BIO-DETOXIFICATION OF SECONDARY METABOLITES OF FILAMENTOUS FUNGI

MANAL SULEIMAN ESHELLI

A Thesis Submitted To Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), the University Of Strathclyde, In Fulfillment Of the Requirements for the Degree of Doctor of Philosophy

October 2010

DECLARATION

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

October , 2010

ACKNOWLEDGEMENT

The compilation of this Ph.D study was only possible through the many supports I have gained throughout the years. I express my sincere gratitude and appreciation to Prof. Brian McNeil and Dr. Linda Harvey, the supervisors of the research team at the University of Strathclyde. Their wide knowledge and logical way of thinking have been of great value for me to start my career in research/academia. His understanding, her motivation and their personal guidance have provided a good basis for this present thesis.

I am deeply grateful to our collaborators, Dr. RuAngelie Edrada- Ebal for her dedicated help in HPLC/MS analysis and Dr .Elizabeth Ellis for permitting me to work in her lab. I acknowledge all my friends and colleagues in fermentation group Mr.Walter McEwan, Ms. Ebtihaj Jambi, Mr. Ioannis Voulgaris, Mr. Pete Gardner, Ms. Tantima Kumlung, Mr. Ivo Kretzers and to Drs Mariana Fazenda, Payal Roychoudhury, Qiang Li and Andrew O'Donnell. All your constructive comments have been a great help to me especially during my difficult moments. Thank you.

I am grateful to the members of departmental staff and colleagues; Mrs.Janette Young, Mr. David McNeill, Ms. Samar Azeer, Ms. Nabila Hamed, and to Drs Angzzas Kassim, Vinod Jyothikumar and too many to list, for their untiring help, secretarial work and wonderful friendship throughout the years.

I owe my loving thanks to my small family Mr. Naser Asheen, Adam and Elaf, without their understanding and encouragement it would have been impossible for me to finish this work. My special gratitude is due to my parents, sister and brothers. They have lost a lot due to my research abroad and I thank them for their unconditional love and support.

The financial support by Ministry Of Libyan Higher Education, Tripoli, Libya is gratefully acknowledged.

LIST OF PUBLICATIONS

I. Eshelli, M., Harvey,M., L., and McNeil, B. Ability of probiotic bacteria to remove aflatoxin B1 from liquid Culture, Scottish Microbiology Society (SMS) Conference, Napier University, Edinburgh, UK. (Poster) April 27th 2007.

II. Eshelli, M., Harvey, M., L., and McNeil, B. Ability of some of lactic acid bacteria to remove of aflatoxin B1 from contaminated liquid culture. Scottish Toxicology Interest Group meeting. University of Strathclyde. Glasgow, UK, (Poster) June 22th 2007

III. Eshelli, M., Harvey, M., L., and McNeil, B. Biodetoxification of ochratoxin A by *Rhodococcus.erythropolis* cultures. The 3th Symposium for Libyan Students in UK Universities. Sheffield Hallam University. UK (Platform presentation). June 12th 2010.

IV. Eshelli, M, Harvey,M., L., and McNeil, B. Biological detoxification of patulin by *Rhodococcus erythropolis* cultures. 9th International Mycological Congress. Edinburgh International Conference Centre (EICC), Edinburgh. UK. (Poster) 1-6 August 2010.

V. Eshelli, M., Edrada-Ebel, R., Harvey,M., L., and McNeil, B. Bio-Degradation of Aflatoxin B1 by Actinomycete Cultures. Society for General microbiology (SGM) conference. University of Nottingham. (Poster) 6-9 September 2010.

VI. Eshelli, M., Edrada-Ebel, R., Harvey, M., L., and McNeil,B. Biological detoxification of aflatoxin B_1 by *Rhodococcus erythropolis* ATCC 4277, *Streptomyces lividans* TK 24, and *Streptomyces aureofaciens* ATCC 10762 Cultures. 8-10 6th conference of the World Mycotoxin, Netherlands, November (Abstract) 2010.

VII. Eshelli, M., Edrada-Ebel, R., Harvey, M., L., and McNeil, B., (2010) Bio-Metabolomics of Aflatoxin B1 Degradation. Applied and Environmental Microbiology, paper in draft.

ABSTRACT

There is great interest in methods of detoxifying food containing toxic fungal metabolites while still retaining the nutritional value. Successful de-contamination implies increased food supplies in regions where food supply is poor, and mycotoxin contamination is prevalent. Biological detoxification is generally likely to be milder and maintain food/ feed nutrient levels better.

