sequences to confirm the identity of positive *S. cerevisiae* transformants (*A. niger* F567; F474).

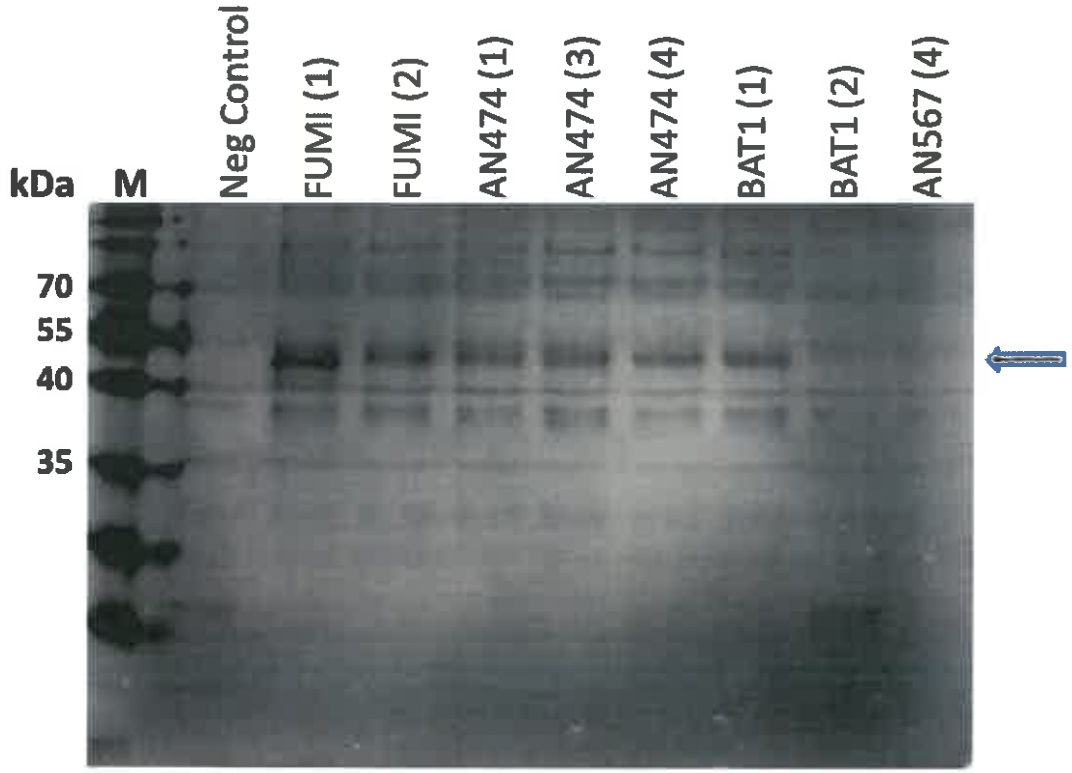


Fig. 4. SDS-PAGE analysis of aminotransferase enzymes extracellularly expressed in *S. cerevisiae* Y294 (YIL).

Discussion

The focus of this research project was to develop practical, affordable and environmentally sound methods to biologically detoxify the fumonisin mycotoxins. The aim was to eliminate FB_1 contamination from food and feed grain commodities by treatment with food-grade recombinant enzyme preparations. Fungi capable of degrading FB_1 were selected by screening fungal isolates from culture collections and by enrichment procedure from compost-rich soil. Selection was based on the ability to utilize molecules containing groups related to the terminal end (2-amino-3-hydroxy butyl moiety) of FB_1 as nitrogen source. *A. niger* SU 10864 was able to grow in minimal culture media with FB_1 as nitrogen source. Species identification by microscopic examination, sequencing of the ITS regions and BLAST sequence comparison resulted in 100% homology with *A. niger* CBS 513.88. In subsequent experiments a dual approach, including induction of *A. niger* enzymes as well as cloning of relevant genes was used to produce enzymes capable of degrading FB_1. Enzymes were induced by cultivating the most promising *A. niger* CBS 513.88 isolates in a medium with 1,4 diaminobutane as sole nitrogen source. Fungal growth and concomitant decrease in 1,4 diaminobutane and FB_1 concentrations indicated the formation of possible transaminase enzymes produced by the fungal isolates. Growth of fungal strains with 1,4 diaminobutane as sole nitrogen source will be further evaluated to enhance enzyme activity. Several gene sequences, originating from fungal (*A. niger*) and bacterial sources, encoding aminotransferase and carboxylesterase enzymes were selected, synthesized and successfully expressed in *S. cerevisiae* Y294. *S. cerevisiae,* a food-grade yeast, is frequently employed as host organism in genetic engineering studies and has a long association with the food and beverage industries mainly because of its GRAS status,

natural robustness in industrial processes, ability to ferment naturally and ease of genetic manipulation. The genes were cloned utilizing vector pRDH122 under control of the strong ENO1 promoter and terminator. Expression of microbial enzymes originating from *A. niger* strains in *S. cerevisiae* could support the development of safe recombinant cultures and enzymatic preparations for degrading FB_1 in food and feed. A future study will focus on evaluation and optimization of enzyme activities as well as a treatment method to reduce fumonisin levels in contaminated maize in cooperation with researchers at BIOMIN, Austria. Recombinant enzyme preparations will be incorporated into a washing method established and customized by the PROMEC Unit for reducing fumonisin exposure in rural populations exposed to high levels of these carcinogenic mycotoxins. Subsequent utilisation of the enzyme preparations for industrial use in other food and feed commodities will be investigated.

