BIOLOGICAL AND PHOTOCATALYTIC DEGRADATION OF MYCOTOXINS IN CORN FOR USE IN BIO-FUEL PRODUCTION

A Thesis

by

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ABSTRACT

Mycotoxin contamination of grains is a serious global problem with feed stocks often being contaminated with multiple, different mycotoxins. Many studies have successfully isolated microorganisms or used chemical and physical methods to degrade these compounds separately. It is unclear if the presence of other mycotoxins will interfere with the degradation efficiency of a particular microorganism. Chemical and physical treatments typically require high energy inputs and are linked with safety concerns. Two studies were conducted to evaluate effectiveness of two separate methods to decontaminate aqueous and corn samples containing multiple, different mycotoxins.

The first study was conducted using mixed mycotoxin substrates and an aflatoxin-degrading microorganism, *Rhodococcus corynebacterioides* DSM 20151. The bacterium was tested in a complex liquid medium containing: 1) 5 mg/L of AFB1, 5 mg/L of ZEA, or 5 mg/L of OTA or 2) combinations of AFB1 with each of the other two mycotoxins at 5 mg/L each. The addition of ZEA and OTA had no significant effect on AFB1 degradation by *R. corynebacterioides* with 100% of AFB1 degraded within 72 hours. The bacterium was also able to degrade 95% of OTA in the presence of AFB1. However, ZEA showed no degradation with or without AFB1 within 72 hours. This lack of effect on degradation and inability to degrade ZEA is likely due to the specificity of the enzymes and genes responsible for AFB1 and OTA degradation.

A second study was conducted using UV-C and the photocatalyst TiO_2 to degrade AFB1, ZEA, and OTA. Aqueous and corn slurry samples containing 3 mg/L of AFB1, 3 mg/L of ZEA, and 3 mg/L OTA were tested for degradation with the following treatments: 1) UV-C only and 2) UV-C with the addition of 0.02 g/mL TiO_2. After 30 minutes, UV-C was able to degrade

71% of AFB1, 61% of ZEA, and 79% of OTA in aqueous samples, whereas, with the TiO_2 addition, UV-C was able to degrade 94% of AFB1, 100% of ZEA, and 97% of OTA. However, little to no degradation was observed after each treatment in corn slurry samples. These results suggest that photocatalytic degradation may be a promising method for remediation of mycotoxin-contaminated corn.

DEDICATION

This thesis is dedicated to my grandparents Roy Jackson and Penne Rodgers for all of their support throughout my educational journey.

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Contributors

This work was supervised by a thesis committee consisting of Professor Terry J. Gentry, Scott A. Senseman, and Youjun Deng of the Department of Soil and Crop Sciences and Professor Elizabeth Pierson of the Department of Horticultural Sciences.

All work for the thesis was completed by the student, under the advisement of Dr. Terry J. Gentry and Dr. Scott A. Senseman of the Department of Soil and Crop Sciences.

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1. INTRODUCTION

The United States' ethanol production industry generates profitable co-products mainly in the form of dried distiller's grains (DDGs). Approximately 90% of the co-products of corn-based ethanol are fed to livestock (USDA, 2007). One potential risk of increased intake of DDGs by livestock is the increased ingestion of mycotoxins. During the ethanol fermentation process mycotoxins are not degraded, but rather enriched up to three times in DDGs (Bothast, 1992). Due to the toxicity of these mycotoxins to livestock, it is necessary to develop a safe, cost effective method for their removal from bio-fuel feed stocks.

Mycotoxins are naturally occurring secondary metabolites produced by some filamentous fungi found in a wide range of feed stocks such as corn, wheat, soybeans, and peanuts that cause adverse health effects in humans and animals when ingested (Whitlow et al., 2011). Annually, it is estimated that 25% of feed stocks are contaminated with mycotoxins worldwide (CAST, 1989). In the United States alone, mycotoxins are responsible for approximately $932 million annually from crop losses and an additional $466 million from regulatory enforcement, testing, and other quality control methods (CAST, 2003). To address contamination issues, a large amount of research on degradation methods has been pursued. Some of these methods are of chemical and physical nature such as ammoniation, ozonation, and thermal treatments requiring high energy inputs (Dupuy, 1993; Jouany, 2007; McKenzie et al., 1997). Others have also isolated and utilized mycotoxin degrading microorganisms to decrease concentrations (Alberts et al., 2006; Ciegler, 1966; Petchkongkaew et al., 2008)

Currently, corn is the primary feed stock for ethanol fermentation (Kelderman, 2007).

Several mycotoxins are potential contaminants of corn including aflatoxins, fumonisins, zearalenone, ochratoxins, and deoxynivalenol (Whitlow et al., 2011). Due to the toxicity of these mycotoxins to human and animal health, stringent regulations restrict contaminated food products from being utilized in the food production industry. One possible alternative use for corn containing high levels of mycotoxins is in corn-based ethanol production. Although utilizing these wastes in the bio-fuel industry seems to be a remedy to the monetary losses caused by mycotoxin contamination, other negative impacts have become apparent including the contamination of corn-based ethanol fermentation co-products, DDGs (Bothast, 1992). These co-products are valuable to the ethanol industry, but utilizing mycotoxin contaminated corn is increasing the mycotoxin concentrations found in DDGs, causing detrimental effects in livestock (Bothast, 1992).

The objective of my research was to address the toxigenic issue of mycotoxins in the bio-fuel industry. The objective was accomplished by determining the efficiency of a mycotoxin-degrading bacterium, *Rhodococcus corynebacterioides* DSM 21051, to degrade mixed mycotoxin substrates. Due to the complexity of some mycotoxin structures and the co-occurrence of mycotoxins in foodstuffs, using a biological treatment solely to address the contamination issue did not look promising. This circumstance prompted the development of an alternative method to accomplish the objective set forth. The alternate method tested determined the efficacy of a titanium dioxide (TiO_2) photocatalyst treatment on the degradation of mycotoxins.

2. LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are secondary metabolites, bioactive low molecular weight compounds, produced by a wide range of filamentous fungi or moulds (Keller et al., 2005). These compounds are naturally occurring environmental contaminants widely found in foodstuffs such as corn, peanuts, almonds, figs, and a variety of other foods (Fung & Clark, 2004). Mycotoxin effects on human and animals were first revealed by the occurrence of the "Turkey X Disease" outbreak in England in 1960 (De Iongh et al., 1962). The disease resulted in the death of 100,000 turkey poults and a number of other small animals. The outbreak was traced back to a mixture of complex lactones, or what is now known as aflatoxins, produced by some strains of *Aspergillus flavus* growing on peanut meal. This was a major event leading to the realization that fungal secondary metabolites could pose hazards to human and animal health. Mycotoxins and their effects have become a growing concern in the feedstuff industry due to their reported elevation in occurrence (Rodrigues & Nahrer, 2008). Table 1 lists the most common mycotoxins found in feedstuffs and the fungi associated with its production.

Of the naturally occurring mycotoxins, there are five groups of toxins that are of greatest concern. These are aflatoxins, ochratoxins, zearalenone, deoxynivalenol, and fumonisins (Bianchini & Bullerman, 2014). The primary fungi that produce these secondary metabolites include *Aspergillus, Penicillium,* and *Fusarium* spp.

Table 1. Naturally occurring mycotoxins associated with feedstuffs

Mycotoxin	Fungi	Host
Aflatoxin B1	*Aspergillus flavus; Aspergillus parasiticus; Aspegillus nomius*	Corn, sorghum, pearl millet, rice, wheat, groundnut, soybean, sunflower, cotton, chilies, black pepper, coriander, turmeric, zinger, almonds, pistachio, walnuts, coconut, milk
Aflatoxin B2		
Aflatoxin G1		
Aflatoxin G2		
Rubratoxin B	*Penicillium rubrum; Penicillium purpurogenum*	Cereal grains, rice, wheat
Ochratoxin A	*Asperillus ochraceus , Penicillium verrucosum*	Cereal grains, coffee, dried fruit, red wine (grapes)
Ochratoxin B		
Ochratoxin C		
Fumonisin B1	*Fusarium moniliforme, Fusarium proliferatum*	Corn, Rice
Fumonisin B2		
Fumonisin B3		
Vomitoxin (Deoxynivenol)	*Fusarium graminearum (Gibberella zeae); Fusarium culmorum*	Wheat, barley, oats, rye, and corn; less often in rice, sorghum, and triticale
Nivalenol	*Fusarium nivale; Fusarium cerealis; Fusarium Poae*	Wheat, corn, barley, oats, and rye
Zearalenone	*Fusarium graminearum (Gibberella zeae)*	Corn, barley, oats, wheat, rice, and sorghum

Vincelli and Parker (2002);Scott (1993); Thirumala-Devi et al. (2001); Richard (2000)

Aflatoxins. Aflatoxins are primarily produced by *Aspergillus flavus* but have also been found to be produced by *Aspergillus parasiticus* and *Aspergillus nomius*. Aflatoxins were isolated and characterized after the death of 100,000 turkey poults due to the "Turkey X Disease" in England in 1960 and traced to the ingestion of mouldy peanut meal (De Iongh et al., 1962). There are six aflatoxins associated with the group including B1, B2, G1, G2, M1, and M2. Aflatoxin B1 (AFB1; Fig. 1) receives the most attention due to its higher toxicity and higher frequency of occurrence. It is a potent carcinogen and is usually the major aflatoxin produced by toxigenic strains (Bennett & Klich, 2003). Other associated health effects to animals include liver disease, immune system deficiencies, decreased breeding efficiency, and hypoprotenemia.

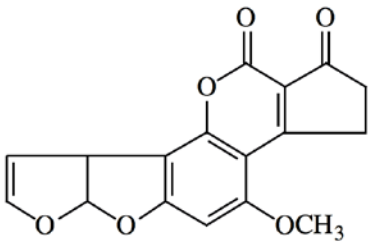


Figure 1. Chemical structure of aflatoxin B1 (AFB1)

Exposure to aflatoxins is typically by ingestion of contaminated foodstuffs but exposure routes can also include inhalation and dermal absorption. Currently the maximum concentration allowed for human consumption set by the United States (U.S.) Food and Drug Administration (FDA) is 20 micrograms per kilogram (µg/kg). Higher levels up to 300 µg/kg are allowed in feed utilized for livestock.

Ochratoxins. Ochratoxins are a group of related compounds produced by *Aspergillus ochraceus* and related species, as well as *Penicillium verracosum* (Bianchini & Bullerman, 2014). The most important mycotoxin in this group is ochratoxin A (OTA; Fig. 2). It was discovered in 1965 during a large screening of fungal metabolites that was designed specifically to identify new mycotoxins (Van der Merwe et al., 1965). Ochratoxin has been found to be a potent nephrotoxin and animal studies indicate that it is also a liver toxin, an immune suppressant, a potent teratogen, and a carcinogen (Beardall et al., 1994; Kuiper-Goodman & Scott, 1989). Of the ochratoxin group, OTA is the most toxic and primary contaminant found in foodstuffs.

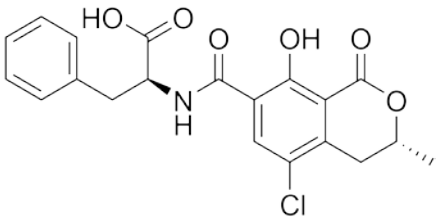


Figure 2. Chemical structure of ochratoxin A (OTA)

Exposure to OTA is primarily due to consumption of contaminated foodstuffs. The Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) Joint Expert Committee recommends a provisional tolerable weekly intake of 100 μg/kg body weight of OTA.

Zearalenone. Zearalenone (ZEA; Fig. 3) is a mycotoxin produced mainly by fungi belonging to the genus *Fusarium* in foods and feeds. It is frequently implicated in reproductive disorders of livestock and occasionally in hyperoestrogenic syndromes in humans (Zinedine et al., 2007).

