MICROBIAL DETOXIFICATION OF GROUNDNUT MEAL NATURALLY CONTAMINATED WITH AFLATOXIN USING *RHODOCOCCUS ERYTHROPOLIS*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÖNAY BURAK DOĞAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

AUGUST 2015

Approval of the thesis:

MICROBIAL DETOXIFICATION OF GROUNDNUT MEAL NATURALLY CONTAMINATED WITH AFLATOXIN USING *RHODOCOCCUS ERYTHROPOLIS*

submitted by **ÖNAY BURAK DOĞAN** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Alev Bayındırlı _____
Head of Department, **Food Engineering**

Assoc. Prof. Dr. Deniz Çekmecelioğlu _____
Supervisor, **Food Engineering Dept., METU**

Prof. Dr. Tahsin Faruk Bozoğlu _____
Co-supervisor, **Food Engineering Dept., METU**

Examining Committee Members:

Prof. Dr. Zümrüt Begüm Ögel _____
Food Engineering Dept.,KGTÜ

Assoc. Prof. Dr. Deniz Çekmecelioğlu _____
Food Engineering Dept., METU

Assoc. Prof. Dr. İlkay Şensoy _____
Food Engineering Dept., METU

Asst. Prof. Dr. Baran Önal Ulusoy _____
Food Engineering Dept., ÇKÜ

Asst. Prof. Dr. Yeşim Soyer _____
Food Engineering Dept., METU

Date: 21.08.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Önay Burak Doğan

Signature:

ABSTRACT

MICROBIAL DETOXIFICATION OF GROUNDNUT MEAL NATURALLY CONTAMINATED WITH AFLATOXIN USING *RHODOCOCCUS ERYTHROPOLIS*

Doğan, Önay Burak

M.S., Department of Food Engineering

Supervisor: Assoc. Prof. Dr. Deniz Çekmecelioğlu

Co-Supervisor: Prof. Dr. Tahsin Faruk Bozoğlu

August 2015, 103 pages

Aflatoxins are highly mutagenic toxins with carcinogenic effects produced as secondary metabolites by fungal species *Aspergillus flavus* and *Aspergillus parasiticus* under certain conditions. Chronic or acute consumption of aflatoxins found in food and feed products possesses great health risks. It is particularly an important problem in animal feed from food waste and by-products. Therefore there is growing need to eliminate aflatoxins from contaminated products.

In this study, first the optimum growth conditions of gram-positive, aerobic bacterium *Rhodococcus erythropolis*, which is known to be degrading aflatoxin, were determined in synthetic media. One factor at a time approach was adopted to determine the most effective carbon and nitrogen sources for growth. Plackett-Burman design was used to screen other variables (temperature, pH, liquid culture volume, agitation speed and concentrations of nitrogen and carbon sources)

important for growth. Three variables determined as significant by Plackett-Burman design was then further evaluated with Box-Behnken response surface optimization method and optimum conditions were defined for growth of *R. erythropolis.*

For better understanding of aflatoxin degrading ability of *R. erythropolis,* viable cells and crude extracellular enzymes were compared. Process conditions for detoxification of Aflatoxin B_1 were optimized by Box-Behnken response surface method with three variables (solid concentration, inoculum volume and time). Decrease in toxicity of treated groundnut meal was assessed by sheep liver glutathione-S-transferase (GST) assay.

The results showed that peptone and glucose are the best nitrogen and carbon sources for growth of *R. erythropolis,* respectively. Optimal culture conditions were found as 22.5 °C of temperature, pH 7, 100 mL of liquid volume in 500 mL flasks, 1% (v/v) of inoculum volume, 135 rpm of agitation speed, 5 g/L of glucose concentration and 5 g/L of peptone concentration.

Viable cells were found to be more effective for Aflatoxin B_1 degradation and used for rest of the study. It was observed that *R. erythropolis* cells and extracellular enzymes are able to degrade aflatoxin even when grown in absence of the toxin. It was observed that viable cell cultures of *R. erythropolis* performed better detoxification activity than extracellular enzymes. Optimum conditions for detoxification were found as 27.4 %(w/v) of solid concentration, 4.88 %(v/v) of inoculum volume and 24 h of time by Box-Behnken response optimization. At these conditions maximum reduction in AFB_1 was predicted as 92.2% and verified as 87.3% Toxicity of treated groundnut meal extracts were found to be decreased significanty by GST assay. Treated samples inhibited the enzyme activity 64.5% and untreated samples inhibited 86.6%.

As a result, viable cell cultures of *R. erythropolis* was suggested as an effective detoxification agent for aflatoxin contaminated groundnut meal used for animal feed.

Keywords: Mycotoxins, aflatoxin, detoxification, *Rhodococcus erythropolis*, groundnut meal.

ÖZ

DOĞAL OLARAK AFLATOKSİN İLE KONTAMİNE OLMUŞ YERFISTIĞI KÜSPESİNİN *RHODOCOCCUS ERYTHROPOLIS* KULLANILARAK MİKROBİYAL DETOKSİFİKASYONU

Doğan, Önay Burak
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Doç. Dr. Deniz Çekmecelioğlu
Ortak Tez Yöneticisi: Prof. Dr. Tahsin Faruk Bozoğlu

Ağustos 2015, 103 sayfa

Aflatoksinler *Aspergillus flavus* ve *Aspergillus parasiticus* türü küflerin belirli koşullar altında ürettiği kanserojen etkiye sahip mutajenik ikincil metabolitlerdir. Gıda ve yemlerde bulunan aflatoksinlerin kronik veya akut tüketimi büyük sağlık risklerine neden olmaktadır. Özellikle gıda artıkları ve yan ürünlerinden üretilen yemlerde aflatoksin önemli bir sorundur. Bu nedenle kontamine olmuş ürünlerde aflatoksinleri giderme gereksinimi her geçen gün artmaktadır.

Bu çalışmada öncelikle aflatoksin parçaladığı bilinen gram-pozitif ve aerobic *Rhodococcus erythropolis* bakterisinin en uygun gelişim koşulları sentetik ortamda belirlenmiştir. Gelişim için en etkili olan karbon ve azot kaynaklarının belirlenmesi için her seferinde bir faktör değiştirme yaklaşımı kullanılmıştır. Gelişim için önemli faktörler (sıcaklık, pH, sıvı kültür hacmi, çalkalama hızı ile azot ve karbon kaynakları derişimi) Plackett-Burman yöntemi ile taranmıştır. Plackett-Burman tasarımı sonucunda belirlenen önemli üç faktör (sıcaklık, sıvı

hacmi ve çalkalama hızı) daha sonra Box-Behnken tepki yüzey optimizasyonu yöntemiyle incelenmiş ve *R. erythropolis* gelişimi için gerekli en uygun koşullar belirlenmiştir.

R. erythropolis'in aflatoksin parçalama yeteneğinin daha iyi anlaşılması için canlı hücre kültürleri ve hücredışı enzimleri karşılaştırılmıştır. Aflatoksin B_1 detoksifikasyon işlemi üç faktörlü (katı derişimi, aşılama hacmi ve süre) Box-Behnken tepki yüzey yöntemi ile incelenmiştir. İşlenmiş yerfıstığı küspesinde toksisite değişimi koyun karaciğeri glutathione-S-transferase enzimatik yöntemiyle değerlendirilmiştir.

Sonuçlar, *R. erythropolis* gelişimi için en uygun azot ve karbon kaynaklarının pepton ve glikoz olduğunu göstermiştir. En uygun gelişim koşulları; 22.5°C sıcaklık, 7 pH, 500 mL erlen içerisinde 100 mL sıvı hacmi, %1 aşılama hacmi, 135 dev/dak çalkalama hızı, 5 g/L glikoz derişimi ve 5 g/L pepton derişimi olarak bulunmuştur.

Canlı hücrelerin Aflatoksin B_1 parçalamada daha etkili olduğu saptanmış ve çalışmanın geri kalanında bakteri kültürü bu şekilde kullanılmıştır. *R. erythropolis* hücreleri ve hücre dışı enzimlerinin aflatoksin yokluğunda geliştirildiğinde bile AFB_1'i parçalamada etkili olduğu gözlemlenmiştir. *R. erythropolis'*in canlı hücre kültürlerinin hücre dışı enzimlere göre daha iyi detoksifikasyon etkisi sağladığı gözlemlenmiştir. Box-Behnken deney tasarımının analizi sonucunda en uygun detoksifikasyon koşulları ise 27.4 %(w/v) katı derişimi, % 4.88(v/v) aşılama hacmi ve 24 saat işlem süresi olarak belirlenmiştir. Bu koşullar altında teorik olarak %92.2 detoksifikasyon sağlanması öngörülmektedir ve bu koşullarda tekrarlanan doğrulama deneylerinde %87.2 detoksifikasyon sağlanmıştır. İşlenmiş yerfıstığı küspesinin toksisitesinin önemli derecede azaldığı da saptanmıştır. İşlenmiş örnekler enzim aktivitesini %64.5 oranında inhibe ederken, işlenmemiş örnekler %86.6 oranında inhibe etmiştir.

Sonuç olarak, *R. erythropolis*'in canlı hücre kültürleri, aflatoksinlerle kontamine olmuş ve hayvan yeminde kullanılacak yerfıstığı küspesinin detoksifikasyonunda etkili detoksifikasyon ajanı olarak önerilmektedir.

Anahtar Kelimeler: Mikotoksinler, aflatoksin, detoksifikasyon, *Rhodococcus erythropolis,* yerfıstığı küspesi.

To my family…

ACKNOWLEDGEMENTS

I would like to express my gratitude and respect to my advisor and mentor, Assoc. Prof. Dr. Deniz Çekmecelioğlu for his guidance, support and encouragement through this study. I would like to thank my co-advisor Prof. Dr. Faruk Bozoğlu for his kind support.

I would also like to thank the examining committee members Prof. Dr. Zümrüt Begüm Ögel, Assoc. Prof Dr. İlkay Şensoy Assist. Prof. Dr. Baran Önal Ulusoy, and Assist. Prof Dr. Yeşim Soyer.

METU-BAP coordination (BAP-03-14-2014-008) kindly funded this study and The Scientific and Technological Council of Turkey (TÜBİTAK) provided financial support during my thesis studies.

I would like to thank very much to Assist. Prof. Dr. Baran Önal Ulusoy for aflatoxin analysis in HPLC and Assoc. Prof. Dr. Gülçin Sağdıçoğlu Celep for her help in sheep liver cytosol preparation.

I owe a debt of gratitude to my lab mates Gözde Ören Yardımcı and Sibel Uzuner for their valuable contributions. I would like to thank all my colleagues and friends; Ece Bulut, Ceren Kuvvet, Meltem Karadeniz, Elçin Bilgin, Elif Yıldız Bulut, Betül Çilek Tatar, Oya Nihan Uncu Bilau and Armağan Cabadağ.

I would like to say thanks to Ali Übeyitoğulları, Ahmet Yıldız and Sertan Cengiz for their friendship, they always cheered me up in dark days. Thanks to my wife-to-be Ezgi Ulusoy for her endless love and support.

Finally I would like to express my deepest gratitudes to my parents Gülay-Önder Doğan and my aunt Ayşegül Kırış for their invaluable support, belief and unconditional love.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

Aflatoxins are toxic metabolites, which can be found on some very important agricultural commodities. Aflatoxin contamination is a widespread threat for human and animal health and it causes a considerable loss of natural and economic resources.

Groundnut meal is an important feed source especially for poultry and other livestock. This raw material is rich in protein and fibers but highly susceptible to fungal contamination. High levels of aflatoxins in groundnut meal may result in discard of large amounts.

To overcome health effects and economic loss of aflatoxin contamination, detoxification strategies are gaining importance. In current literature, several physical, chemical and biological methods for detoxification were suggested. In this study, efficiency of microbial detoxification by direct use of gram-positive bacterium *R. erythropolis* was evaluated.

The aim of this study was to decrease the amount of aflatoxins in naturally contaminated groundnut meal using *R. erythropolis* as an efficient detoxifying agent. By this way, it was also aimed to recover this valuable by-product to agricultural economy.

Before observing the aflatoxin degrading ability of this microorganism, growth characteristics and optimum conditions were determined (Chapter 4). First, one at a time approach was adopted to decide on which carbon and nitrogen sources were utilized best by the organism (Chapter 3). After that, different growth

conditions (temperature, pH, liquid culture volume, agitation speed and concentrations of nitrogen and carbon sources) were screened using Plackett-Burman design method. Three process variables (temperature, liquid volume, agitation speed) determined to be significant were then used for optimization by Box-Behnken response surface method.

In chapter 2, current literature on aflatoxins, detoxification approaches, target raw material groundnut meal and biocatalyst *R. erythropolis* are reviewed for better understanding.

For better understanding of the aflatoxin detoxification, the effect of extracellular enzymes or bacterial cultures were also examined (Chapter 4). Three independent variables (solid concentration, inoculum volume and time) were selected for optimization of aflatoxin detoxification using the Box-Behnken design. Decrease in toxicity was evaluated by glutathione-S-transferase assay under optimal conditions.

In chapter 5, overall conclusions are made about this study, and recommendations are given for those who will study this topic or similar in future. Also, applicability of this method to the industry was evaluated.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by several mold species. Common groups of mycotoxins are Aflatoxins, Ochratoxins, Citrinin, Ergot Alkaloids, Patulin and Fumonisins. Food and feed products colonized by mycotoxin producing molds can be exposed to different types of mycotoxins which may cause cancer or liver deterioration and thus provide a great threat for human and animal health and global economy.

Along rapid development of food and feed production industry, the concept of food safety is also gaining importance. Although foodborne infections and intoxications are subjects studied for hundreds of years, risks belonging to fungal toxins is fairly a new topic. The first case of mycotoxicosis was reported as “Turkey X disease” in the United Kingdom in 1960 in which more than 100,000 turkeys were fed with aflatoxin contaminated peanut meal (Wannop, 1961). Only after this date, studies on mycotoxins gained speed and the last major group of mycotoxins, fumonisins were discovered in 1988 (Yiannikouris & Jouany, 2002). Mycotoxin contamination can begin on any stage of the production including natural raw material, processing and storage.

2.1.1 Aflatoxins

Aflatoxins are furanocoumarin type of secondary metabolites produced by some strains of fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. There

are 18 derivatives of aflatoxins discovered so far, and four of them, namely B_1, B_2, G_1 and G_2 are produced by *Aspergillus*. These fatal molecules are named upon their fluorescence behavior under long wave ultraviolet light. B type of aflatoxins give blue and G type of aflatoxins give green color when excited by UV. Toxicity of aflatoxins is in the order of $B_1>G_1>B_2>G_2$ (McLean & Dutton, 1995). The chemical structures of aflatoxins B_1, G_1, B_2, G_2 and also M_1 are given in Figure 2.1.

Mutagenic and toxigenic properties of aflatoxins are associated with lactone ring structure in the toxin and according to Lee et al. (1981), fluorescence behavior and toxic effects of aflatoxins are directly related. Cleavage of the lactone ring, reduces the mutagenity of the molecule by 450-fold and toxicity by 18-fold. This reduction can be monitored by loss of fluorescence under UV excitation.

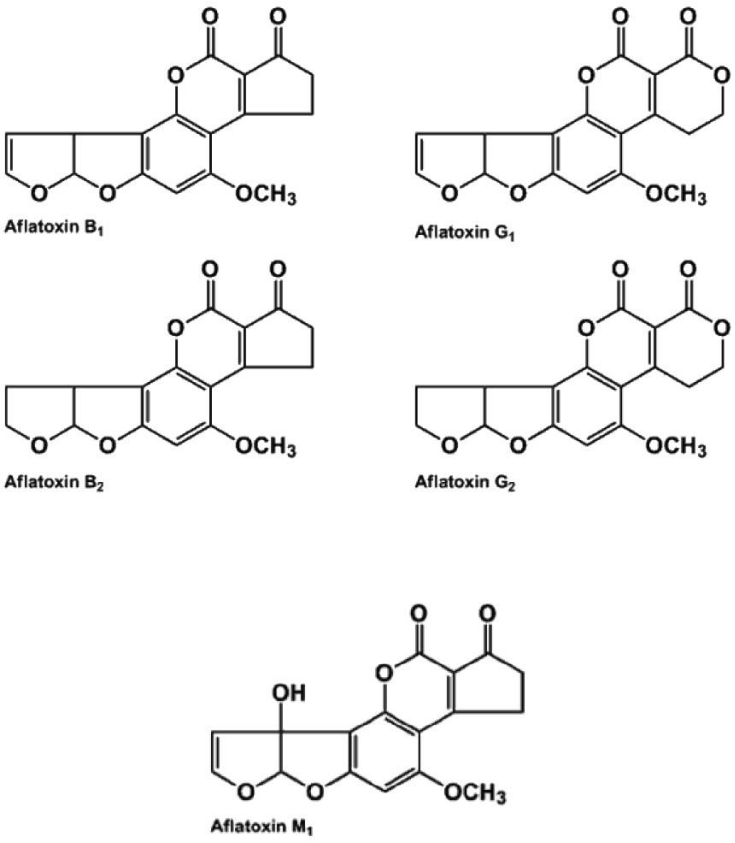

Figure 2.1 Chemical structures of Aflatoxins B_1, B_2, G_1, G_2 and M_1. (FDA, 2012)

2.1.1.1 Metabolism of aflatoxin in human and animal bodies

In living organisms, aflatoxin acts as toxigenic, carcinogenic, mutagenic and teratogenic agent. Poisoning due to aflatoxin consumption is named as "aflatoxicosis". Consuming high levels of aflatoxins results in acute aflatoxicosis. On the other hand, consuming low levels of aflatoxins for long periods of time results in chronic aflatoxicosis. Signs of acute toxicosis can be clearly monitored by congestion and bleeding triggered by liver lesions which are the result of fatty acid accumulation in the liver. A rapid death (usually a few hours or days) occurs.

Chronic toxicosis is more common in animal and human. DNA changes induced by aflatoxins cause slow death of liver cells or formation of tumor cells. In addition, aflatoxin causes immune system deficiency in livestock, which can lead to other diseases (Yiannikouris & Jouany, 2002).

Aflatoxicosis is very common in mammals, however abovementioned effects can vary from one organism to another. Some species can be highly susceptible, however others like mice are more resistant to adverse effects of aflatoxins in the liver (Ellis et al., 1991).

AFB_1 or other major aflatoxins do not exhibit harmful behavior on their primary states. Microsomal enzyme sets convert AFB_1 to its AFB_1-8,9-epoxide (AFBO). This intermediate molecule is then forwarded to detoxification mechanisms to conjugate with proteins and glutathione. Detoxified Glutathione-Aflatoxin conjugate (GSH-AFBO) is excreted from the system but remaining toxic parts are tend to interact with DNA, RNA and enzymes. Binding of AFB_1 to DNA or RNA causes mutations and errors in protein synthesis. AFB_1 also known to inhibit the activity of important enzymes such as adenosine triphosphatase, enzymes responsible for glycogenesis and RNA polymerase (Mishra & Das, 2003; Yu, 1977).

Figure 2.2 represents an overview of AFB_1 metabolism. By oxidation in the rumen and liver, AFB_1 is converted into another very toxic metabolite, aflatoxicol. Liver enzyme systems are responsible of oxidation or epoxidation of AFB_1. If toxin is oxidized, other toxic derivatives such as M_1, Q_1, B_2 and P_1 are formed. Particularly important AFM_1 is excreted in milk. In epoxidation pathway, AFBO is formed which is later detoxified by liver enzymes. However, when high amounts are consumed, all of the epoxide is not detoxified and binds to nucleic acids or proteins in liver, causing enzyme inhibition and mutations.

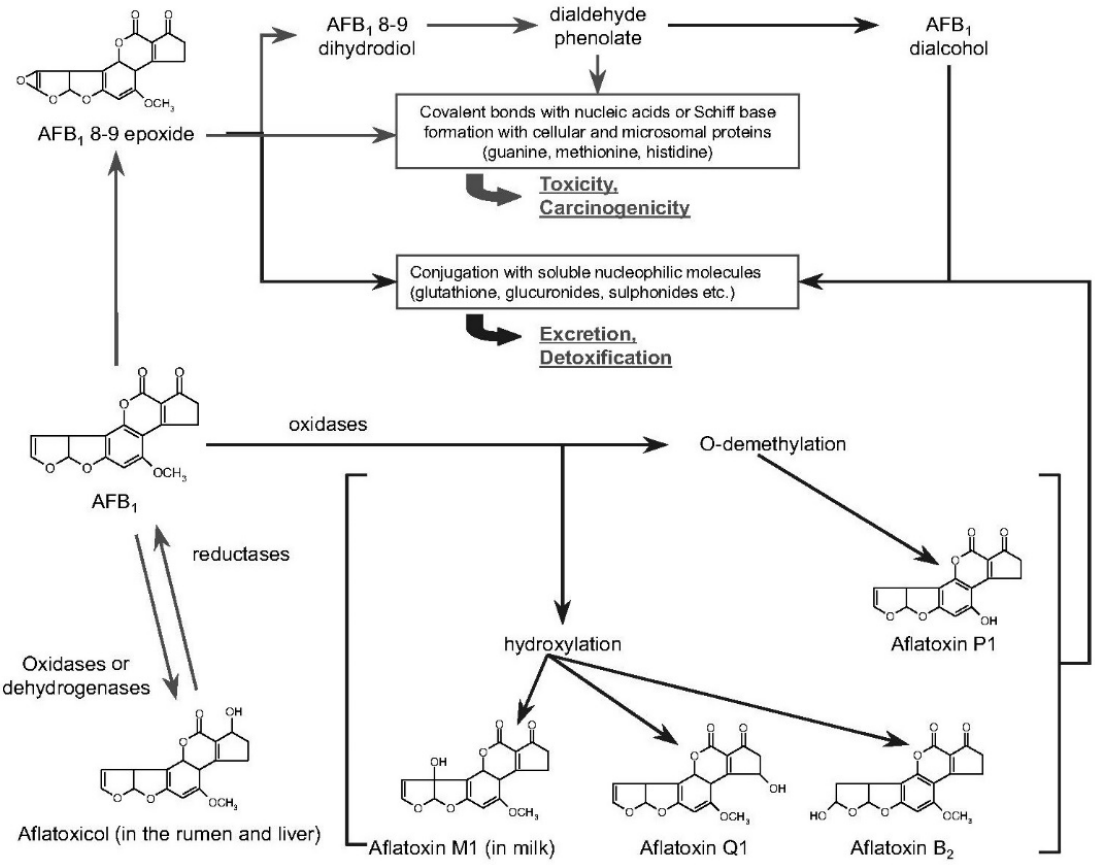

Figure 2.2 An overview of AFB1 metabolism. (Yiannikouris & Jouany, 2002)

2.1.1.2 Effect of aflatoxin on liver enzymes

Reaching the liver, AFB_1 is due to a detoxification process, like many other xenobiotic compounds, where activated toxic compounds are converted into their water soluble derivatives. Microsomal and cytosolic glutathione-S-transferases (GSTs) are responsible for detoxifying xenobiotic compounds by catalyzing the reaction between xenobiotic compounds and –SH group of glutathione (GSH).