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9. Scientific outputs

9.1. Expected outputs of project as stated in the application

Outputs	Date of completion	Nature of output e.g. number and title of papers
Scientific papers:	December 2013	2 papers in international refereed journals
Technical reports	Annually	3 annual progress reports
Databases	Not applicable	
Procedures/methods	December 2013	If successful, will be captured in patents
Human capacity development	December 2013	Technical skills by Dr. JF Alberts and Ms. H Burger regarding the development of intervention strategies
Technology transfer:	December 2013	Development of community based intervention to reduce mycotoxins in maize. Patent protected technologies
Other outputs:	Research dissemination	1x national and international conferences

9.2. Actual outputs of project

Outputs	Date of completion	Nature of output e.g. number and title of papers
Scientific papers	-	In progress
Book Chapters	August 2012	Marasas WFO, Gelderblom WCA, Shephard GS, Vismer HF, Alberts JF, van der Westhuizen L. History and relevance of fumonisins. In: World Nutrition Forum NutriEconomics, Balancing Global Nutrition & Productivity, Binder EM., Ed., Anytime Publishing, Leicestershire, UK, 2012, pp.213-240.
Technical reports	Annually (2011-2013)	3 annual progress reports submitted to the Maize Trust
Procedures/methods	11 July 2012	Workshop: LC-MS for small molecules. Central Analytical Facility, Stellenbosch University.

	26 February 2013	Workshop: Mycotoxins in Food and Feed, STIAS, Stellenbosch University.
	28-29 February 2013	Workshop: Real-time PCR. Bio-Rad.
	17-19; 24-25 October 2013	Course: DBText/Inmagic database levels 1, 2 and Advanced.
Human capacity development		Technical skills by Dr JF Alberts and Ms L du Plessis.
Other outputs	Research dissemination:	
	3-6 April 2011	MycoRed International Conference, Cape Town (Oral presentation).
	9-11 November 2011	SASM Conference, Cape Town (Oral presentation).
		Alberts JF, Gelderblom WCA, Van Zyl WH. Degradation of aflatoxin B_1 by *Rhodococcus erythropolis* enzymes through extracellular expression in *Escherichia coli*.
	1 March 2012	Maize Trust Mycotoxin Research Symposium, Pretoria (Oral presentation).
	20-24 January 2013	SASPP Conference, Bela-Bela (Oral presentation).
		Alberts JF, van Zyl WH, Vismer HF, Gelderblom WCA. Biological detoxification of mycotoxins by treatment with microbial enzymes.
	27-31 May 2013	MycoRed International Conference, Italy (Oral presentation).
		Alberts JF, Van Zyl WH, Vismer HF, du Plessis L, Gelderblom WCA. Reduction of mycotoxins in grains by treatment with microbial enzymes.

9.3. Outputs not achieved (give reasons)

Manuscript preparation: In progress

10. Successful institutional and inter-institutional collaboration

Researcher	Institution	Role
Prof WH van Zyl	Stellenbosch University	Consultation: Molecular cloning
Dr G Schatzmayr	BIOMIN, Austria	Evaluation of enzymatic maize wash method
Prof A Botha	Stellenbosch University	Cultivation media and conditions for fungi; Fungal culture collection
Ms L du Plessis	Stellenbosch University	Molecular cloning

11. Benefit of the outputs to the Maize Industry

Contributions to all facets of the maize industry concerned with fumonisin contamination and its possible reduction in maize and maize products by understanding the postharvest burden of fumonisin contamination of the maize supply. Project addresses the decontamination of fumonisins in maize and maize food products which will have major health impact on a population heavily reliant on maize as a staple foodstuff, consuming both home-grown maize and commercially-bought maize-based products. The objectives of this project are in alignment with the final strategic objectives of the maize trust.

12. Progress with regards to human resource development

(e.g. Training of post-graduate students in mycotoxin research)

13. Funds available to complete the execution of the proposed tasks according to the expenditure statement of the project (Were the funds adequate?)

YES

14. Comments (Discuss anything you wish to share with The Maize Trust)

I hereby wish to express my sincere gratitude to the Maize Trust for their support and funding of this project.

16. Signature of the Project Leader

Project Leader

Tygerberg

Place

31/07/2013

Date