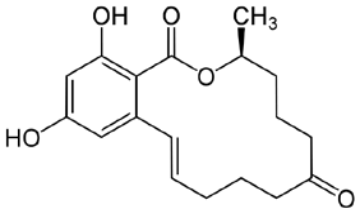


Figure 3. Chemical structure of zearalenone (ZEA)

The Joint FAO/WHO Expert Committee on Food Additives established a provisional maximum tolerable daily intake for ZEA of 0.5 μg/kg of body weight.

Deoxynivalenol. Deoxynivalenol (DON; Fig. 4) is part of a family of mycotoxins called trichothecenes and is the most commonly occurring of the group. High doses of DON ingested by animals have shown to cause nausea, vomiting, and diarrhea; at lower doses animals have exhibited weight loss and food refusal (Rotter, 1996).

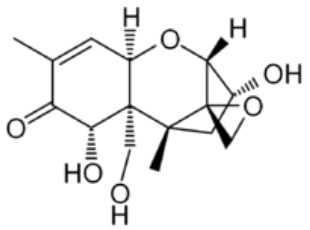


Figure 4. Chemical structure of deoxynivalenol (DON)

FAO/WHO Committee on Food Additives recommends tolerable daily intakes (TDIs) ranging from 1 to 5 µg/kg body weight.

Fumonisins. Fumonisins are a group of mycotoxins produced by *Fusarium* species and are common contaminants of corn. Fumonisin B1 (FB1; Fig. 5) is the most important and most potent in this group.

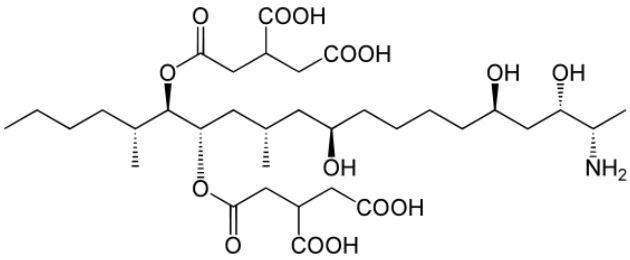


Figure 5. Chemical structure of fumonisin B1 (FB1)

FAO/WHO Committee on Food Additives recommends a maximum intake of 2 μg/kg body weight per day consumption on the basis of a no observed effect level (NOEL) and safety factor of 100.

2.2 Mycotoxin Occurrence

Because mycotoxins pose hazardous effects on human health, the World Health Organization (WHO) constantly monitors food safety programs and evaluates mycotoxin levels in various food sources (Fung & Clark, 2004). BIOMIN GmbH, a leading company focusing on Health in Animal Nutrition, in collaboration with Romer Labs Singapore conducts a quarterly Mycotoxin Survey Program. The analyses are carried out for the most important mycotoxins in terms of animal production- AFB1, ZEA, DON, FB1, and OTA. Samples range from corn, wheat and rice to processing by-products such as soy meal, DDGS, and other fodder. Based on the 2005 through 2008 reports, an increasing trend of mycotoxins has been observed (Rodrigues & Nahrer, 2008).

Annually, it is estimated that 25% of feed stocks are contaminated with mycotoxins worldwide (CAST, 1989). The primary economic losses can be attributed to the effects on livestock productivity, crop losses and the costs of regulatory programs directed toward mycotoxins (Whitlow et al., 2011). In the United States alone, mycotoxins are responsible for approximately $932 million annually from crop losses and an additional $466 million from regulatory enforcement, testing, and other quality control methods (CAST, 2003). The occurrence and concentrations of mycotoxins is variable based on climatic conditions and plant stresses that affect mycotoxin formation each growing year (Coulombe, 1993).

The occurrence of mycotoxins is dependent on many factors and contamination may occur at different phases of the production process. There are a number of factors that are conducive to mycotoxin production including temperature, relative humidity, moisture,

substrate, pH, competitive and associative growth of other fungi and microorganisms, and plants stress such as drought (Bianchini & Bullerman, 2014). Contamination can occur during pre-harvest and post-harvest. Pre-harvest production of mycotoxins is associated with plant stress conditions and post-harvest production of mycotoxins with storage conditions.

Most mycotoxin problems originate in the field although they can be formed during the storage of grains. For example, field infection of corn with *A. flavus* can be expected when temperatures are high and there is drought stress (Wicklow & Shotwell, 1983). Corn is susceptible to *A. flavus* infection via the silks (Marsh & Payne, 1984), and stress conditions present at the time of the pollination (anthesis) lead to pre-harvest aflatoxin contamination. These growth conditions are frequently observed in the southern U.S. and results in routine aflatoxin contamination in crops. Aflatoxins can also occur in other regions in years where weather conditions are conducive. Therefore, mycotoxin problems are widely distributed regionally.

2.3 Prevention and/or Elimination of Mycotoxins

A number of studies on the prevention and elimination of mycotoxin contamination in foods and feeds have been done which include physical, chemical and biological methods. The first line of defense to prevent mycotoxin contamination is minimizing mycotoxin production itself by using best management practices. Best management practices would include harvesting the grain at low moisture conditions and storing it at cool and dry conditions. Due to the limitations in some countries, best management practices may be difficult to execute such as those regions in warm, humid climates. Subsequently, the growth of fungi and the production of mycotoxins must be limited utilizing alternative methods. Physical methods can be employed as an alternative such as sorting bad grain from the good grain before storage to reduce the potential for mycotoxin formation. Visually inspecting commodities for the presence of fungal growth

will decrease the opportunity for contamination to occur but fungal growth does not always correspond to the presence of aflatoxins (Hocking et al., 2001). Biological control methods have been investigated to prevent contamination such as using a non-toxigenic strain of *Aspergillus flavus* to biocompete with the toxigenic strains. By doing so, the non-toxigenic strain occupies the niche and does not allow the toxigenic strain to propagate (Cotty, 1990; Cotty, 1989). The non-toxigenic strain of *Aspergillus flavus* has been commercialized for biological control of aflatoxin contamination in corn under the product name Aflaguard.

Once contamination occurs in the commodity, decontamination methods must be employed to decrease concentrations to allowable levels or completely eliminate the concentration. Different treatments have been used to decrease concentration levels that are of physical or chemical nature including thermal treatments, use of adsorbents, ammoniation, and ozonation. A variety of studies have investigated the effectiveness of using adsorbents for binding mycotoxins. These studies have been focused on mineral clays (Barrer, 1989; Deng et al., 2012; Dixon et al., 2008; Mumpton, 1999). Studies utilizing clay binders as feed additives to decrease the bioavailability of aflatoxin during digestion of contaminated feeds have proven to be a successful method. According to Deng et al. (2012), aflatoxin molecules have the ability to occupy the interlayer space of smectite with exchange cations and water molecules and the stability and selectivity of aflatoxin adsorption would be enhanced when the size and the polarity of aflatoxin molecule match those of the adsorbing nanoscale domains in the interlayer of smectite. The mechanism for aflatoxin adsorbtion to smectite was suggested to be through direct ion-dipole interactions and coordination between exchange cations and the carbonyl oxygens and, at high humidity, through hydrogen bonding between cation hydration-shell water and carbonyl groups (Deng et al., 2012). Hydrated sodium calcium aluminosilicates (HSCAS) shows a high affinity for AFB1 forming a complex which was stable at temperatures of 25 and 37°C, in

a pH range of 2-10 (Huwig et al., 2001). When added to chicken diets at a rate of 0.5% with an AFB1 concentration of 7.5 milligram per kilogram, the growth inhibitory effects were significantly decreased. The use of aluminosilicates for the adsorption of other mycotoxins was also investigated, but with little success (Bauer, 1994; Lemke et al., 1998; Ramos et al., 1996).

Chemically, mycotoxins can be destroyed with the use of ammoniation (Park, 1993) and ozone (McKenzie et al., 1997), among other techniques. Triatomic oxygen, or ozone, is a potent oxidizer capable of oxidizing a wide array of chemical compounds but has a strong affinity for the olefinic double bond (Criegee, 1975). McKenzie et al. (1997), investigated the use of ozone as an oxidizer of a variety of mycotoxins with a focus on aflatoxins. Multiple matrices were used including water, corn, and rice powder. Each matrix was fortified with a standard concentration of the mycotoxin of interest then treated with ozone. The study found that the reaction between ozone and aflatoxin reduced the aflatoxin concentration in aqueous solution over time with a constant supply of ozone. In corn slurry samples, the aflatoxin concentration was reduced by 72% compared to the standard with a constant supply of 20% weight ozone. FB1, OTA, ZEA, as well as a few other mycotoxins underwent the same treatment process with 10% weight ozone. All mycotoxins were undetectable by high performance liquid chromatography (HPLC) after treatment.

Although these physical and chemical methods have been proven effective against aflatoxins, the main drawbacks to using these methods is the ineffectiveness against other mycotoxins and the possible deterioration of animal health by excessive residual chemical in feed (Huwig et al., 2001), the safety concerns associated with them during application (i.e. ozone and other chemicals), and the cost effectiveness of the implementation.

2.4 Biological Degradation of Mycotoxins

Biological degradation, or also termed biodegradation, with the use of microorganisms to decrease mycotoxin concentrations is a useful method to remediate contaminated grains and grain products. In order for this method to be successful, a microorganism with the ability to degrade the compound of interest is required and complete degradation is favorable.

A number of microorganisms have been isolated with the ability to degrade AFB1 and other common mycotoxins. Ciegler (1966) isolated an AFB1-degrading actinomycete deposited as *Flavobacterium aurantiacum* NRRL B-184. The purpose of their study was to screen for microorganisms with the capability to detoxify AFB1 and aflatoxin G1. The microorganisms were grown with the presence of AFB1 in the media. Viable cells of *F. aurantiacum* NRRL B-184 were able to decrease the concentration of AFB1 from ground samples of corn and peanuts by 100% of the initial concentration and 86% from ground soybean (Ciegler, 1966). Although this study suggested AFB1 was being degraded by *F. aurantiacum*, more evidence was necessary to confirm breakdown of the compound into less toxic compounds was occurring. This was confirmed with the use of carbon-labelled aflatoxin in a study conducted by Line et al. (1994). Using carbon-labelled AFB1, it was determined that the aflatoxin was being metabolized by *F. aurantiacum* rather than being solely bound to the cells. Later *F. aurantiacum* was redeposited as *Nocardia corynebacterioides* DSM 12676 and now reclassified as *Rhodococcus corynebacterioides* DSM 44601. Further investigation of microbial degradation of AFB1, identified an additional strain of *Nocardia corynebacterioides* (formerly deposited as *Corynebacterium rubrum* and redeposited as *Rhodococcus corynebacterioides* DSM 20151) was proven to be capable of degrading AFB1 to a greater extent than that of *R. corynebacterioides* DSM 44601. Furthermore, Holzapfel et al. (2002) found *N. corynebacterioides* to also degrade OTA in liquid cultures. Unfortunately, the specific strain of *N. corynebacterioides* determined to

degrade OTA could not be confirmed during literature review. However, these findings indicate a single microorganism could be utilized to decontaminate feedstocks containing more than one mycotoxin.

Other studies have also indicated that one microorganism is able to degrade multiple, different mycotoxins but the degradation process may be more specific than initially thought. One study isolated *Bacillus* spp. from soybean and fresh Thua-nao (one of the oldest traditionally fermented soybean products) collected from the north of Thailand (Petchkongkaew et al., 2008). Studies on the inhibition of *A. flavus* and *A. westerdijkiae* NRRL 3174 growth by all isolates of *Bacillus* spp. were conducted by dual culture technique on agar plates (Petchkongkaew et al., 2008). These isolates were then tested for AFB1 and OTA detoxification ability on both solid and liquid media. The results found that most of the strains were able to detoxify aflatoxin but only some of them could detoxify OTA. This study further indicates the specificity of the microorganisms and perhaps the specificity of the enzymes produced to degrade the mycotoxin of interest.