Therefore, GST enzymes protect the cells from the toxigenic and carcinogenic compounds by forming GSH conjugates (Habig et al., 1974; Strange et al., 2001).

Tests show that GST activity in liver and kidneys is decreased when animals are fed with AFB_1 contaminated feed. Meki et al. (2001) reported that GST activity in the livers of rats fed with 50 μg AFB_1/kg body weight were decreased significantly. Devendran and Balasubramanian (2011) also reported decreasing activity of in liver and kidney GST enzymes while increasing AFB_1 administration to the rats.

GST assay is widely used to measure resistance of animal tissues oxidative damage caused by foreign toxic compounds and medicines *in vitro*. GST activity is important when studying effects of antioxidant properties of medicinal herbs (Coruh et al., 2007). It is also used for studying the effects of aflatoxins and chemopreventive compounds on liver tissues (Gao et al., 2010).

2.1.1.3 Cost of aflatoxin contamination

In addition to adverse health effects of aflatoxins on human and animals, they cause a great damage on global economics. Besides the costs of cancer cases related to aflatoxins, trade restrictions, discarding of contaminated materials, cost of detection and research activities are important factors comprising the aflatoxin economy loss.

Liver cancer is the third most deadly type of cancer. Each year, from 550,000 to 600,000 people are estimated to be diagnosed with liver cancer worldwide. Among these cases, 25,200 to 155,000 are related to chronic aflatoxin consumption. Aflatoxin related cancer cases are more prevalent in developing countries, rather than developed countries (Liu & Wu, 2010). According to American Cancer Society report (2010), liver cancer accounts for 8.6% of the total cancer cases and the total cost of cancer patients worldwide was estimated as US$

895 billion in 2008. Therefore, cost of aflatoxin related cancer can be estimated as US$ 12.5 billion worldwide.

Trade barriers due to aflatoxin limits is a great problem for developing countries whose economies are dependent on agricultural exports to developed countries mostly Europe and America. The European Union aflatoxin regulations aim to reduce health risks by 1.4 deaths per billion of their population annually. However, these strict regulations are estimated to decrease African agricultural exports by 64% which means a loss of US$ 670 million per year (Otsuki et al., 2001).

Management of aflatoxin problem, by detection and prevention researches also put stress on agricultural industry. In the US, where mycotoxin control mechanisms are well implemented, cost of combined pre and post-harvest biocontrol mechanisms is US$ 42-79 per hectare of crop field (Khlangwiset & Wu, 2010). This figure is subject to change with the type of crop and also severity of the aflatoxin problem. For the peanut industry in Southern US, Lamb and Sternitzke (2001) estimates an average of US$ 69.34 management costs per hectare of peanut fields. Thus, cost effective and sustainable methods are still required.

2.1.1.4 Aflatoxin management and regulations

A three stage mechanism is suggested for control and prevention of mycotoxins in human or animal feed. Primary prevention is to take preliminary measures against contamination at pre-harvest stage. Secondary prevention step is to eliminate fungal growth while the product can still be used. However, when the food material is highly contaminated, tertiary prevention measures must be taken to eliminate toxins and inhibit fungal growth. (Suttajit, 1991)

By the emerge of mycotoxin problem in food and feed stuff at 1960's, many countries began to establish legislations in order to control contamination starting from late 1970's. This topic was first reviewed comprehensively by Food and

Agriculture Organization (1997) in 1995 and it was updated for the last time in 2003 (FAO, 2004). In 2003, among 117 investigated countries, at least 100 countries had regulations for mycotoxin levels in food and feed. Although there is 30% increase compared to 1995, there are still countries which don't limit mycotoxins. Many of those countries applying limits on mycotoxin, pay a great attention to aflatoxins since they have specific limits on only AFB_1, total aflatoxins ($B_1+B_2+G_1+G_2$) or both (van Egmond & Jonker, 2004).

In Turkey, aflatoxin limits are updated in accordance with the European Union limits. According to Turkish Food Codex, groundnuts and other oily seeds can contain at maximum, 8.0 μg/kg AFB_1 and 15.0 μg/kg total aflatoxin (2011). For animal feed, only AFB_1 limits are applied and maximum allowance is 20 μg/kg, however, mixed formulations for dairy cattle and calves, dairy sheep and lamb, dairy goats, porklings and young poultry can only contain 5 μg/kg AFB_1 (2014). These limits are in complete accordance with European Commission regulations (2006).

Exposure of human and animals to aflatoxin is particularly a great problem for Africa because of the climate conditions and primitive agricultural practices. In 2002, only 14 countries in Africa were known to have specific mycotoxin limits and these limits are often applied only to exported products. This number represents a coverage of only 54% of the population of the continent, and the rest of the African population is under the risk of consuming aflatoxin contaminated products (Magan et al., 2011; van Egmond & Jonker, 2004).

Although mycotoxin contamination doesn't seem to be the greatest problem for crop producers in Europe and Middle East, due to global climate change at its utmost pace, a higher rate of mycotoxigenic contamination is expected in these regions in near future (Magan et al., 2011).

2.2 Detoxification of Aflatoxins

Health issues and economic losses due to aflatoxin contamination led science and industry to find ways to reduce aflatoxin content of food and feed stuff to acceptable levels. Detoxification studies have been conducted on three subtitles; physical, chemical and biological methods.

2.2.1. Physical methods of detoxification

Physical methods of detoxification refer to decontamination of food and feed materials by means of separation and degradation. The most commonly suggested methods are extraction of aflatoxins by solvents, adsorption to solids or using electromagnetic waves such as γ-rays, UV-light or microwaves.

Gardner et al. (1968) studied separation of aflatoxins from cottonseed and peanut meals using tertiary and binary solvent mixtures containing different ratios of acetone, hexane and water. Peanut meal was initially naturally contaminated with 400 to 700 μg/kg total aflatoxins. Using tertiary system containing 54% acetone, 44% hexane and 2% water and binary system containing 90% acetone and 10% water, 85% and 95% reduction in aflatoxins were reported respectively.

In another study, aqueous isopropanol was suggested for removal of aflatoxin from cottonseed and peanut meals (Rayner & Dollear, 1968). It was reported that aflatoxins were not detectable by thin-layer chromatography after extraction with 6 passes of 80% isopropanol at 60°C for both oilseed meals. Trials with 88% isopropanol-water azeotrope, however, removed only 88% of aflatoxins and it was concluded that efficiency of this method was dependent on the concentration of isopropanol and temperature.

Fonseca and Regitano-d'Arce (1993) suggested 90°, 93° and 96° commercial ethanol by Soxhlet extraction for peanut meal contaminated with 400 μg/kg AFB_1. They reported that pure ethanol did not reduce the amount of AFB_1 significantly.

However, using 93° and 96°, a complete removal of aflatoxins were detected after 210-240 minutes of extraction.

Use of solid adsorbents is another common method to decrease the adverse effect of aflatoxins in animal feeds. Adsorbents added to the feed, binds the toxin in the gastrointestinal tract of animal, lowering the bioavailability during digestion. Huwig et al. (2001) made a comparison between most commonly used groups of mycotoxin adsorbents; aluminosilicates, activated charcoal and special polymers. Although these materials have high capacity for binding mycotoxins in vitro, they exhibited different interactions in living systems.

Reduction in the mutagenity of AFB_1 due to the susceptibility to ionizing radiation was first reported by Dyck et al. (1982). Aziz et al. (2004) studied the effect of γ radiation on fungus growth and aflatoxin detoxification on naturally contaminated maize, chickpeas and groundnuts. It was reported that 4.0 kGy of irradiation inhibited the growth of mycotoxin producing fungi significantly and at a dose of 5.0 kGy, growth was totally inhibited. At the same time, application of 6.0 kGy radiation decreased the amount of AFB_1 by 74.3-76.7%. Ghanem et al. (2008) made trials on different food and feed raw materials at 4.6 and 10 kGy radiation. In peanuts, only 56.6% reduction in AFB_1 was achieved and it was observed that the oil content of the material interferes with the efficiency of γ-irradiation process.

Herzallah et al. (2008) investigated the effect of solar radiation, γ-radiation and microwave heating on aflatoxin residues in poultry feed. Aflatoxin residues after treatments were analyzed by ELISA method. More than 60% of photodegradation was observed in samples exposed to direct sunlight for 30 hours and the efficiency of this method was found to be independent of the initial toxin concentration. In samples treated by irradiation doses ranging from 5 to 20 kGy 34 to 40% reduction were detected. Only microwave heating was the least effective method with a degradation rate of 22 to 32%.

Although physical methods seem feasible, there are limitations and drawbacks. Use of solvents may leave residues in the product and also affects the

overall quality by extracting some important nutrients. Adsorbents are good binders theoretically, but under the rumen conditions, efficiency of binding stays limited. Electromagnetic degradation methods, on the other hand, require high amount of initial investment, also limited penetration depth and uneven treatment risks limits the efficiency.

2.2.2. Chemical methods of detoxification

Chemical methods of detoxification refer to degradation of aflatoxins by chemical alterations with addition of chemicals.

Ammoniation was suggested as an efficient and economically feasible way of decontamination for oilseeds. Lee and Cucullu (1978) were able to achieve a high decontamination rate that only 0.36% of aflatoxin B_1 residue was detected in cottonseed and peanut meal samples. However, despite the high decontamination rates, there are concerns about the safety of ammonia application for aflatoxin decontamination. In their study, investigating the in vivo effects of ammonia treated peanut meal, Neal et al. (2001) reported a decrease in the growth rate of male rats fed with ammonia treated peanut meal for 90 days. Also, lesions and tumors due to breakdown products were detected in rat livers.

Ozone treatment is another chemical decontamination method for aflatoxins. Dwarakanath et al. (1968) were able to achieve 78% destruction of AFB_1 in high moisture peanut meal by ozone treatment at 100°C for an hour. AFB_1 and AFG_1 were easily destroyed by ozone treatment but AFB_2 was more resistant to the treatment. Akbas and Ozdemir (2006) ozonated artificially contaminated ground and kernel pistachio. AFB_1 in samples were reduced by 23% and the total aflatoxins were reduced by 24% when treated with 5 mg/L ozone for 140 minutes; indicating that AFB_1 is especially more susceptible to ozone than its other derivatives. Yet, negative changes in sensory attributes were detected in ozone treated pistachios.

Samarajeewa et al. (1991) applied 11, 16 and 35 mg chlorine gas for each grams of corn meal, copra meal and peanuts and achieved more than 75% degradation of AFB_1. Together with the reduction in AFB_1 levels, a reduction in the mutagenicity was also detected.

Li et al. (2009) studied the effect of citric acid treatment on B-type aflatoxins in peanuts. Treating for 30 minutes with 80g/L citric acid solution decreased the aflatoxin content from 98.60 µg/kg to below 20 µg/kg. Méndez-Albores et al. (2007) decontaminated duckling feed initially containing 110 µg/kg AFB_1 with citric acid by up to 86% and reported a decrease of toxicity in treated samples.

Chemical methods are suggested for cost efficiency, however there are many risks associated with chemically treated food and feed stuff. Ammoniation and ozonation are very effective, yet they are also toxic and undesirable chemical changes can occur depending on the type of contaminated product. When using other powerful chemicals, it is possible that nutritive and sensory properties are also changed.

2.2.3. Biological methods of detoxification

Biological detoxification methods are based on conversion of aflatoxins to less toxic metabolites by direct use of microorganisms or enzymes.

2.2.3.1. Microbiological methods

Ciegler et al. (1966) screened about a thousand different microorganisms including yeasts, molds, bacteria, actinomycetes, algae and fungal spores for their ability to degrade aflatoxin. Among those, only gram-negative bacterium *Flavobacterium aurantiacum* was able to detoxify contaminated milk, oil, peanut butter, peanuts and corn partially and the mechanism of action was suggested as enzymatic (Smiley & Draughon, 2000). Duckling assays showed that no new toxic

metabolites were produced by this bacterium. However, an orange color pigmentation is reported with the use of this microorganism (Line et al., 1994). *F. aurantiacum* is later reclassified as *Nocardia corynebacterioides* (Teniola et al., 2005). Interestingly, this bacterium was again reclassified as *Rhodococcus corynebacterioides,* which has a 16S rRNA gene sequence consistent with the family *Rhodococcus* (Yassin & Schaal, 2005).

Teniola et al. (2005) first described aflatoxin degrading ability of *R. erythropolis* by using cell free extracts in liquid culture. *R. erythropolis* offered a high degradation rate with less toxic metabolites under relatively milder conditions. Aflatoxin degradation ability of this microorganism is further reviewed in Chapter 2.4.1.

Some *Pseudomonas* strains are also reported as aflatoxin degraders. Sangare et al. (2014) identified *Pseudomonas aeruginosa* among 25 other bacterial isolates, to have capability of degrading aflatoxins in liquid culture media. Maximum degradation of 90.2% was achieved at temperature of 55°C. Samuel et al. (2014) were able to reduce AFB_1 levels in liquid culture to non-detectable levels by incubating two *P. putida* strains for 24 hours at 37°C.

Guan et al. (2010) examined aflatoxin degradation efficiency of gram-negative bacteria *Myxococcus fulvus.* This microorganism was able to transform 80.7% of AFB_1 incubated in liquid culture media at 30°C for 72 hours. It was observed that the supernatant of the culture, therefore the extracellular enzymes were responsible for the degradation process. During the degradation, it was observed that the lactone ring structure was disturbed.

Some members of Lactic Acid Bacteria (LAB) family is known to bind aflatoxins physically to their cell walls. While studying the growth inhibitory effect of *Streptococcus lactis* on aflatoxin producing fungi *A. flavus,* Coallier-Ascah and Idziak (1985) observed a so-called “degradation” of previously formed aflatoxin in liquid culture. However, this phenomenon was later explained as physical binding rather than a biochemical conversion (El-Nezami et al., 1998). Haskard et al. (2001)

reported that *Lactobacillus rhamnosus* strain GG and *L. rhamnosus* strain LC-705 were the most efficient binders of aflatoxins among 12 different LAB strains. Although bound toxins were stable under temperature, pH deviations and physical stress, adsorption is still considered as a reversible process. Therefore, LAB species can be used as biological adsorbents in the gastrointestinal tracts of animals to reduce the bioavailability of the toxin during digestion of feed (Shetty & Jespersen, 2006).

Baker's yeast, *Saccharomyces cerevisiae* is also known to bind mycotoxins. Kusumaningtyas et al. (2006) inoculated chicken feed with *A. flavus, S. cerevisiae* and *Rhizopus oligosporus.* They detected an inhibition on the production rate of AFB_1. This phenomenon was explained by both the yeasts' competition with *A. flavus* and binding of the toxin to the cell walls. Therefore, *S. cerevisiae* is recommended as a feed additive to reduce the effects of aflatoxin contamination in animal feed (Shetty & Jespersen, 2006).

In brief, use of microorganisms is a promising method of detoxification. Process is natural with the least nutritional and sensory loss possible and maximum efficiency. Of course, there can be risks associated with microorganisms but benefits are outweighing. Microbial processes can often be proceeded under milder conditions with no or minimum amount of other additives.

2.2.3.2. Enzymatic methods

Das and Mishra (2000) used horseradish peroxidase enzyme to detoxify groundnut meal samples artificially contaminated with AFB_1. Treating with 10 IU enzyme, 100 g of groundnut meal was detoxified by 53%. After enzyme treatment, samples were exposed to 1kW microwave radiation for 15 minutes and a final of 97% detoxification was achieved. It was observed that enzyme treatment had effects on the protein structure of the meal, altering the nitrogen solubility. Also, a reduction in death rate was reported for rats fed with decontaminated groundnut

samples. Tripathi and Mishra (2009) used peroxidase extracted from garlic bulbs to detoxify red chili powder and achieved 70% reduction in AFB_1.

Motomura et al. (2003) screened 19 types of edible mushrooms against AFB_1 degradation activity. An extracellular enzyme from *Pleurotus ostreatus* was reported to be decreasing the fluorescence of the toxin by opening up the lactone ring, therefore reducing the carcinogenic action of this deadly molecule.

Alberts et al. (2009) examined the aflatoxin degrading ability of laccase enzymes isolated from different white rot fungi species in liquid culture media. *Peniphora* species showed the highest laccase activity and degraded 40.45% of AFB_1 in the medium. Purified fungal lactase enzymes between 0.05 to 1 IU/mL, on the other hand, were able to degrade 87.34% of AFB_1.

Liu et al. (1998) reported that multienzyme extracts from edible and medicinal mushroom *Armillariella tabescens* are able to detoxify AFB_1 in liquid solution. Later, one enzyme in this multienzyme mixture was classified as "aflatoxin oxidase" and purified using ammonium sulfate precipitation, hydrophobic interaction chromatography and metal ion affinity chromatography (Cao et al., 2011).

Use of microbial enzymes has many advantages of direct use of microorganisms. In addition, isolated enzymes are highly specific to substrate, therefore there are no risks of production of other metabolites or any undesired chemical changes. However, complex enzyme systems are responsible for degradation of AFB_1 and isolation of one single enzyme is not always as efficient as direct use of microorganisms. Also, enzyme isolation and purification is a cost and labor intensive process.

2.3. Groundnut

Groundnut or peanut (*Arachis hypogaea L.*) is a member of the *Fabaceae* family, together with legumes, peas and beans. Groundnut is widely used as a snack

and oil source. Groundnut oil is produced by either cold pressing or solvent extraction. After pressing or extraction, defatted part, groundnut meal is left. This part is low in oil content but contains a high amount of proteins and dietary fibers for animal feed use. However, it is not always possible to feed animals with groundnut meal due to high levels of aflatoxin contamination (IMF, 1986).

Although 40% of global production of groundnuts are consumed directly as nuts, they supply 10% of the World's oilseed demand. China, India, the USA and Brazil are the main players in World's groundnut production. The USA is the net exporter of this crop, however, most part of the groundnuts produced in China and India are consumed in domestic markets (Diop et al., 2004).

Groundnuts, one of the major oilseeds, has a great production capacity all over the world. Groundnut oil is mainly produced in Asia and Africa. Asia produces 73.5% and Africa produces 21.3% of the World's groundnut oil supply. On average, China produced 1 million and 869 thousand metric tons and India produces 1 million and 635 thousand tons of groundnut oil from 1993 to 2013 when worldwide average production was 5 million and 149 thousand tons (FAO, 2014). Turkey, where the oilseed market is dominated by sunflower seed, produces 6,000 tons of peanut oil annually (USDA, 2015). Amount of peanut oil production is still limited but with increasing demand to peanut oil and advances in peanut agriculture in Çukurova region, it is expected to develop more in coming years.

For the last five years (2011-2015) an average of 6.79 million tons of groundnut meal was produced worldwide with an increasing trend. USDA estimates annual 6.98 million tons of production for 2015-2016 season. About two-third of this amount is consumed domestically as an important protein rich meal in oil producing countries. Global trade is limited on this commodity due to high levels of aflatoxin contamination (USDA, 2015).

Groundnut meal is especially important for poultry production. Nitrogen-corrected total metabolizable energy (TME_n) of solvent extracted groundnut meal is 2,664 kcal/kg average and the protein content ranged between 40.1% and 50.9%

with a mean of 45.6%. Other nutritional values for groundnut meal is given in Table 2.1. (Batal et al., 2005)

Table 2.1 Nutrient composition of groundnut meal.

Nutrient	**Mass fraction on dry basis (%)**
Crude protein	45.6 ± 2.8
Crude fat	2.47 ± 2.47
Crude fiber	8.30 ± 2.05
Ash	5.02 ± 0.59

Since the first ever detected aflatoxicosis case happened to be related to groundnut meal, research was focused on detoxification of groundnut meal. Groundnut meal, by its nature, is highly susceptible to fungal attack and therefore aflatoxin contamination. Not all types of fungi, or especially strains of *A. flavus* are aflatoxigenic but most isolates from groundnut meal of this species are able to produce aflatoxins under favorable conditions before, during and after the harvest, processing or storage phases. On the other hand, invasion of groundnut seeds and products by *Aspergillus* species does not only cause aflatoxin contamination but also there are nutrient losses detected in terms of oil degradation, reduction of protein content and alterations in carbohydrate structures (Mehan et al., 1991).

2.4. *Rhodococcus erythropolis*

Belonging to the order of *Actinomycetales* and the family *Nocardiaceae*, *Rhodococci* are Gram-positive, aerobic, non-sporulating and non-motile bacteria (Conville & Witebsky, 2007). *Rhodococcus* is fairly a new organism for bacterial microbiology, but its use in modern biotechnology is promising. Due to their mobile, large and linear plasmid, members of this genus are capable of producing a large variety of enzymes for degrading different organic compounds (Bell et al., 1998; Gűrtler & Seviour, 2010).

