ANALYSIS OF PLASTIC DEGRADATION BY THE FILAMENTOUS FUNGUS *ASPERGILLUS ORYZAE*

by

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ABSTRACT

The present research is focused on the application of *Aspergillus oryzae* for the degradation of biodegradable plastics and conventional plastics. By addition of inducing agents and altering the initial pH of the culture media, the biodegradation process of biodegradable film was enhanced. The use of inducing substrates olive oil and yeast extract significantly increased the esterase activity of *A. oryzae*. The alkaline initial pH levels resulted in 20% mass loss of the PBAT/PLA film and significantly increased the esterase activity of *A. oryzae*. By addition of pro-oxidants, conventional plastics are made more susceptible to UV irradiation and subsequent biodegradation. Colorimetry analysis showed significant changes in the surface of the recalcitrant plastics with pro-oxidant exposure, UV irradiation, and incubation with *A. oryzae*. Fourier transform infrared analysis showed the production of carbonyl groups with pro-oxidant exposure and UV irradiation, followed by degradation of these groups after incubation with *A. oryzae*. The generation of these functional groups as a result of oxidation (whether catalyzed by pro-oxidants and UV irradiation or just atmospheric) are an indication of abiotic degradation and chemical transformation of the film surface. The presence of these functional groups also indicate susceptibility to degradation by microorganisms, as the degradation of these functional groups was observed following incubation with *A. oryzae*. The percent change in mass of the R94W film was found to be greater than that of the RU88X-6 film for both respective untreated and pro-oxidant treated samples, at all respective QUV exposures, omitting pro-oxidant untreated at 0 hours QUV exposure, which resulted in the greatest change in mass.

LIST OF ABBREVIATIONS

Abbreviation	Definition
A. flavus	*Aspergillus flavus*
A. oryzae	*Aspergillus oryzae*
AIC	Akaike information criterion
ANOVA	Analysis of variance
Ao	*Aspergillus oryzae*
AR(1)	AutoRegressive
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CD	Czapek-Dox
CDA	Czapek-Dox Agar
cm	Centimeters
CS	Compound symmetry
CV	Coefficient of variation
Da	Daltons
F. solani	*Fusarium solani*
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate
GCEIMS	Gas chromatography electron impact ionization mass spectrometry
GCMSD	Gas chromatography mass selective detector
GLM	General linear model
H_2O	Water
HSD	Honestly significant difference
IPG	Intertape Polymer Group
IPTG	isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	Potassium phosphate, dibasic (dipotassium)
KCL	Potassium chloride
KH_2PO_4	Potassium phosphate, monobasic (monopotassium)
m/z	Mass to charge ratio
mL	Milliliter
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
mmol	Millimole
Mn	Maganese
MSTFA	N-Trimethylsilyl-N-methyl trifluoroacetamide
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
nm	Nanometer
No.	Number
PBAT	Poly(butylene adipate-co-terephthalate)
PCL	Poly(capro lactone)
PE	Polyethylene
PFTBA	Perfluorotributylamine
pKa	Dissociation Constant
PLA	Poly(lactic acid)

pNP	p-nitrophenol
pNPB	p-nitrophenyl butyrate
QUV	Q-panel laboratory ultra violet testing
rpm	Revolutions per minute
SBC	Schwarz's Bayesian Criterion
UN	Unstructured
UV	Ultra violet
v/v	Volume to volume ratio (percent volume)
Vis	Visible
$W/nm/m^2$	Watt per nanometer per square meter
w/v	Weight to volume ratio (percent weight)
°C	Degrees Celsius
µg	Micrograms
µL	Microliters
µmol	Micromoles

ACKNOWLEDGEMENTS

I would like to first and foremost thank my supervisor, Dr. Gordon W. Price, who took me on as a third year undergraduate student with an idea to work with a plastic degrading fungus as a fourth year thesis project. This project has developed in ways I never would have imagined when I first began my research as a summer student research assistant. Your continued mentorship and advising has been invaluable throughout this entire process. Thank you to my committee members, Dr. Balakrishnan Prithiviraj and Dr. Chibuike Udenigwe, for their continued support with recommendations and direction with experimental design and when troubleshooting experimental procedures. Thank you to the Plant, Food, and Environmental Science Department lab technicians, including Paul, Anne, Kalyani, and Debbie, for their help in finding lab equipment, chemicals, and suggestions at any time they were needed.

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To all of my funguys and fungals, this is for you.

Chapter 1: Introduction

1.1. The Global Plastics Problem

Plastics are a worldwide problem that humans alone have caused. Since its introduction in the 1860s, plastic products have become an indispensable part of day to day life. Plastics are an ingenious anthropogenic development, with properties that allow widespread applications from toys to technology to packaging. The problem with the increasing production of plastics is their proper disposal; recycling can recover some of this waste, but the most common fate of plastics is landfilling. This has had an undesirable effect on the environment, causing dangerous levels of pollution in terrestrial, marine, and atmospheric environments (Boettner et al., 1973; Webb et al., 2013). Due to their inexpensive production and desired physical-chemical properties, these non-biodegradable polymers are universally used in packaging, building and construction, medical, agricultural, and electronic applications (Cho et al., 2011; Plastics Europe, 2016). Following the industrialization of plastics more than half a century ago, plastics synthesized from non-renewable resources have replaced many natural materials and their production has grown, placing increasing pressure on disposal facilities' capacities for plastic waste disposal. In 2016, the global plastics production was estimated to be 335 million tons, a 4% increase from that of 322 million tons in 2015 (Plastics Europe, 2017), and the Canadian plastics industry was valued at $19.7 billion (Statistics Canada, 2011). Global analysis showed that of the 8500 million tons of plastic produced between 1905 and 2015, 2500 million tons are currently in use, 4900 million tons have been discarded (via landfilling or in the natural environment), 800 million tons have been incinerated, and 600 million tons have been recycled (Geyer et al., 2017). Approximately 50% of plastics are intended for single use, e.g. packaging, agriculture films (Hopewell et al., 2009) and subsequent disposal. The majority of these synthetic polymers do not fully degrade in the biosphere as they are recalcitrant to microorganisms (Cho et al., 2011), thus the fate of plastics is important to consider. The increased production of plastics and the lack of biodegradability of commercial polymers (especially those used in packaging, industry, and agriculture) has led to a potentially enormous environmental pollution problem that could persist for centuries (Albertsson et al., 1987, Lambert and Wagner, 2017). Degradation of waste plastics through various means has become one of the alternatives to deal with such problems (Singh and Sharma, 2008). In current events, countries around the world are banning single use plastics bags and plastic drinking straws in an effort to move towards zero plastic waste.

1.2. Plastics Industry

Since the industrialization of plastics in the 1930s, continuous innovation has led to an annual increase in global plastic production of approximately 10%, with production growing from 1.3 million tons in 1950 to 335 million tons in 2016 (Panda et al., 2010; Plastics Europe, 2017). By the 1960s, the key commodity resins and a large number of engineering resins had been developed, and there was sufficient machinery infrastructure to allow for the plastics industry to grow exponentially (Statistics Canada, 2011). This abundance of plastics led to the displacement of paper, glass, and metal from their traditional applications.

The production of plastic begins with natural products, such as cellulose, coal, natural gas, and crude oil. Plastics are generally derived from petrochemicals and require energy for production. Approximately 4% of the world's oil and gas production is converted to plastics and the production process for plastics consumes the energy equivalent to an additional 3–4% (Al-Salem et al., 2009). The production of plastics begins with distillation to separate heavy crude oil into lighter fractions. Each fraction comprises a mixture of hydrocarbon chains that differ in size and structure. Of these fractions, naphtha is used to produce plastics (Plastics Europe, 2017). Plastics are produced through two major processes, polymerization and polycondensation, each of which require specific catalysts. In polymerization, monomers (e.g. ethylene) are linked together to form long polymer chains with unique properties, structures, and sizes that depend on the type of monomer used. Polycondensation polymers are formed through condensation reactions in which molecules are joined together in a step-growth polymerization via reactive functional groups, during which small molecules are lost as byproducts such as water or methanol (Cheremisinoff, 1989).

1.2.1. Canadian Plastics Industry

The Canadian synthetic plastic industry produces approximately 2% of the world's volume of plastics, of which more than one third are intended for single-use (e.g. packaging, agricultural mulch films) and are consequently disposed of. The other main end-uses of Canadian plastics are construction and automotive parts (Statistics Canada, 2011). The Canadian plastics industry is focused towards sustainable development, replacing many products in packaging, automotive, and construction with plastics, however this comes with the challenge of solid waste reduction

and management in order to minimize environmental pollution. Companies are encouraged to use higher recycled resin content in new products and to reduce the amount of materials used to form products (Statistics Canada, 2011).

1.2.2. Agricultural Applications

Plasticulture, a technology of using plastics in agriculture, dates back to 1938 when British scientists first used polyethylene as a sheet film. This approach is used early in the growing season to modify the soil microclimate to benefit crop growth. Cropping has been revolutionized through the use of these films by providing additional crop protection, and has given rise to the modern plasticulture systems. Plasticulture includes not only plastic mulch films, but also drip irrigation tape, row covers, low tunnels, high tunnels, silage bags, hay bale wraps, and pots/trays (Kasirajan and Ngouajio, 2012). The largest volume of agricultural plastics is used for plastic mulch films, creating environmental concerns when it comes to their removal and disposal.

The removal of the plastic, which requires both machinery and manual labor, has high costs of pickup and disposal, resulting in onsite burning to be considered more economically friendly to farmers. This open burning does not result in complete combustion of the plastics, releasing air pollutants such as carbon monoxide. Moreover, other pollutants are also released into the environment due to contamination of the film with fertilizers and pesticides, particularly dioxins (known carcinogens and endocrine disruptors). Proper incineration of plastics to ensure complete combustion requires burning at temperatures of 1000 to 1200 ^{0}C (open burning will only reach temperatures of 200 to 315 ^{0}C), which will reduce the emission of pollutants (Kasirajan and Ngouajio, 2012). However, incinerators are not typically designed to burn the dirt and debris contaminants that cover the plastics so operators are reluctant to accept such wastes. Recycling of agricultural plastics is not done directly from the field due to the presence of dirt and debris, which are present up to 40 to 50% by weight in the plastics, and plastics with more than 5% contaminants by weight are not accepted for recycling. The effects of photo-degradation on the plastics due to UV exposure in the field also make them unacceptable for recycling. Landfilling is the most common disposal method for agricultural plastics, in spite of increasing cost and environmental hazard associated with this method of disposal (Kasirajan and Ngouajio, 2012).