This research examined the potential of various microbial strains to destroy or reduce the toxicity of aflatoxin B1 (AFB1), ochratoxin A(OTA) and patulin (PAT) when cultured in mycotoxin containing liquid media. Initial studies were focused upon the potential of pro-biotic bacteria. Five strains were used (three strains of *Lactobacillus,* and one each of *Lactococcus and Bifidobacterium*) to detoxify AFB1. The amount of AFB1 adsorbed by the pro-biotic bacteria was influenced by both microbial numbers and toxin concentration. In addition, the temperature had a significant effect on the adsorption of AFB1. A series of experiments was used to investigate whether or not physical or chemical treatments would induce any marked alteration in the adhesion of aflatoxin B1. The results indicate that AFB1 adhesion is mediated by means of binding to cell wall polysaccharide and peptidoglycan, probably by hydrophobic interactions and intermolecular forces of attraction to the peptidoglycan element in the cell wall. Lipoteichoic acid may also be involved in the adhesion process.

Later studies involved the use of Actinomycete bacteria for degrading AFB1, PAT and OTA. Controlled liquid cultures of these bacteria (*Rhodococcus erythropolis* ATCC 4277, *Streptomyces lividans* and *Streptomyces aureofaciens)* were employed and tested against fungal toxins in liquid media. The amount of the fungal toxin remaining after various durations of treatment was measured by reverse phase HPLC, LCMS, and TLC to confirm the degradation of the AFB1. The pathway of degradation or chemical alteration of the toxin was followed by means of high resolution FTMS analysis to unravel the degradative pathway for AFB1, HPLC was used to confirm the degradation of PAT and OTA due to the costs associated with LCMS/MS analysis, and time constraints on its use. No significant differences were

found between the three cultures in terms of their ability to degrade AFB1. Analysis of the degradative route indicated that each microorganism may have a different way of degrading AFB1. As the metabolites produced during AFB1 degradation by *R. erythropolis* are significantly different from the other two microorganisms, the biodegradation process was investigated under a variety of conditions in order to optimise its degradation. Toxins were detoxified at all the pHs and incubation temperatures used in this experiment.

Moreover, to confirm toxins degradation by these bacteria, cytotoxicity assays were performed by two different methods: MTT and LDH, using the human cell line HepG2. Testing liquid media containing fungal toxins following the biological detoxification, a significant reduction in the toxicity of the treated fluids was noted as reflected in a steady rise in the percentage of viable HepG2 cells with increased treatment time. Results confirmed that *R. erythropolis* was able to degrade AFB1 into a non-toxic compound while *Streptomyces* strains reduced AFB1 toxicity not did not destroy it completely.

In an attempt to discover the enzyme or enzymes responsible for the bio-detoxification, culture fluids of *R. erythropolis* where detoxification had occurred were analysed. Mixtures of enzymes were purified and identified by using two columns, DEAE Sepharose and PhenylSepharose. Preliminary studies of the addition of inducers and inhibitors to the intracellular cultures indicated that enzymes involved in toxin degradation may be of the mono-oxygenase / dehydrogenase type.

LIST OF TEXTUAL ABBREVIATIONS AND CHMICAL FORMATION

AFB1	Aflatoxin B1
AFB2	Aflatoxin B1
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNase	deoxyribonuclease
DNase	deoxyribonuclease
DPBS	Dulbecco's Phosphate buffered saline
FBS	fetal bovine serum
HCl	Hydrochloric acid
$HepG_2$	human liver carcinoma cell line
LDH	Lactate dehydrogenase
Mg	Magnesium
Mn	Manganese
MRS	Man, Rogosa and Sharpe broth
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$NaIO_4$	Sodium metaperiodate
NaOH	Sodium hydroxide
OTA	Ochratoxin A
PAHS	polycyclic aromatic hydrocarbons
PAT	Patulin
PBS	phosphate buffer
PPM	Part Per Million
RNase	Ribonuclease
SD	Standard deviations
SDS	Sodium dodecyl. Sulphate
TCA	Trichloroacetic acid
THAM	Tris (hydroxymethyl) aminomethane hydrochloride
Zn	Zinc

STANDARD UNITS

amu	atomic mass unit
C^o	Centigrade
Da	Dalton units
K	kilo
l	Litre
mg	milligram
ml	Millilitre
mM	MilliMolar
µg	Microgram
µl	Microlitre
v	volume
vvm	volume of air per volume of culture per minute