In many cases, microorganisms have been used to successfully convert mycotoxins to harmless degradation products. Unfortunately, the conversions are generally slow and incomplete (Arici, 1999; Bata & Lásztity, 1999; Karlovsky, 1999; Sweeney & Dobson, 1998). Specificity of these processes may also pose a problem when applying this method to a system containing a variety of different mycotoxins.

2.5 Photocatalytic Degradation of Organic Contaminants

Photocatalytic treatment of organic pollutants is commonly used in water/wastewater treatment research and applied technologies. Advanced oxidative processes (AOPs) constitute an effective technology for the treatment of wastewaters containing non-easily removable organic compounds, and among these AOPs photocatalysis is the most promising

(Philippopoulos & Nikolaki, 2010). Light driven AOPs, such as photocatalysis, involve the formation of hydroxyl radicals. Hydroxyl radicals react almost non-selectively with the organic pollutants at very high rates and can result in complete mineralization of the pollutants to carbon dioxide, water, inorganic compounds or in their transformation to harmless end products (Philippopoulos & Nikolaki, 2010). Photocatalytic treatment of AFB1 has been previously investigated in water and in peanut oil (Liu et al., 2011; Liu et al., 2010). These studies included subjecting peanut oil and water samples fortified with 0.2 milligram per liter (mg/L), 2.0 mg/L, and 5.0 mg/L of AFB1 then subjecting the samples to various UV irradiation intensities. Several observations resulted from their study including complete degradation of AFB1 in water after 100 minutes of irradiation time without variation in degree of degradation with increasing concentrations of AFB1, increased UV intensities and irradiation times were more effective on the photodegradation of AFB1, and the photodegradation of AFB1 was proved to follow first-order reaction kinetics.

Among photocatalytic treatments of wastewater, the most commonly used process is the decontamination of organic pollutants in aqueous media in the presence of a semiconducting solid catalyst. The catalyst most widely used is titanium dioxide (TiO_2) due to its high oxidative power, low cost, photostability, and nontoxicity (Lee et al., 2003; Markowska-Szczupak et al., 2011; Shephard et al., 1998).

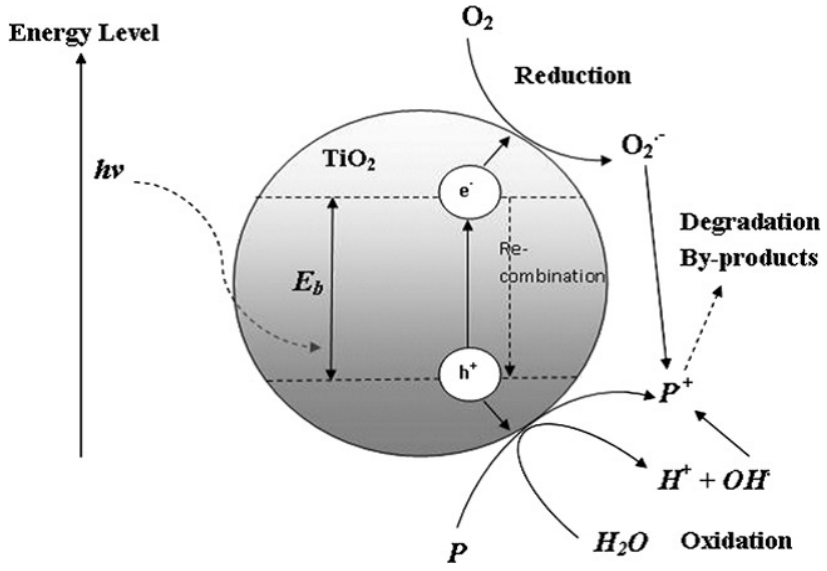


Figure 6. Photocatalysis mechanisms for degrading organic pollutants (P) (from Chong et al. (2010))

Direct photolysis is the transformation occurring from direct light absorption whereas indirect photolysis can occur when light-induced, reactive species, degrade the compound of interest. An addition of a photocatalyst in the presence of UV light is considered to be an indirect photolysis process (Lee et al., 2003; Schwarzenbach et al., 1995). The TiO_2 photocatalyst shows outstanding UV light photocatalytic activity in the decomposition of dyes in paper mill effluents and other organic pollutants (Markowska-Szczupak et al., 2011). A study conducted by Shephard et al. (1998) documents the degradation of the cyanobacterial toxin microcystin-YR in water. At a level of 0.2 gram per liter (g/L) TiO_2, microcystin-YR levels of approximately 64 nanogram per milliliter (ng/ml) decreased to below the detection limit of 10 ng/ml after 12 minutes, while at a level of 1.0 g/L, a similar reduction in toxin level was achieved within 6 minutes (Shephard et al., 1998). Also, the utilization of a TiO_2 photocatalyst as a pre-treatment of lignocellulosic materials prior to fermentation degrades lignin without decreasing the efficiency of ethanol production (Yasuda et al., 2011). Upon excitation by light,

the photon energy generates an electron hole pair on the TiO_2 surface (Linsebigler et al., 1995). This highly unstable state has strong oxidation power and converts water and oxygen into reactive oxygen species (ROS) (Linsebigler et al., 1995). This mechanism can be compared with that of the ozonation of aflatoxins in previous studies. Ozone, or triatomic oxygen (O_3), is a powerful disinfectant and oxidizing agent (McKenzie et al., 1997). It reacts across the 8, 9 double bond of the furan ring of aflatoxin through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids (McKenzie et al., 1997). Although photocatalysis with the addition of TiO_2 has not been attempted on mycotoxins, these findings, along with the results of photocatalytic treatment of AFB1(Liu et al., 2011; Liu et al., 2010), support the hypothesis that utilizing a TiO_2 photocatalyst pre-treatment for corn prior to production of ethanol has the potential to degrade mycotoxins in corn feed stocks.

2.6 Mycotoxins Found in Dried Distiller's Grains (DDGs)

In the United States, ethanol is almost entirely produced from corn (Kelderman, 2007). The production of ethanol from corn has primarily been achieved through dry- and wet-milling processes. The majority of U.S. ethanol production is from dry-grind technology. The traditional dry-grind process grinds the whole corn kernel and mixes it with water and enzymes. The mash is then cooked to liquefy the starch further. The mash is then cooled and mixed with more enzymes to convert the remaining sugar polymers to glucose before fermenting to ethanol (Murthy et al., 2005). The components of the kernel not fermented include the germ, fiber, and protein, and are concentrated in the DDGs that are produced as co-products. DDGs are then used as feed additives due to their high nutritional value.

A potential health risk of utilizing corn as a primary feedstock for ethanol production is the potential for mycotoxin exposure in DDGs when fed to livestock as a supplement in feed.

This risk is dependent on the fate of the mycotoxin present in the original corn batch. Studies have been conducted to determine the fate of various mycotoxins during processing of commodities such as ethanol fermentation and brewing (Bennett et al., 1981; Bennett & Richard, 1996; Bothast, 1992; Chu et al., 1975; Lillehoj, 1978). After fermentation, mycotoxins are not found in the ethanol but rather remain in the other fractions, including wet distiller's grains and other fractions combined into DDGs (Bothast, 1992). The concentration in the other fractions can be as high as three times the initial concentration of mycotoxins present in the original grain. This is because these fractions represent a smaller mass than the original grain.

It has also been found that many of the feedstock sources are contaminated with multiple, different mycotoxins whether from mixing the sources on receipt or from being contaminated with multiple strains of fungi (Zhang et al., 2009). In that study, DDGs from 20 ethanol fermentation plants were collected and analyzed for a variety of mycotoxins. Further analysis of these samples indicated that DDGs were frequently contaminated with a combination of mycotoxins rather than one mycotoxin. This poses a problem because most remedial methods investigated and tested have dealt with the destruction of a single mycotoxin. It would be advantageous to the biofuel industry if a method capable of degrading multiple mycotoxins was available for treatment of contaminated feedstocks and co-products.

3. BIOLOGICAL DEGRADATION OF MYCOTOXINS

3.1 Overview

A study was conducted using a biological degradation method with *R. corynebacterioides* DSM 20151 to decrease mycotoxin concentrations in aqueous samples. Cultures were prepared to confirm degradation of AFB1 and OTA by *R. corynebacterioides* DSM 20151 and determine the efficacy of *R. corynebacterioides* DSM 20151 to degrade ZEA. Following degradation confirmation, cultures were prepared to determine the effect of mixed substrates of A) 5 mg/L AFB1 and 5 mg/L OTA and B) 5 mg/L AFB1 and 5 mg/L ZEA on the degradation process by *R. corynebacterioides* DSM 20151. Results from this study determined the effect of multiple mycotoxin substrates on the degradation process and the feasibility of utilizing *R. corynebacterioides* DSM 20151 in the ethanol fermentation process to decrease multiple mycotoxin concentrations simultaneously.

3.2 Introduction

A potentially cost effective method for mycotoxin decontamination is the use of mycotoxin-degrading microorganisms. Many microorganisms have been successfully isolated with the ability to degrade these compounds (Alberts et al., 2006; Ciegler, 1966; Petchkongkaew et al., 2008; Teniola et al., 2005). Currently, it is unclear if the presence of other mycotoxins will interfere with the degradation efficiency of a particular microorganism or if a microorganism can simultaneously degrade a mixed substrate of mycotoxins. This is essential information for the ethanol industry because many of the feed stock sources are contaminated with multiple, different mycotoxins whether from mixing the sources on receipt or from being contaminated with multiple strains of fungi as shown in a study conducted by Zhang et al. (2009). In that study, DDGs, a co-product of ethanol fermentation commonly fed to livestock,

were collected from 20 ethanol fermentation plants and analyzed for a variety of mycotoxins. Analysis of these samples indicated DDGs were frequently contaminated with a combination of mycotoxins rather than one mycotoxin.

One particular organism, *R. corynebacterioides* DSM 20151, is an actinomycete determined to degrade AFB1 by Teniola et al. (2005). A later study found that a similar strain of microorganism (*N. corynebacterioides)* could degrade OTA in liquid cultures (Holzapfel et al., 2002). Unfortunately the mechanism used for this process has yet to be determined but is suggested to be due to extracellular enzymes, as has been found to be the mechanism for other mycotoxin-degrading microorganisms (Alberts et al., 2006; Teniola et al., 2005). Because many of the microorganisms classified as mycotoxin degraders produce extracellular enzymes to breakdown the mycotoxins into less toxic forms, it is important to determine the extent to which they are effective against the mycotoxins and the specificity of the enzymes for effective and efficient removal (Alberts et al., 2006). *R. corynebacterioides* DSM 20151 has already been identified as an AFB1 degrader, and a similar bacterial strain has been identified as an OTA degrader. Therefore, these two mycotoxins were used along with ZEA, a frequent contaminant found in DDGs (Zhang et al., 2009), to conduct this investigation. The maximum permissible level of AFB1 in corn is 20 µg/kg for human consumption and 300 µg/kg for animal consumption. Currently, there are no set standards for OTA and ZEA in the United States (Egmond & Jonker, 2004). Although standards have not been set in the United States, in other countries permissible levels of ZEA are between 50 and 1000 µg/kg and OTA are between 3 and 50 µg/kg (Egmond & Jonker, 2004). It was determined that in order for this method to be successful, levels of these three mycotoxins must be below these maximum permissible values after treatments.