CleanFARMS (2012), along with the Canadian Plastics Industry Association (CPIA) and the Resource Recovery Fund Board (RRFB) Nova Scotia, prepared a quantitative report of agricultural plastics used in the Maritime Provinces. This report estimated that 2,124 tons of agricultural plastic packaging wastes are generated on Maritime farms every year. Of this, approximately 1,479 tons per year is plastic film waste that must be managed by individual farmers. These plastic films (used for silage film, bale wrap, greenhouse film, mulch film, row covers, fertilizer bags, grow bags, and packaging) are manufactured from low density polyethylene (LDPE). LDPE plastics accounted for the largest amount of plastic waste (tons/year) in each of Nova Scotia, New Brunswick and Prince Edward Island when compared to other types of plastic used in agriculture, such as polypropylene (PP), high density polyethylene (HDPE), polystyrene (PS) and polyethylene terephthalate (PET) (CleanFARMS, 2012).

Nova Scotia is the largest user of mulch films and row covers in the Maritimes (30 of the estimated 37 tonnes/year generated). Currently, Prince Edward Island is the sole Maritime province that has a recycling program for agricultural plastics that was implemented across the country by CleanFARMS. The pilot study being used in Prince Edward Island (from February to July 2012, that was then extended another six months) accepted silage wrap, greenhouse film and net wrap in loads of up to ½ ton at no cost to the farmer unless the plastic was contaminated or dirty (CleanFARMS, 2012). New Brunswick and Nova Scotia have not implemented silage or bale wrap recycling programs.

1.3. Classification of Plastics

Generally speaking, there are two types of polymers: thermoplastics and thermosetting polymers. Thermoplastics are the plastics that do not undergo chemical change in their composition when heated and can be molded again and again upon heating. Thermoset, or thermosetting, plastics will undergo a chemical change upon heating and formation, and thus cannot be reheated and reformed. Thermosets will retain their strength and shape under heat and pressure.

Thermoplastics include polyethylene, polypropylene, polyvinyl chloride, polystyrene, polyamines, fluoropolymers, and others. Thermoset plastics include polyurethane, polyester, silicone, and epoxy resins (Plastics Europe, 2017). Thermoplastics constitute approximately 80%

of total plastic consumption and are commonly used in packaging applications (Al-Salem et al., 2009).

1.3.1. Classification by Degradation

Recalcitrant (Conventional Synthetic) Plastics

Commercial polyethylene (PE) (Fig. 1.1), a common component of consumer products, is the largest globally produced polymer, at about 90 million tons per year (Plastics Europe, 2016). PE is composed of long hydrocarbon chains that are hydrophobic and without any functional groups, making them inaccessible to microorganisms. They are resistant to photo-oxidation and biodegradation because they contain antioxidants and stabilizers, thus leading to a potentially massive environmental pollution problem.

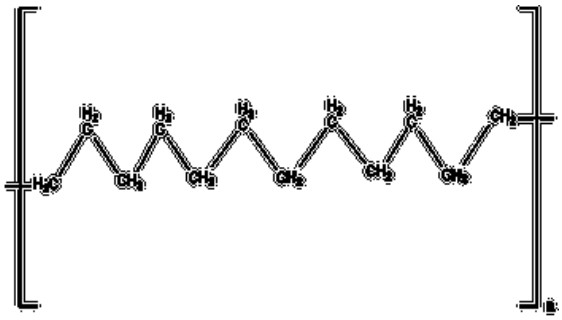


Figure 1.1: Monomer Structure of Polyethylene (PE) (Drawn on ChemSketch).

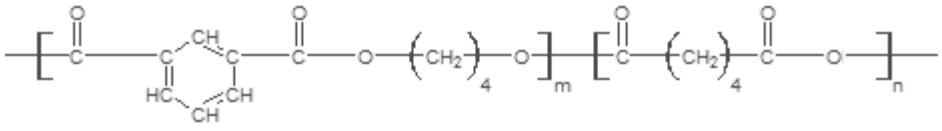
Biodegradable Plastics

Biodegradable plastics were introduced in the late 1980s to mitigate the growing problem of plastic disposal (Biodegradable Plastics Institute, 2003). Plastics deemed biodegradable include synthetic plastics (petroleum-based, though the majority of these do not degrade readily), bioplastics (from natural, renewable resources), or their blends (synthetic plastics blended with natural products or with bioplastics) (Plastics Europe, 2016). The term bioplastics comes from the concepts of biodegradable plastics and bio-based plastics. Bio-based plastics may be derived through modification of naturally occurring polymers (e.g. polusaccharides and cellulose from agricultural resources), polymers obtained through microbial production (e.g. polyhydroxyalkanoates (PHAs)), or polymers synthesized from biological feedstocks (e.g. polylactic acid (PLA)) (Lambert and Wagner, 2017). Initially, there was confusion and skepticism on the claims and performance of these products that did not biodegrade as expected. Early on, a lack of scientifically based test methods or standards allowed manufacturers to make unsubstantiated claims regarding the biodegradability of their products (Biodegradable Plastics Institute, 2003). Standards and methods have since been developed by the American Society for Testing and Materials (ASTM) to evaluate plastic degradability.

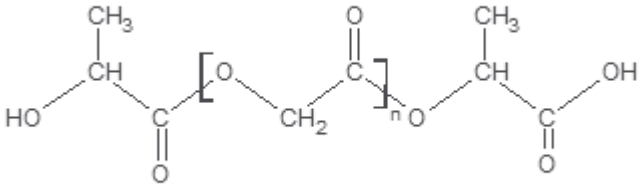
A biodegradable plastic is a material that is capable of being degraded by naturally occurring microorganisms such as fungi, bacteria, or algae (ASTM D20, 2012). In order to be considered compostable, the degradation of the plastic polymer in a compost setting must result in production of carbon dioxide, water, inorganic compounds and biomass at a rate that is consistent with the known materials being composted (ASTM D20, 2012). Under aerobic

conditions, such as in composting processes, microorganisms will use oxygen to oxidize carbon into carbon dioxide, which can be used as an indicator of plastic polymer degradation. This is the most common method of evaluating polymer degradation in laboratory tests (Shah et al. 2008). To be considered biodegradable, 90% of the organic components of the plastic (or of each organic constituent with a concentration higher than 1%) must be converted into carbon dioxide within the time frame of the trial (ASTM D20, 2012). It should be noted that a small portion of the polymer being degraded will be converted into microbial biomass, humus, and other natural products so it is rare that degradation of the plastic reaches 100% (Shah et al., 2008). These plastics also burn with a lower calorific value than that of synthetic plastics when incinerated. However, the production cost of biodegradable plastics are higher than those of synthetic plastics, and the current disposal systems cannot recover or equal the cost of production, so these biodegradable plastics are not an economically feasible recycling option for most manufacturing companies (Maeda et al., 2005).

Aliphatic polyesters are a promising type of biodegradable polymers due to their susceptibility to degradation by microorganisms. Poly(butylene adipate-co-terephthalate) (PBAT) (Fig. 1.2 A) is an aliphatic aromatic co-polyester that can easily be processed from petroleum-based resources while having similar mechanical properties to that of polyethylene. PBAT is certified as compostable by the Biodegradable Plastic Institute (BPI) according to the American Society for Testing and Materials (ASTM) specifications (Kijchavengkul et al., 2010; ASTM D20, 2012). It has a fast biodegradation rate, showing significant degradation within a year of inoculation in soil, water with activated sludge, and seawater (Cai et al., 2012). Poly(lactic acid) (PLA) (Fig. 1.2 B) is an aliphatic polyester that has good mechanical properties while being made from renewable resources and being easily degraded under aerobic conditions (Cai et al., 2012).



A)



B)

Figure 1.2: Monomer Structures of Poly(butylene adipate-co-terephthalate) (PBAT; A) and Poly(lactic acid) (PLA; B) (Drawn on ChemSketch).

1.4. Plastic Waste Management

A Statistics Canada survey of the waste management industry showed an increase in waste diversion expenditures from 2008 to 2010 (Statistics Canada 2013). This increase was primarily due to the increasing costs of landfills post closure/maintenance funds and operation of recycling facilities. However, the waste diverted to recycling/organics processing facilities showed a 3% decrease from 2008 to 2010, which was the first decrease in diversion since 2002 (Statistics Canada, 2013). The lifecycle of plastics products (Fig. 1.3) is more commonly a linear lifespan with landfilling being the final stage. However, with the proper use of recycling, incineration, and polymer degradation, the cycle can come full circle once again.

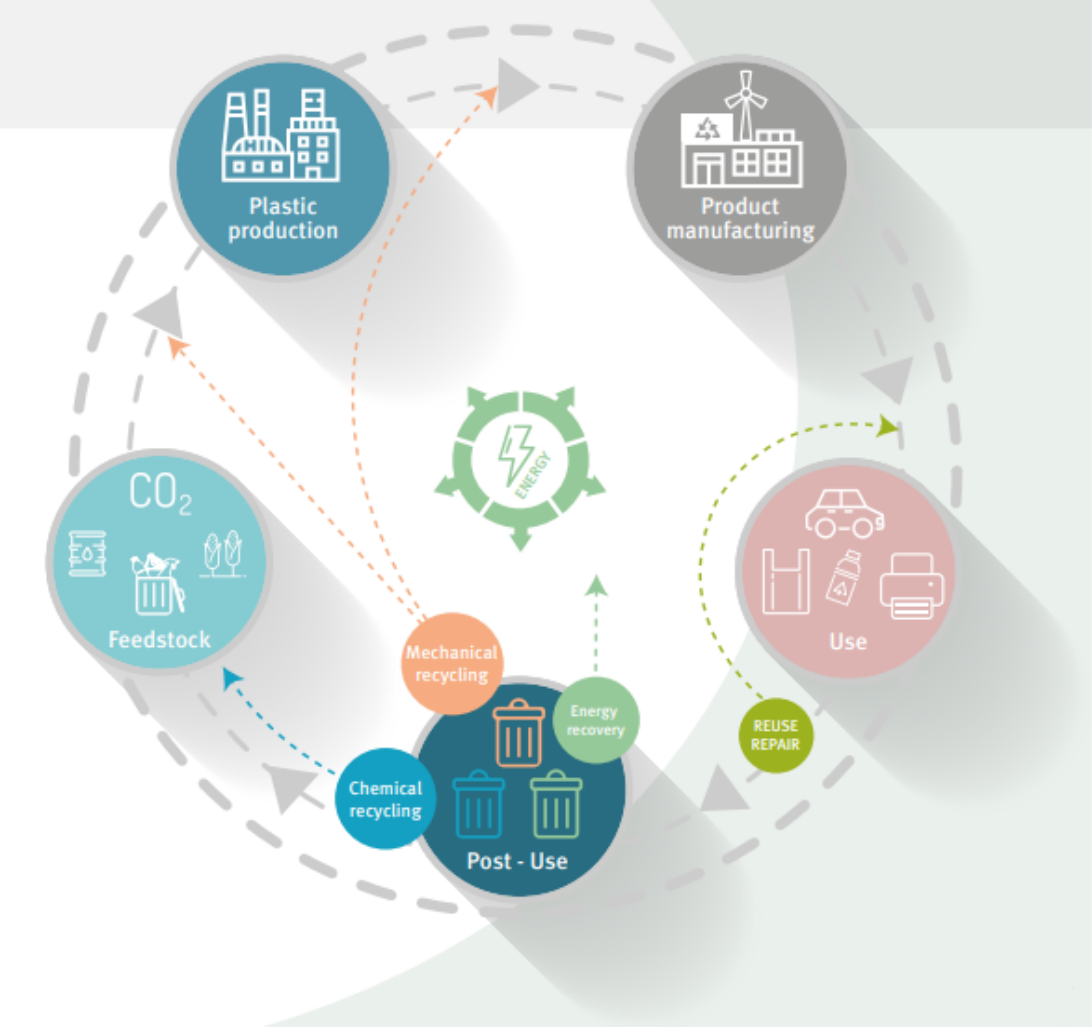


Figure 1.3: Lifecycle of Plastic Products (Plastics Europe, 2017).