R. erythropolis in particular, has many possible applications in biotechnology. Set of enzymes produced by *R. erythropolis* are reported to be capable of catalyzing many technologically important biochemical reactions including oxidation, dehydrogenation, epoxidation, hydroxylation, hydrolysis, dehalogenations and desulfurizations (de Carvalho & da Fonseca, 2005). Some examples of biotechnological applications of *R. erythropolis* are; limonene degradation (Werf et al., 1999), n-alkanes and alcohols degradation at extreme conditions (Chih-Wen et al., 2012; de Carvalho, 2012), desulfurization of dibenzothiopene (Izumi et al., 1994), microbial bioflocculant production (Bicca et al., 1999; Pirog et al., 2004), degradation of dinitrophenol (Lenke et al., 1992), cholesterol oxidase activity (Sojo et al., 1997) and last but not least, degradation of aromatic mycotoxins, especially AFB_1 (Alberts et al., 2006; Cserháti et al., 2013; Eshelli et al., 2015; Teniola et al., 2005).

R. erythropolis is not considered a common human or animal pathogen. However, it should be noted that there were 7 medical cases where people with immune deficiency due to some other diseases were diagnosed with *R. erythropolis* infection (Bagdure et al., 2012).

2.4.1. Aflatoxin degradation ability of *R. erythropolis.*

Teniola et al. (2005) treated liquid cultures supplemented with 2.5 ppm (mg/L) AFB_1 with cell free extracts of 3 different microorganisms, *N. corynebacterioides, Mycobacterium fluoranthenivorans* and *R. erythropolis. R. erythropolis* extracts showed an efficient reduction in AFB_1, only 3-6% residue remaining in the liquid culture after 72 hours of incubation. Also, it was observed that *R. erythropolis* has a wider temperature range for biological detoxification. Alberts et al. (2006) confirmed abovementioned results by using cell free extracts of *R. erythropolis* cultures starting from an initial AFB_1 concentration of 1.75 ppm (mg/L). In their study, 33.2% of AFB_1 residue was left in the liquid culture after 72 hours of treatment and loss of mutagenicity was detected by the Ames test of mutagenicity.

Kong et al. (2012) suggested optimum conditions for AFB_1 degradation by using viable cell cultures of *R. erythropolis* in synthetic media by Plackett-Burman design, central composite design and response surface analysis methods. At temperature 23.2°C, pH 7.17, 24.6 mL liquid volume in 100-mL flasks, 10% inoculum volume, 180 rpm agitation speed and 81.9 hours of incubation time, 95.8% reduction in AFB_1 was reported.

Eshelli et al. (2015) reported 95% reduction in AFB_1 in liquid culture starting with an initial load of 20 µg/mL in 24 hours. In this study, degradation efficiency of three Actinomycetes, *R. erythropolis, Streptomyces lividans* and *Streptomyces aureofaciens* were compared and no significant difference was reported between the efficiency of these microorganisms. Degradation products were also analyzed by HR-FTMS and MS^2 fragmentation methods and a possible pathway of degradation of AFB_1 was suggested. As the peak area for AFB_1 decreases, an increase in a metabolite with 236 atomic mass unit (amu) was observed and it was thought that this low molecular weight compound was then participated in the citrate cycle. During biodegradation lactone carbonyl ring and cyclopentenone ring of AFB_1 was opened. Suggested degradation mechanism is shown in Figure 2.3. First, lactone ring is hydrolyzed and open lactone ring is decarboxylated yielding 268 amu

molecular weight aflatoxin derivative, also known as Aflatoxin D_1. This molecule is then converted to Aflatoxin D_2 (MW: 206 amu) where the difuran property is protected but lactone ring is still missing. At the last stage, unsaturated part of one furan ring is cleaved yielding the final degradation metabolite with 236 amu molecular mass.

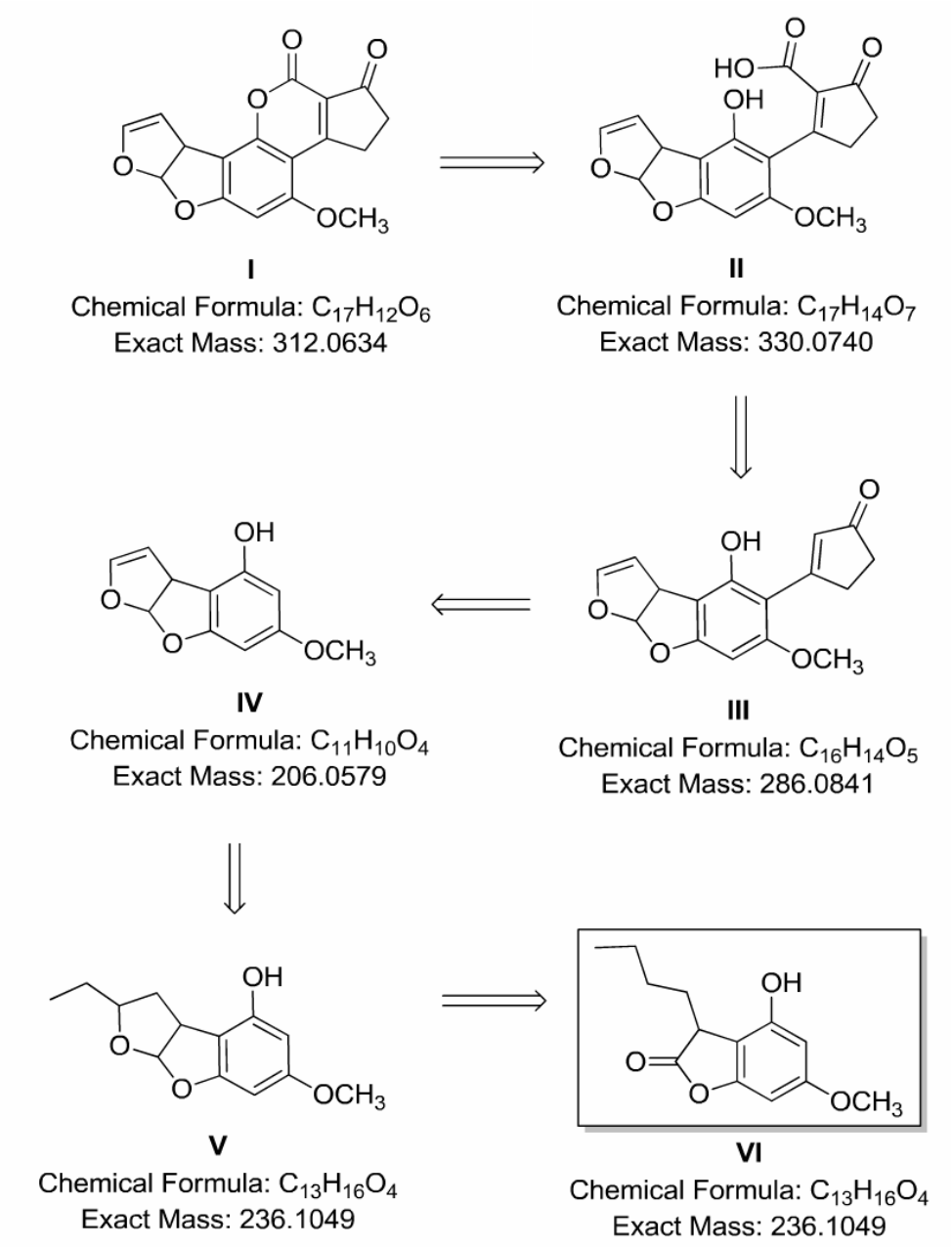

Figure 2.3 Suggested degradation pathway of AFB_1 by *R. erythropolis.* (Eshelli et al., 2015)

2.5 Objectives of the study

Aflatoxins have detrimental effects on many aspects. The most efficient way to reduce these effects is to apply post-harvest management procedures. However, in practice it is not always possible to completely inhibit growth of fungi. Therefore, detoxification methods are always necessary for food safety.

Groundnut meal is an important commodity for agricultural supply chains, however aflatoxin problem risks the public health and economy. Reducing the aflatoxin levels in groundnut meal makes it possible to use it in feed ration mixtures instead of discarding.

Physical and chemical methods have been suggested to detoxify contaminated food and feed products. Nonetheless, as mentioned in this literature review, most of them are inefficient, expensive or causing undesired changes in the final product. On the other hand, biological methods offer effective detoxification rates by little or no changes in the physicochemical structure of the product. Within these biological methods *R. erythropolis* is a promising detoxifying agent since it can degrade AFB_1 in a short time with a broader range of mild processing conditions. Detoxification studies using *R. erythropolis* were only conducted in liquid culture so far as stated in Chapter 2.4.1. In this study, detoxification in a real feed material is addressed.

In this work, detoxification of groundnut meal, a byproduct of vegetable oil production, was studied. For this purpose, first the growth characteristics of *R. erythropolis* were investigated and optimized using response surface methodology (RSM). Aflatoxin degrading ability of the microorganism was also addressed by making a comparison of viable cell cultures and extracellular enzyme portions. AFB_1 detoxifying ability of the organism in groundnut slurry was then optimized using RSM. Decrease in the toxicity of degradation byproducts were investigated by GST assay.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw materials

Defatted groundnut meal (GNM) was provided by a local groundnut oil production factory from Osmaniye, Turkey. The meal was ground in a laboratory scale grinding mill (Thomas-Wiley Laboratory Mill, Model 4, Arthur H. Thomas Company, Philadelphia, PA, USA) equipped with a 1 mm diameter sieve and kept in plastic bags at room temperature. Meal was naturally contaminated with 270.1 ± 4.9 μg/kg of AFB_1.

Rhodococcus erythropolis NRRL B-16531 was kindly provided by the Agricultural Research Service (ARS) culture collection of United States Department of Agriculture in lyophilized form. The microorganism was activated in nutrient broth and stored in 20% glycerol-water as culture stock at -82°C in freezer (Revco Elite Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA). For short term storage, it was maintained on nutrient agar at +4°C.

Aflatest® inmunoaffinity columns (VICAM, Watertown, MA, USA) were used for clean-up of aflatoxins during aflatoxin determination by HPLC.

Sheep liver for GST assay was purchased from Kazan slaughterhouse in Ankara, Turkey.

3.1.2 Chemicals

Chemicals used in this study are listed in Table A.1.

3.1.3. Buffers and solutions

Preparations of buffers and solutions are given in Appendix B.

3.1.4. Growth medium

Pre-mix nutrient broth and agar was dissolved in deionized water for activation and preliminary growth of microorganism. Other media were prepared considering the amounts of ingredients in the experimental design matrix. At optimal growth conditions, 5 g/L glucose, 5g/L peptone and 5g/L yeast extract were dissolved in deionized water and sterilized at 121°C for 15 minutes in autoclave (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan).

3.2. Methods

3.2.1 Sample preparation

Defatted GNM was first weighed and sterilized by autoclaving at 121°C for 15 minutes in 500 mL flasks in solid form.

Sterile liquid supplemented with yeast extract was added to the flask according to the solid concentration (Table 3.5) after separate sterilization. This fermentation medium was inoculated with 48 hours grown cultures of *R. erythropolis.* For determination of extracellular enzyme activity, growth medium was centrifuged at 2000 x g for 15 minutes for separation of extracellular part from the suspension. Liquid portion was filtrated by filter cloth after the fermentation and discarded. Remaining meal was then dried in a laboratory scale tray dyer (Eksis

Endustriyel Kurutma Sistemleri, Isparta, Turkey) for 3 hours with 70°C air blowing at 1m/s and trays rotating at 6 rpm. An overview of the detoxification process is given in Figure 3.1.

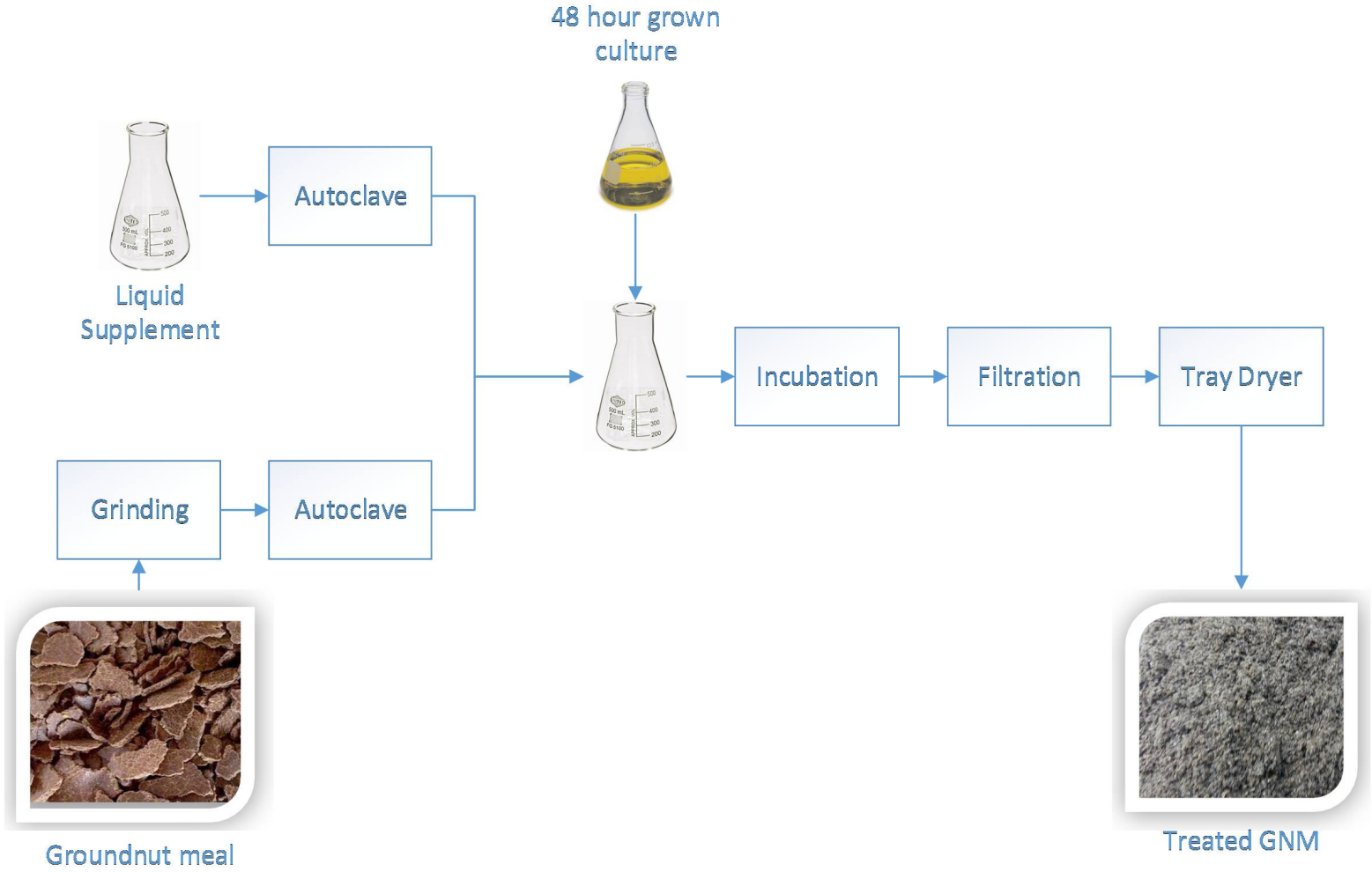

Figure 3.1 Overview of the detoxification process

3.2.2 Bacterial counts and optical density

Growth of the bacteria was monitored by measuring optical density at wavelength of 600 nm (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). Samples were diluted with growth medium in order to read absorbance values between 0.000 and 1.000. To relate optical density with the number of organisms, plate counts were carried out by overnight incubation of plate count agars inoculated by spread plate method. Standard curve for optical density versus log number of cells is presented in Figure D.1.

3.2.3 Aflatoxin analysis

3.2.3.1 Aflatoxin extraction

AOAC Official Method 999.07 (2012) was adopted for extraction and clean-up of aflatoxins. Previously weighed and processed groundnut cake was extracted with methanol-water (8:2) plus 50 mL of hexane in 500 mL flasks shaking at 150 rpm (0.314 x g) for an hour. 10 mL of the methanolic phase of the extract was filtered through filter paper and diluted with 60 mL of PBS solution.

3.2.3.2 Immunoaffinity column chromatography

Preparatory clean-up was done according to AOAC Official Method 997.07 (2012). Immunoaffinity cleanup columns were first adjusted to room temperature and conditioned by passing 10 mL of PBS solution prior to sample loading. Filtrate was then passed through the column at a rate of 3mL/min by using a vacuum manifold. Column was washed with deionized water. Aflatoxins which remained on the column were eluted with 1.25 mL HPLC grade methanol and diluted with 1.75 mL water prior to HPLC injection.

3.2.3.3 HPLC analysis

Aflatoxin analysis were done based on AOAC Official Method 997.07 (2012). High performance liquid chromatograph (Agilent 1100, Agilent Technologies, CA, USA) equipped with Hichrom ODS-2 column 250 x 4.6 mm (Hichrom Limited, Berkshire, UK) was used. Coring cell was used for electrochemical post-column derivatization. Samples (100 µL) were injected into the mobile phase consisting of water-acetonitrile-methanol (6:2:2) plus KBr and HNO_3 at a flow rate of 1mL/min at 40°C. Fluorescence of aflatoxin was detected by fluorescence detector at 360 nm excitation and 440 nm cut-off emission wavelengths.

Concentration of sample solutions were calculated using Equation (1).

$$C_{smp}(ng/mL) = a \times signal_{smp} + b \quad (1)$$

Concentration of AFB_1 in samples was calculated according to Equation (2)

$$AFB1\ Concentration = \frac{C_{smp} \times solvent \times elution}{W_t \times aliquot} \quad (2)$$

Where a & b are coefficients for linear equation, C_{smp} is the toxin concentration in the sample extract, $signal_{smp}$ is the peak area from the chromatogram and W_t is the test portion taken from the product.

3.2.4 Preparation of sheep liver cytosol

Sheep liver was homogenized in 10 mM potassium phosphate buffer (pH=7.0) containing 0.15 M KCl, 1 mM EDTA and 1mM DTT. Resulting homogenate was centrifuged at 4°C at 10,000 x RPM (21801 x g) for 30 minutes by Sigma 3K30 refrigerated centrifuge (Sigma-Zentrifugen GMBH, Harz, Germany). Supernatant was then taken and centrifuged again at 4°C at 21,000 x RPM (45782 x g) for 90 minutes. Supernatant was frozen and stored at -84°C up to three months(İscan et al., 1998). Protein content of the cytosols were measured by

Lowry's method of protein measurement with Folin-Ciocalteu's phenol reagent as 30mg/mL (Lowry et al., 1951).

3.2.5 Sheep liver glutathione-S-transferase assay

Glutathione-S-transferase (GST) activity was determined spectrophotometrically at 340 nm wavelength using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Sheep liver cytosol was diluted 100-folds with 10 mM potassium phosphate buffer (pH=6.5). Enzymatic reaction was carried out in 100 mM phosphate buffer (pH=6.5) containing 1mM reduced L-glutathione (GSH), 1mM CDNB and groundnut extract. Reaction mixture without extract was used as the control. Reaction was started by adding enzyme source. Thioether formation was observed by monitoring absorbance change for 5 minutes at 340 nm and at 30°C in a Shimadzu spectrophotometer. Molar extinction coefficient for CNDB conjugate at 340 nm was taken as 0.0096 μM^{-1} cm^{-1} (Habig et al., 1974). Specific GST activity was determined using Equation (3).

$$\text{EA(IU/mL)} = \frac{OD_{340}}{\text{time}} \times \frac{1}{\varepsilon_{340}} \times \frac{V}{V_{enz}} \times \text{dilution factor} \quad (3)$$

where OD_{340} is absorbance at 340 nm, ε_{340} is the molar extinction coefficient for CNDB conjugate at 340 nm, V is reaction volume in mL and V_{enz} is volume of the enzyme in mL and time is in minutes.

3.2.6 Statistical methods

3.2.6.1 One factor at a time approach (OFAT)

Conventional OFAT approach was used to decide on which carbon and nitrogen sources are best for the optimum growth of *R. erythropolis.* For carbon sources, glucose, xylose, lactose and sucrose were tested, whereas for nitrogen sources peptone, tryptose and ammonium sulfate were compared. At each trial,

concentration of other ingredients were hold constant while adding each carbon or nitrogen source separately. For statistical analysis, analysis of variance (ANOVA) at 95% confidence level was performed using Minitab 16 (Minitab Inc., State College, PA, USA).

3.2.6.2 Plackett-Burman design (PB)

Plackett-Burman design is a first order polynomial model to screen a large number of independent variables instead of time consuming full factorial model (Plackett & Burman, 1946). In this study, two level PB design was applied with 12 runs and two replications at 95% confidence level. Variables and range of values for PB design are given in Table 3.1. Given ranges were decided based on preliminary experiments and previous studies.

The experimental design matrix for temperature, pH, liquid volume, inoculum volume, agitation speed, glucose concentration and peptone concentration was constructed by Minitab 16 software. Results were analyzed by ANOVA table and Pareto chart to screen the most significant variables.