3.3 Materials and Methods

3.3.1 Materials

Mycotoxins, including AFB1, OTA, and ZEA were purchased from Sigma Aldrich (USA). Solvents were purchased from Fisher Scientific (USA). *R. corynebacterioides* DSM 20151 was purchased from DSMZ (Germany). Nylon filters and HPLC vials were purchased from Fisher Scientific (USA). Other materials were taken from current laboratory inventory.

3.3.2 Confirmation of Afatoxin B1 and Ochratoxin A Degradation by R. Corynebacterioides DSM 20151

To confirm the documented degradation of AFB1 and OTA by *R. corynebacterioides* DSM 20151, the organism was cultured in 10 mL standard I broth (Goodfellow, 1986) for 24 h in a shaking incubator at 28°C and 200 rounds per minute (rpm). After 24 h, 100 µL of culture was transferred to 10 mL of 5 mg/L AFB1 standard I broth in replicates of three. The three cultures were incubated under the same conditions as the primary incubation. At 24, 48, and 72 h, a 2-mL sample was taken and filtered through a 0.22-µM nylon filter then 1 mL was extracted and analyzed by HPLC. This procedure was also performed to confirm degradation of OTA by *R. corynebacterioides* DSM 20151 and determine the ability of *R. corynebacterioides* DSM 20151 to degrade ZEA.

3.3.3 Degradation Efficacy and Efficiency of Mixed Mycotoxin Substrates by R. Corynebacterioides DSM 20151

After confirming degradation of AFB1, OTA, and ZEA by *R. corynebacterioides* DSM 20151, mixed substrates of mycotoxins were tested using the same protocol as above. A culture of *R. corynebacterioides* DSM 20151 was grown in 10-mL standard I broth at 28°C for 24 h on a rotator set at 200 rpm. After 24 h, 100 µL of culture was transferred to 10 mL of a 5 mg/L AFB1 and 5 mg/L OTA mixture in standard I broth in replicates of three. The three cultures were

incubated under the same conditions as the primary incubation. At 24, 48, and 72 h, a 2-mL sample was taken and filtered with a 0.22-µM nylon filter then 1 mL of each was extracted and analyzed by HPLC. This protocol was repeated with a 5 mg/L AFB1 and 5 mg/L ZEA mixture in standard I broth.

3.3.4 Extraction of Samples

To analyze samples taken at 24, 48, and 72 h, AFB1, OTA, and ZEA were extracted from the broth samples. To extract these mycotoxins, ethyl acetate was added to the 1-mL samples at a 1:1 (vol:vol) quantity. The samples were then placed on a horizontal shaker for 15 minutes. Following agitation, the supernatant was transferred to a glass test tube. The two previous steps were repeated three times and each sample's supernatant was combined. The test tubes with collective supernatant were placed on an evaporator, under a stream of nitrogen gas, until dryness. The extracted mycotoxins were then dissolved in 1 mL of methanol, filtered with a 0.22-µM nylon filter, and analyzed by HPLC (Perkin Elmer model Elan DRCII).

3.3.5 HPLC Analysis

Table 2 contains the analytical parameters that were used for HPLC analysis of each mycotoxin.

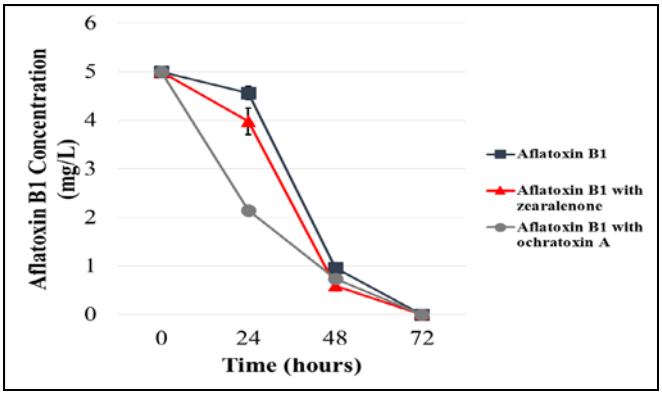
Table 2. Analytical parameters for HPLC analysis of mycotoxins

Aflatoxin B1	**Ochratoxin A (OchraTest)**	**Zearalenone (ZearalaTest)**
Column: C-18 **Mobile Phase:** 50:50 Methanol:H_2O **Flow Rate:** 1 mL/min **Injection Volume:** 20 µL **Run Time:** 10 minutes **Wavelength:** 365 nm **Peak:** 6.5 minutes	**Column:** C-18 **Mobile Phase:** 49.5:49.5:1 ACN:H_2O:Acetic Acid **Flow Rate:** 0.8 mL/min **Injection Volume:**50 µL **Run Time:** 12 minutes **Wavelength:** 333 nm **Peak:** 9.7 minutes	**Column:** RP-8 **Mobile Phase:** 46:46:8 ACN:H_2O:Methanol **Flow Rate:** 0.2 mL/min **Injection Volum**e: 20 µL **Run Time:** 20 minutes **Wavelength:** 236 nm **Peak:** 13.6 minutes
Source: Adapted from Teniola et al. (2005)	**Source:** Directly from Vicam's OchraTest Instruction Manual (VICAM, 2008)	**Source:** Adapted from Vicam's ZearalaTest Instruction Manual (VICAM, 2011)

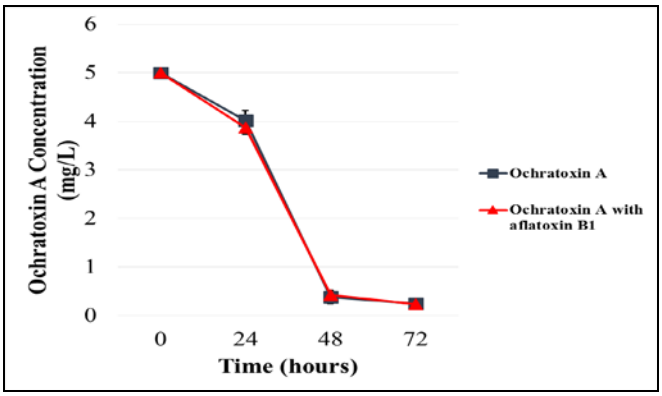
3.4 Results and Discussion

This study indicated that the degradation of mycotoxins by *R. corynebacterioides* DSM 20151 does not seem to be inhibited by the addition of other mycotoxins as shown in Figure 7.

(A)



(B)



(C)

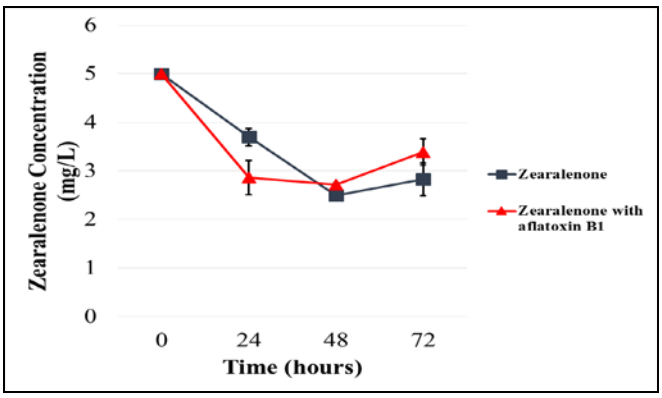


Figure 7. Degradation of aflatoxin B1 (A), zearalenone (B), and ochratoxin A (C) individually and in mixtures. Error bars represent standard error of three replicates.

Data collected for AFB1 indicates that AFB1 is degraded by *R. corynebacterioides* DSM 20151 somewhat steadily over 72 hours of incubation with approximately 90% of AFB1 degraded after 48 hours of incubation and 100% of AFB1 degraded after 72 hours of incubation. Similar AFB1 degradation was observed with the addition of OTA and ZEA with the exception of the AFB1 with OTA sample at 24 hours. Interestingly, the addition of OTA temporarily enhanced the degradation of AFB1 at 24 hours but this effect was diminished by 48 hours. Similar degradation results as AFB1 are seen with the degradation of OTA by *R. corynebacterioides* DSM 20151; however, no change in degradation rate of OTA was observed for the OTA sample with the addition of AFB1 at 24 hours as seen with AFB1 in the presence of OTA. Two main degradation pathways have been reported for AFB1: modification of the difuran ring or modification of the coumarin structure (Vanhoutte et al., 2016). Degradation of AFB1 through modification of the difuran ring moiety into AFB1-8,9- dihydrodiol has been demonstrated by manganese peroxidase from the white rot fungi *Phanerochaetesordida* (Wang et al., 2011) and the aflatoxin-detoxifizyme (ADTZ) of fungus *Armillariella tabescens* (Liu et al., 1998). Cleavage of the lactone ring in the coumarin moiety of AFB1 has been demonstrated by the activity of a *Pseudomonas putida* strain discovered to degrade AFB1 to AFD1 and subsequently into AFD2 (Grove et al., 1984; Samuel et al., 2014). Whereas, the main detoxification pathway of OTA is the hydrolyzation of the amide bond between the isocoumarin residue and phenylalanine by a carboxypeptidase (Vanhoutte et al., 2016). It is likely that the enzyme responsible for AFB1 degradation by *R. corynebacterioides* is different from the enzyme produced for the degradation of OTA due to the differing degradation pathways. It is possible that the enzyme responsible for degradation of OTA by *R. corynebacterioides* is also capable of degrading AFB1 through an alternate pathway not discussed above, which could explain the temporarily enhanced degradation of AFB1 at 24 hours with the presence of OTA.

Our findings also indicate that *R. corynebacterioides* DSM 20151 is capable of degrading 95% of OTA even in the presence of AFB1 and that the bacterium is not capable of effectively degrading ZEA under the tested conditions.

Degradation activities by *R. corynbacterioides* DSM 20151 have been previously reported for AFB1 (Mann & Rehm, 1976; Teniola et al., 2005). *R. corynebacterioides* DSM 20151 degraded more than 99% of AFB1 (1.48 μg/mL) in fortified liquid culture after 4 days of incubation (Mann & Rehm, 1976) and more than 90% of AFB1 (2.5 μg/mL) in fortified phosphate buffer containing cell free extracts of *R. corynebacterioides* DSM 20151 liquid culture after 4 hours of incubation (Teniola et al., 2005). In contrast, our study found that *R. corynebacterioides* DSM 20151 degraded more than 90% of AFB1 (5 μg/ml) in fortified liquid culture after 2 days of incubation and complete degradation after 3 days. No studies have provided evidence of OTA degradation by *R. corynebacterioides* DSM 20151; however, a strain similar to *R. corynebacterioides* DSM 20151 identified as *Nocardia corynebacterioides* has been shown to degrade OTA (Holzapfel et al., 2002).

Based on our study results, use of *R. corynebacterioides* DSM 20151 does not appear to be a promising strategy if it is necessary to degrade multiple, different mycotoxins in commodities. It may be possible to identify other microorganisms which can degrade multiple mycotoxins, but due to the variety and complexity of possible mycotoxins, this approach would probably require the use of multiple microorganisms in order be successful. Several enzymes produced by mycotoxin-degrading microorganisms have been identified for the degradation of AFB1, OTA, and ZEA. In a study conducted by Alberts et al. (2009), a pure laccase enzyme from *Trametes versicolor* and a recombinant laccase enzyme produced by *Aspergillis niger* degraded, respectively, 87% and 55% of AFB1with a significant loss of mutagenicity evaluated in the Ames *Salmonella*-based assay. Extracellular enzymes of *Rhodococcus erythropolis* were

also able to degrade AFB1 with a loss of mutagenicity (Alberts et al., 2006). Additionally, two classes of carboxypeptidases have been associated with degradation of OTA namely Carboxypeptidase A (CPA) (Chang et al., 2015; Stander et al., 2001) and Carboxypeptidase Y (CPY) (Dridi et al., 2015). Almost all strains that are reported to degrade OTA result in the formation of L-b- phenylalanine and OTα, the former being less toxic than OTA (Bruinink & Sidler, 1997). Finally, two notable microorganisms have been identified capable of ZEA detoxification with use of enzymes including *Clonostachys rosea* IFO 7063 (isogenic strain of NRRL1859) through the activity of a ZEA lactonohydrolase enzyme (zhd101) (Kakeya et al., 2002) and *Trichosporum mycotoxinivorans* through the activity of an unspecified a/b-hydrolase (Molnar et al., 2004). These microorganisms are notable among the reported microorganisms capable of degrading ZEA due to their abilities to detoxify ZEA to breakdown products exhibiting the loss of estrogenic activities.