1.4.1. Recycling

Recycling of plastics is a method of recovering the polymeric material and/or energy of the product. In order to properly recycle plastics, their collection and separation from other wastes is required, as well as separation of types and colors of plastic (Hopewell et al., 2009). Recycling consists of two processes: chemical and mechanical processing. Chemical processing proceeds by chemolysis of the plastic material using a compound that results in either total depolymerisation into monomers or partial depolymerisation into oligomers. This reaction can occur by hydrolysis (water), methanolysis (methanol), or glycolysis (ethylene glycol) (Awaja and Pavel, 2005). Mechanical processing is more cost effective than chemical processing, and uses several individual steps. These steps include the removal of contaminating materials that

can inhibit the recycling process and result in deterioration of the recovered product, drying to minimize the moisture content to reduce hydrolytic degradation of the recovered product, and melt processing to produce useful granules, though in the presence of contaminants this can result in thermal degradation causing a reduction in the molecular weight of the plastic (Awaja and Pavel, 2005). Though it makes sense environmentally to recycle, the economic losses due to the price of the recycled product (which is typically less than the original product) and the cost of recycling processes will favor landfill disposal.

1.4.2. Incineration

An alternate method of plastic disposal is incineration, which will result in either complete or incomplete combustion, with the resulting emissions depending on the composition of the polymer. Incineration does not require significant space and there is oppotunity for energy recovery in the form of heat (Webb et al., 2013). However, incomplete combustion can result in emissions such as carbon monoxide, hydrogen cyanide, ammonia, nitrous oxides, hydrogen chloride, sulfur dioxide, dioxins, and furans that can be hazardous in cases open burning or accidental fire. Even with complete combustion, the amount of released carbon dioxide and dioxins into the atmosphere contributes to global warming of the climate and pollution of the environment (Boettner et al., 1973).

1.4.3. Landfilling

Landfilling of plastic wastes not only prevents the recovery of their polymeric materials, but also contributes to the loss of space in landfills. Landfills occupy space that could otherwise be used for productive processes, such as agriculture. Plastics in landfills experience slow degradation and can persist for more than twenty years. This is due to the limited availability of oxygen, as landfills are designed to be anaerobic. The partial degradation plastics may experience would be due to thermal degradation, though this can be a source of many secondary environmental pollutants such as volatile compounds and endocrine disruptors (Webb et al., 2013). The leaching of these compounds into the surrounding environment is a significant concern.

1.5. Polymer Degradation

Polymer degradation is characterized by any physical or chemical change as a result of environmental factors such as light, temperature, moisture, chemical conditions (e.g. pH), or biological activity. These physical, chemical, and biological reactions result in deterioration of functionality due to bond scissions, destruction and formation of functional groups, and other subsequent chemical transformations. These reactions cause changes in material properties such as mechanical, optical, or electrical characteristics through cracking, erosion, discoloration, and phase separation (Singh and Sharma, 2008).

1.5.1. Photodegradation

Photodegradation uses UV light or high energy radiation to activate the electrons of the polymer, inducing degradation through oxidation, cross-linking and bond scissions (Chmela, 2013). UV radiation (290 to 400 nm) is responsible for direct photodegradation, causing photolysis (photo-chemical reactions) when light is absorbed by the polymer and directly causes chemical reactions, and is strong enough to cleave C–C bonds. Visible sunlight (400 to 760 nm) will accelerate degradation by heating, and infrared radiation (760 to 2500 nm) will accelerate thermal oxidation (Shah et al., 2008). Photo-oxidative degradation is considered one of the primary sources of damage exerted upon polymeric substrates at ambient conditions. In the presence of oxygen or ozone, photo-oxidative degradation is facilitated by radiation, leading to the formation of radicals that induce oxidation of the polymer. Oxygen in the form of carbonyl is incorporated into the polymer through Norrish Type I and II mechanisms (Fig. 1.4). This produces aldehydes, ketones and carboxylic acids from oxygenated species during photolysis reactions such as bond scission, cross-linking and secondary oxidation (Chmela, 2013). Norrish type II reactions lead to the formation of double bonds in the polymer chain (Sudhakar et al., 2007). Photodegradation reactions occur on the surface of the polymer, causing changes in the physical and optical properties of the plastic. The most damaging effects are the visual effect (yellowing), the loss of mechanical properties of the polymers, and the changes in molecular weight and the molecular weight distribution (Singh and Sharma, 2008). Photodegradation is slow in initiation, but fast in propagation (subsequent reactions) and is considered to be environmentally friendly without the use of high energy radiation (Shah et al., 2008).

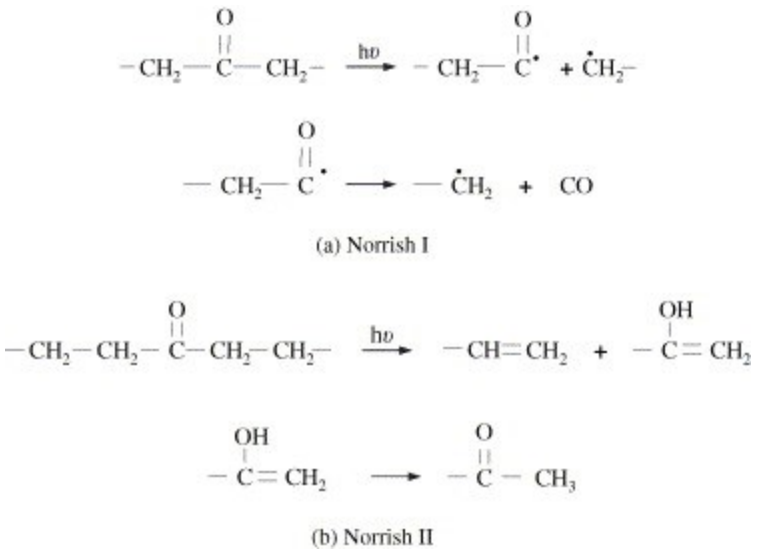


Figure 1.4: Norrish I and Norrish II Degradation Mechanisms: (a) Norrish I degradation can lead to chain scission or crosslinking; (b) Norrish II degradation leads to chain scission and formation of double bonds (Albertsson et al., 1987).

1.5.2. Thermal (Oxidative) Degradation

Thermal degradation of polymers causes molecular degradation as a result of overheating. The high temperatures cause the molecular backbone to cleave and react with itself, changing the properties of the polymer. These reactions occur throughout the bulk of the polymer, resulting in changes in physical and optical properties, such as reduced ductility and embrittlement, chalking, color changes, cracking, and a general reduction in most other desirable physical properties. Thermal degradation occurs rapidly, and involves changes to the molecular weight and molecular weight distribution of the polymer (Shah et al., 2008). Thermal degradation is considered oxidative degradation as it requires the presence of oxygen. Two main reactions occur during thermal degradation: random scission of links causing molecular weight reduction, and chain end scission of C–C bonds that generate volatile products (Singh and Sharma, 2008). The random scission will occur at any point of the chain, breaking off fragments with a low yield of monomers. The chain end scission occurs when the backbone bonds are weaker than the bonds of the side groups, and will successively release monomer units as the C–C bonds are broken along the backbone (Singh and Sharma, 2008).

1.5.3. Ozone-Induced Degradation

Under normal conditions, oxidative aging processes are very slow and the polymer retains its properties longer, but the presence of ozone (even in small concentrations) will accelerate the polymer aging process. Ozone induced degradation proceeds by intensive formation of oxygenated compounds on main chains containing C=C bonds, aromatic rings or saturated hydrocarbon links, causing changes to the molecular weight and mechanical and optical properties of the polymer. The reactions will then proceed through unstable intermediates that cause decomposition of macromolecules (Singh and Sharma, 2008).

1.5.4. Hydrolysis

Polyesters are degraded mainly by simple hydrolysis. The first stage of the degradation process involves non-enzymatic, random hydrolytic ester cleavage and its duration is determined by the initial molecular weight of the polymer as well as its chemical structure (Shah et al., 2008). Hydrolysis of the polymer backbone is most desirable since it produces low molecular weight by-products. Natural polymers undergo degradation by hydrolysis whereas synthetic polymers are water insoluble. They tend to be more crystalline and this property accounts for their water-insolubility. The primary mechanism for the biodegradation of high molecular weight polymers is the oxidation or hydrolysis by enzyme to create functional groups that improves its hydrophylicity. Consequently, the main chains of polymers are degraded resulting in polymers of low molecular weight and feeble mechanical properties, thus, making it more accessible for further microbial assimilation (Albertsson et al., 1987). Hydrolysis occurs at the ester linkages and this results in a lowering of the polymer's molecular weight, allowing for further degradation by microorganisms.

1.5.5. Biodegradation

Biodegradation is the biochemical transformation of compounds through mineralization by microorganisms such as bacteria, fungi, and algae. Biodegradation can be defined as a change in surface properties or loss of mechanical strength, assimilation by microorganisms, degradation by enzymes, and/or backbone chain breakage, resulting reduction in average molecular weight of the polymer (Singh and Sharma, 2008). Biodegradation can occur through any of these mechanisms alone or in combination with one another.

Abiotic degradation such as photodegradation, thermal degradation, oxo-biodegradation, and hydrolytic degradation of polymers may enhance their biodegradation. Microbial colonization can be promoted hrough physical disintegration, an increased surface area, a reduction in molecular weight, and/or the production of accessible functional groups. Biodegradation of polymers involve at least two classes of enzymes: extracellular and intracellular depolymerases (Gu, 2003). Extracellular enzymes secreted by microorganisms are responsible for the initial breakdown of long chain polymers into their smaller constituent molecules of short chains (e.g. oligomers, dimers, monomers). The short chain molecules can then be absorbed by microorganisms and mineralized by intracellular enzymes, producing water, carbon dioxide, and biomass under aerobic conditions, and methane, carbon dioxide, and biomass under anaerobic conditions (Shah et al., 2008).