Table 3.1 Ranges of process variables used for PB design

Variable	Low level (-1)	High level (+1)
Temperature (°C)	20	40
pH	6	8
Liquid volume (mL)	50	150
Inoculum volume (% v/v)	1	5
Agitation (rpm)	80	160
Glucose concentration (g/L)	5	15
Peptone concentration (g/L)	5	15

Table 3.2 PB design parameters for screening of major factors for *R. erythropolis* growth. (Coded factors)

Run Order	Temperature (°C)	pH	Liquid volume (mL)	Inoculum volume (% v/v)	Agitation (rpm)	Glucose concentration (g/L)	Peptone concentration (g/L)
1	+1	-1	-1	-1	+1	+1	+1
2	+1	+1	-1	+1	-1	-1	-1
3	-1	+1	-1	-1	-1	+1	+1
4	-1	+1	+1	+1	-1	+1	+1
5	-1	-1	+1	+1	+1	-1	+1
6	-1	+1	+1	-1	+1	-1	-1
7	+1	-1	+1	+1	-1	+1	-1
8	+1	-1	+1	-1	-1	-1	+1
9	+1	+1	-1	+1	+1	-1	+1
10	-1	-1	-1	-1	-1	-1	-1
11	+1	+1	+1	-1	+1	+1	-1
12	-1	-1	-1	+1	+1	+1	-1

3.2.6.3 Response surface optimization

Response surface optimization with Box-Behnken design was used to find optimum process conditions for both bacterial growth and aflatoxin detoxification. Box-Behnken design makes use of three level rotatable response surface design to make a quadratic model which can analyze individual and combined effects of variables (Box & Behnken, 1960). Experimental design matrices with two replications were constructed and ANOVA was performed. Results were analyzed by response optimizer tool of Minitab 16 software. Experimental data were fit to the quadratic equation (4).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (4)$$

where b's are regression coefficients and X_1, X_2 and X_3 are independent variables as given in Table 3.3 and 3.5.

3.2.6.3.1 Box Behnken (BB) design for optimization of culture conditions

Design variables for growth medium optimization (temperature, liquid volume, agitation speed) were decided by the results of PB design. For these three variables a set of 15 runs was carried out in low, center and high level conditions with two replications. Levels of the variables are given in Table 3.3 and the experimental design matrix with coded factors are given in Table 3.4.

Table 3.3 Ranges of variables for BB optimization

	Variable	Low level (-1)	Center (0)	High level (+1)
X_1	Temperature (°C)	20	30	40
X_2	Liquid volume	100	150	200
X_3	Agitation speed (rpm)	80	120	160

Table 3.4 Experimental design matrix of BB design for growth optimization (coded factors)

Run Order	Temperature (°C)	Liquid volume (mL)	Agitation speed (rpm)
1	0	-1	+1
2	0	+1	-1
3	-1	0	-1
4	+1	+1	0
5	0	0	0
6	0	+1	+1
7	-1	-1	0
8	+1	-1	0
9	+1	0	+1
10	-1	0	+1
11	+1	0	-1
12	-1	+1	0
13	0	-1	-1
14	0	0	0
15	0	0	0

3.2.6.3.2 Box-Behnken (BB) design for optimization of AFB_1 detoxification

Design variables for AFB_1 degradation optimization were chosen as solid concentration, inoculum volume and fermentation time. For these three variables a set of 15 runs was carried out in low, center and high level conditions with two replications. Levels of the variables are given in Table 3.5 and the experimental design matrix with coded factors are given in Table 3.6. Given ranges were decided based on preliminary experiments and previous studies.

Table 3.5 Ranges of variables for BB design for optimization of AFB_1 degradation.

	Variable	Low level (-1)	Center (0)	High level (+1)
X_1	Solid concentration (g/100 mL)	10	20	30
X_2	Inoculum volume (% v/v)	1	3	5
X_3	Time (h)	24	48	72

Table 3.6 Experimental design matrix of BB design for AFB_1 degradation optimization (coded factors)

RunOrder	Innoculum size (% v/v)	Solid conc. (%(w/v))	Time (h)
1	0	0	0
2	0	+1	-1
3	-1	-1	0
4	0	-1	+1
5	-1	0	-1
6	0	-1	-1
7	+1	+1	0
8	+1	-1	0
9	0	+1	+1
10	-1	+1	0
11	+1	0	-1
12	-1	0	+1
13	+1	0	+1
14	0	0	0
15	0	0	0

3.2.6.4 Model verification

The predicted models were verified by additional experiments at optimum points. Performance of quadratic models were evaluated by calculating coefficient of determination (R^2), root mean square error (RMSE) (Eqn. 5) and mean absolute error (MAE) values (Eqn. 6).

$$RMSE = \left[\frac{1}{N}\sum_{i=1}^{N}(X_{pred,i} - X_{exp,i})^2\right]^{0.5} \quad (5)$$

$$MAE = \frac{1}{N}\sum_{i=1}^{N}\left|X_{pred,i} - X_{exp,i}\right| \quad (6)$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth curve of *R. erythropolis* NRRL B-16531

Growth curve of *R. erythropolis* NRRL B-16531 was constructed under optimum growth conditions (Table 4.2) and presented in Figure 4.1. Cells were observed to reach stationary phase after the 44^{th} hour of incubation with a specific growth rate of 0.33 h^{-1}.

Although it is not clearly known on which growth phase aflatoxin degrading enzymes are produced, there are several advantages of using cells at stationary phase. Most of the enzymes promoting the survival of the microorganism are produced during the stationary phase (Fanget & Foley, 2011). Most number of cells per mL of inoculum is achieved at this phase which means a higher concentration of biocatalyst available in the reaction medium. Also, cell lysis and modifications in the cell wall during stationary phase makes intracellular enzymes free in the medium, increasing the effectiveness of the process (Reed, 1966).

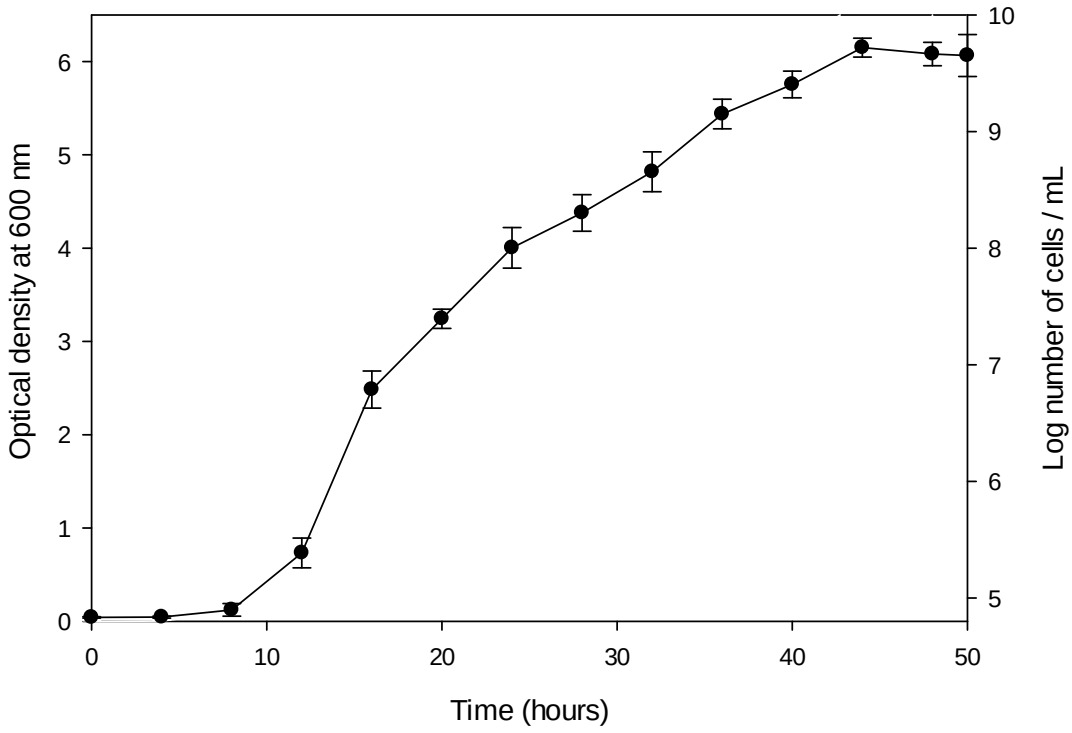

Figure 4.1 Growth curve of *R. erythropolis*

4.2 Optimization of growth conditions

4.2.1 One factor at a time

To evaluate the effect of carbon source on *R. erythropolis* growth, glucose, xylose, lactose and sucrose were compared as shown in Figure 4.2. There were significant differences among carbon sources and glucose was found the best carbon sources giving the highest number of cells (10.135±0.047 log number of cells/mL).

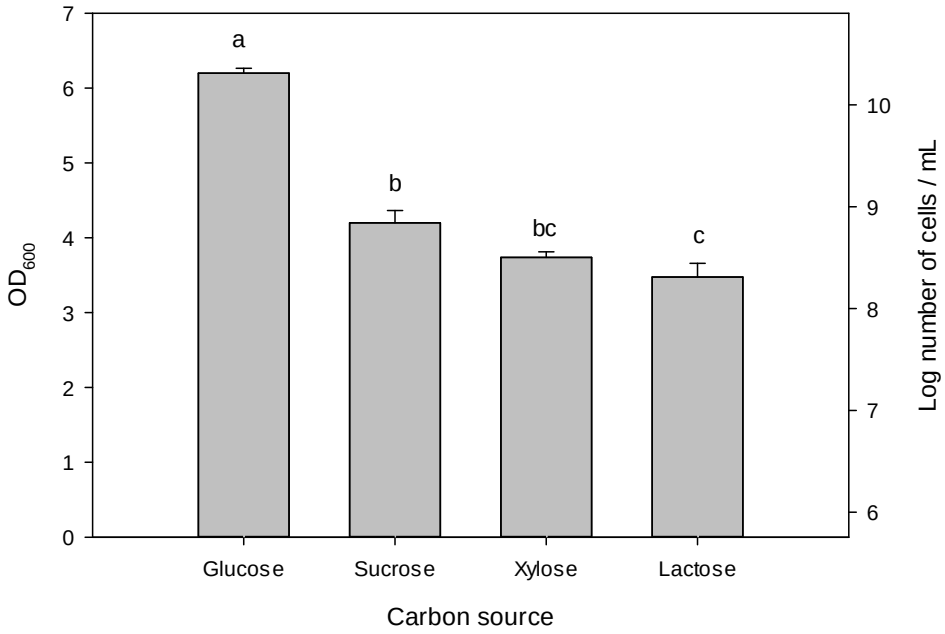

Figure 4.2 Effects of different carbon sources on *R. erythropolis* growth.

R. erythropolis can grow on a large variety of carbon sources including saccharides, alcohols, organic acids and cyclic hydrocarbons (Kurane et al., 1994b). However, Moumita et al. (2009) reported that different types of carbon metabolism was observed for different strains of *R. erythropolis.* Growth of some strains are favored by glucose or fructose, however some other reach higher number of cells on sucrose, glycerol or other carbon sources. Results of this study implies that *R. erythropolis* NRRL B-16531 favors glucose rather than xylose, lactose or sucrose but other strains of this microorganism would not necessarily exhibit the same behavior.

To evaluate the effect of nitrogen source on growth, peptone, tryptose and ammonium sulfate were used as shown in Figure 4.3. There was no significant

difference between peptone (9.475±0.019 log number of cells/mL) and tryptose, but number of cells was significantly lower when ammonium sulfate was used as sole nitrogen source.

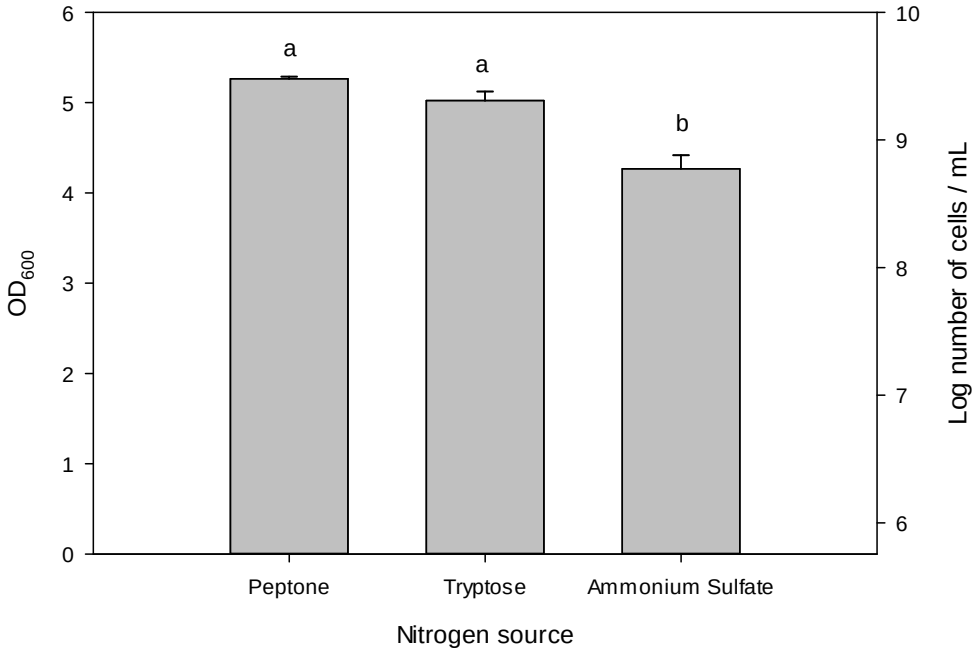

Figure 4.3 Effects of different nitrogen sources on *R. erythropolis* growth

Nitrogen metabolism of *R. erythropolis* is more selective than its carbon metabolism. Kurane et al. (1986) remarked that as an inorganic nitrogen source, ammonium sulfate was favorable for cell growth, however addition of organic nitrogen sources also increased cell growth as reported for *R. erythropolis* S-1

strain. Moumita et al. (2009) also confirmed that using inorganic nitrogen sources for six different strains of *R. erythropolis* resulted in very low cell mass.

Therefore, peptone was chosen as the nitrogen source and glucose was chosen as carbon source for growing *R. erythropolis* in future trials.

4.2.2 Plackett-Burman (PB) design

The PB design is a quick way to screen and select important process variables prior to response surface optimization. There are many examples of screening cell growth and other bioprocess variables using PB design method. Waśko et al. (2010) used this method for optimization of medium components for *Lactobacillus rhamnosus* biomass production. Kong et al. (2012) utilized PB design for optimization of AFB_1 degradation of *R. erythropolis* in liquid culture. Zhang et al. (2014) optimized the aflatoxin production of *A. flavus* on peanuts using PB design.

The PB design matrix was constructed to determine which factors (liquid volume, agitation speed, temperature, pH, glucose concentration, peptone concentration and inoculum volume) are significant for growth of *R. erythropolis*. Liquid volume, agitation speed and temperature were found significant ($P<0.05$). Also, pareto chart in Figure 4.4 shows the standardized effect of seven process variables where the effect of liquid volume, agitation speed and temperature were higher. These three factors were further evaluated for optimization of cultivation conditions using Box-Behnken response surface method.

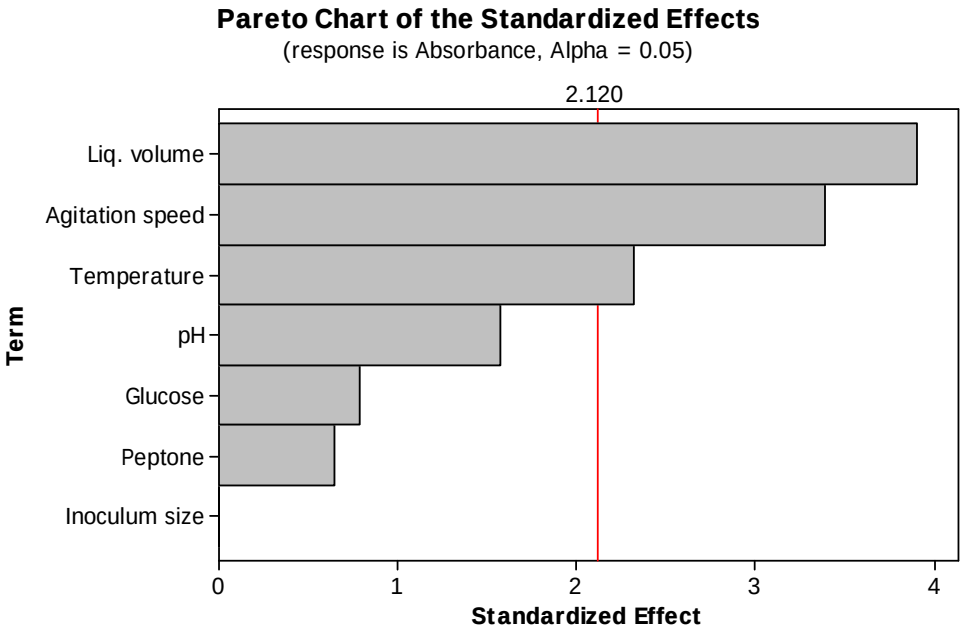

Figure 4.4 Pareto chart for screening important factors for *R. erythropolis* growth.

4.2.3 Box-Behnken (BB) response surface optimization of *R. erythropolis* growth

Box-Behnken design was selected for response surface optimization of the growth conditions because it offers less number of experimental runs than central composite design (CCD) and full factorial designs with 3 factors by utilizing center points and midpoints. Thus, Box-Behnken design is more efficient than CCD and factorial designs (Ferreira et al., 2007).

An overview of experimental results for BB design is given in Table 4.1. At minimum conditions 7.56±0.09 log number of cells per mL were detected in the growth medium. Highest log number of cells per mL (10.66±0.04) measured at run order 4. Comparing the maximum and minimum growth, at optimized conditions,

log number of cells was 1.41 times higher than non-optimized conditions which means that the actual number of cells in the medium was 2290 times higher. In their optimization study, Zhai et al. (2013) could reach 10^8 CFU/mL biomass density under optimal conditions where initial biomass density was reported as 10^6 CFU/mL.

Table 4.1 Results of Box-Behnken design for culture growth

Run Order	Temperature (°C)	Liquid volume (mL)	Agitation speed (rpm)	Log number of cells /mL
1	0	-1	+1	9.24±0.10
2	0	+1	-1	9.57±0.11
3	-1	0	-1	9.37±0.12
4	+1	+1	0	10.66±0.04
5	0	0	0	8.20±0.16
6	0	+1	+1	9.85±0.07
7	-1	-1	0	8.39±0.59
8	+1	-1	0	10.33±0.16
9	+1	0	+1	10.32±0.07
10	-1	0	+1	8.09±1.19
11	+1	0	-1	8.30±1.35
12	-1	+1	0	9.52±0.15
13	0	-1	-1	8.47±0.01
14	0	0	0	7.56±0.09
15	0	0	0	8.14±0.09

Equation (7) describes growth of *R. erythropolis* in liquid culture as a function of coded factors X_1 (temperature), X_2 (liquid volume) and X_3 (agitation speed) and their interactions. By looking at coefficients in Eqn. (7), liquid volume is more effective on cell growth than other two independent variables followed by agitation speed and temperature. Among interactions, liquid volume & agitation speed and the square of temperature are important factors in this equation.

$$Y = 9.3909 - 0.4256X_1 - 0.9888X_2 + 0.5642X_3 + 0.2218X_1X_2 - 0.1854X_1X_3 + 0.4081X_2X_3 - 0.4751X_1^2 + 0.2621X_2^2 - 0.3953X_3^2 \quad (7)$$

The RMSE and MAE values were calculated as 0.20 and 0.25 respectively and the insignificant lack-of-fit ($P>0.05$) was detected indicating that this model makes effective prediction of the real situation. The R^2 value was found as 0.9696 meaning that 96.96% of the response variable variation can be explained by the model in equation (7). For validation, a comparison of experimental and predicted values is given in Figure 4.5 with R^2 value of 0.98.

This model was then evaluated with the response optimizer tool of Minitab 16. Optimum conditions were computed as temperature of 22.5°C, liquid volume of 100 mL in 500 mL flasks and agitation speed of 135 rpm. At these conditions, it was predicted that a maximum of 10.92 log number of cells/mL growth can be achieved. Overall optimum conditions combining the results of PB and BB designs are given in Table 4.2.

Table 4.2 Optimum conditions for maximum growth of *R. erythropolis*

Variables (units)	Values
Temperature (°C)	22.5
pH	7
Liquid volume (mL)	100
Inoculum volume (%)	1
Agitation speed (rpm)	135
Glucose concentration (g/L)	5
Peptone concentration (g/L)	5

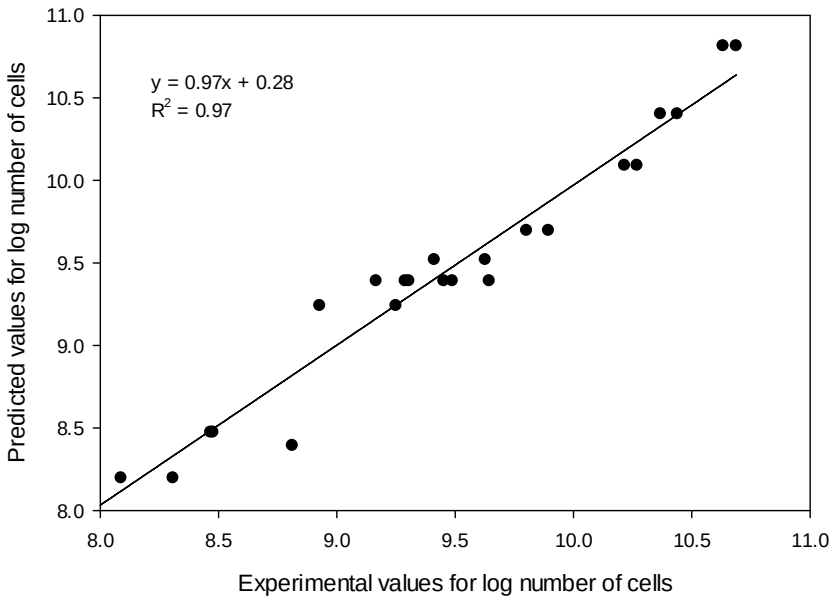

Figure 4.5 Experimental versus predicted values of log number of cells

Figure 4.6 represents the surface plot for the effect of temperature and agitation speed when liquid volume is held constant at 150 mL. Increase in cell growth was observed with increase in agitation speed to around 135 rpm but further increase reduced the growth. A peak in cell growth was observed at 22.5°C. A slight decrease was observed at lower temperatures but decrease was more drastic at higher temperatures. Optimum temperature for *R erythropolis* CS98 was reported as 25°C confirming the results of this study and 20% lower growth rates was observed at 20 and 30°C, and it was significantly reduced by further increasing the temperature to 35°C(Tomioka et al., 1994). Kong et al. (2012) reported that optimum AFB_1 degradation was observed at 23.2°C for *R. erythropolis.* On the other hand, Zhai et al. (2013) expressed the optimum growth temperature of *R. erythropolis* 4.1491 as 15.3°C which implies that the optimum temperature of this microorganism is strain dependent.