As indicated above, extracellular enzymes have been proven to degrade mycotoxins without the presence of microbial cells or nutrient requirements, therefore, enhancing their applicability to feedstocks as opposed to direct application of microbial cell cultures. Furthermore, the use of extracted extracellular enzymes appears to enhance the degradation rates observed for AFB1 as shown by Teniola et al. (2005) as compared to the results of our study and the results of Mann and Rehm (1976) when utilizing liquid culture. Due to the ability for extracellular enzymes to degrade mycotoxins without cellular function or nutrient requirements, they prove to be a promising treatment for degradation and detoxification of mycotoxins in feedstocks used for ethanol production. It is important to note, though, that there are specific temperature requirements under which enzymes function best (Abrunhosa et al., 2014; Patharajan et al., 2011; Péteri et al., 2007; Teniola et al., 2005). Also, as is the case with microorganisms, extracellular enzymes are specific to the mycotoxin(s) that the host

microorganism is shown to degrade; therefore, this approach would probably require the use of multiple enzymes in order to be successful at degrading multiple, different mycotoxins. However, the use of multiple enzymes may prove to be less challenging than multiple microorganisms due to the specific growth and nutrient requirements associated with microbial cultures.

4. PHOTOCATALYTIC DEGRADATION OF MYCOTOXINS

4.1 Overview

A study was conducted to determine the efficacy of TiO_2 to detoxify AFB1, OTA, and ZEA in aqueous samples and ground corn samples with UV-C irradiation. The TiO_2 mixture quantities were optimized for determining the highest rate of detoxification. The results of this study determined the applicability of utilizing TiO_2 with UV-C irradiation as a pre-treatment of corn used for ethanol production.

4.2 Introduction

Phototransformations are either direct or indirect and involve photolysis of the compound of interest, breaking it down into smaller chemical species (Schwarzenbach et al., 1995). Direct photolysis is the transformation occurring from direct light absorption whereas indirect photolysis can occur when light-induced, reactive species, degrade the compound of interest. An addition of a photocatalyst in the presence of UV light is considered to be an indirect photolysis process (Lee et al., 2003; Schwarzenbach et al., 1995). UV light generally varies the reaction efficacy and efficiency depending on the wavelength. There are three forms of UV light applicable to photocatalysis: UV-A or long-wave UV light, UV-B or medium-wave UV light, and UV-C or short-wave UV light. UV-C has the highest intensity of UV light, increasing reactivity. Recent interest has increased in utilizing TiO_2 as a photocatalyst for the degradation of organic pollutants, specifically the anatase form because it appears to be the most efficient semiconductor for environmental applications (Lee et al., 2003; Markowska-Szczupak et al., 2011; Shephard et al., 1998). Although this seems to be the case, it also appears that mixtures of the anatase and rutile forms of TiO_2 have a synergistic effect, aiding in the degradation of these compounds (Ohno et al., 2003). The TiO_2 photocatalyst shows outstanding

UV light photocatalytic activity in the decomposition of dyes in paper mill effluents and other organic pollutants (Markowska-Szczupak et al., 2011).

Many studies have been conducted utilizing TiO_2 to degrade a wide variety of organic contaminants. A study conducted by Shephard et al. (1998) documents the degradation of the cyanobacterial toxin microcystin-YR in water. At a level of 0.2 g/L TiO_2, microcystin-YR levels of approximately 64 ng/ml decreased to below the detection limit of 10 ng/ml after 12 minutes, while at a level of 1.0 g/L, a similar reduction in toxin level was achieved within 6 minutes (Shephard et al., 1998). Also, the utilization of a TiO_2 photocatalyst as a pre-treatment of lignocellulosic materials prior to fermentation degrades lignin without decreasing the efficiency of ethanol production (Yasuda et al., 2011). Upon excitation by light, the photon energy generates an electron hole pair on the TiO_2 surface (Linsebigler et al., 1995). This highly unstable state has strong oxidation power and converts water and oxygen into reactive oxygen species (ROS) (Linsebigler et al., 1995). This mechanism can be compared with that of the ozonation of aflatoxins in previous studies. Ozone, or triatomic oxygen (O_3), is a powerful disinfectant and oxidizing agent (McKenzie et al., 1997). It reacts across the 8, 9 double bond of the furan ring of aflatoxin through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids (McKenzie et al., 1997).

Although this photocatalysis with the addition of TiO_2 has not been attempted on mycotoxins, these findings support the hypothesis that utilizing a TiO_2 photocatalyst pre-treatment for corn prior to production of ethanol has the potential to degrade mycotoxins in corn feed stocks. TiO_2 is one of the most viable materials for photocatalysis because of its high oxidative power, low cost, photostability, and nontoxicity. These characteristics show high potential for the removal of mycotoxins in current bio-fuel systems (Linsebigler et al., 1995).

4.3 Materials and Methods

4.3.1 Materials

Mycotoxins, including AFB1, OTA, and ZEA, and TiO_2 catalysts in the anatase and rutile form were purchased from Sigma Aldrich (USA). Solvents were purchased from Fisher Scientific (USA). Nylon filters and ultrahigh performance liquid chromatography (UPLC) vials were purchased from Fisher Scientific (USA). UV-C lamp (62 Watt, 18 inch compact lamp) and assembly were purchased from Universal Light Source, Inc. (San Francisco, CA). Other materials, including polytetrafluoroethylene (PTFE) cuvettes, were taken from current laboratory inventory.

4.3.2 TiO_2 Concentration Optimization for Mycotoxin Degradation

To determine the optimal concentration of TiO_2 to degrade mycotoxins with UV-C irradiation, TiO_2 was added in a variety of quantities to aqueous samples containing ZEA. ZEA was used for determining the optimal TiO_2 concentration due to its efficacy to degrade when encountering free radicals in solution from preliminary studies.

Aqueous Samples. A stock solution of 3 mg/L ZEA in water was prepared. Approximately half of the stock solution was divided into twenty 2-mL PTFE cuvettes containing the 1-mL samples. TiO_2 was added to the other half of the stock solution for a TiO_2 concentration of 1.0 g/mL. The 1.0 g/mL TiO_2 stock solution was equally split into five glass beakers and diluted into substock solutions to concentrations of 0.002 g/mL, 0.2 g/mL, 0.6 g/mL, 1.0 g/mL, and 1.4 g/mL TiO_2. Each substock solution was divided into five 2-mL cuvettes containing 1-mL samples. One cuvette without TiO_2 and one cuvette with the addition of TiO_2 were placed securely on a stir plate set at 1000 rpm, the cuvette without TiO_2 serving as a UV-C control. The UV-C lamp was secured 2.5 cm in front of the cuvettes. The samples were irradiated for 9 minutes with 1-mL samples being removed with and without TiO_2 at set time

intervals. The intervals consisted of 0, 3, 6, and 9 min. The 0 time interval sample served as the no UV-C treatment control. After irradiation, the samples were extracted, filtered using 0.22-µM nylon filter membranes, and analyzed by UPLC. The experiment was replicated three times.

Corn Slurry Samples. A stock solution of 3 mg/L ZEA in water was prepared. Approximately half of the stock solution was divided into twenty 2-mL PTFE cuvettes containing 1-mL samples. TiO_2 was added to the other half of the stock solution for a TiO_2 concentration of 1.0 g/mL. The 1.0 g/mL TiO_2 stock solution was split into five glass beakers and diluted into sub stock solutions to concentrations of 0.05 g/mL, 0.1 g/mL, 0.15 g/mL, 0.2 g/mL, and 0.5 g/mL TiO_2. Each sub stock solution was divided into five 2-mL cuvettes containing 1-mL samples and 0.1 g of ground corn was added to each cuvette. One cuvette without TiO_2 and one cuvette with the addition of TiO_2 were placed securely on a stir plate set at 1000 rpm, the cuvette without TiO_2 serving as a UV-C control. The UV-C lamp was secured 2.5 cm in front of the cuvettes. The samples were irradiated for 9 minutes with 1-mL samples being removed with and without TiO_2 at set time intervals. The intervals consisted of 0, 8, 16, 24, and 32 min. The 0 time interval sample served as the no UV-C treatment control. After irradiation, the samples were extracted, filtered using 0.22-µM nylon filter membranes, and analyzed by UPLC. The experiment was replicated three times.

4.3.3 Photocatalytic Degradation of Mycotoxins

To determine the efficacy and efficiency of TiO_2 photocatalysis to degrade mycotoxins with UV-C irradiation, TiO_2 was added to aqueous and ground corn samples containing mycotoxins in a variety of quantities. Again AFB1, OTA, and ZEA degradation was assessed.

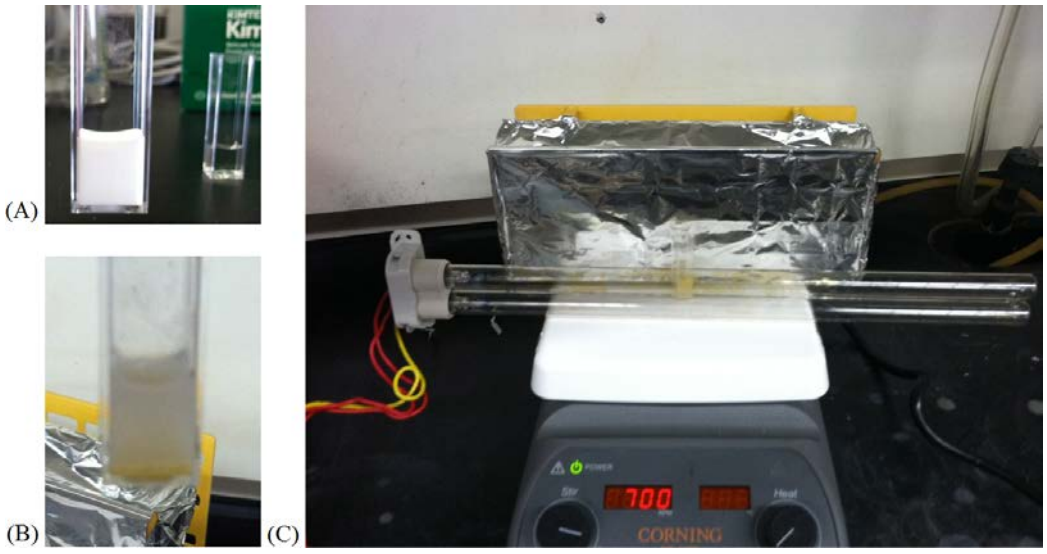


Figure 8. Photocatalytic treatment set-up. Samples were prepared with the addition of TiO_2 without ground corn (A) and with ground corn (B). Samples were secured in front of a UV-C light bulb for irradiation (C).