The characteristics of the polymer and environmental conditions will affect the biodegradation process. Polymer characteristics such as mobility, crystallinity, molecular weight, type of functional groups, substituents present in the structure, and use of plasticizers or additives will affect polymer degradation. Generally, higher molecular weight polymers show decreased biodegradation. This is because a high molecular weight results in a sharp decrease in solubility, making the polymer unfavorable for microbial attack due to the inability of the microorganism to absorb large molecules through the cellular membrane (Gu, 2003). Dominant groups of microorganisms and degradative pathways of polymer degradation are determined by environmental conditions. Under aerobic conditions, aerobic microorganisms are responsible for degradation of polymers, with microbial biomass, carbon dioxide, and water as the final products. In contrast, under anoxic conditions, anaerobic microorganisms are responsible for polymer deterioration, producing microbial biomass, organic acids, carbon dioxide, methane gas, and water (Gu, 2003). Other environmental factors affecting biodegradation of polymers include temperature, moisture, and pH. The type of organisms involved also affect biodegradation due to the various functions of different enzymes used by the organisms and the substrate specificity of these enzymes. Enzymes are biological catalysts with high substrate specificities, meaning a particular enzyme will catalyze a particular reaction with high efficiency (Mueller, 2006).

1.6. *Aspergillus oryzae*

1.6.1. History of *A. oryzae*

Aspergillus oryzae is an ascomycete fungus of the family Trichococomacea that has been used for traditional Japanese fermentation products (such as rice wine, soy sauce, and soybean paste) as a koji mold, which consisted of both *A. oryzae* and the fermented material (indicating it had been cultivated without the knowledge that it is a microorganism). *A. oryzae* was first isolated from koji in 1876 by H. Ahlburg (Machida et al. 2008). *A. oryzae* is a filamentous fungus that is capable of secreting large amounts of enzymes, e.g. cutinase, making it well suited for biodegradable plastic degradation systems. It is presumed to be a domesticated species as a result of its use in fermentation processes, implying that it would not be found in nature though there have been documented cases. The presence of *A. oryzae* in nature could be due to dispersal of its spores after domestication because of the lack of controlled processes in earlier years. Two possible originations of the domesticated species are that it was imported from China during the Yayoi period (300 B.C. to A.D. 300) with fermentation technology, or that it has been isolated from *Aspergillus flavus*, which is a more dangerous species that produces aflatoxin, the most potent natural carcinogen (Machida et al., 2008). *A. oryzae* has no record of producing aflatoxins or other carcinogenic metabolites, and resulting fermented food products are aflatoxin-free. If it did originate from *A. flavus*, it is possible that the industrial use of *A. oryzae* has resulted in selection for genes that are safe for human consumption, or that *A. oryzae* had originally been selected for use in fermentation because it is a safe mutant. Zhang et al. (2005) compared *A. oryzae* and *A. flavus* and found that *A. oryzae* lacked the gene cluster required for aflatoxin biosynthesis. *A. oryzae* is generally regarded as safe (GRAS) by the Food and Drug Administration (FDA) in the USA, and its safety is supported by the World Health Organization (WHO) (Michida et al. 2008). In Canada, it is classified as a Risk Level 2 by the Public Health Agency of Canada (PHAC), which is defined as having a moderate risk to the individual and a low community risk. *A. oryzae* "can cause human disease but, under normal circumstances, is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures rarely cause infection leading to serious disease; effective treatment and preventive measures are available, and the risk of spread is limited." (PHAC, 2004).

1.6.2 Enzymatic Activity of *A. oryzae*

Cutinase

A. oryzae is a fungal phytopathogen that uses cutinase to hydrolyze the natural polyester material, cutin, which is present in the plant cuticle. The action of cutinase allows the fungus to penetrate the cuticular barrier on the surface of the host plant during the initial phase of infection (Carvalho et al., 1998).

Cutinase, a polyester-hydrolyzing enzyme, was first purified and characterized from fungi, and has also evolved in other microorganisms such as bacteria. Phytopathogenic fungi, such as *A. oryzae*, use cutinase to penetrate the cuticular barrier in the first steps of fungal infection. The enzymatic degradation of this cross-linked lipid-polyester matrix is caused by the hydrolysis of the ester bonds, producing smaller hydroxyl acids (Liu et al., 2010). Cutinases are used by fungi for either pathogenicity or saprophytic growth on cutin as a carbon source (Chen et al., 2013).

Structure and Function

Cutinases are carboxylic ester hydrolases, belonging to the class of serine esterases and are the smallest member in the superfamily of the α/β hyrolases (α/β proteins is a class of structural domains in which the secondary structure is composed of alternating α-helices and β-strands along the backbone; the β-strands are therefore mostly parallel). These enzymes are specific for the degradation of primary alcohol esters, the dominant linkage in cutin (Carvalho et al., 1998). Cutinase is a one domain molecule consisting of 197 residues with a hydrophobic core. This core includes a slightly twisted five-parallel-stranded β-sheet that is surrounded by four α-helices. It has a molecular weight of approximately 22 000 Da, and contains four invariant cysteines that form two disulfide bridges that aid in structure stability (Carvalho et al., 1999). The oxyanion hole is comprised of serine and glutamine backbone amides that are critical in polarizing the ester bond of the substrate and stabilizing the transition state of the formed substrate oxyanion (Liu et al., 2009).

The catalytic pocket of cutinase is divided into carboxylic acid and alcohol recognition sites. It contains serine in the active sites which are not buried under an amphipathic loop (hydrophobic lid), making them accessible to solvent and substrate even without an interfacial phase (Dutta et al., 2009). The lack of this hydrophobic lid (which is present in true lipases) exposes the active

site serine, making the active site larger and therefore capable of hydrolyzing high molecular weight substrates such as cutin and polyesters. The catalytic triad in the active site consists of a nucleophile (serine), a base (histidine), and an acid (aspartate) (Longhi and Cambillau, 1999).

Though cutinase is not a true lipase, it is able to hydrolyze lipid substrates, which is uncommon of esterases (Chen et al., 2013). Sharing the catalytic properties of lipases and esterases, cutinase shows activity with or without the presence of an oil-water interface. This feature makes cutinase an interesting biocatalyst in processes involving hydrolysis, esterification, and transesterification reactions (Pio and Macedo, 2009).

The action of the catalytic triad begins when the aspartate is hydrogen-bonded to the histidine, which increases its acid dissociation constant (pKa) and allows the histidine to act as a strong base that will deprotonate serine. The serine, acting as a nucleophile, will attack the carbonyl carbon of the ester and force its oxygen to accept an electron. This forms a tetrahedral intermediate that is stabilized by the positive charge of the amide group on the serine backbone. The intermediate collapses as the carbonyl is restored, causing histidine to donate its proton to the oxygen attached to the carbonyl, which then acts as an alcohol leaving group. A water molecule is introduced to donate a proton to the nitrogen of the histidine. The hydroxyl group then attacks the carbonyl carbon and forces its oxygen to once again accept an electron. This forms a second tetrahedral intermediate that collapses as the carbonyl is restored. The hydroxyl group isn't a good leaving group, so serine acts as the leaving group that forms a hydrogen bond between its oxygen attached to the carbonyl and the protonated nitrogen of the histidine. The resulting products are the catalytic triad and a carboxylic acid.

Stability and Applicability

The utility of enzymes (cutinases or others) in chemical reactions is commonly restricted by intolerance to high temperatures and the constraints of the substrate recognition pocket (Longhi and Cambillau, 1999).

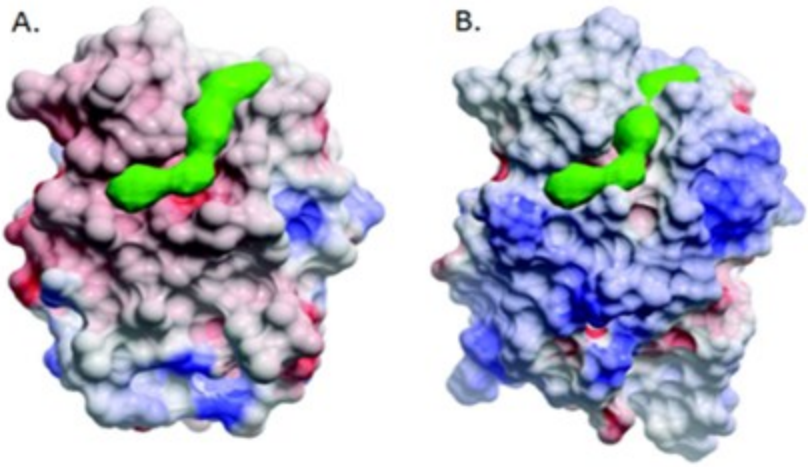


Figure 1.5: Electrostatic Surface Rendering of (A.) *A. oryzae* and (B.) *F. solani* Cutinases, Indicating the Continuous Groove (Green Mass) at the Active Site (Liu et al. 2009).

The cutinase structure of *A. oryzae* presents a continuous groove by the active site, as seen in Figure 1.5(A). This groove is longer in comparison to the widely studied cutinase from *Fusarium solani* (Fig. 1.5(B)), in which the groove is shallower and stops abruptly.

Ensuring that the cutinase enzyme is stable is important in its activity. There are two factors contributing to the cutinase enzyme's stability – an overall neutral surface charge in the crowning area by the active site and the presence of two disulfide bonds in the structure (Baker et al., 2012).

The overall surface charge of the protein is neutral at a neutral pH, but changes with changing pH. Under basic conditions, the surface charge will have a net negative charge, and under acidic conditions, it will have a net positive charge. However, the active site surrounding the crowning area will maintain a more neutral charge even in acidic conditions when the protein surface is positively charged. The cutinase structure will remain stable in a pH range of 4 to 9, though there is no activity at pH 4 to 5. This can be explained by the ionization state of the catalytic histidine, which must be deprotonated to then stabilize the serine of the catalytic triad (Carvalho et al.,1999). The two disulfide bridges between the invariant cysteines plays an important role in structure stability of cutinase, disruption of which will strongly affect the enzyme's conformation and activity when the catalytic triad is distorted and no longer close enough for catalysis (Carvalho et al., 1999).

A study by Liu et al. (2009) explored the structure and function of *A. oryzae* cutinase in comparison to the well-studied *F. solani* cutinase enzyme. It was observed in the crystallographic analysis that the *A. oryzae* cutinase possessed an additional disulfide bond and a topologically favored catalytic triad with a continuous and deep groove. The cutinase of *A. oryzae* was able to recognize and degrade longer chain substrates than the *F. solani* cutinase, and showed a preference for the hydrolysis of these longer chains. It was speculated that this preference could be attributed to the deep, continuous groove that extends across the active site of the enzyme. An enhanced thermotolerance was also observed in the *A. oryzae* cutinase, maintaining its level of activity when incubated even up to 40 ^{0}C, whereas the *F. solani* cutinase activity decreased with rising temperatures. This improved stability could be explained by the additional disulfide bond present in the *A. oryzae* structure (Liu et al., 2009).