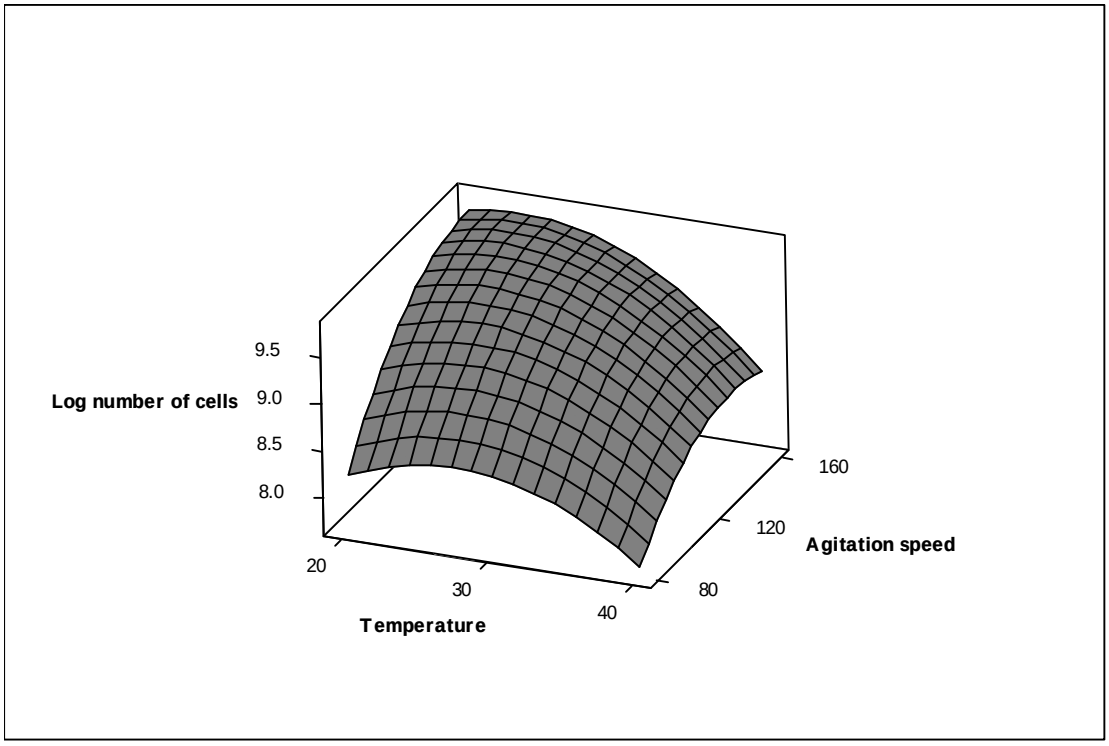

Figure 4.6 Surface plot showing the effect of temperature and agitation speed at constant liquid volume

In Figure 4.7 effect of temperature and liquid volume was plotted while holding agitation speed at the center of point 120 rpm. Cell growth was increased by decreasing the liquid volume due to effective oxygen transfer to the medium but amount of dissolved oxygen is also dependent on temperature where the solubility of oxygen is higher at lower temperatures. Therefore, at lower temperatures higher biomass density could be observed even at higher liquid volumes.

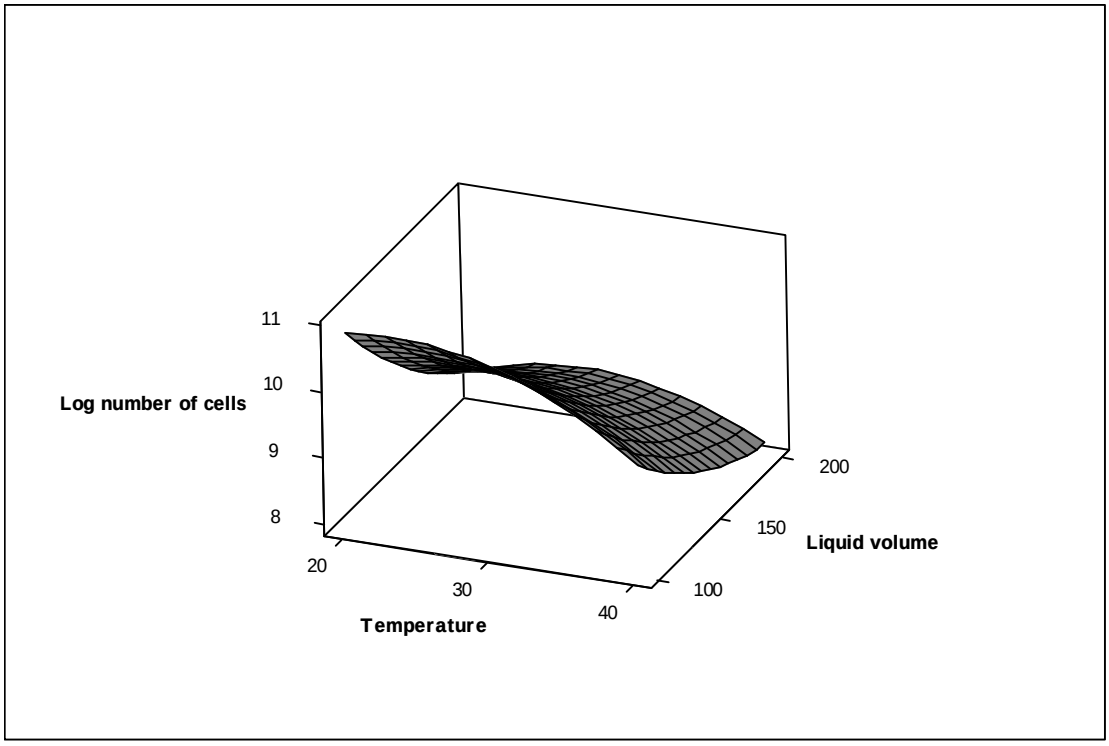

Figure 4.7 Surface plot showing the effect of temperature and liquid volume at constant agitation speed

Figure 4.8 shows the effects of liquid volume and agitation speed where temperature was held constant at 30°C. A maxima was observed where the liquid volume is minimum while agitation speed was around 135 rpm. The reasons for higher growth at lower liquid volumes are mainly effective oxygen dissolution in the medium and effective mixing of cells and nutrients.

Effect of agitation speed is clearly seen from both figures 4.6 and 4.8. As the agitation increases, amount of dissolved oxygen increases making it available for aerobic aspiration of cells and flocculation was prevented. *R. erythropolis* is known to be a flocculating agent for bioremediation processes (Kurane et al., 1994a). However, flocculating activity of this organism negatively affects the growth rate

by forming cell floccules and biofilms, reducing efficiency of aeration and agitation (Kamble & Meena, 2010).

Furthermore, as the agitation speed was further increased, limitation in growth was observed. This limitation could be explained by both oxygen toxicity and damage on cell wall structures at high shear rates. Oxidative stress caused by excess oxygen and other oxides limits the growth of aerobic bacterial species, as it causes oxidative damage on all living organisms (Cabiscol et al., 2000). Oxidative and high-shear damage on *R. erythropolis* was also observed by Kamble and Meena (2010) while increasing agitation speed from 200 rpm to 300 rpm.

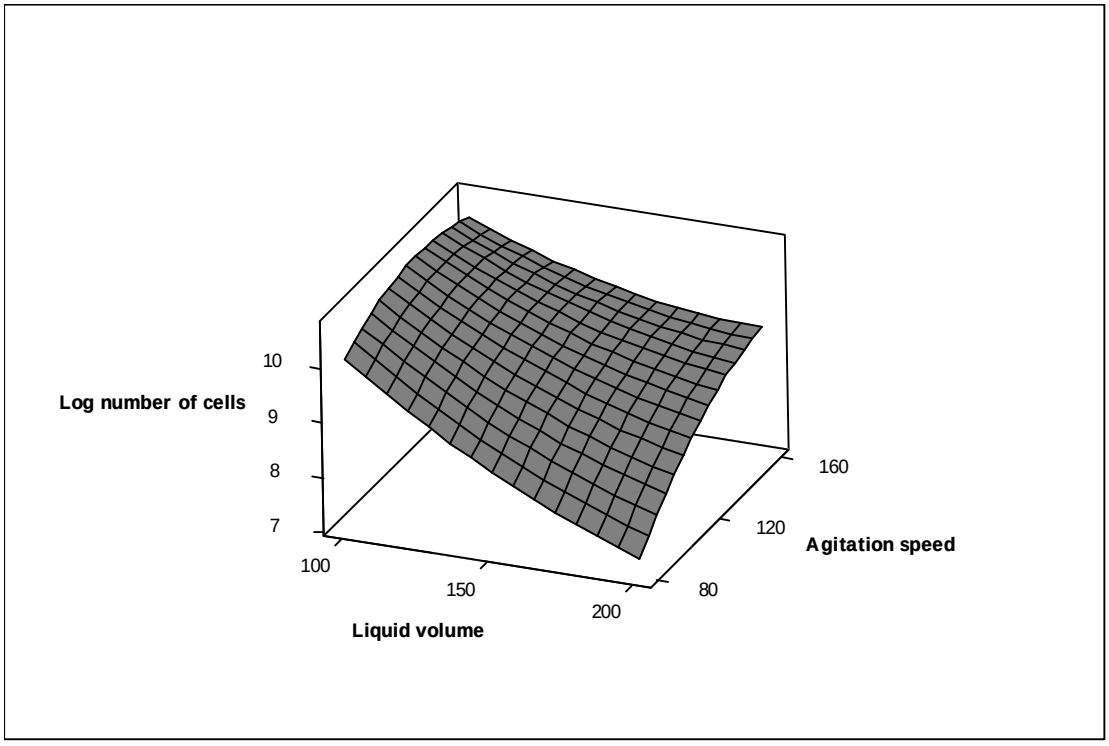

Figure 4.8 Surface plot showing the effect of liquid volume and agitation speed at constant temperature

Figure 4.9 is also given to better interpret the dual interactions between independent variables while the other variable was kept constant at center level. Elliptic formation in contour plots represents a perfect interaction between variables (Muralidhar et al., 2001). Elliptical shape was observed in contour plot of agitation speed vs. temperature, indicating a stronger interaction between these two variables. However contour plot of agitation speed vs liquid volume indicates that there are fewer interactions between these two variables.

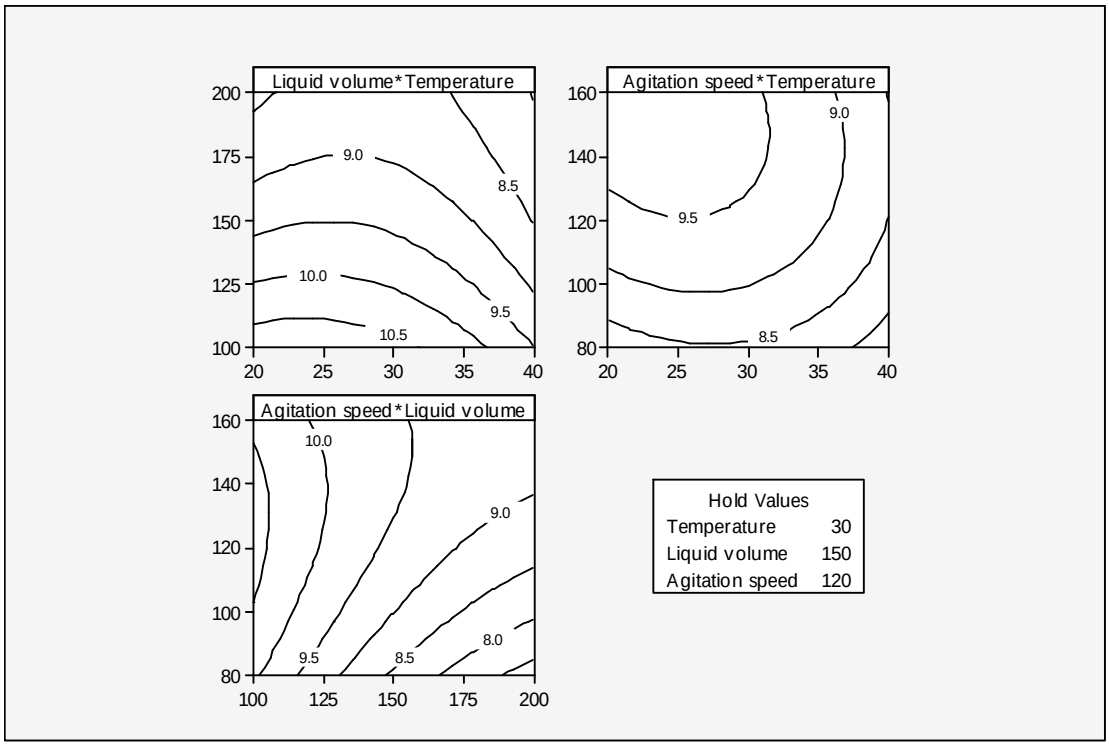

Figure 4.9 Contour plots for growth optimization

4.3 Evaluation of AFB_1 degradation by viable cells and extracellular enzymes

A comparison of AFB_1 degradation capacity between viable cell cultures and crude extracellular part of the culture was made to clarify the mechanism of degradation. In control group with no inoculation of cells or extracellular enzymes, 6.67±1.57% reduction was achieved after 72 hours (Figure 4.10). Extracellular enzymes reduced AFB_1 by 55.18±0.52% and viable cells reduced 68.52±1.05% after 72 hours (Figure 4.10). In liquid culture with an initial AFB_1 concentration of 2.5 ppm (mg/kg) Teniola et al. (2005) achieved more than 90% degradation in 4 hours and complete degradation after 24 hours. In this study, more than 55% degradation of AFB_1 was observed within 24 hours indicating that the biodegradation is fastest at first 24 hours of the process. However, there are two main reasons for slower action in this case. First, initial AFB_1 concentration was as low as 270 µg/kg for the product and further diluted with the addition of liquid medium to GNM. Therefore, enzymatic reaction was being carried on a slower phase. Second, complex biochemical matrix of GNM, particularly the oil content caused a barrier between the liquid medium and AFB_1 reducing the availability of the toxin for biodegradation. It should also be noted that initial evaluation was carried out under non-optimized conditions.

There was significant difference between control, viable cells and extracellular enzymes, therefore viable cell cultures were selected for detoxification of AFB_1. In liquid cultures, Teniola et al. (2005) reported AFB_1 degradation by both extracellular and intracellular enzymes of *R. erythropolis* cell-free extracts. However, the effect of cell growth on AFB_1 reduction was not studied. Kong et al. (2012) could achieve 95.8% degradation of AFB_1 in liquid cultures using viable cells of *R. erythropolis* after 81.9 hours.

Alberts et al. (2006) reported that biodegradation of AFB_1 occurs even when the organism was grown in the absence of AFB_1, concluding that this ability is a

constitutive property of *R. erythropolis*. In this study, also cells grown in the absence of AFB_1 was able to degrade the toxin.

Combining the existing knowledge and results of this study, the mechanism of action of *R. erythropolis* on AFB_1 can be evaluated. Degradation of AFB_1 is a constitutive ability for *R. erythropolis* which means that enzymes responsible for degradation are produced with or without the toxin present in the medium. Degradation is enzymatic, rather than physical or chemical means. Set of enzymes responsible for degradation are both extracellular and intracellular, however combination of these two while cells are growing can increase the efficiency of degradation.

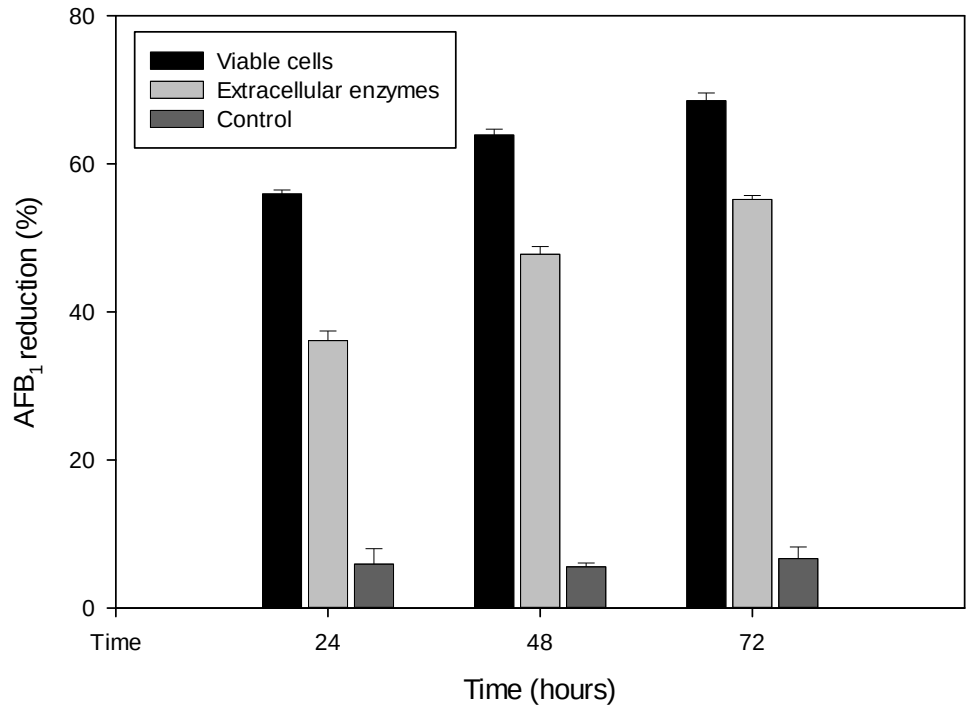

Figure 4.10 Evaluation of AFB_1 degradation capacity of viable cells and extracellular enzymes

4.4 Optimization of AFB_1 degradation

4.4.1 Box-Behnken (BB) response surface optimization of AFB_1 detoxification

In this section, optimal conditions for AFB_1 degradation of *R. erythropolis* in GNM slurry was presented. Results and conditions of BB experimental design are given in Table 4.3. A maxima was observed at run order 9, with 91.1±0.8% detoxification. Minimum detoxification was observed as 71.7±2.5% which corresponds to 27.05 % increase from the minimum detoxification rate.

Using the results in Table 4.3 a quadratic model was constructed using response surface method of Minitab 16 software. The quadratic model representing the response of percent AFB_1 reduction against solid concentration (X_1), inoculum volume (X_2) and process time (X_3) is given in equation (8). The R^2, RMSE and MAE values were found as 0.9825, 1.84 and 1.47 respectively. Lack-of-fit was insignificant (P>0.05) indicating that this model represents the system well.

$$Y = 46.3382 + 0.3515X_1 - 0.2340X_3 + 0.0057X_1X_2 - 0.0185X_2X_3 - 0.0007X_1^2 - 0.2434X_2^2 + 0.0034X_3^2 \quad (8)$$

Table 4.3 Results of Box-Behnken design for AFB_1 degradation

Run Order	Innoculum size (% v/v)	Solid conc. (%(w/v))	Time (h)	Remaining AFB_1 (µg/kg)	Detoxification (%)
1	0	0	0	38.2±1.8	85.8±0.7
2	0	+1	-1	26.1±1.9	90.3±0.7
3	-1	-1	0	76.5±6.7	71.7±2.5
4	0	-1	+1	67.3±3.1	75.1±1.2
5	-1	0	-1	36.6±2.6	86.4±1.0
6	0	-1	-1	75.2±4.7	72.1±1.7
7	+1	+1	0	29.4±4.2	89.1±1.5
8	+1	-1	0	82.9±3.1	69.3±1.1
9	0	+1	+1	24.0±2.2	91.1±0.8
10	-1	+1	0	35.4±1.7	86.9±0.4
11	+1	0	-1	30.6±0.8	88.7±0.3
12	-1	0	+1	31.6±1.4	88.3±0.5
13	+1	0	+1	28.2±2.0	89.6±0.7
14	0	0	0	33.3±0.6	87.7±0.2
15	0	0	0	31.8±3.4	88.2±1.2

The mathematical model in equation (8) was also evaluated for optimum points using response optimizer tool of Minitab 16 and the optimum conditions were found as 27.4 %(w/v) of solid concentration, 4.88 % (v/v) of inoculum volume and 24 hours of time as shown in Table 4.4. At optimal conditions, the predicted maximum AFB_1 reduction was 92.21%. However, the verification experiments yielded 87.25±0.79% of detoxification. Although actual detoxification was lower than the predicted maximum, it was still in agreement with the predicted value in 95% confidence interval.

Table 4.4 Optimal conditions for maximum detoxification

Variables (units)	Values
Solid concentration %(w/v)	27.4
Inoculum volume % (v/v)	4.88
Time (h)	24

For further validation of the model, a comparison of experimental and predicted values is given in Figure 4.11. This plot represents the variation in experimental and predicted detoxification where the R^2 is 0.95 meaning that 95% of variations between values can be explained by the quadratic model. High R^2 value also means that predicted and experimental values are in accordance with each other.