Aqueous Samples. A 20-mL stock solution of 3 mg/L AFB1, 3 mg/L OTA, and 3 mg/L ZEA in water was prepared. Approximately half of the stock solution was divided into nine 2-mL PTFE cuvettes containing 1-mL samples. TiO_2 was added to the other half of the stock solution for a concentration of 0.02 g/mL and divided into nine 2-mL cuvettes containing 1-mL samples. One cuvette without TiO_2 and one cuvette with the addition of TiO_2 were placed securely on a stir plate set at 1000 rpm, the cuvette without TiO_2 serving as a UV-C control. The UV-C lamp was secured 2.5 cm in front of the cuvettes. The samples were irradiated for each specified time interval, a set of two for each time interval (one with and one without TiO_2). The intervals consisted of 0, 2, 4, 6, 8, 10, 15, 20, 30 min. The 0 time interval sample served as the no UV-C treatment control. After irradiation, the samples were extracted, filtered using 0.22-µM nylon filter membranes, and analyzed by UPLC. The experiment was replicated three times.

Ground Corn Samples. A corn sample was milled to a powder using a Wiley mill to a particle size of less than 1 mm and stored in an aluminum canister for use. A stock solution

volume of 60 mL was prepared composed of 3 mg/L AFB1, 3 mg/L OTA, and 3 mg/L ZEA in methanol. The stock solution was divided equally into two beakers and a concentration of 0.5 g/mL TiO_2 added to one of the sub stock solution beakers. Each of the two beakers were then filled and mixed with 30 g of milled corn and let dry for an hour in a chemical hood. Once dry, both were divided into 1 g subsamples in ten plastic weigh boats, evenly distributed. The weigh boats were placed under the UV-C lamp, 2.5 cm above the weigh boats, alternating samples with and without TiO_2. The samples were irradiated for 30 minutes, and samples were removed with and without TiO_2 at set time intervals. The intervals consisted of 0, 2, 4, 6, 8, 10, 15, 20, 30 minutes. Time interval 0 served as the no UV treatment for analysis. After irradiation, the samples were extracted, filtered through 0.22-µM nylon filter membranes, and analyzed by UPLC. The experiment was replicated three times.

Due to negative analytical results with dry corn samples (data not shown), water was introduced to form a corn slurry. Additionally, time intervals were increased due to negative results. A sample of 0.1 g ground corn (based on a preliminary study; data not shown) containing no mycotoxin was added to eighteen 2-mL PTFE cuvettes. Following the corn addition, 1 mL of mycotoxin stock solution composed of 3 mg/L AFB1, 3 mg/L OTA, and 3 mg/L ZEA in water was added to nine cuvettes and 1 mL of mycotoxin stock solution with the addition of 0.5 g/mL TiO_2 was added to the remaining nine cuvettes. One cuvette without TiO_2 and one cuvette with the addition of TiO_2 were placed securely on a stir plate set at 1000 rpm, the cuvette without TiO_2 serving as a UV-C control. The UV-C lamp was secured 2.5 cm in front of the cuvettes. The samples were irradiated for their specified time (0, 8, 16, 24, 32, 40, 48, 56, and 64 minutes). After irradiation, the samples were extracted, filtered through 0.22-µM nylon filter membranes, and analyzed by UPLC. The experiment was replicated three times.

4.3.4 Extraction of Samples

After irradiation, the aqueous samples with and without the addition of ground corn were extracted in the same manner as the biological samples (Chapter 3) by adding ethyl acetate to the 1-mL samples at a 1:1 (vol:vol) quantity. The samples were then placed on a horizontal shaker for 15 minutes. Following agitation, the supernatant was transferred to a glass test tube. The two previous steps were repeated three times and each sample's supernatant combined. The test tubes with collective supernatant were placed on an evaporator, under a stream of nitrogen gas, until dryness. The extracted mycotoxins were then dissolved in 1 mL of methanol, filtered with a 0.22-µM nylon filter, and analyzed by UPLC.

4.3.5 UPLC Analysis

Table 3 and 4 contain the analytical parameters that were used for UPLC analysis of AFB1, OTA, and ZEA.

Table 3. Analytical parameters for UPLC analysis of mycotoxins

UPLC Conditions		**MS Conditions**	
LC system:	Waters ACQUITY UPLC System	**MS system:**	Waters ACQUITY TQ Detector
Column:	ACQUITY UPLC HSS T3	**Ionization mode:**	ESI Positive
Column temp.:	40^0C	**Capillary voltage:**	4 Kv
Flow rate:	400 µL/min	**Cone voltage:**	Various
Mobile phase:		**Desolvation gas:**	Nitrogen
A	H_2O+0.1% formic acid	**Cone gas:**	Nitrogen
B	Acetonitrile	**Source temp.:**	120^0C
Gradient:		**Acquisition:**	Multiple reaction monitoring (MRM)
Time 0 min	70% A	**Collision gas:**	Argon
Time 2 min	90% A		
Total run time:	9.2 minutes		
Injection volume:	5 µL		
Source: Adapted from Morphet et al. (2007)			

Table 4. ACQUITY TQD MRM mycotoxin parameters

Mycotoxin	MRM Transitions	Typical ion ratio	Dwell time	Cone voltage (V)	Collision energy (eV)
Aflatoxin B1	313>285	0.21	0.03	50	23
	313>241				37
Ochratoxin A	404>239	0.90	0.03	31	19
	404>358				14
Zearalenone	319>187	0.60	0.03	20	19
	319>185				23
Source: Morphet et al. (2007)					

4.4 Results and Discussion

TiO_2 Optimization. A TiO_2 optimization study was conducted to determine the optimal concentration of TiO_2 in an aqueous samples and corn slurry samples for enhanced degradation of mycotoxins. ZEA was used for determining the optimal TiO_2 concentration due to its efficacy to degrade when encountering free radicals in solution from preliminary studies.

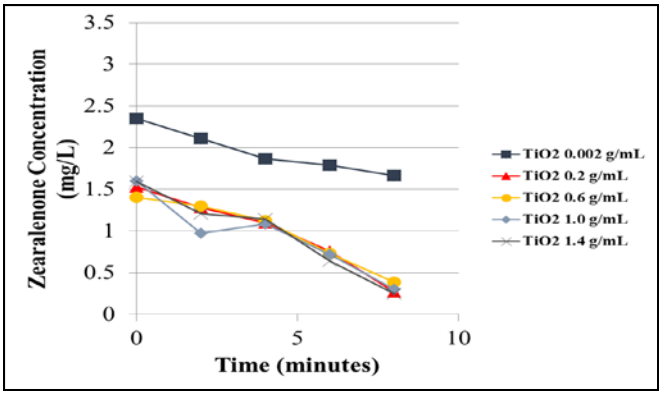


Figure 9. TiO2 concentration optimization for aqueous samples trial 1 with the use of zearalenone.

Based on the results above, concentrations between 0.2 g/mL and 1.4 g/mL have similar degradation rates. Due to the similarity between degradation rates, a second TiO_2 concentration

optimization trial was conducted that included TiO_2 concentrations of 0.002 g/mL, 0.02 g/mL, and 0.2 g/mL to determine the optimal concentration of TiO_2 that had the highest degradation rate and lowest TiO_2 concentration requirement.

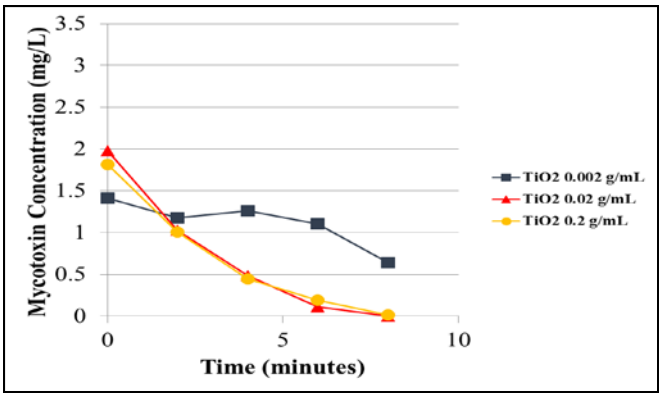


Figure 10. TiO_2 concentration optimization for aqueous samples trial 2 with the use of zearalenone.

Results of the second TiO_2 concentration optimization trial suggests that the optimal TiO_2 concentration that had the highest degradation rate combined with the lowest TiO_2 requirement was 0.02 g/mL for the given mycotoxin concentration level. Based on these results, a TiO_2 concentration of 0.02 g/mL was used in all studies involving aqueous samples and TiO_2 photocatalysis of mycotoxins.

TiO_2 optimization was also performed for corn slurry samples with the use of ZEA. The optimization results for corn slurry samples are shown below in Figure 11.

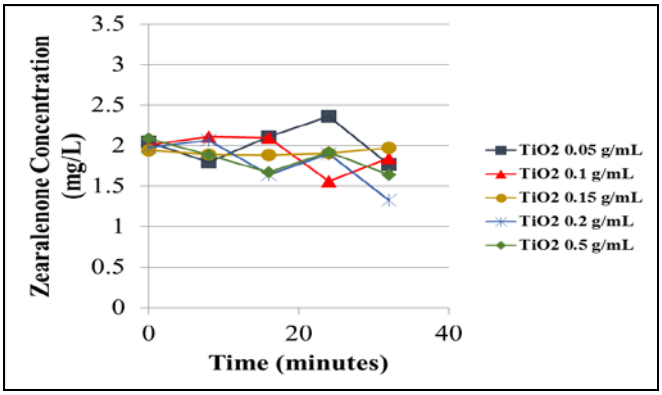


Figure 11. TiO_2 concentration optimization for corn slurry samples with the use of zearalenone.

Based on the results above, the TiO_2 concentration of 0.2 g/mL appeared to have the most significant effect on ZEA concentration in corn slurry samples; however, ZEA was not completely degraded with any of the TiO_2 concentrations and the differences between to concentrations tested were not significant. Therefore, a TiO_2 concentration of 0.5 g/mL was used for studies involving corn slurry samples and TiO_2 photocatalysis of mycotoxins. The low degradation rates observed may have been due to inadequate UV activation of TiO_2 from ground corn interference or due to an overload of organics with the addition of ground corn. A preliminary study determining the best ground corn volume was conducted prior to the TiO_2 optimization study. Results of the study indicated that ZEA degradation increased as the volume of corn decreased; however, the lowest volume of corn used in the study was determined to not be a feasible volume for implementation in an ethanol fermentation system. Therefore, no further optimization was deemed worthwhile.

TiO_2-Photocatalytic Treatment of Aqueous Samples. The addition of TiO_2 with UV-C irradiation to aqueous samples of AFB1, OTA, and ZEA showed increased degradation in comparison with the irradiation of samples with UV-C light alone. Treatment time varied depending on the mycotoxin of concern. AFB1 and OTA required more treatment time

compared to ZEA in order to observe a significant decrease in concentration. The degradation results for the three mycotoxins are shown below in Figure 12.