The presence of the additional disulfide bond explained the refoldability of *A. oryzae* cutinase and its ability to degrade poly(capro lactone) (PCL) at a pH of 3.0, even though the enzyme's melting temperature is below 40 ^{0}C (the required temperature for PCL degradation experiments; Baker et al., 2012). This additional disulfide bond had not been previously reported for any cutinase or hydrolase structures (Liu et al., 2009).

Cutinases present unique characteristics, such as having catalytic properties of both lipases and esterases and consequently not requiring an interfacial phase. Additionally, they show stability in organic solvents and ionic liquids, and in free and microencapsulated in reverse micelles (Pio and Macedo, 2009), allowing for application in a wide variety of industries.

Effect of Mycelial Morphology on Enzymatic Activity

The morphological growth patterns of biofilms developed by filamentous fungi can affect the enzymatic activity of the fungus. In submerged cultures, filamentous fungi will cultivate themselves as freely dispersed mycelia (hyphal elements) or as pellets that are agglomerates of several hyphal elements. Pellet formation occurs by the coagulation of spores and/or hyphal elements (Carlsen et al., 1996). The morphology of the filamentous fungus can influence not only the growth rate of the fungus, but also the production process of proteins such as enzymes (Papagianni, 2004). Chemical factors that affect morphology include pH, temperature, carbon substrate type and concentration, levels of nitrogen and phosphate, and dissolved oxygen and

carbon dioxide. Physical factors include agitation systems, rheology of the medium, geometry of the fermentation reactor, and whether the system is batch, batch-fed, or continuous fed (Papagianni, 2004).

In production processes, particular morphological forms will increase or decrease the performance of the filamentous fungus. In a submerged culture, changes in morphology affect nutrient consumption and oxygen uptake. Filamentous growth as freely dispersed hyphal elements may result in highly viscous media which has a negative impact on the mass transfer properties of the fungus. Pelleted growth may result in a decrease in growth rate due to substrate and oxygen limitation in the dense core parts of the pellet (Jin et al., 1999).

The microscopic and macroscopic morphology of *A oryzae* was characterized as a function of pH by Carlsen et al. (1996). The agglomeration process of *A. oryzae* is pH dependent – pellets are formed at pH levels higher than 5, whereas the fungus exists as freely dispersed hyphal elements at lower pH values. The specific maximum growth rate of *A. oryzae* occurs between pH 3 to 7.

1.6.3. *A. oryzae* and Plastic Biodegradation

A. oryzae has been used in studies pertaining to the biodegradation of biodegradable plastics (e.g. PBAT) (Maeda et al., 2005; Hatheway and Price, 2014) and conventional plastics (e.g. PE) exposed to pro-oxidant pretreatment and UV irradiation (Konduri et al., 2011). *A. oryzae* was found to be more active towards PBAT in alkaline conditions (Maeda et al., 2005) and demonstrated increased esterase activity correlated with decreasing mass of PBAT/PLA film when incubated on solid substrate (Hatheway and Price, 2014).

1.7. Objectives

For the past three decades, worldwide research has focused on the biodegradation of plastics in order to remediate the large volumes of recalcitrant waste the human race has created.

The objective of the current study was to investigate the degradation activity of *A. oryzae* on both recalcitrant conventional polyethylene plastics and biodegradable poly(butylene adipate-co-terephthalate)/poly(lactic acid) blend plastics.

The objective of the first study was to evaluate the susceptibility of PBAT/PLA film to degradation by *A. oryzae*. The objectives were to assess how the i) addition of an enzyme inducing agent and ii) change in the initial pH of the growth medium impacted biodegradation of the plastics. The degradation was evaluated in terms of mass loss, changes in physical properties of the film surface, enzymatic activity of the fungus, and production of chemical degradation products. The degradation was described in terms of UV/Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), colorimetry, and gas chromatography mass spectrometry (GC-MS).

It was hypothesized that the addition of inducing agents would increase the degradation activity of *A. oryzae* by inducing the release of the extracellular esterase enzyme, cutinase, in the presence of the PBAT/PLA film. It was also hypothesized that by altering the initial pH levels of the growth medium, the degradation activity of *A. oryzae* would be affected. At lower pH levels (acidic conditions), there was expected to be less cutinase enzyme activity due to the overall neutral surface charge of the enzyme being disrupted.

The objective of the second study was to evaluate the susceptibility of polyethylene film to degradation by *A. oryzae* after different levels of UV exposure, with or without the presence of pro-oxidants. The susceptibility to biodegradation following pro-oxidant pretreatment and UV irradiation was assessed by correlating the generation of oxidation products on the surface of the film to the action of pro-oxidant pretreatment and UV irradiation, and the consequent loss of oxidation products following incubation with *A. oryzae.* The degradation was evaluated in terms of mass loss, enzymatic activity, production and consumption of oxidation products, and changes in physical properties of the film. The degradation was described in terms of UV/Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), and colorimetry.

It was hypothesized that when exposed to pro-oxidant pretreatment and UV irradiation would increase susceptibility of the PE film to biodegradation by *A. oryzae*. It was also hypothesized that upon increasing UV irradiation exposure, the generation of oxidation products would increase and thus enhance biodegradation of the PE film.

Chapter 2: Effect of Inducing Agents and pH on Biodegradation of PBAT/PLA by *Aspergillus oryzae*

2.1. Introduction

Biodegradable plastic bags are accepted in some municipalities as part of the source separated organics stream. However, anecdotal evidence suggests that degradation of the plastic bags is inconsistent leading to concerns with equipment malfunctions (clogging machinery) and contamination of compost piles. There is evidence that agricultural mulch films composed of poly(butylene adipate-co-terephthalate) (PBAT) and poly(lactic acid) (PLA) plastics are biodegradable but standards for optimum conditions for field-scale applications are limited in the scientific literature. The standardization of biodegradability of these plastics will lead to their large-scale application in agriculture. Currently, field studies have shown significant degradation of these films due to UV irradiation through photo-oxidation after periods of just two weeks (Kijchavenkul et al., 2008)

Biodegradable plastics (e.g. PLA) are materials capable of being degraded by naturally occurring microorganisms, and are an environmentally friendly alternative to nondegradable plastics (e.g. PE). The induction of an extracellular cutinase from *Aspergillus nidulans* was studied by Castro-Ochoa et al. (2012), in which it was determined that olive oil (the main component being triolein, a long-chain triglyceride) at a concentration of 0.1% caused the largest increase in carboxylic ester hydrolase activity. The concentration of the inducer is important, as shown when olive oil used at a concentration of 1.0% inhibited hydrolase activity. This inhibitory effect has been attributed to the presence of hydrolysis products generated, to feedback inhibition, and to oxygen transfer interference. The cutinase of *A. nidulans* showed high sequence identity and similarity to other fungal cutinases, particularly those of the *Aspergillus* genus. When the sequences were compared to *A. oryzae* cutinases, the highest percentages of similarity (88.7%) were obtained (Castro-Ochoa et al., 2012). Optimum culture medium for production of cutinase was studied by Li et al. (2010) using a fractional factorial design. Ultrasonication was used to disrupt the cell in order to measure the intracellular cutinase activity, an optimum production of 145.27 U/mL was obtained with a culture media containing 33.92 g/L glucose, 30.92 g/L yeast extract, and 0.76 g/L isopropyl β-D-1-thiogalactopyranoside (IPTG) (Li et al., 2010). Other studies examining the effect of added nutrients to culture media to influence cutinase production have found that starch

had a negative influence, while yeast extract and potassium phosphate had a strong positive influence (Rispoli and Shah, 2007).

The present research is focused on a specific fungus, *A. oryzae*, that has demonstrated the ability to degrade biodegradable plastics, using the plastic as a source of nutrition for its growth. The application of this fungus in the degradation of biodegradable plastics used in agricultural mulch films was investigated. The study examined addition of inducing substrates to culture media and variation of initial pH levels of culture media to influence enzymatic activity on biodegradable plastics. The present research analyzed enzymatic activity using spectrophotometry to determine optimal conditions for degradation, as well as degradation products formed and metabolites released by the fungus during the process using gas chromatography-mass spectrometry. The data collected from this study will have applications in the diversion of biodegradable plastics from the municipal waste stream, and potential elimination of this plastic waste. This fungus may prove to be an organism capable of handling problems associated with biodegradable plastics in the composting system, allowing for more efficient and successful degradation of these plastics that would otherwise get stuck in machinery or remain in composting piles/landfills for years.

2.2. Objectives and Hypothesis

The objective of the current study was to evaluate the susceptibility of PBAT/PLA film to degradation by *A. oryzae*. The objectives were to assess how the i) addition of an enzyme inducing agent and ii) change in the initial pH of the growth medium impacted biodegradation of the plastics. The degradation was evaluated in terms of mass loss, changes in physical properties of the film surface, enzymatic activity of the fungus, and production of chemical degradation products. The degradation was described in terms of UV/Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), colorimetry, and gas chromatography mass spectrometry (GC-MS).

It was hypothesized that the addition of inducing agents would increase the degradation activity of *A. oryzae* by inducing the release of the extracellular esterase enzyme, cutinase, in the presence of the PBAT/PLA film.

It was also hypothesized that by altering the initial pH levels of the growth medium, the degradation activity of *A. oryzae* would be affected. At lower pH levels (acidic conditions), there was expected to be less cutinase enzyme activity due to the overall neutral surface charge of the enzyme being disrupted.

2.3. Materials and Methods

2.3.1. Cultivation Methods

Fungal Strain

Aspergillus oryzae RIB40 (ATCC-42149) (Machida 2002) was used as the enzyme source for all studies with PBAT/PLA degradable plastics. A pure culture of *A. oryzae* (RIB40) was obtained from the American Type Culture Collection (ATCC No. 42149) and rehydrated in sterilized water to produce a spore suspension. The suspension was cultured on potato dextrose agar (PDA). The cultures were incubated at 21 °C and biomass was observed microscopically after 7 days.

Media Preparation

Potato dextrose agar (PDA) (Difco) was prepared by adding 39.0 g PDA powder per 1 L distilled H_2O and autoclaving at 121 °C. The autoclaved media was poured onto petri dishes in a laminar flow hood and allowed to solidify. PDA was used for preparation of colonies from the rehydrated spore suspension. In addition, 312-Czapek's Dox Agar (CDA) fungal minimal media containing 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 30.0 g commercial grade sucrose, and 15.0g Agar No. 1 powder per 1 L distilled H_2O and autoclaving at 121°C (ATCC) was prepared for cultivation of colonies for use in the incubation studies. In the biodegradation assays, 312-Czapek's Dox (CD) broth fungal minimal media containing 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 30.0 g commercial grade sucrose per 1 L distilled H_2O and autoclaving at 121°C was prepared (omitting the sucrose when plastic film was used as a carbon source).