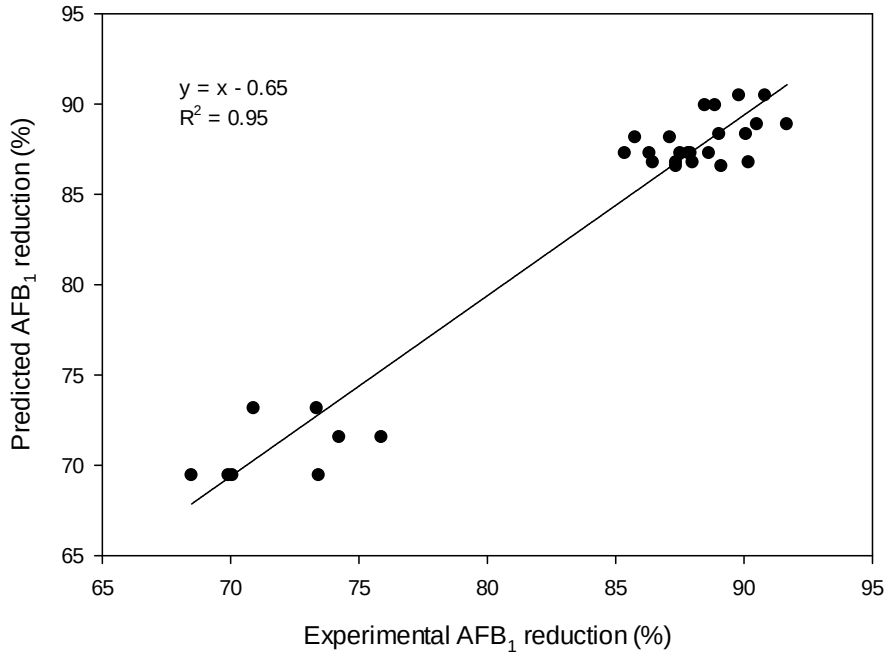

Figure 4.11 Experimental versus predicted values of AFB_1 reduction

The optimal conditions for detoxification suggests that *R. erythropolis* is an efficient agent for reducing AFB_1 concentration in contaminated products. Also, *R. erythropolis* has some advantages over other microorganisms suggested in current literature. For instance, *R. corynebacterioides* is also an efficient AFB_1 degrader but pigmentation and slow reaction limits the use of this organism (Teniola et al., 2005). *P. aeruginosa* is another good option with maximum 90.2% reduction, however optimum temperature is 55°C (Samuel et al., 2014). On the other hand, *R. erythropolis* is able to grow and detoxify at milder temperature and other process conditions while maintaining a high detoxification rate even when initial AFB_1 concentration is very low.

Figure 4.12 indicates the effect of solid concentration and inoculum over AFB_1 reduction while keeping time constant at 48 h. A dramatic increase was

observed when solid concentration was increased up to 270 g/L and a slight decrease was observed after this point where concentration of AFB_1 in liquid culture was calculated as 0.0729 µg/mL. This rapid increase can be explained by higher initial concentration of substrate AFB_1 and also the amount of nutrients from GNM. Very low AFB_1 concentration was used throughout this study compared to liquid culture experiments, Teniola et al. (2005) used 2.50 ppm (mg/L) and Alberts et al. (2006) used 1.75 ppm (mg/L) AFB_1 concentrations.

In inoculum volume, slight increase was observed until 4.8% and no more increase was observed thereafter. Higher amount of inoculum means higher amount of biomass and also extracellular enzymes. Since microorganism was taken from the growth culture at early stationary phase, growth rate was still enough for a faster growth and enzyme production. Therefore, further increasing the inoculum volume had little effect on AFB_1 degradation. In fact, the effect of inoculum volume was insignificant on AFB_1 degradation which was confirmed by the study of Kong et al. (2012). However, effect of inoculum volume was significant by interactions with other two independent variables.

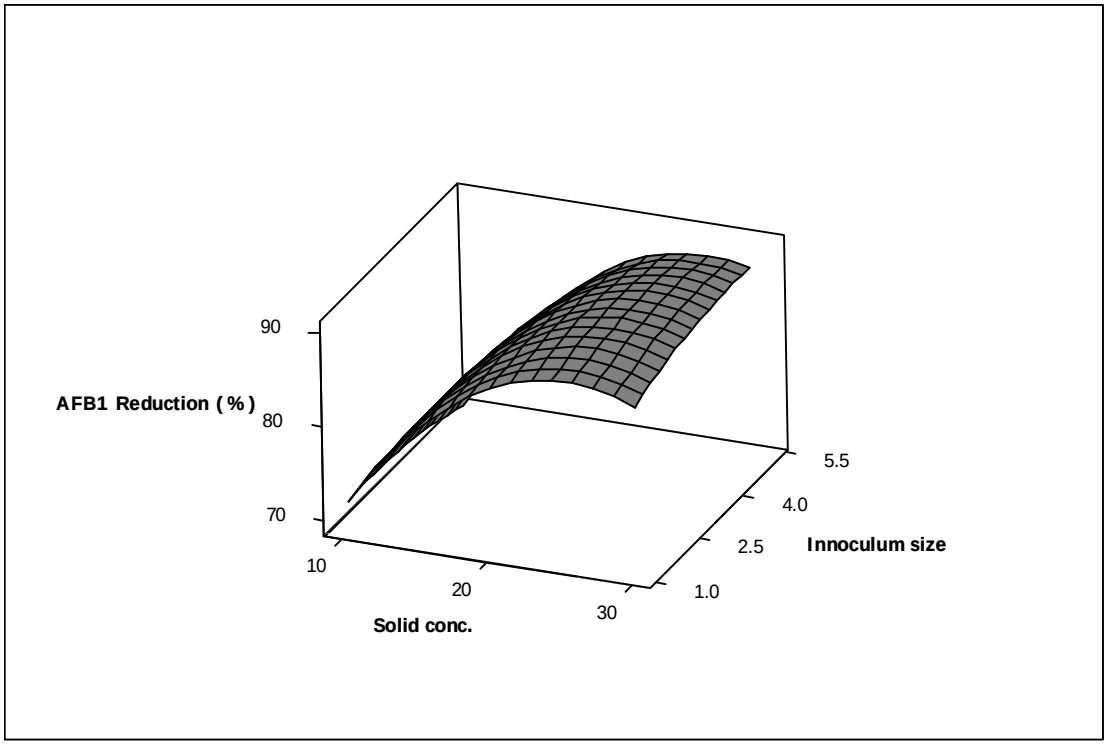

Figure 4.12 Surface plot showing the effect of solid concentration and inoculum volume at constant time

In Figure 4.13 concave up shape was observed for time changes and concave down for inoculum volume forming a saddle shape when solid concentration was kept constant at 20 g/L. Negative interaction between inoculum and time explains this phenomena. There are not exact maxima or minima for this surface. A minimum was observed around 30 hours and a maximum was observed around 4 % v/v. The effect of process time is clearly seen from figure 4.13 where the percent reduction was higher at the edges. However, this doesn't necessarily mean that the amount of toxin was increased after 24^{th} hour and decreased after passing 48^{th} hour. The time course of AFB_1 degradation was presented in chapter 4.3. However, in response surface analysis, interactions play an important role so that extreme points were observed at two edges of the time scale. A similar case was observed while

optimization in liquid culture by Kong et al. (2012) where the optimal incubation time was 81.9 h, however in this case degradation was lowered at longer and shorter incubation times.

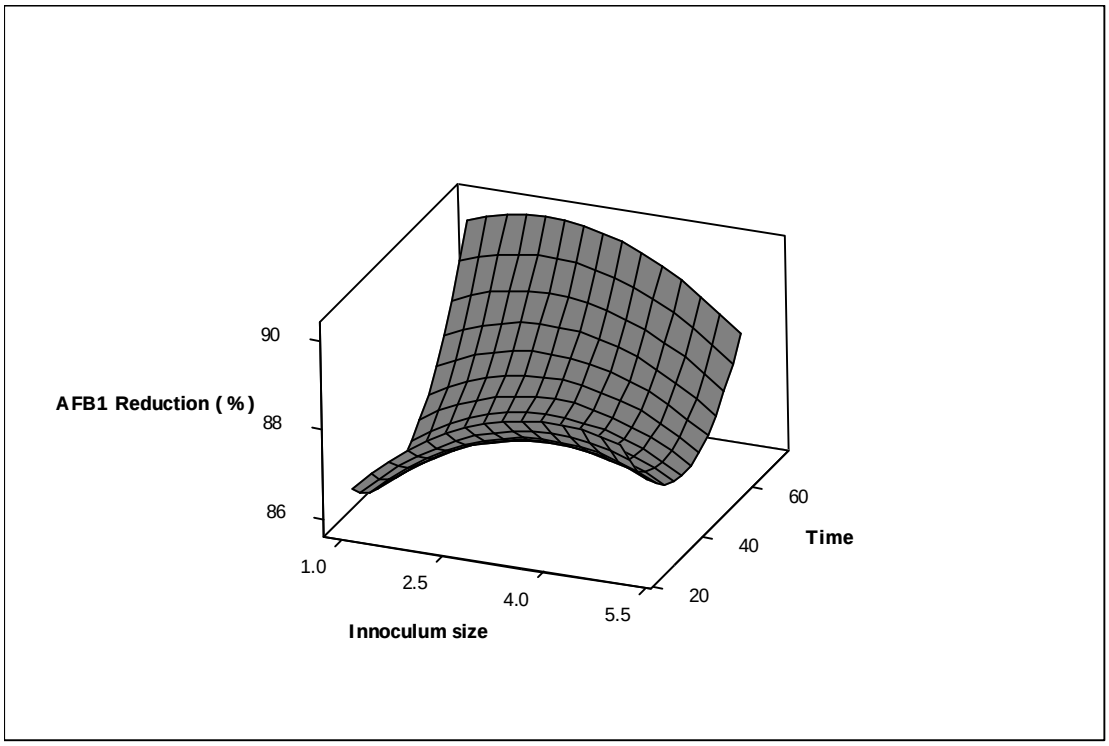

Figure 4.13 Surface plot showing the effect of inoculum volume and time at constant solid concentration

In Figure 4.14 surface plot of solid concentration and time is presented at constant inoculum volume at 3 % v/v. Valley shape was observed with a minimum at around 48 hours. AFB_1 reduction was increased until the maximum point until 270 g/L but slightly reduced by further increasing the solid concentration.

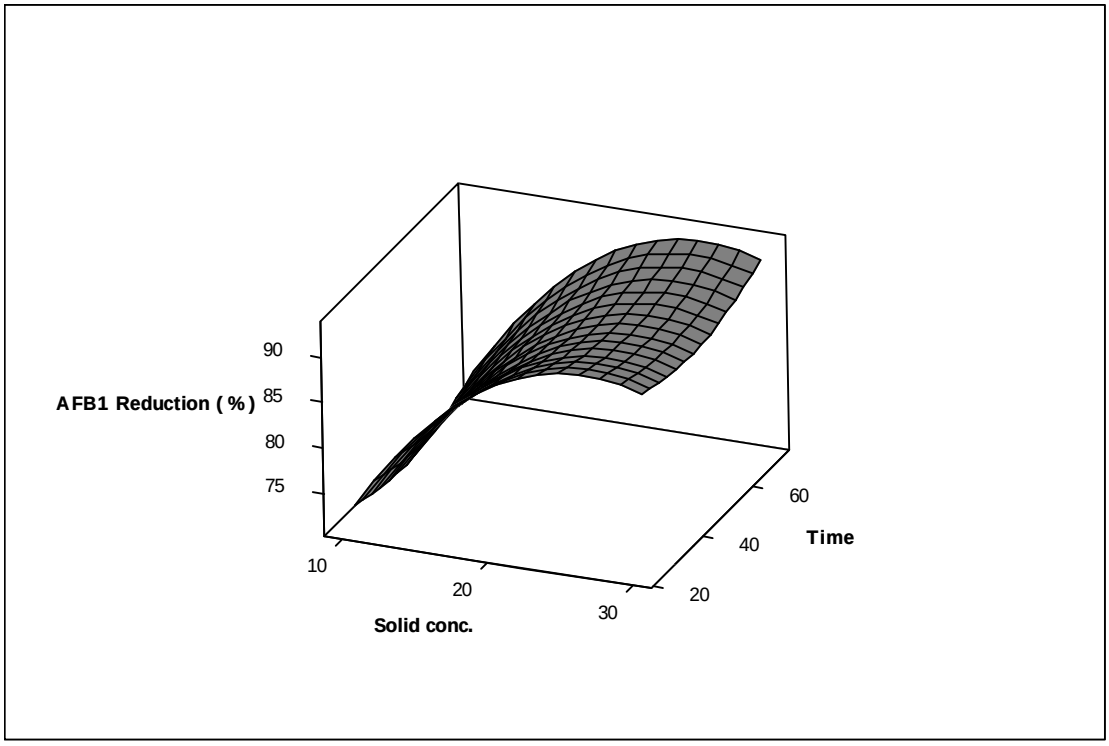

Figure 4.14 Surface plot showing the effect of solid concentration and time at constant inoculum volume

Figure 4.15 shows the contour plots for AFB_1 degradation of *R. erythropolis.* Inoculum volume vs. solid concentration exhibits the characteristics of elliptical contour at 90% which is the indication of a perfect interaction between them. Contour plot of time vs. solid liquid ratio resembles a cross shape, a 2D representation of the saddle formation where no single maximum or minimum points was observed. This shape indicates that the interaction between time and solid concentration are insignificant. This interpretation is also confirmed by insignificance of this interaction in analysis of variance ($P>0.05$). Only a single contour was observed in contour plot of time versus inoculum volume. This interaction is significant for our observations, however, effect is very low, explained by -0.0185 regression coefficient back in equation (8).

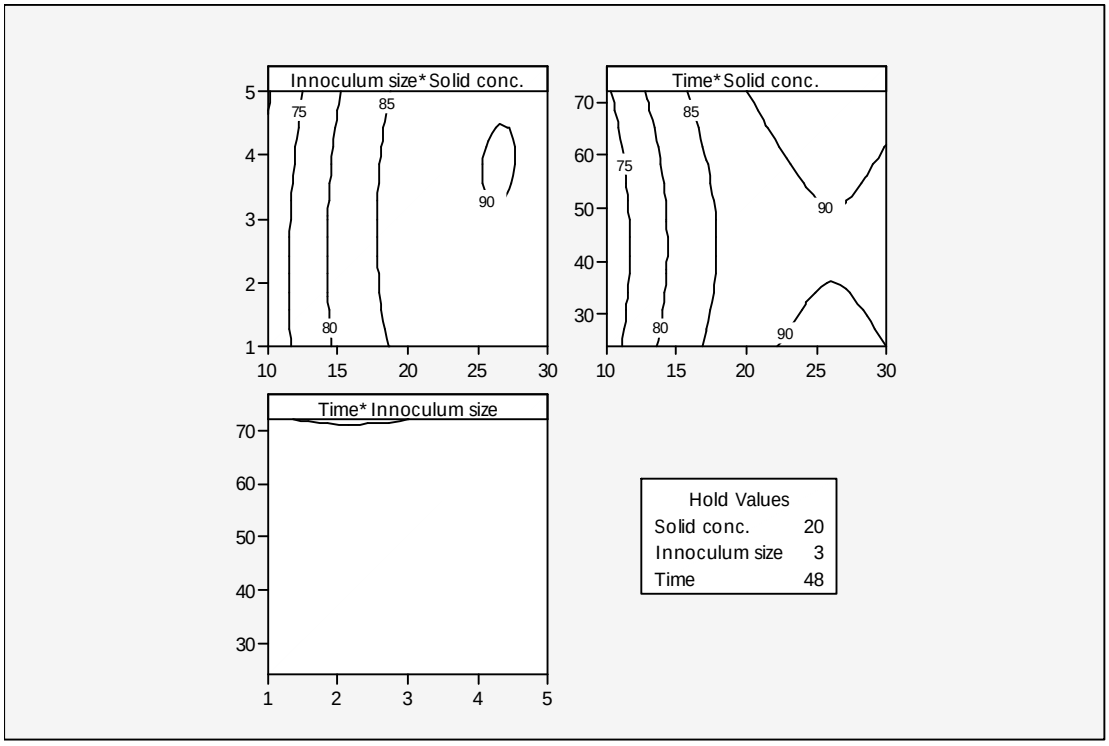

Figure 4.15 Contour plots for AFB_1 degradation

4.5 Sheep liver glutathione-S-transferase (GST) assay

To detect reduction in toxicity of detoxified samples, GST activity towards the substrate CDNB was observed. Figure 4.16 represents the percent enzyme inhibition using the methanol-water extracts of samples treated with *R. erythropolis* culture and untreated ones. Results of the GST assay showed that aflatoxin has an inhibitory effect on cytosolic GST enzymes. Undiluted extracts of untreated GNM almost completely inhibited the enzymatic reaction, therefore several dilutions were made to observe correct enzyme activity. Control group was used to determine the enzyme activity without toxin source. Treated GNM extracts were determined by HPLC to contain 5.97 µg/L AFB_1 and untreated extracts 45 µg/L AFB_1. Treated samples inhibited the enzyme activity 64.47 ± 0.32 % and untreated samples

inhibited 86.64 ± 0.52 % as shown in Figure 4.15. Significant difference was found between control, untreated and treated groups at 95% confidence level. This results suggests that the activation of binding of GSH to AFB_1 disturbed the conjugation of GSH with CDNB. By microbial detoxification of GNMs, significant loss of GST activity was detected.

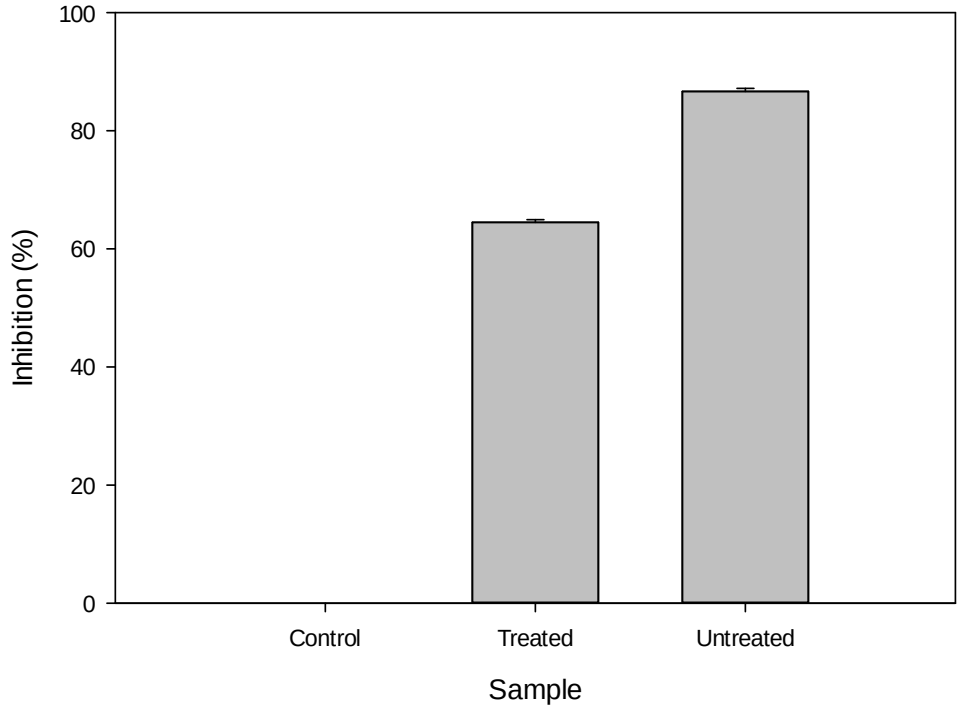

Figure 4.16 AFB_1 Inhibition of GST activity towards CDNB.

4.6 Industrial applicability of detoxification by *R. erythropolis*

In laboratory scale, maximum 87% detoxification could be achieved and 92% was predicted. However it should be noted that this system was only tried in flask conditions. Under more precisely controlled systems such as laboratory or industrial scale fermenters, results would be much closer to the predicted model.

Aflatoxin contamination, in nature, has many variations caused by crops, fields and geographic conditions. In Africa, where the risks are particularly higher, aflatoxin contamination at rates above 1000 µg/kg was detected in groundnut and groundnut products. However, feed industry usually tries to deal contaminated products below 200 µg/kg (Mehan et al., 1991). Therefore 90% detoxification is enough to solve the problem of industry, where a raw material of 200 µg/kg AFB_1 content could be detoxified below the legal limit of 20 µg/kg.

One major drawback of this system would be the need of fresh water for preparing slurry and energy for drying the product after the treatment. Water treatment system should be established in order to cope with the high water requirements of this process. In regions like Africa, where accessibility to freshwater is limited, assessing the opportunity for solid-state fermentation systems could be an efficient solution.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

In this study, aflatoxin degradation potential of gram positive bacterium *R. erythropolis* was investigated. First, optimum growth conditions were determined and then detoxification of GNM using *R. erythropolis* was optimized.

For optimum growth conditions, two different statistical methods, Plackett-Burman design and Box-Behnken design were sequentially used. As a result, the highest growth (10.92 log number of cells / mL) was observed at temperature of 22.5°C, pH 7, liquid volume of 100 mL in 500 mL flasks, inoculum volume of 1%, agitation speed of 135 rpm, glucose concentration 5 g/L and peptone concentration 5 g/L. By finding optimum growth conditions, efficient use of *R. erythropolis* was suggested for all relevant fields of biotechnology.

To determine the aflatoxin biodegradation behavior of *R. erythropolis*, efficacy of viable cell cultures and extracellular enzymes were compared under non-optimized conditions. It was found that viable cell cultures reduced the amount of AFB_1 in the samples significantly compared to extracellular enzymes. Three variables (solid concentration, inoculum volume and time) were selected for optimization of detoxification of GNM. A theoretical optimum of 92.21% reduction was achieved at as 27.4 %(w/v) solid concentration, 4.88 % v/v inoculum volume and 24 hours time. Efficiency of this process was also determined by sheep liver GST inhibition. It was observed that inhibition of the enzyme was significantly lower with treated sample extracts.

Results of this study shows that *R. erythropolis* is an effective detoxification agent for aflatoxin management purposes. Theoretical 92% and practical 87% rates

are effective enough to detoxify mid-level contaminated products. Suggested process would decrease the amount of AFB_1 from about 200 μg/kg to below 20 μg/kg regulatory limit. This makes possible that discarded GNM can be directly used as feed or added to mixed formulations.