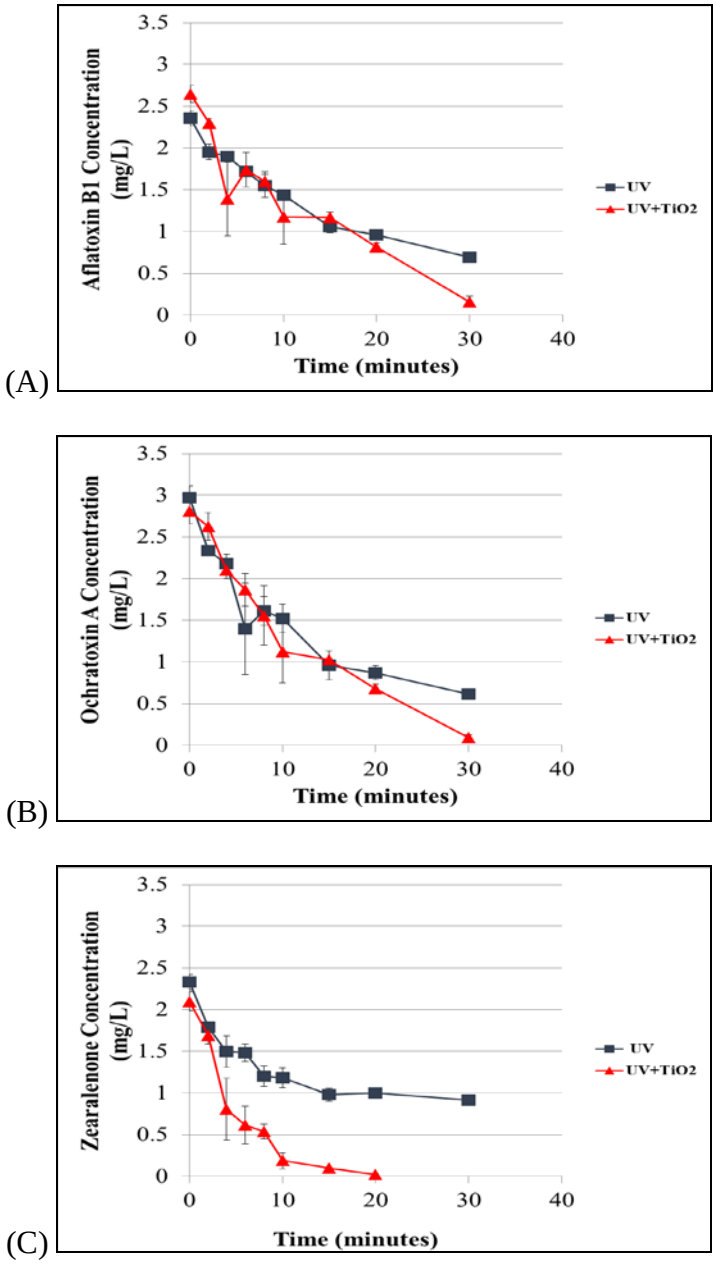


Figure 12. Photocatalytic degradation of aflatoxin B1 (A), ochratoxin A (B), and zearalenone (C) in aqueous solution with and without the addition of TiO_2 (0.02 g/mL). Error bars represent standard error of three replicates.

Data collected for AFB1 indicates UV-C light alone and with the addition of TiO_2 appears to decrease steadily until approximately 20 minutes of irradiation time. After 20 minutes, increased degradation was observed in samples with the addition of TiO_2. The TiO_2-photocatalytic treatment was able to degrade 94% of AFB1 after 30 minutes of irradiation. A difference of approximately 30% was observed between the two treatments for AFB1 after a total of 30 minutes irradiation.

A similar trend to AFB1 was observed with OTA. The concentration of OTA in solution appears to decrease steadily until approximately 20 minutes of irradiation time. A significant decrease in concentration was observed between 20 and 30 minutes of irradiation time with the addition of TiO_2. The TiO_2-photocatalytic treatment was able to degrade 97% of OTA after 30 minutes of irradiation. A difference of approximately 18% between the two treatments for OTA after a total of 30 minutes irradiation.

Conversely, ZEA showed a higher degradation efficacy with the addition of TiO_2. The concentration of ZEA in solution appears to decrease rapidly with the addition of TiO_2. A significant decrease in concentration was observed at 20 minutes of irradiation time with the addition of TiO_2 and 100% degradation by 30 minutes of irradiation time. A difference of approximately 39% between the two treatments was observed at 20 minutes. As shown in Chapter 2, the ZEA structure (Figure 3) appears to be the least complex compound of the mycotoxins used in this study. Due to the lower complexity of the structure of ZEA, the compound may be more readily oxidized by free radicals generated when TiO_2 is activated by the UV-C light as opposed to AFB1 and OTA.

Supporting results have been reported with other mycotoxin degradation studies when comparing similar chemical or physical degradation methods to degradation efficacy of multiple mycotoxins. One such study includes a study conducted by McKenzie et al. (1997) investigating

the oxidative degradation effect of ozone on multiple mycotoxins. As part of their study, AFB1 and ZEA were added to aqueous samples at equimolar concentrations and treated with ozone for 15 seconds with 2% weight ozone and 20% weight ozone for AFB1 and 10% weight ozone for ZEA. Results of the study found that weight percentage of ozone is required to be doubled in order for 100% loss of AFB1 to occur within the set treatment time of 15 seconds as compared to ZEA. Additionally, thermal processing/treatment of mycotoxin-contaminated grains has shown results indicating the stability of ZEA at high temperatures is less than the stability of other mycotoxins including AFB1 and OTA. Melting points have been reported for AFB1, OTA, and ZEA as 237 to 306ºC, 169ºC, and 164 to 165ºC, respectively (EFSA, 2004; Rustom, 1997; Tsubouchi et al., 1987). Studies investigating thermal extrusion processing of grains have reported a reduction in AFB1 ranging from 10% to 25%, a reduction in OTA ranging from 8% to 35%, and a reduction in ZEA ranging from 66% to 83% with temperatures ranging from 120 to 196 ºC (Cazzaniga et al., 2001; Ryu et al., 1999; Scudamore et al., 2004). These results indicate that ZEA is significantly more susceptible to degradation than AFB1 and OTA and also demonstrates the structural stability of AFB1 and OTA as compared to ZEA. It is important to note that, although these melting points indicate destruction can occur at high temperatures, studies investigating the fate of mycotoxins during extrusion processing of grains (discussed above) have shown that there are variable degradation rates when treated with this method. These variable results are due to several factors including extruder temperature, screw speed, moisture content of the extrusion mixture, and residence time in the extruder (Bullerman & Bianchini, 2007).Therefore, the photocatalytic method in our study may have been impacted by factors such as stir plate speed and light intensity, which may play a role in the difference in degradation results for ZEA in comparison to AFB1 and OTA. These factors were minimally investigated during our study and, therefore, should be further investigated to determine their

role in degradation rates for these compounds or considered for optimization of the method for use in an ethanol fermentation system.

An additional noteworthy finding of our study includes the absence of byproducts observed within samples during TiO_2-photocatalytic treatment in comparison with UV-C treatment alone. In a study conducted by Liu et al. (2010), discussed previously in Chapter 2, three byproducts were noted during photocatalytic treatment of AFB1 in deionized water samples. Further investigation was conducted to determine the toxicity of the byproducts identified in deionized water samples and to determine any toxicological properties of treated peanut oil during various stages of photocatalytic treatment (Liu et al., 2011; Liu et al., 2012). The double bond in position 8, 9 of the furo-furan ring, the site where aflatoxin-DNA and aflatoxin-protein interactions occur, and the lactone ring in the coumarin moiety are the two important sites for toxicological activity of AFB1 (molecular structure is shown in Figure 1) (Liu et al., 2011). Therefore, removing the double bond of the terminal furan ring or opening the lactone ring is the major aim of detoxification (Samarajeewa et al., 1990). Two toxicological studies were conducted including the Ames test for mutagenicity (Liu et al., 2011) and a cell viability assay with HepG2 cells (Liu et al., 2012).

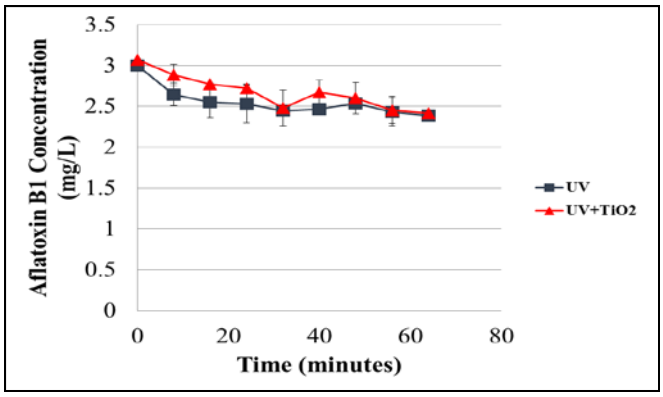
Based on the Ames test results and the HepG2 cell assay results using a peanut oil sample matrix, the analyses did not reveal formation of any toxic byproducts suggesting that AFB1 was most likely degraded to products with chemical properties different from that of AFB1 (Liu et al., 2011; Liu et al., 2012; Samarajeewa et al., 1990). The Liu et al. (2011) study also suggested photodegradation products R-COOH, R-CHO, R-CO-R', and CO_2 to be the final photodegradation products of AFB1 under UV irradiation in peanut oil as previously reported by McKenzie et al. (1997) under ozonation. However, the HepG2 cell assay results reported in the Liu et al. (2012) study using a deionized water sample matrix indicate that the toxigenic

properties (although somewhat weakened by treatment) of the byproducts generated during photocatalytic treatment are similar to the toxigenic properties associated with AFB1.

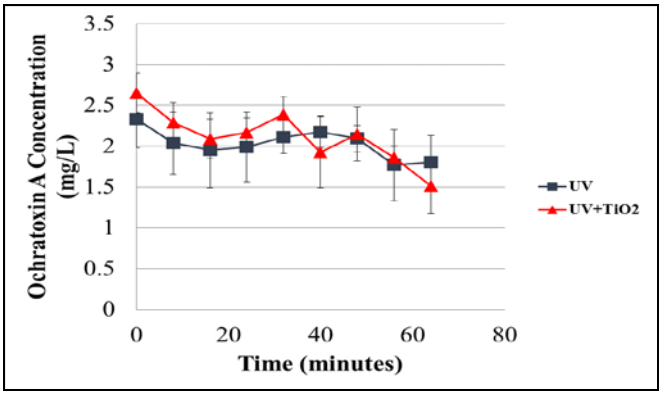
As stated above, no byproducts for AFB1, OTA, or ZEA were observed in aqueous (deionized water) samples during TiO_2-photocatalytic treatment in our study, indicating that the breakdown products are likely similar to those suggested by Liu et al. (2011) and by McKenzie et al. (1997). However, further investigation of the toxigenic properties of potential photodegradation products generated during TiO_2-photocatalytic treatment should be conducted using a toxicity test similar to that performed by Liu et al. (2011) and Liu et al. (2012) for confirmation purposes.

TiO_2-Photocatalytic Treatment of Corn Slurry Samples. The addition of TiO_2 with UV-C irradiation to corn slurry samples of AFB1, OTA, and ZEA appeared to not increase degradation effects. The degradation results for the three mycotoxins are shown below in Figure 13.

(A)



(B)



(C)

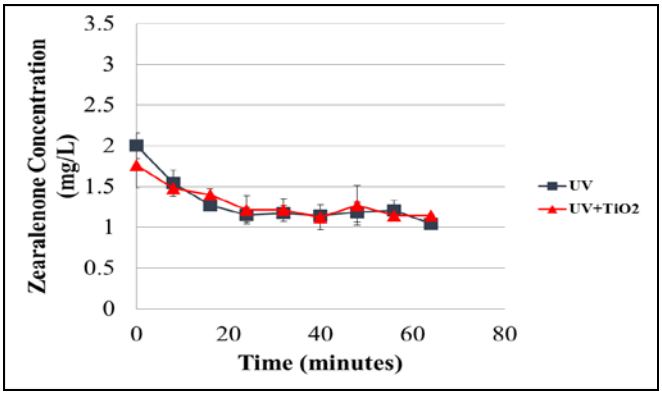


Figure 13. Photocatalytic degradation of aflatoxin B1 (A), ochratoxin A (B), and zearalenone (C) in corn slurry with and without the addition of TiO_2 (0.5 g/mL). Error bars represent standard error of three replicates.