Spore Suspension Preparation

Conidiospores of *A. oryzae* RIB40 were prepared from colonies on CDA plates. The conidiospore suspension (1×10^9 conidiospores in 0.025% Tween 80 and 0.8% NaCl) was

inoculated into 100 ml CD broth medium, and the final concentration of conidiospores was 1×10^6 spore/mL (approximated using a Haemocytometer).

Plastic Samples

Plastic samples were obtained from Indaco (Ontario, Canada). Indaco Manufacturing has been producing biodegradable film since the late 1980's. Their agricultural mulch film is made from a blend of biodegradable polymers that are certified to ASTM D6400-12 standard as biodegradable and compostable under laboratory conditions. The films were composed of a PBAT and PLA blend. The PBAT/PLA film was cut into 10cm x 10cm pieces, massed, washed in 70% ethanol for sterilization, and dried in a laminar flow hood prior to incubation with *A. oryzae*.

Inducers

Three inducing agents were tested (virgin olive oil, yeast extract, and KH_2PO_4) to determine the effect of added inducing agents on biodegradation. Olive oil (at 0.5% and 1.0% v/v), yeast extract (at 0.8% w/v), and KH_2PO_4 (at 0.5% w/v) were added to the growth medium prior to incubation with *A. oryzae*. A positive (*A. oryzae* inoculated CD broth with plastic film) and negative (uninoculated CD broth with plastic film) control, without addition of inducing agents, were also examined.

pH

Five initial pH levels were tested, including 5.5, 6, 6.5, 7, and 7.5, to determine the effect of initial pH on biodegradation. A positive control (*A. oryzae* inoculated with plastic film) with unadjusted pH (8.0) and a negative control (*A. oryzae* inoculated without plastic film) with unadjusted pH (8.0) were used.

Biodegradation Assay

The biodegradation assay was performed in 250-mL Erlenmeyer flasks by adding 100 µL of pure active *A. oryzae* culture into 100 mL of CD broth containing PBAT/PLA samples as a carbon source in separate flasks. The biodegradation assay was performed with positive (*A. oryzae* inoculated CD broth with plastic film) and negative (uninoculated CD broth with plastic film) controls. The flasks were randomly assigned to locations within the agitator and incubated at 21

°C at 100 rpm for 15 days with constant agitation. Three replicates were prepared for each inducing agent and initial pH level.

2.3.2. Analytical Methods

Film Harvest

After exposure to *A. oryzae* for 15 days, PBAT/PLA films were harvested, washed in 70% ethanol to remove as much biomass as possible, dried at 45°C, and weighed. Each of the films was compared with the corresponding uncultured material (negative control) as well as with the cultured material.

Determination of Mass Loss

Recovered PBAT/PLA films were analyzed for degradation by weight loss before and after microbial treatment using an electronic balance. The percentage change in mass of the inoculated PBAT/PLA samples is given by the formula:

$$\% \text{ change in mass} = (\text{initial mass} - \text{final mass})/\text{initial mass} \times 100\% \qquad \text{Eq. (2.1)}$$

pH Change

The pH of the culture media was measured using a Accumet Excel XL50 Dual Channel pH/Ion/Conductivity Meter (Fisher Scientific) prior to and following incubation with *A. oryzae* to determine the change in pH of the culture media.

Enzymatic Activity

Esterase activity was assayed in a substrate solution containing 100 mM Tris-HCl buffer (pH 7.3), 0.03% TritonX-100 and 0.01% p-nitrophenyl butyrate (pNPB) (Verma et al. 2013). Following incubation, 2 mL of liquid media was removed by pipet and filtered through Whatman No. 1 filter paper to remove solids. The extract was then added to the pNPB substrate solution, a known esterase substrate that produces p-nitrophenol (pNP) via enzymatic hydrolysis, which can then be measured spectrophotometrically at 405 nm. The enzyme assay was carried out for 20 minutes (absorbance measured in 5 minute intervals) in triplicate. A control containing only the pNPB substrate solution was assayed with the experimental units in order to account for any error due to the substrate solution itself.

Standards of pNP were prepared at concentrations of 0.0, 0.84, 1.68, 2.52, 3.36, 4.20, 8.40, 16.80, 25.20, and 42.0 µmol/L in order to perform a linear regression analysis of pNP concentration versus absorbance at 405 nm.

The linear regression analysis of concentration of pNP (µmol) from absorbance at 405nm was found to significantly contribute to the model ($p < 0.001$) (Table A-1). The regression equation for the concentration of pNP (µmol) from absorbance at 405 nm was found to be $y = 0.6667 + 58.67x$ (Table A-2). The measurements for absorbance at 405 nm were then used to calculate the production of pNP in µmol through the regression equation.

Visual Observation of Morphology

The macroscopic morphology of the fungal growth was visually observed at the end of the incubation period to qualitatively evaluate the effect of initial pH on growth morphology and subsequently degradation and enzymatic activity. The morphology was classified as i) free filamentous, indicating free floating, dispersed hyphal elements within the submerged culture; ii) pellets (small, large), indicating spherical agglomerates of several hyphal elements; and iii) biofilm, indicating fungal adhesion to the plastic sample.

Gas Chromatography Mass Spectrometry Analysis of Metabolomics

Indaco film was inoculated in four samples of the CDA biomass cultivars and growth on the film was observed after 7 days. Biomass found on the film was collected for metabolomic analysis in replicates of three and analyzed in free form. Fungal growth from separate CDA cultivars containing solely media was also collected as a control in replicates of three and analyzed in free form. Samples were prepared using a method adopted from Liebeke and Bundy (2012), which consisted of the addition of two extraction solutions (1:1 acetonitrile:methanol and 2:2:1 acetonitrile:methanol:water) where the samples were each sonicated and centrifuged. The supernatants were the nitrogen evaporated in a fume hood, and the internal standards were added (Leucine D-3, Glucose U ^{13}C, Myristic Acid, at 1 mmol concentrations). The samples were again nitrogen evaporated, followed by addition of the derivitizing agent (hydroxylamine hydrochloric acid in pyridine) and MSTFA.

Samples were analyzed by GC-EIMS with Autosampler (Agilent 5975 GC-MSD system) in negative ion mode. The instrument was tuned using PFTBA and the MS operating characteristics verified using the tune evaluation in the Agilent ChemStation software before each run. A 1 µL sample was injected in splitless mode, following five pre-injection solvent washes (4 µL acetone), two sample washes (1 µL) and three sample pumps (1 µL), followed by five post-injection solvent washes (4 µL acetone). Helium was the carrier gas, and the instrument was set at a constant flow of 1 mL/min (pressure = 8.2317 psi). The column was an Agilent DB-5MS 5% phenyl 95% methylpolysiloxane (dimensions of 30 m length × 0.25 mm i.d. × 0.25 µm film thickness) with 10 m of DuraGuard column. Temperature program for the run time of 37.5 minutes was as follows: 60°C hold 1 minute, ramp 10°C min^{-1} to 325°C, hold 10 min. The injection port was held at 250°C. Transfer line, ion source, and quadrupole temperatures were 290°C, 230°C, and 150°C respectively. The mass spectrometer was operated in full scan mode (m/z 50-650) following a 5.9 minute solvent delay, with a resolution of at least 1 scan per second. Chromatograms were obtained using the Agilent ChemStation software, and it was also used for preliminary analysis to determine retention times and identifying ions for each standard, and to confirm the presence of metabolites in the samples using the NICD Mass Spectral Library.

To facilitate the use of bioinformatics tools to manage data, chromatograms were converted into .netCDF format using OpenChrom, a freely available, multi-platform chromatography-MS workstation. Another free program, MZmine 2, was used for data pre-processing. Peaks were extracted using the retention times and quantifying ions determined from analysis of standards. Peaks were smoothed prior to deconvolution using the baseline cut-off method. Peak alignment was achieved using the Join aligner, with an m/z tolerance of 0.001 and a 2.5 minute retention time window, since there was some variability in the retention time of certain metabolites as determined during preliminary analysis.

Gap filling was accomplished through MZmine by looking for missed peaks in the raw data, and a retention time correction was also applied. Data was filtered by removing duplicate peaks with the same retention time and m/z. The final aligned and integrated peak list from MZmine was exported into .CSV format where it was manually checked for correctness (based on expected retention times and quantifying ions for each metabolite) prior to statistical analysis. The abundance for each metabolite was divided by the mass of fungus extracted for each sample.

This was then divided by the abundance of the internal standard leucine-d3 in each sample. This results in an abundance corrected for mass and expressed relative to the abundance of the internal standard. These values were then expressed as a percentage of the media treatment for visual comparison.

2.3.3. Statistical Analysis

Assumptions

To ensure the validity of a statistical test, the assumptions of normality, constant variance, and independence must be considered and met. If the assumptions are not met, the appropriate transformations were applied to the individual datasets, and the results were back transformed.

Paired Comparison

A paired comparison design was used to determine significant differences between the initial and final mass of the PBAT/PLA film and the pH of the growth medium. The observations for mass and pH before and after incubation with *A. oryzae* were paired due to being from the same experimental unit, thus eliminating variability from the difference between objects and improving precision. The before and after sampling on the same experimental unit allowed for a high covariance due to high similarity, and this design did not require randomization (Montgomery, 2013). A paired t-test was performed for each dataset that included i) the initial and final mass of the PBAT/PLA film to determine if there was a significant difference in the mass loss after incubation with *A. oryzae* in the presence of inducing agents, ii) the initial and final mass of the PBAT/PLA film to determine if there was a significant difference in the mass loss after incubation with *A. oryzae* with different initial pH levels of the culture medium, and iii) the initial and final pH of the culture medium to determine if there was a significant difference in the pH after incubation with *A. oryzae* with different initial pH levels of the culture medium. The significance level was set at 95% ($\alpha = 0.05$). The datasets were analyzed using Minitab® 18 Statistical Software. The assumptions were met without transformation for all datasets.

One-Way Analysis of Variance

The analysis of variance (ANOVA) is used when there are more than two treatments or levels of simultaneous interest. It involves the analysis of a single factor of interest with more than two treatments or levels of the factor by testing the equality of several means. The one-way ANOVA

was run for the response variable percent change in mass to compare the means for each treatment (or level).