Although the results of this study are very significant, there are still gaps to be filled. Suggested process is still laboratory scale, therefore; scale up studies should be addressed for large scale industrial processing. Solid state fermentation opportunities should also be evaluated. Other food or feed materials can be detoxified by *R. erythropolis*. Even though decrease of toxicity was detected by GST assay, still safety of the treated feed material should be evaluated by *in vivo* tests. Effects of this process on human health should also be addressed by *in vitro* tests and clinical trials.

REFERENCES

2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, (Ed.) E. Comission, Vol. 1881/2006, OJ L 364.

2010. The Global Economic Cost of Cancer. American Cancer Society.

2012. Official Methods of Analysis of AOAC International. 19 ed, AOAC INTERNATIONAL. Gaithersburg, MD, USA.

2011. Türk Gıda Kodeksi Bulaşanlar Yönetmeliği, (Ed.) G.T.v.H. Bakanlığı, Resmi Gazete. Ankara.

2014. Yemlerde İstenmeyen Maddeler Hakkında Tebliğ. in: *2014/11*, (Ed.) G.T.v.H. Bakanlığı, Resmi Gazete. Ankara.

Akbas, M.Y., Ozdemir, M. 2006. Effect of different ozone treatments on aflatoxin degradation and physicochemical properties of pistachios. *Journal of the Science of Food and Agriculture*, **86**, 2099-2104.

Alberts, J., Engelbrecht, Y., Steyn, P., Holzapfel, W., van Zyl, W. 2006. Biological degradation of aflatoxin B1 by Rhodococcus erythropolis cultures. *International Journal of Food Microbiology*, **109**(1-2), 121-126.

Alberts, J.F., Gelderblom, W.C.A., Botha, A., van Zyl, W.H. 2009. Degradation of aflatoxin B1 by fungal laccase enzymes. *International Journal of Food Microbiology*, **135**(1), 47-52.

Aziz, N.H., Moussa, L.A.A., Far, F.M.E. 2004. Reduction of fungi and mycotoxins formation in seeds by gamma-radiation. *Journal of Food Safety*(24), 109-127.

Bagdure, S.R., Fisher, M.A., Ryan, M.E., Khasawneh, F.A. 2012. Rhodococcus erythropolis Encephalitis in Patient Receiving Rituximab. *Emerging Infectious Diseases*, **18**(8), 1377-1379.

Batal, A., Dale, N., Café, M. 2005. Nutrient composition of peanut meal. *The Journal of Applied Poultry Research*, **14**(2), 254-257.

Bell, K.S., Philp, J.C., Aw, D.W.J. 1998. The genus Rhodococcus. *Journal of Applied Microbiology*, **85**(2), 195-210.

Bicca, F.C., Fleck, L.C., Ayub, M.A.Z. 1999. Production of biosurfactant by hydrocarbon degrading Rhodococcus ruber and Rhodococcus erythropolis. *Revista de Microbiologia*(30), 231-236.

Box, G.E.P., Behnken, D.W. 1960. Some New Three Level Designs for the Study of Quantitative Variables. *Technometrics*, **2**(4), 455-475.

Cabiscol, E., Tamarit, J., Ros, J. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*, **3**(1), 3-8.

Cao, H., Liu, D., Mo, X., Xie, C., Yao, D. 2011. A fungal enzyme with the ability of aflatoxin B 1 conversion: Purification and ESI-MS/MS identification. *Microbiological Research*(166), 475-483.

Chih-Wen, L., Mao-Shih, L., Yun-Chi, C., Luis, A.S.-S., Hwai-Shen, L. 2012. Biodegradation of n-alkanes at high concentration and correlation to the accumulation of H+ ions in Rhodococcus erythropolis NTU-1. *Biochemical Engineering Journal*, **63**.

Ciegler, A., Lillehoj, E., Peterson, R., Hall, H. 1966. Microbial detoxification of aflatoxin. *Applied Microbiology*, **14**(6), 934-939.

Coallier-Ascah, J., Idziak, E.S. 1985. Interaction between Streptococcus lactis and Aspergillus flavus on production of aflatoxin. *Applied and Environmental Microbiology*, **49**(1), 163-7.

Conville, P.S., Witebsky, F.G. 2007. Nocardia, Rhodococcus, Gordonia, Actinomadura, Streptomyces, and other aerobic actinomycetes. 9 ed. in: *Manual of clinical microbiology*, (Eds.) J. Versalovic, K.C. Carroll, G. Funke, J.H. Jorgensen, M.L. Landry, D.W. Warnock, Vol. 1, ASM Press, 2011. Washington, DC, pp. 515-542.

Coruh, N., Celep, A.G.S., Özgökçe, F. 2007. Antioxidant properties of Prangos ferulacea (L.) Lindl., Chaerophyllum macropodum Boiss. and Heracleum persicum Desf. from Apiaceae family used as food in …. *Food Chemistry*(100), 1237-1242.

Cserháti, M., Kriszt, B., Krifaton, C., Szoboszlay, S., Háhn, J., Tóth, S., Nagy, I., Kukolya, J. 2013. Mycotoxin-degradation profile of Rhodococcus strains. *International Journal of Food Microbiology*, **166**(1), 176-185.

Das, C., Mishra, H.N. 2000. Effect of aflatoxin B1 detoxification on the physicochemical properties and quality of ground nut meal. *Food Chemistry*, **70**(4), 483-487.

de Carvalho, C.C., da Fonseca, M. 2005. The remarkable Rhodococcus erythropolis. *Applied Microbiology and Biotechnology*, **67**(6), 715-726.

de Carvalho, C.C.C. 2012. Adaptation of Rhodococcus erythropolis cells for growth and bioremediation under extreme conditions. *Research in Microbiology*, **163**(2), 125-136.

Devendran, G., Balasubramanian, U. 2011. Biochemical and histopathological analysis of aflatoxin induced toxicity in liver and kidney of rat. *Asian Journal of Plant Science and Research*, **1**(4), 61-69.

Diop, N., Beghin, J.C., Sewadeh, M. 2004. Groundnut Policies, Global Trade Dynamics, and the Impact of Trade Liberalization. in: *Global Agricultural Trade and Developing Countries*, (Eds.) M.A. Aksoy, J.C. Beghin, World Bank Publications.

Dwarakanath, C.T., Rayner, E.T., Mann, G.E., Dollear, F.G. 1968. Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization. *Journal of the American Oil Chemists' Society*, **45**(2), 93-95.

Dyck, V.P.J., Tobback, P., Feyes, M. 1982. Sensitivity of aflatoxin B1 to ionizing radiation. *Applied and Environmental Microbiology*, **43**(6), 1317-1319.

El-Nezami, H., Kankaanpaa, P., Salminen, S., Ahokas, J. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin

B1. *Food and Chemical Toxicology : an international journal published for the British Industrial Biological Research Association*, **36**(4), 321-326.

Ellis, W.O., Smith, J.P., Simpson, B.K., Oldham, J.H. 1991. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Critical Reviews in Food Science and Nutrition*, **30**(4), 403-39.

Eshelli, M., Harvey, L., Edrada-Ebel, R., McNeil, B. 2015. Metabolomics of the bio-degradation process of aflatoxin B1 by actinomycetes at an initial pH of 6.0. *Toxins*, **7**(2), 439-456.

Fanget, N.V., Foley, S. 2011. Starvation/stationary-phase survival of Rhodococcus erythropolis SQ1: a physiological and genetic analysis. *Archives of Microbiology*, **193**(1), 1-13.

FAO. 2014. FAOSTAT, Food and Agriculture Organization of the United Nations. Rome, Italy.

FAO. 1997. *Worldwide Regulations for Mycotoxins in food and feed in 2003. A Compendium, FAO Food and Nutrition Paper 64*. FAO, Rome.

FAO. 2004. *Worldwide Regulations for Mycotoxins in food and feed in 2003. A Compendium, FAO Food and Nutrition Paper 81*. FAO, Rome.

FDA. 2012. Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins. Second ed, (Eds.) K.A. Lampel, S. Al-Khaldi, S.M. Cahill, Food and Drug Administration.

Ferreira, S.L.C., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., Silva, E.G.P.d., Portugal, L.A., Reis, P.S.d., Souza, A.S., Santos,

W.N.L. 2007. Box-Behnken design: an alternative for the optimization of analytical methods. *Analytica Chimica Acta*, **597**(2), 179-186.

Fonseca, H., Regitano-d'Arce, M. 1993. Aflatoxin removal of peanut meals with aqueous ethanol. *Scientia Agricola*, **50**(1), 154-156.

Gao, S.S., Chen, X.Y., Zhu, R.Z., Choi, B.M., Kim, B.R. 2010. Sulforaphane induces glutathione S-transferase isozymes which detoxify aflatoxin B(1)-8,9-epoxide in AML 12 cells. *Biofactors*, **36**(4), 289-96.

Gardner, H.K., Koltun, S.P., Vix, H.L.E. 1968. Solvent extraction of aflatoxins from oilseed meals. *Journal of Agricultural and Food Chemistry*, **16**(6), 990-993.

Ghanem, I., Orfi, M., Shamma, M. 2008. Effect of gamma radiation on the inactivation of aflatoxin B1 in food and feed crops. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, **39**(4), 787-791.

Guan, S., Zhao, L., Ma, Q., Zhou, T., Wang, N., Hu, X., Ji, C. 2010. In vitro efficacy of Myxococcus fulvus ANSM068 to biotransform aflatoxin B1. *International Journal of Molecular Sciences*, **11**(10), 4063-4079.

Gűrtler, V., Seviour, R. 2010. Systematics of Members of the Genus Rhodococcus (Zopf 1891) Emend Goodfellow et al. 1998. in: *Biology of Rhodococcus*, (Ed.) H.M. Alvarez, Vol. 16, Springer Berlin Heidelberg, pp. 1-28.

Habig, W.H., Pabst, M.J., Jakoby, W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*, **249**(22), 7130-9.

Haskard, C.A., El-Nezami, H.S., Kankaanpaa, P.E., Salminen, S., Ahokas, J.T. 2001. Surface binding of aflatoxin B(1) by lactic acid bacteria. *Applied and Environmental Microbiology*, **67**(7), 3086-91.

Herzallah, S., Alshawabkeh, K., Al Fataftah, A. 2008. Aflatoxin decontamination of artificially contaminated feeds by sunlight, γ-radiation, and microwave heating. *Journal of Applied Poultry Research*, **17**(4), 515-521.

Huwig, A., Freimund, S., Käppeli, O., Dutler, H. 2001. Mycotoxin detoxication of animal feed by different adsorbents. *Toxicology Letters*(122), 179-188.

IMF. 1986. *Primary Commodities Market Deveopments and Outlook*. International Monetary Fund, Washington D.C.

İscan, M., Çoban, T., Bülbül, D., Eke, B.C., Aygörmez, S., Berberoglu, U. 1998. Xenobiotic metabolizing and antioxidant enzymes in normal and neoplastic human breast tissue. *European Journal of Drug Metabolism and Pharmacokinetics*, **23**(4), 497-500.

Izumi, Y., Ohshiro, T., Ogino, H., Hine, Y. 1994. Selective desulfurization of dibenzothiophene by Rhodococcus erythropolis D-1. *Applied and Environmental Microbiology*, **60**(1), 223-226.

Kamble, A.L., Meena, V.S. 2010. Effect of agitation and aeration on the production of nitrile hydratase by Rhodococcus erythropolis MTCC 1526 in a stirred tank reactor. *Letters in Applied Microbiology*(51), 413-4120.

Khlangwiset, P., Wu, F. 2010. Costs and efficacy of public health interventions to reduce aflatoxin–induced human disease. *Food Additives and Contaminants*, **27**(7), 998-1014.

Kong, Q., Zhai, C., Guan, B., Li, C., Shan, S., Yu, J. 2012. Mathematic modeling for optimum conditions on aflatoxin B_1degradation by the aerobic bacterium Rhodococcus erythropolis. *Toxins*, **4**(11), 1181-1195.

Kurane, R., Hatamochi, K., Kakuno, T. 1994a. Production of a bioflocculant by Rhodococcus erythropolis S-1 grown on alcohols. *Bioscience, Biotechnology and Biochemistry*, **58**(2), 428-429.

Kurane, R., Hatamochi, K., Kakuno, T., Kiyohara, M., Hirano, M., Taniguchi, Y. 1994b. Production of a bioflocculant by Rhodococcus erythropolis S-1 grown on alcohols. *Bioscience, Biotechnology and Biochemistry*, **58**(2), 428-429.

Kurane, R., Toeda, K., Takeda, K., Suzuki, T. 1986. Culture Conditions for Production of Microbial Flocculant by Rhodococcus erythropolis. *Agricultural and Biological Chemistry*, **50**(9), 2309-2313.

Kusumaningtyas, E., Widiastuti, R., Maryam, R. 2006. Reduction of aflatoxin B1 in chicken feed by using Saccharomyces cerevisiae, Rhizopus oligosporus and their combination. *Mycopathologia*(162), 307-311.

Lamb, M., Sternitzke, D. 2001. Cost of aflatoxin to the farmer, buying point, and sheller segments of the Southeast United States peanut industry. *Peanut Science*, **28**(2), 59-63.

Lee, L.S., Cucullu, A.F. 1978. Conversion of aflatoxin B1 to aflatoxin D1 in ammoniated peanut and cottonseed meals. *Journal of Agricultural and Food Chemistry*, **26**(4), 881-884.

Lee, L.S., Dunn, J.J., DeLucca, A.J., Ciegler, A. 1981. Role of lactone ring of aflatoxin B1 in toxicity and mutagenicity. *Experientia*, **37**(1), 16-17.

Lenke, H., Pieper, D.H., Bruhn, C. 1992. Degradation of 2, 4-dinitrophenol by two Rhodococcus erythropolis strains, HL 24-1 and HL 24-2. *Applied and Environmental Microbiology*, **58**(9), 2928-2932.

Li, J.-h., Wei, Y.-m., Guo, B.-l., Pan, J.-r., Lu, W. 2009. Decontamination of B-aflatoxins in Peanut with Citric Acid Treatment. *Food Science*, **30**(13), 37-40.

Line, J.E., Brackett, R.E., Wilkinson, R.E. 1994. Evidence for Degradation of Aflatoxin B1 by Flavobacterium aurantiacum. *Journal of Food Protection*, **57**(9), 788-791.

Liu, D.L., Yao, D.S., Liang, R., Ma, L., Cheng, W.Q. 1998. Detoxification of Aflatoxin B1 by Enzymes Isolated from Armillariella tabescens. *Food and Chemical Toxicology*(36), 563-574.

Liu, Y., Wu, F. 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental health perspectives*, **118**(6), 818-824.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*(193), 265-275.

Magan, N., Medina, A., Aldred, D. 2011. Possible climate-change effects on mycotoxin contamination of food crops pre-and postharvest. *Plant Pathology*(60), 150-163.

McLean, M., Dutton, M.F. 1995. Cellular interactions and metabolism of aflatoxin: an update. *Pharmacology & Therapeutics*, **65**(2), 163-92.

Mehan, V.K., McDonald, D., Haravu, L.J., Jayanthi, S. 1991. *The Groundnut Aflatoxin Problem: Review and Literature Database*. International Crops Research Institute for the Semi-arid Tropics, Patancheru, India.

Meki, A.-R.M.A., Abdel-Ghaffar, S., El-Gibaly, I. 2001. Aflatoxin B1 induces apoptosis in rat liver: protective effect of melatonin. *Neuroendocrinology Letters*, **22**(6), 417-426.

Méndez-Albores, A., Del Río-García, J.C., Moreno-Martínez, E. 2007. Decontamination of aflatoxin duckling feed with aqueous citric acid treatment. *Animal Feed Science and Technology*, **135**(3–4), 249-262.

Mishra, H.N., Das, C. 2003. A review on biological control and metabolism of aflatoxin. *Critical Reviews in Food Science and Nutrition*, **43**(3), 245-64.

Motomura, M., Toyomasu, T., Mizuno, K., Shinozawa, T. 2003. Purification and characterization of an aflatoxin degradation enzyme fromPleurotus ostreatus. *Microbiological Research*, **158**(3), 237-242.

Moumita, P.P., Bhalchandra, K.V., Kiran, M.D., Renuka, M.J., Sanjay, N.N., Bhaskar, D.K. 2009. Media optimization for biosurfactant production by Rhodococcus erythropolis MTCC 2794: artificial intelligence versus a statistical approach. *Journal of Industrial Microbiology & Biotechnology*(36), 747-756.

Muralidhar, R.V., Chirumamila, R.R., Marchant, R. 2001. A response surface approach for the comparison of lipase production by Candida cylindracea

using two different carbon sources. *Biochemical Engineering Journal*(9), 17-23.

Neal, G., Judah, D., Carthew, P., Verma, A., Latour, I., Weir, L., Coker, R., Nagler, M., Hoogenboom, L. 2001. Differences detected in vivo between samples of aflatoxin-contaminated peanut meal, following decontamination by two ammonia-based processes. *Food Additives and Contaminants*, **18**(2), 137-149.

Otsuki, T., Wilson, J.S., Sewadeh, M. 2001. Saving two in a billion: quantifying the trade effect of European food safety standards on African exports. *Food Policy*(26), 495-514.

Pirog, T.P., Shevchuk, T.A., Voloshina, I.N. 2004. Production of surfactants by Rhodococcus erythropolis strain EK-1, grown on hydrophilic and hydrophobic substrates. *Applied Biochemistry and Microbiology*, **40**(5), 470-475.

Plackett, R.L., Burman, J.P. 1946. The Design of Optimum Multifactorial Experiments. *Biometrika*, **33**(4), 305-325.

Rayner, E., Dollear, F.G. 1968. Removal of aflatoxins from oilseed meals by extraction with aqueous isopropanol. *Journal of the American Oil Chemists' Society*, **45**(9), 622-624.

Reed, G. 1966. *Enzymes in Food Processing (1966)*. Elsevier Science.

Samarajeewa, U., Sen, A.C., Fernando, S.Y. 1991. Inactivation of aflatoxin B1 in corn meal, copra meal and peanuts by chlorine gas treatment. *Food and Chemical Toxicology*, **29**(1), 41-47.

Samuel, M.S., Sivaramakrishna, A., Mehta, A. 2014. Degradation and detoxification of aflatoxin B1 by Pseudomonas putida. *International Biodeterioration & Biodegradation*, **86**, 202-209.

Sangare, L., Zhao, Y., Folly, Y., Chang, J., Li, J., Selvaraj, J., Xing, F., Zhou, L., Wang, Y., Liu, Y. 2014. Aflatoxin B1 Degradation by a Pseudomonas Strain. *Toxins*, **6**(10), 3028-3040.

Shetty, P., Jespersen, L. 2006. Saccharomyces cerevisiae and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology*, **17**(2), 48-55.

Smiley, R.D., Draughon, F.A. 2000. Preliminary evidence that degradation of aflatoxin B1 by Flavobacterium aurantiacum is enzymatic. *Journal Of Food Protection*, **63**(3), 415-418.

Sojo, M., Bru, R., Lopez-Molina, D. 1997. Cell-linked and extracellular cholesterol oxidase activities from Rhodococcus erythropolis. Isolation and physiological characterization. *Applied Microbiology and Biotechnology*(47), 583-589.

Strange, R.C., Spiteri, M.A., Ramachandran, S. 2001. Glutathione-S-transferase family of enzymes. *Mutation Research*, **408**, 21-26.

Suttajit, M. 1991. Prevention and control of mycotoxins. in: *Mycotoxin Prevention and Control in Foodgrains*, (Ed.) A.S.F. R.L. Semple, P.A. Hicks and J.V. Lozare, AGPP Publication. Bangkok, Thailand.

Teniola, O., Addo, P., Brost, I., Färber, P., Jany, K.D., Alberts, J., van Zyl, W., Steyn, P., Holzapfel, W. 2005. Degradation of aflatoxin B1 by cell-free

extracts of Rhodococcus erythropolis and Mycobacterium fluoranthenivorans sp. nov. DSM44556(T). *International Journal of Food Microbiology*, **105**(2), 111-117.

Tomioka, N., Uchiyama, H., Yagi, O. 1994. Cesium Accumulation and Growth Characteristics of Rhodococcus erythropolis CS98 and Rhodococcus sp. Strain CS402. *Applied and Environmental Microbiology*, **60**(7), 2227-2231.

Tripathi, S., Mishra, H.N. 2009. Modeling and Optimization of Enzymatic Degradation of Aflatoxin B1 (AFB1) in Red Chili Powder Using Response Surface Methodology. *Food and Bioprocess Technology*(4), 770-780.

USDA. 2015. Oilseeds: World Markets and Trade. Foreign Agricultural Service, United States Department of Agriculture.

van Egmond, H.P., Jonker, M.A. 2004. 3 - Current regulations governing mycotoxin limits in food. in: *Mycotoxins in Food*, (Eds.) N. Magan, M. Olsen, Woodhead Publishing, pp. 49-68.

Wannop, C.C. 1961. The Histopathology of Turkey "X" Disease in Great Britain. *Avian Diseases*, **5**(4), 371-381.

Waśko, A., Monika, K.-W., Podleśny, M., Magdalena, P.-B., Targoński, Z., Agnieszka, K.-K. 2010. The Plackett-Burman design in optimization of media components for biomass production of Lactobacillus rhamnosus OXY. *Acta Biologica Hungarica*, **61**(3), 344355.

Werf, M.J.v.d., Swarts, H.J., Bont, J.A.d. 1999. Rhodococcus erythropolis DCL14 contains a novel degradation pathway for limonene. *Applied and Environmental Microbiology*, **65**(5), 2092-2102.

Yassin, A.F., Schaal, K.P. 2005. Reclassification of Nocardia corynebacterioides Serrano et al. 1972 (Approved Lists 1980) as Rhodococcus corynebacterioides comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, **55**(3), 1345-1348.

Yiannikouris, A., Jouany, J.-P. 2002. Mycotoxins in feeds and their fate in animals: a review. *Animal Research*, **51**(2), 81-100.

Yu, F.L. 1977. Mechanism of aflatoxin B1 inhibition of rat hepatic nuclear RNA synthesis. *Journal of Biological Chemistry*, **252**(10), 3245-3251.