Based on Figure 13, AFB1, OTA and ZEA concentrations trend downward over time with the application of UV-C light treatment with no enhanced degradation from the addition of TiO_2. A reduction in AFB1, OTA, and ZEA degradation of approximately 72%, 54%, and 64%, respectively, was observed between the aqueous solutions and the corn slurry samples. The results indicate that corn interferes with the efficiency of the catalytic degradation process. Similar observations were made by McKenzie et al. (1997) when corn slurries mixed with AFB1 underwent a treatment of 20% weight ozone. In their study, 1 g of ground corn was mixed with a 4-mL water standard (STD) and with a 4-mL contaminated rice powder (CRP) solution (1 mg of CRP in 4 mL of water), both fortified with 2 mg/L AFB1. The samples were treated with ozone by bubbling the gas through the slurry mixture then extracting the AFB1 from the corn slurries. The addition of ground corn to the two samples resulted in an approximate degradation reduction of 28% for the STD sample and an approximate degradation reduction of 33% for the CRP solution sample.

The results observed from this study indicate that in order to obtain positive results utilizing a photocatalytic treatment with TiO_2, it may be necessary for mycotoxins to be in an aqueous solution. This may be due to inadequate UV activation of TiO_2 from ground corn interference or due to an overload of organics with the addition of ground corn. Photocatalysis treatment of organic pollutants with the addition of TiO_2 has been commonly investigated for use in water and wastewater treatment industries. Several studies suggest the use of photocatalysis treatment for the degradation of naturally occurring organic matter (NOM) and humic acids in drinking water sources (Bekbölet & Özkösemen, 1996; Eggins et al., 1997; Liu et al., 2008; Stevenson, 1994). In a study conducted by Liu et al. (2008), photocatalysis with the addition of TiO_2 was investigated for the degradation of NOM. In their study, water collected from the Myponga Reservoir in Australia was subjected to UV-A/TiO_2 treatment in a photoreactor

equipped with a blacklight blue fluorescent lamp (maximum emission at 365 nm). A concentration of 0.1 g/L TiO_2 was added to the water for treatment and samples were irradiated for a period of time, during which 30 mL of water was removed at 30-minute time intervals. Degradation was monitored using two methods including change in dissolved organic carbon (DOC) and UV absorbance at 254 nm (UV_{254}) in the water. Results observed included a higher percentage of UV_{254} removal as compared to that of DOC removal, indicating that loss of aromaticity was easier to achieve than mineralization of NOM. The study also determined complete mineralization could not be achieved using their method of degradation due to the complexity and heterogeneity of the NOM composition. Although complete mineralization could not be achieved, this study proves the capacity for free radicals to interact with other organic compounds, especially other aromatic compounds, within a sample. Interestingly, their study also included an addition of hydrogen peroxide which resulted in faster kinetics of photocatalytic degradation compared to the oxidation with TiO_2 alone. Improved photocatalytic degradation rates of mycotoxins could potentially be achieved with the use of hydrogen peroxide additions.

As described previously, a primary purpose for our study is to determine if a photocatalytic treatment with the use of TiO2 (TiO_2-photocatalytic treatment) could be incorporated into an ethanol fermentation system for mycotoxin degradation and improved value of DDGs. Based on corn slurry results obtained through this study, in order for the method to be incorporated into an ethanol fermentation system, the mycotoxins may need to be extracted and then undergo TiO_2-photocatalytic treatment. This could potentially be accomplished by utilizing the ethanol produced during fermentation by recirculating the ethanol from the corn batch through a photocatalytic treatment process. The ethanol production by the corn dry-grind process is shown in Figure 14.

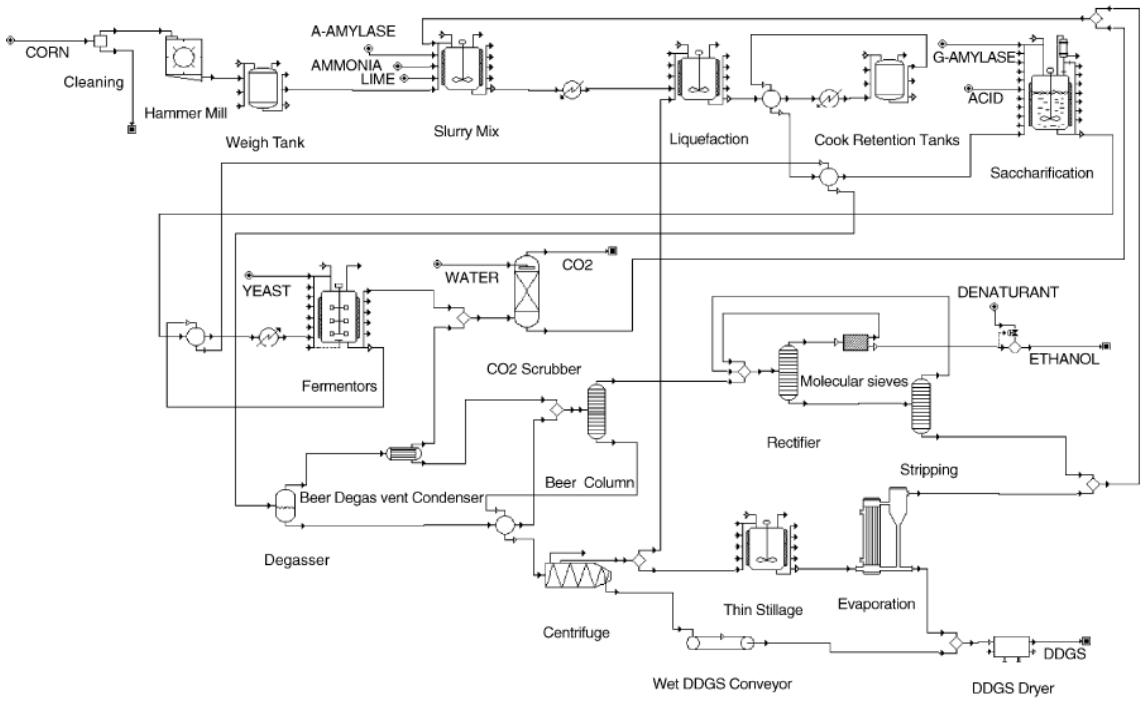


Figure 14. Ethanol production by the corn dry-grind process (from Kwiatkowski et al. (2006))

A majority of the mycotoxins are found in the stillage after fermentation (Bothast, 1992; Johnston et al., 2012; Lillehoj et al., 1979). Therefore, there are two locations within the ethanol production process that a TiO_2-photocatalytic treatment could be employed: 1) a pre-treatment batch tank at the cleaning stage of the production process with the use of ethanol produced during previous batches and 2) post ethanol fermentation with treatment of the thin stillage and DDGs with the use of ethanol produced during previous batches. For successful integration of a TiO_2-photocatalytic method as a treatment within the ethanol production process, further investigation of several factors must be achieved including solubility of multiple, different mycotoxins in ethanol, extraction efficiency with the use of ethanol from whole kernel batches, DDG batches and thin stillage batches, and the effects of ethanol on the effectiveness of TiO_2 degradation of mycotoxins. As shown in our study, ground corn appears to interfere with the

effectiveness of the TiO_2-photocatalytic process; therefore, implementation at the cleaning stage of the production process would decrease the potential interference of organic material from ground corn. At the cleaning stage in the ethanol production process, the corn is whole kernel, which allows for an ethanol "washing" process to be conducted prior to the grinding step of the process. The ethanol "wash" water/ethanol-mycotoxin solution could then be collected and treated with the TiO_2-photocatalytic treatment method. Implementation of the TiO_2-photocatalytic treatment post fermentation will require extraction of mycotoxins from the DDGs and thin stillage by-products. Thin stillage (usually a slurry form containing ground corn organic matter) and DDG extraction solutions would then be combined and treated with the TiO_2-photocatalytic treatment. Extraction may be achieved with the use of ethanol; however, further investigation of ethanol extraction efficiency from wet or slurry matrices should be conducted. In this case, an additional, stronger solvent such as ethyl acetate may be necessary for successful extraction. Otherwise, the wet DDGs and thin stillage may be dried/evaporated and then undergo mycotoxin extraction with ethanol; however, further investigation of ground corn organic matter within the extraction solution should be conducted. Based on this evaluation of TiO_2-photocatalytic treatment integration into an ethanol production process, the most promising stage of implementation would be at the cleaning stage of the process.

5. SUMMARY AND CONCLUSIONS

Mycotoxin contamination of food and grains is a serious global problem with feed stocks often being contaminated with multiple, different mycotoxins. Due to the toxicity of these mycotoxins to human and animal health, stringent regulations restrict contaminated food products from being utilized in the food production industry. One possible alternative use for corn containing high levels of mycotoxins is in corn-based ethanol production. Although utilizing these wastes in the bio-fuel industry seems to be a remedy to the monetary losses caused by mycotoxin contamination, other negative impacts have become apparent including the contamination of corn-based ethanol fermentation co-products, DDGs (Bothast, 1992). These co-products are valuable to the ethanol industry, but utilizing mycotoxin-contaminated corn is increasing the mycotoxin concentrations found in DDGs, causing detrimental effects in livestock (Bothast, 1992). Many studies have successfully isolated microorganisms able to degrade these compounds separately. It is unclear if the presence of other mycotoxins will interfere with the degradation efficiency of a particular microorganism or if a microorganism can simultaneously degrade a mixed substrate of mycotoxins. Other studies have successfully detoxified these compounds using chemical and physical methods such as ammoniation, ozonation, and thermal treatments. These treatments typically require high energy inputs and are linked with many safety concerns. Two studies were conducted to evaluate effectiveness of two separate methods to decontaminate corn samples containing multiple, different mycotoxins.

The first study was conducted to determine the effect of mixed mycotoxin substrates on an aflatoxin-degrading bacterium, *R. corynebacterioides* DSM 20151. The addition of ZEA and OTA had no significant effect on the degradation of AFB1 by *R. corynebacterioides* DSM 20151. With and without the addition of ZEA and OTA, the bacterium was capable of degrading

100% of AFB1 within 72 hours. The bacterium was also able to degrade 95% of OTA even in the presence of AFB1. However, *R. corynebacterioides* DSM 20151 did not degrade ZEA with or without AFB1 within the 72 experiment. This lack of effect on the degradation of AFB1 and inability to degrade ZEA is likely due to the specificity of the enzymes responsible for degradation of AFB1 as well as OTA.

A second study was conducted to determine the effectiveness of an alternate method, high intensity ultraviolet light (UV-C) and the photocatalyst TiO_2, to degrade a variety of mycotoxins in aqueous and corn slurry samples. Mycotoxins included in the study were AFB1, ZEA, and OTA. In aqueous solutions after 30 minutes, UV-C was able to degrade AFB1, ZEA, and OTA but not to below regulatory or recommended levels, whereas the addition of TiO_2 seemed to have a significant effect on the reaction improving the degradation to under or close to regulatory levels. In corn slurry samples after 30 minutes, UV-C treatment and UV-C treatment with the addition of titanium dioxide were unable to degrade AFB1, ZEA, and OTA. The low efficiency of degradation in corn slurry samples could be due to low contact between titanium dioxide and UV-C light from corn interference or absorption.

These results suggest that photocatalytic degradation might be a promising method for remediation of mycotoxin-contaminated corn; however, modifications to the process will need to be investigated to enhance efficiency. As seen in the studies conducted, photocatalytic degradation was observed to be effective in aqueous samples but not in corn slurry samples. In order for the method to be incorporated in an ethanol production method, the mycotoxins may need to be extracted and then undergo photocatalytic treatment. This could potentially be accomplished by utilizing the ethanol produced during fermentation by recirculating the ethanol from the corn batch through a photocatalytic treatment system. If ethanol production increases, there will be a substantial increase in the amount of ethanol co-products including DDGs. This

method could become an increasingly important remedy as DDGs and other ethanol co-products play an important role in animal feed.

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