If the ANOVA produces a p-value that is less than the level of significance and the null hypothesis is rejected, a multiple means comparison must be carried out to determine which mean is different. Tukey's test is a conservative test that uses pairs of means to determine significant differences and form letter groupings while controlling experimental error. Fisher's least significant difference (LSD) test is the least conservative test and is based on pairs of means to form letter groupings. The magnitude of experimental error should be used to determine which pairwise comparison to employ in multiple means comparison. This can be evaluated by using the coefficient of variation ($\mathrm{CV} = \frac{S}{\bar{y}}$) or by considering the nature of the experiment. If the coefficient of variation is less than 10% (low experimental error), then Tukey's Honestly Significant Difference (HSD) method would be appropriate. If the coefficient of variation is greater than 10% (moderate to high experimental error), the Fisher's Least Significant Difference (LSD) method would be appropriate. It is also important to consider the numerical size of the values; if the values are small, the coefficient of variation would be inflated due to a small $\bar{y}$ as the denominator.

The significance level was set at 95% ($\alpha = 0.05$). The datasets were analyzed using Minitab® 18 Statistical Software.

One-Way Analysis of Variance of Effect of Inducing Agent on Percent Change in Mass of Film

The single factor being analyzed was inducing agent with $a = 6$ levels of treatment and with $n = 3$ replicates. This allowed for the comparison of percent change in mass for all treatments. A one-way ANOVA was performed on the percent change in mass (Eq. 2.1) data in order to determine significant differences between the treatments. The assumptions of normality and constant variance were satisfied without transformation. The assumption of independence was satisfied through randomization.

One-Way Analysis of Variance of pH Effect on Percent Change in Mass of Film

The single factor being analyzed was initial pH with $a = 7$ levels of treatment and with $n = 3$ replicates. This allowed for the comparison of percent change in mass for all treatments. A one-way ANOVA was performed on the percent change in mass (Eq. 2.1) data in order to determine significant differences between the initial pH treatments. The assumptions of normality and constant variance were satisfied without transformation. The assumption of independence was satisfied through randomization.

One-Way Analysis of Variance of pH Effect on ΔpH

The single factor being analyzed was initial pH with $a = 7$ levels of treatment and with $n = 3$ replicates. This allowed for the comparison of percent change in mass for all treatments. A one-way ANOVA was performed on the ΔpH (initial pH – final pH) data in order to determine significant differences between the initial pH treatments. The assumptions of normality and constant variance were satisfied without transformation. The assumption of independence was satisfied through randomization.

One-Way Analysis of Variance of Location Effect on Metabolites

The single factor being analyzed was location with $a = 2$ levels of treatment (plastic and media; for each significant metabolite) and with $n = 3$ replicates. This allowed for the comparison of abundance for all treatments. A one-way ANOVA was performed on the metabolite data in order to determine significant differences between the plastic and the media. The assumptions of normality and constant variance were satisfied without transformation. The assumption of independence was satisfied through randomization.

Linear Regression Analysis

The linear regression model with one independent variable was used to describe the relationship between absorbance at wavelength 405 nm and the concentration of pNP in μmol by means of pNP standards. The linear regression model is described as $y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$ where the parameter β_0 is the intercept of the plane, β_1 is the partial regression coefficient that measures the expected unit change in y per unit change in x_1, and ε is the error terms associated with the model and is assumed to follow a normal distribution with a mean of 0 and a variance of σ^2 ($\varepsilon_i \sim NID(0, \sigma^2)$), with a constant variance, and be independent of one another (Montgomery, 2013).

Repeated Measures Analysis

Repeated measures are used when the same response variable is measured on the same experimental unit on two or more occasions. This repetition can be either temporal (different points in time) or spatial (different points in space). Repeated measures analysis is common in biological and agricultural research when studying growth/decay curves, or examining the long-term effect of treatments. However, repeated measures violates the assumption of independence of the error terms. This is because time, which is included in the model as a factor, cannot be randomized. As measurements are taken, subsequent measurements will depend on the previous measurement. The consequence of this violation is the same as that of violating constant variance; the mean squares error (MSE) will be inflated, causing the F-value to be deflated, and subsequently causing the p-value to be inflated. This violation will result in difficulty detecting significant differences between treatment effects.

The proc mixed method in SAS® employs univariate analysis using the most appropriate covariance structure. Identifying the most appropriate covariance structure is a key step in the proc mixed method, as the covariance structure embodies the type of relationship of the values over time. The most common temporal covariance structures are i) Autoregressive order 1 (AR(1)), when the correlation between time points is ρ, which is constant, both ρ and σ^2 stay constant, but is not appropriate for unequally spaced time points; ii) Compound symmetry (CS), when a group of time points depends on another group of time points and/or there is different dependence within different groups of time points; and iii) Unstructured (UN), when there is no pattern to ρ, but variance is constant (verified with transformation). To determine the most appropriate covariance structure, the model should be run in SAS with several possible covariance structures. The structure that gives the smallest absolute value for Akaike's Information Criterion (AIC) or Schwarz's Bayesian Criterion (SBC) (depending on statistical religion) is the most appropriate structure. Both AIC and SBC are model-fit criteria, which are basically log values that penalize for the number of parameters estimated. Note that while SBC imposes a heavier penalty than AIC, it is less commonly used by statisticians.

Repeated measures analysis was carried out on the enzymatic activity data collected from the pNP assay using the proc mixed procedure in SAS® 9.3. In order to determine the appropriate covariance structure, the model was run for each AR(1), CS, and UN covariance structures. The

models were evaluated based on the convergence criteria being met, the number of iterations (*i*), the AIC value, and the assumptions of normality and constant variance being met.

Repeated Measures Analysis of Enzymatic Activity of Inducer Effect

The response variables for all treatments at Time = 0 was omitted. Due to the large number of data points being equal to zero, the normality and constant variance were skewed.

The assumptions of normality and constant variance were met when a cube-root transformation was applied to the response variables. The autoregressive order 1 (AR(1)) covariance structure was found to be the most suitable, having the lowest |AIC| and meeting convergence criteria (Table A-7). The compound symmetry (CS) covariance structure was also found to be suitable, having an equal |AIC| and meeting convergence criteria, however the AR(1) covariance structure was selected due to the time points being equally spaced. The proc mixed procedure was then carried out using SAS® on the transformed data. The untransformed data is presented below.

Repeated Measures Analysis of Enzymatic Activity of Initial pH Effect

The response variables for all treatments at Time = 0 was omitted. Due to the large number of data points being equal to zero, the normality and constant variance were skewed.

The assumptions of normality and constant variance were met when a square-root transformation was applied to the response variables. The unstructured (UN) covariance structure was found to be the most appropriate, having the lowest |AIC| (Table A-17). The proc mixed procedure was then carried out using SAS® on the transformed data. The untransformed data is presented below.

2.4. Results and Discussion

2.4.1. Effect of Inducing Agent on Biodegradation

Effect of Inducing Agent on Percent Change in Mass

A paired t-test was performed on the initial and final mass measurements of the PBAT/PLA film to determine if there was a significant difference in the mass loss after incubation with *A. oryzae*. The paired t-test is summarized in Tables A-3 and A-4. The mass loss determined between the initial and final PBAT/PLA plastic samples was found to be significant ($p < 0.001$).

The percent change in mass was then further analyzed to examine differences between the inducing agent treatments. The one-way ANOVA is summarized in Table A-5. The percent change in mass resulting from the inducing agent treatments and controls was found to be not significant ($p = 0.195$). This information indicates that there were no significant differences when comparing all treatments, including inducing agents and both positive and negative controls (with and without inoculation of *A. oryzae*, respectively).

Measurement of changes in mass can help support other indications of biodegradation via enzymatic degradation of the film. In the present study, mass loss in the film was recorded even without exposure to *A. oryzae*, but this was not significantly different than the values obtained for the inoculated (positive control) and inducer treatments. This could be due to a number of factors, including residual fungal biomass on the plastic surface, residual fungal biomass within the plastic film, or residual moisture on the plastic surface.

Effect of Inducing Agent on Enzymatic Activity

The proc mixed procedure was performed on the response variables (μmol pNP) of enzymatic activity. The type three test of effects is summarized in Table A-8. It is evident that the two-way interaction between the main effects of inducing agent and time significantly affected the production of pNP as a result of esterase activity ($p < 0.001$). A multiple means comparison was performed on the data using Fisher's LSD method. It is also important to note that the experiment was carried out in a lab setting, minimizing the effects of uncontrollable factors. A larger production of μmol pNP over time indicates increased esterase activity as a result of the hydrolysis of pNPB. The increased esterase activity indicates an increased production of esterase enzymes by *A. oryzae*.

Table 2.1: Grouping Information of Effect of Inducing Agent on Means of pNP Production (µmol) of *A. oryzae* (± Standard Deviation) at Four Different Time Points with Six Different Inducing Agent Treatments Using the Fisher's LSD Method and 95% Confidence for Two-Way Interaction of Time*Inducer (N = 3)

	Inducer					
Time (mins)	**Control (-)**	**Control (+)**	**Olive Oil (0.5%)**	**Olive Oil (1.0%)**	**Yeast Extract**	**KH_2PO_4**
5	$0.67\pm0.0_{Ba}$	$0.76\pm0.17_{Ba}$	$7.08\pm1.05_{Ab}$	$5.52\pm2.37_{Ac}$	$7.58\pm1.29_{Ac}$	$0.67\pm0.0_{Ba}$
10	$0.67\pm0.0_{Ca}$	$0.95\pm0.26_{Ca}$	$9.33\pm6.83_{Bb}$	$10.38\pm4.70_{ABb}$	$14.36\pm2.83_{Ab}$	$0.67\pm0.0_{Ca}$
15	$0.74\pm0.04_{Ba}$	$1.26\pm0.34_{Ba}$	$18.15\pm3.31_{Aa}$	$15.30\pm5.93_{Aab}$	$20.48\pm4.32_{Aab}$	$0.67\pm0.0_{Ba}$
20	$0.76\pm0.05_{Ba}$	$1.60\pm0.16_{Ba}$	$23.08\pm3.90_{Aa}$	$20.67\pm7.38_{Aa}$	$25.97\pm4.59_{Aa}$	$0.67\pm0.0_{Ba}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns)

It is evident from Fisher's LSD that the inducing agents olive oil 0.5%, olive oil 1.0%, and yeast extract resulted in the highest production of pNP as a result of increased esterase activity. The KH_2PO_4 inducer displayed no esterase activity, having had an inhibiting effect on *A. oryzae*. As expected, the negative control resulted in the smallest production of µmol pNP (the production of µmol pNP calculated was likely due to a small change in the absorption at 405 nm, and not actual production of µmol pNP, as to the treatment had not been inoculated with *A. oryzae*).

2.4.2. Effect of Initial pH on Biodegradation

Effect of Initial pH of the Culture Media on Percent Change in Mass

A paired t-test was performed on the initial and final mass to determine if there was a significant difference in the mass loss after incubation with *A. oryzae*. The paired t-test is summarized in Tables A-9 and A-10. The difference between the initial and final masses was found to be significant ($p < 0.001$).