Zhai, C., Kong, Q., Guan, B., Lv, Z., You, Y., Gao, Y., Yang, Y. 2013. Statistical experimental methods for optimizing the cultivating conditions for Rhodococcus erythropolis. *African Journal of Biotechnology*, **10**(42), 8502-8510.

Zhang, C., Sun, J., Yu, L., Bi, J., Feng, J., Yang, Q. 2014. Optimization of Aflatoxin Production of Aspergillus flavus on Peanuts. *Advanced Materials Research*, **887-888**, 605-609.

APPENDIX A

CHEMICALS LIST

Table A.1 Table of chemicals

Chemical	Supplier
Aflatoxin B1 from *A. flavus*	Sigma-Aldrich
Acetonitrile (HPLC grade)	Merck
Ammonium sulfate	Merck
Bovine serum albumin	Merck
1-chloro-2,4-dinitrobenzene (CDNB)	Acros Organics
Copper sulphate	Merck
Dipotassium phosphate	Merck
Disodium phospate	Merck
Dithiothreitol (DTT)	Sigma-Aldrich
Ethanol	Merck
Ethylenediaminetetraacetic acid (EDTA)	Merck
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich
Glucose	Merck
Glycerol	Sigma-Aldrich

Hexane	Merck
Hydrochloric acid	Merck
Lactose	Merck
L-glutathione reduced (GSH)	Sigma-Aldrich
Methanol (HPLC grade)	Merck
Monopotassium phosphate	Merck
Nutrient agar	Merck
Nutrient broth	Merck
Peptone	Merck
Potassium chloride	Merck
Sodium carbonate	Merck
Sodium chloride	Merck
Sodium hydroxide	Merck
Sodium potassium tartarate	Merck
Sucrose	Merck
Tryptose	Merck
Xylose	Merck
Yeast extract	Merck

APPENDIX B

BUFFERS AND SOLUTIONS

Phosphate buffered saline solution – pH 7.4 (PBS)

0.20 g potassium chloride, 0.20 g monopotassium phosphate, 1.16 g disodium phosphate and 8.00 g sodium chloride was dissolved in 900 mL deionized water. pH was adjusted to 7.4 with 0.1M hydrochloric acid solution and diluted to 1000 mL.

Potassium phosphate buffer (pH 6.5)

Solution A: 272 g KH_2PO_4 per 1000mL dissolved in water (2 M final)

Solution B: 348 g K_2HPO_4 per 1000mL dissolved in water (2 M final)

68.5 mL solution A and 31.5 mL solution B was mixed to final volume 100 mL and concentration 1M. This stock solution was diluted as needed.

APPENDIX C

LOWRY'S METHOD FOR PROTEIN DETERMINATION

Solution A: 50 mL 2% Sodium carbonate + 50 mL 0.1N NaOH solution

Solution B: 10 mL of 1.56% Copper sulphate + 10 mL 2.37% Sodium potassium tartarate

To construct the standard curve bovine serum albumin (BSA) standard solutions were prepared between concentrations 0.05 and 1 mg/mL. Samples and standard solutions were mixed with solutions A and B and incubated with Folin & Ciocalteau's solution for 30 minutes at room temperature. Optical densities were measured at 660 nm wavelength after zero adjustment with blank.

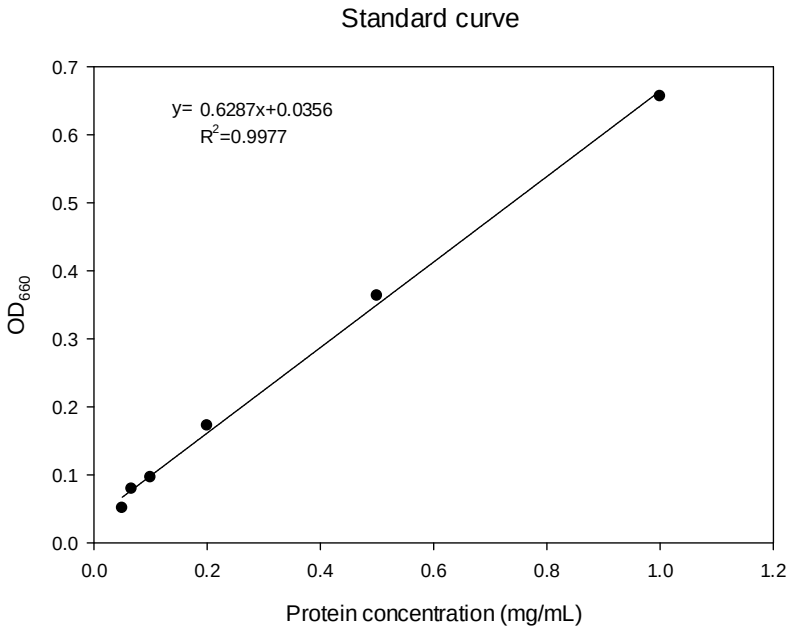

Figure C.1 Standard curve for Lowry's method for protein determination

APPENDIX D

STANDARD CURVE FOR CELL GROWTH

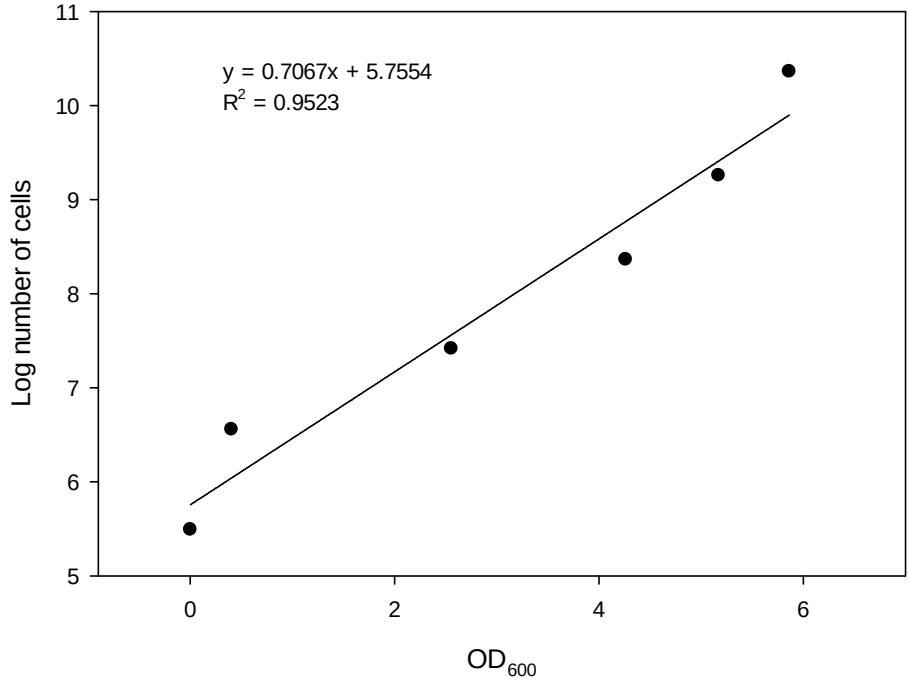

Figure D.1 Standard curve for cell growth monitoring

APPENDIX E

SAMPLE HPLC CHROMATOGRAM

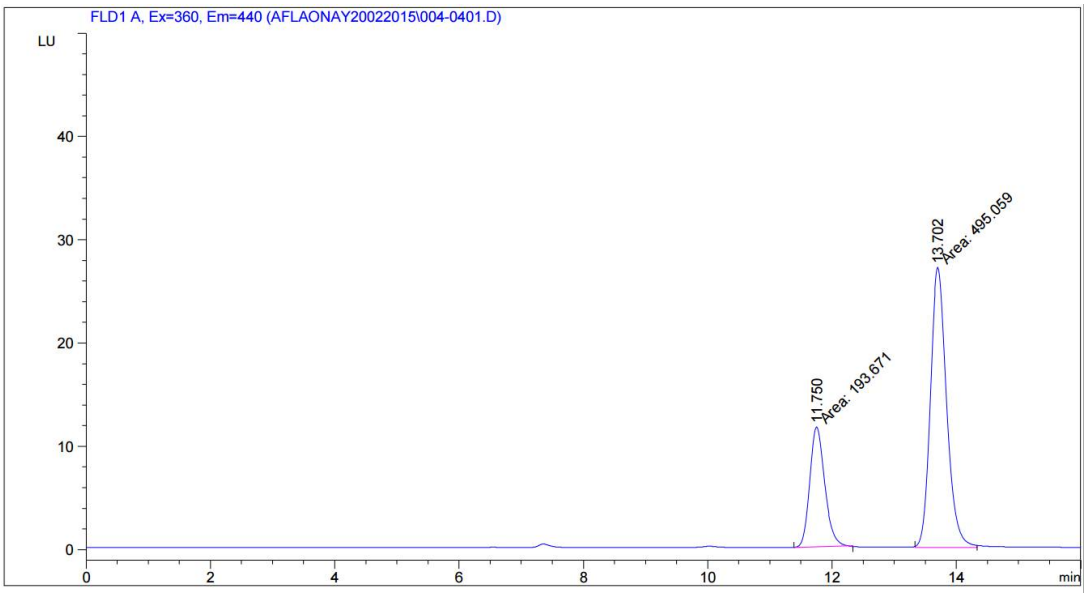

Figure E.1 Sample HPLC chromatogram for aflatoxin determination

Signal 1: FLD1 A, Ex=360, Em=440

RetTime [min]	Sig	Type	Area LU *s	Amt/Area	Amount [ng/100uL]	Grp	Name
8.700	1		-	-	-		G2
10.137	1		-	-	-		G1
11.750	1	MM	193.67134	7.36371e-3	1.42614		B2
13.702	1	MM	495.05896	1.38581e-2	6.86059		B1

APPENDIX F

ANOVA TABLES FOR MULTIPLE COMPARISONS

One-way ANOVA: Absorbance versus Nitrogen source

Source	DF	SS	MS	F	P
Nitrogen source	2	1.0298	0.5149	18.43	0.021
Error	3	0.0838	0.0279		
Total	5	1.1136			

S = 0.1671 R-Sq = 92.47% R-Sq(adj) = 87.46%

Grouping Information Using Tukey Method

Nitrogen source	N	Mean	Grouping
Peptone	2	5.1885	A
Tryptose	2	5.0970	A
NH42SO4	2	4.2675	B

One-way ANOVA: Absorbance versus Carbon Source

Source	DF	SS	MS	F	P
Carbon Source	3	9.1372	3.0457	170.09	0.000
Error	4	0.0716	0.0179		
Total	7	9.2088			

S = 0.1338 R-Sq = 99.22% R-Sq(adj) = 98.64%

Grouping Information Using Tukey Method

Carbon Source	N	Mean	Grouping
Glucose	2	6.1975	A
Sucrose	2	4.1980	B
Xylose	2	3.7365	B C
Lactose	2	3.4745	C

One-way ANOVA: Reduction versus Type

```
Source  DF       SS      MS       F      P
Type     2  10225.0  5112.5  140.55  0.000
Error   15    545.6    36.4
Total   17  10770.6

S = 6.031   R-Sq = 94.93%   R-Sq(adj) = 94.26%


                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N    Mean  StDev  +---------+---------+---------+---------
Cont   6   6.049  1.297  (--*--)
Enz    6  46.358  8.637                    (-*--)
Ferm   6  62.778  5.732                             (-*--)
                        +---------+---------+---------+---------
                        0        20        40        60

Pooled StDev = 6.031


Grouping Information Using Tukey Method

Type  N    Mean  Grouping
Ferm  6  62.778  A
Enz   6  46.358    B
Cont  6   6.049      C

Means that do not share a letter are significantly different.
```

One-way ANOVA: C2 versus C1

```
Source  DF       SS       MS        F      P
C1       2  6177319  3088659  1797.54  0.000
Error    3     5155     1718
Total    5  6182473

S = 41.45   R-Sq = 99.92%   R-Sq(adj) = 99.86%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level      N    Mean  StDev  ------+---------+---------+---------+-
--
Control    2  2761.0   69.2                                     (*-)
Treated    2   980.9   12.5           (*)
Untreated  2   368.9   14.3  (*-)
                            ------+---------+---------+---------+-
--
                                700      1400      2100      2800

Pooled StDev = 41.5


Grouping Information Using Tukey Method

C1         N    Mean  Grouping
Control    2  2761.0  A
Treated    2   980.9     B
Untreated  2   368.9       C

Means that do not share a letter are significantly different.
```

APPENDIX G

CULTURE GROWTH OPTIMIZATION

Response Surface Regression: Log number versus Temperature; Liquid volum; ...

The analysis was done using coded units.

Estimated Regression Coefficients for Log number

Term	Coef	SE Coef	T	P
Constant	9.3909	0.08708	107.844	0.000
Temperature	-0.4256	0.05332	-7.982	0.000
Liquid volume	-0.9888	0.05332	-18.543	0.000
Agitation speed	0.5642	0.05332	10.580	0.000
Temperature*Temperature	-0.4751	0.07849	-6.053	0.000
Liquid volume*Liquid volume	0.2621	0.07849	3.339	0.003
Agitation speed*Agitation speed	-0.3953	0.07849	-5.037	0.000
Temperature*Liquid volume	0.2218	0.07541	2.941	0.008
Temperature*Agitation speed	-0.1854	0.07541	-2.459	0.023
Liquid volume*Agitation speed	0.4081	0.07541	5.412	0.000

S = 0.213297 PRESS = 2.16612
R-Sq = 96.96% R-Sq(pred) = 92.77% R-Sq(adj) = 95.60%

Analysis of Variance for Log number

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	29.0440	29.0440	3.2271	70.93	0.000
Linear	3	23.6347	23.6347	7.8782	173.16	0.000
Temperature	1	2.8983	2.8983	2.8983	63.70	
Liquid volume	1	15.6438	15.6438	15.6438	343.85	
Agitation speed	1	5.0926	5.0926	5.0926	111.93	
Square	3	3.4082	3.4082	1.1361	24.97	
Temperature*Temperature	1	1.6186	1.6669	1.6669	36.64	
Liquid volume*Liquid volume	1	0.6355	0.5072	0.5072	11.15	
Agitation speed*Agitation speed	1	1.1542	1.1542	1.1542	25.37	
Interaction	3	2.0012	2.0012	0.6671	14.66	
Temperature*Liquid volume	1	0.3936	0.3936	0.3936	8.65	
Temperature*Agitation speed	1	0.2750	0.2750	0.2750	6.05	
Liquid volume*Agitation speed	1	1.3325	1.3325	1.3325	29.29	
Residual Error	20	0.9099	0.9099	0.0455		
Lack-of-Fit	3	0.2805	0.2805	0.0935	2.53	
Pure Error	17	0.6294	0.6294	0.0370		
Total	29	29.9539				

Source	P
Regression	0.000
Linear	0.000

```
    Temperature                      0.000
    Liquid volume                    0.000
    Agitation speed                  0.000
  Square                             0.000
    Temperature*Temperature          0.000
    Liquid volume*Liquid volume      0.003
    Agitation speed*Agitation speed  0.000
  Interaction                        0.000
    Temperature*Liquid volume        0.008
    Temperature*Agitation speed      0.023
    Liquid volume*Agitation speed    0.000
Residual Error
  Lack-of-Fit                        0.092
  Pure Error
Total
```

Response Optimization

```
Parameters

            Goal     Lower  Target  Upper  Weight  Import
Log number  Maximum      0      11     11       1       1


Global Solution

Temperature    =   22.4242
Liquid volum   =       100
Agitation sp   =   134.949


Predicted Responses

Log number   =   10.9152  ,   desirability =   0.992288


Composite Desirability = 0.992288
```

APPENDIX H

AFB_1 DEGRADATION OPTIMIZATION

Response Surface Regression: Reduction versus Solid conc., Innoculum size, Time

The analysis was done using uncoded units.

Estimated Regression Coefficients for Reduction

Term	Coef	SE Coef	T	P
Constant	44.1816	3.49591	12.638	0.000
Solid conc.	3.6228	0.20134	17.994	0.000
Innoculum size	1.2481	0.87633	1.424	0.170
Time	-0.1890	0.08389	-2.253	0.036
Solid conc.*Solid conc.	-0.0705	0.00427	-16.517	0.000
Innoculum size*Innoculum size	-0.2434	0.10675	-2.280	0.034
Time*Time	0.0034	0.00074	4.536	0.000
Solid conc.*Innoculum size	0.0573	0.02051	2.795	0.011
Solid conc.*Time	-0.0022	0.00171	-1.314	0.204
Innoculum size*Time	-0.0185	0.00855	-2.169	0.042

S = 1.16040 PRESS = 60.8369
R-Sq = 98.39% R-Sq(pred) = 96.36% R-Sq(adj) = 97.66%

Analysis of Variance for Reduction

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	1644.62	1644.62	182.736	135.71	0.000
Linear	3	1209.66	492.61	164.203	121.94	0.000
Solid conc.	1	1199.35	435.97	435.974	323.77	0.000
Innoculum size	1	0.12	2.73	2.732	2.03	0.170
Time	1	10.19	6.84	6.838	5.08	0.036
Square	3	415.78	415.78	138.592	102.92	0.000
Solid conc.*Solid conc.	1	378.70	367.34	367.338	272.80	0.000
Innoculum size*Innoculum size	1	9.36	7.00	7.002	5.20	0.034
Time*Time	1	27.71	27.71	27.709	20.58	0.000
Interaction	3	19.18	19.18	6.394	4.75	0.012
Solid conc.*Innoculum size	1	10.52	10.52	10.519	7.81	0.011
Solid conc.*Time	1	2.33	2.33	2.326	1.73	0.204
Innoculum size*Time	1	6.34	6.34	6.337	4.71	0.042
Residual Error	20	26.93	26.93	1.347		
Lack-of-Fit	3	0.75	0.75	0.250	0.16	0.920
Pure Error	17	26.18	26.18	1.540		
Total	29	1671.55				

Response Surface Regression: Reduction versus Solid conc., Innoculum size, Time

The analysis was done using uncoded units.

Estimated Regression Coefficients for Reduction (Revised table)

Term	Coef	SE Coef	T	P
Constant	46.3382	3.13978	14.758	0.000
Solid conc.	3.5150	0.18701	18.795	0.000
Innoculum size	1.2481	0.89137	1.400	0.176
Time	-0.2340	0.07792	-3.003	0.007
Solid conc.*Solid conc.	-0.0705	0.00434	-16.238	0.000
Innoculum size*Innoculum size	-0.2434	0.10859	-2.242	0.036
Time*Time	0.0034	0.00075	4.460	0.000
Solid conc.*Innoculum size	0.0573	0.02087	2.748	0.012
Innoculum size*Time	-0.0185	0.00869	-2.133	0.045

S = 1.18032 PRESS = 60.4448
R-Sq = 98.25% R-Sq(pred) = 96.38% R-Sq(adj) = 97.58%

Analysis of Variance for Reduction (Revised table)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	1642.29	1642.29	205.287	147.35	0.000
Linear	3	1209.66	521.78	173.927	124.84	0.000
Solid conc.	1	1199.35	492.15	492.147	353.26	0.000
Innoculum size	1	0.12	2.73	2.732	1.96	0.176
Time	1	10.19	12.56	12.561	9.02	0.007
Square	3	415.78	415.78	138.592	99.48	0.000
Solid conc.*Solid conc.	1	378.70	367.34	367.338	263.67	0.000
Innoculum size*Innoculum size	1	9.36	7.00	7.002	5.03	0.036
Time*Time	1	27.71	27.71	27.709	19.89	0.000
Interaction	2	16.86	16.86	8.428	6.05	0.008
Solid conc.*Innoculum size	1	10.52	10.52	10.519	7.55	0.012
Innoculum size*Time	1	6.34	6.34	6.337	4.55	0.045
Residual Error	21	29.26	29.26	1.393		
Lack-of-Fit	4	3.08	3.08	0.769	0.50	0.737
Pure Error	17	26.18	26.18	1.540		
Total	29	1671.55				

Response Optimization

Parameters

	Goal	Lower	Target	Upper	Weight	Import
AFB1(ppb)	Maximum	60	100	100	1	1

Global Solution

Dilution = 27.3737
Innoculum = 4.87879
Time = 24

APPENDIX I

GST ASSAY

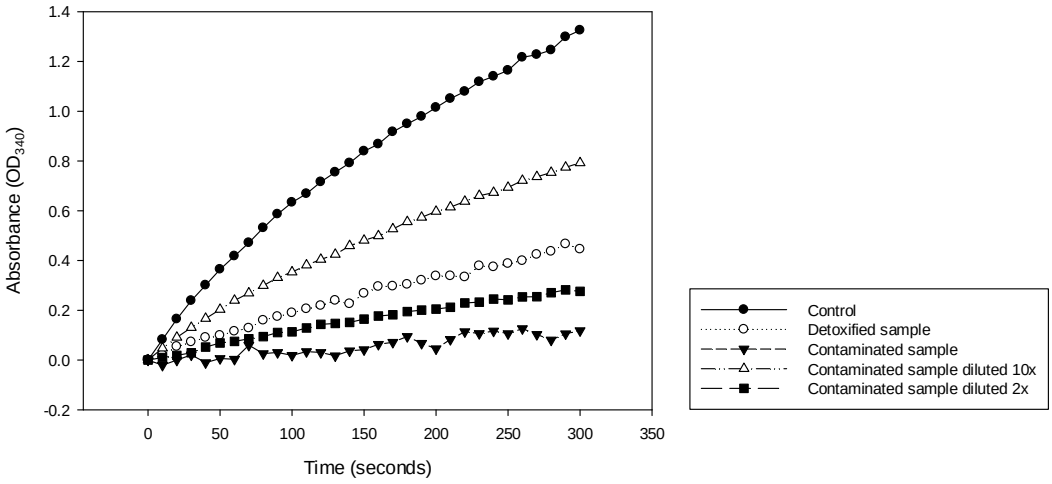

Figure I.1 Optical density at 340nm for GST assay