The percent change in mass was then further analyzed to determine if there were significant differences between the initial pH treatments. The one-way ANOVA is summarized in Table A-11. The percent change in mass resulting from the initial pH and controls was found to be significantly different ($p < 0.001$), indicating that there was a significant difference in percent change in mass for different initial pH treatments.

The coefficient of variation (Table A-12) was used to determine the appropriate multiple means comparison method. A moderate to high coefficient of variation (>10%) suggested the use of Fisher's LSD method. It is also important to note that the experiment was carried out in a lab setting, minimizing the effects of uncontrollable factors.

Table 2.2: Grouping Information of Effect of Initial pH of the Culture Media on Means of Percent Change in Mass (%) of PBAT/PLA Film (± Standard Deviation) Exposed to *A. oryzae* at Five Different Initial pH Levels Using the Fisher's LSD Method and 95% Confidence

pH	N	Mean Percent Change in Mass (± SD)	Grouping
Control + (8.0)	3	17.92 ± 1.76	A
7.5	3	20.85 ± 2.31	A
7.0	3	12.11 ± 2.41	B
6.5	3	12.78 ± 2.06	B
6.0	3	10.62 ± 1.64	B
5.5	3	5.90 ± 2.29	C

Means that do not share a letter are significantly different.

From Fisher's LSD method (Table 2.2), it is evident that the neutral and alkaline initial pH values resulted in a higher percent change in mass than that of the acidic initial pH values. The increase in percent mass loss with increasing initial pH level is due to more complex factors involved in the degradation process, including enzymatic activity and stability, fungal morphology, as well as factors affecting mass measurements such as residual fungal biomass within and on the film and residual moisture on the film. The factors involved in the degradation process are further investigated by means of enzymatic activity assays, changes in the pH of the culture media, and observation of the fungal morphology.

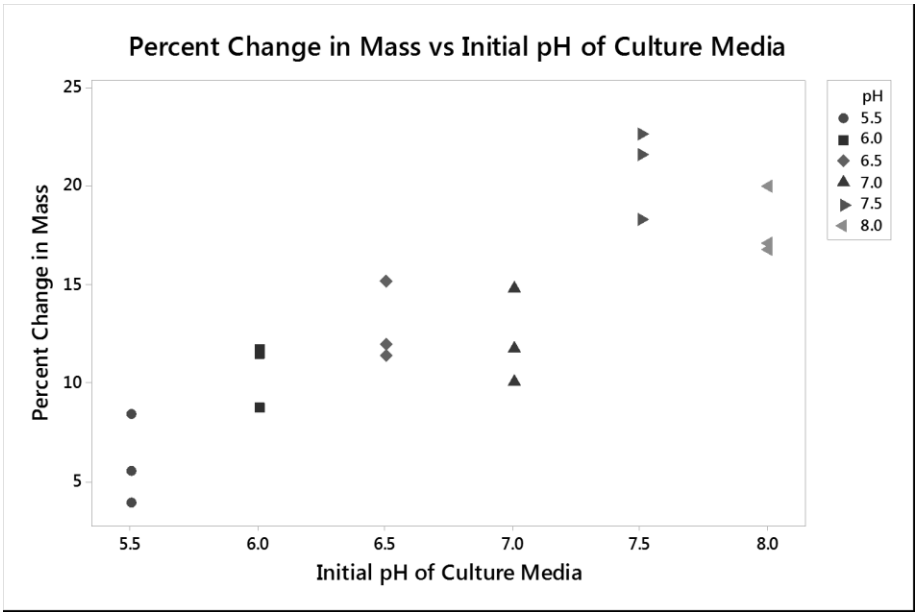


Figure 2.1: Percent Change in Mass as a Function of Differences in Initial pH of the Culture Media

From Figure 2.1, it is evident that the percent change in mass increases as the initial pH of the culture media increases. The percent change in mass appears to plateau at the neutral range, indicating that at lower pH levels, there is some inhibition of degradation in terms of percent mass loss.

Effect of Initial pH of the Culture Media on Change in pH

A paired t-test was performed on the initial and final pH to determine if there was a significant difference in the ΔpH after incubation with *A. oryzae*. The paired t-test is summarized in Tables A-13 and A-14. The difference between the initial and final pH was found to be significant ($p < 0.001$).

The change in pH (ΔpH) was then further analyzed to determine if there were significant differences between the initial pH treatments. The one-way ANOVA is summarized in Table A-15. The change pH resulting from the initial pH and controls was found to be significant ($p < 0.001$), indicating that there was a significant difference in ΔpH for different initial pH treatments.

The coefficient of variation (Table A-16) was used to determine the appropriate multiple means comparison method. A moderate to high coefficient of variation (>10%) suggested the use of Fisher's LSD method. It is also important to note that the experiment was carried out in a lab setting, minimizing the effects of uncontrollable factors.

Table 2.3: Grouping Information of Effect of Initial pH of the Culture Media on Means of ΔpH of the Culture Media (± Standard Deviation) of PBAT/PLA Film Exposed to *A. oryzae* at Five Different Initial pH Levels Using the Fisher's LSD Method and 95% Confidence

pH	N	Mean ΔpH (± SD)	Grouping
Control + (8.0)[1]	3	0.43 ± 0.05	A
Control – (8.0)[1]	3	0.48 ± 0.06	A
7.5[1]	3	0.09 ± 0.02	D
7.0[1]	3	0.01 ± 0.08	D
6.5[2]	3	0.16 ± 0.08	CD
6.0[2]	3	0.28 ± 0.04	B
5.5[2]	3	0.25 ± 0.12	BC

Means that do not share a letter are significantly different.
[1]Decrease in pH presented as absolute values.
[2]Increase in pH presented as absolute values.

The values for ΔpH are given in terms of absolute values for comparison. From Fisher's LSD method (Table 2.3), it is evident that the two control treatments (Control + and Control -) give the highest ΔpH of growth media and are not significantly different from one another. The pH of the controls showed a decrease in pH, resulting in a more neutral pH following incubation, whereas the initially neutral treatments experienced negligible change in pH.

The initial pH treatments of 6.5, 6.0, and 5.5 experienced an increase in pH (presented in Table 2.3 as absolute values), indicating that the pH of the growth media of these treatments increased as a result of incubation with *A. oryzae*. This finding aligns with the hypothesis that at lower initial pH levels, there would be decreased enzymatic activity resulting in a lower impact on the pH of the media. This is due to the overall neutral surface charge of the enzyme being affected by the surrounding acidic environment, resulting in the overall surface charge being in a positive state and the disruption of the stability of the enzyme (Baker et al. 2012).

The higher changes (decreases) in pH can be attributed to increased enzymatic activity, which would align with the findings of the percent change in mass that there was increased activity with increasing pH. These results show that the fungal strain was potentially more active within the unaltered pH treatments, as those treatments showed a greater increase in acidity than that of the more acidic altered pH values. This can be explained by the production of organic acids by *A.*

oryzae as a result of enzymatic activity in biodegradation in the slightly more basic unaltered media, as the production of organic acids by filamentous fungi acidify growth media (Liaud et al. 2014).

Effect of Initial pH of the Culture Media on Enzymatic Activity

The proc mixed procedure was performed on the response variables of enzymatic activity. The type three test of effects is summarized in Table A-18. The two-way interaction between the main effects was not found to be significant ($p > 0.05$), however, the main effects of time and pH individually significantly affected the production of pNP as a result of esterase activity ($p < 0.0001$). A multiple means comparison was performed on the data using Fisher's LSD method. A larger production of μmol pNP over time indicates increased esterase activity as a result of the hydrolysis of pNPB. The increased esterase activity indicates an increased production of esterase enzymes by *A. oryzae*.

Table 2.4: Grouping Information of Effect of Initial pH of the Culture Media on Means of pNP Production (µmol) of *A. oryzae* (± Standard Deviation) at Four Different Time Points Using the Fisher's LSD Method and 95% Confidence for Main Effect of Time

Time (minutes)	N	Mean µmol pNP (±SD)	Grouping
5	21	0.76 ± 0.176	C
10	21	0.86 ± 0.23	C
15	21	1.07 ± 0.44	B
20	21	1.58 ± 0.84	A

Means that do not share a letter are significantly different.

From Fisher's LSD method (Table 2.4), it is evident that there was a significant increase in µmol pNP production over time. The highest production of µmol pNP was observed at the end of the 20 minute time period, as was expected. There appeared to be a delay in µmol pNP production within the first 10 minutes, which is illustrated by the 5 and 10 minute times not being significantly different.

Table 2.5: Grouping Information of Effect of Initial pH of Culture Media on Means of pNP Production (µmol) of *A. oryzae* (± Standard Deviation) at Five Different pH Levels Using the Fisher's LSD Method and 95% Confidence for Main Effect of Initial pH

pH	N	Mean µmol pNP (± SD)	Grouping
Control + (8.0)	12	1.48 ± 1.03	A
Control – (8.0)	12	0.77 ± 0.20	C
7.5	12	1.17 ± 0.40	AB
7.0	12	1.20 ± 0.52	AB
6.5	12	0.91 ± 0.58	BC
6.0	12	1.04 ± 0.33	ABC
5.5	12	0.90 ± 0.47	BC

Means that do not share a letter are significantly different.

From Fisher's LSD method (Table 2.5), it is evident that the initial pH treatments of 7.5, 7.0, 6.0, and the positive control (pH 8.0) resulted in the highest production of µmol pNP and were not significantly different from one another. The more neutral initial pH treatments (7.5, 7.0, positive control) were expected to show an increased production of µmol pNP, as the cutinase enzyme is more active at a neutral range. As expected, the negative control resulted in the smallest production of µmol pNP (the production of µmol pNP calculated was likely due to a small change in the absorption at 405 nm, and not actual production of µmol pNP).

Maeda et al. (2005) studied the effect of pH on the enzymatic activity of cutinase purified from *A. oryzae* cultures on pNPB and found the enzyme exhibited maximum esterase activity at pH 9.0, with stability through the pH range 6.0-11.0. The findings of the current study align with this information as the esterase activity (production of pNP) is greatest at pH 8.0 and only marginally less in the lower pH levels studied.

Visual Observations

The macroscopic morphology of *A. oryzae* grown in submerged cultures at different initial pH values was observed visually and are described in Table 2.6.

Table 2.6: Effect of Initial pH on Visual Observations of Macroscopic Morphology of *A. oryzae* Grown in Submerged Cultures with PBAT/PLA Film

pH	Visual Observations
Control + (8.0)	Opaque media with biofilm and filamentous growth
Control - (8.0)	Translucent media
7.5	Clear media with biofilm and large pellets
7.0	Clear media with biofilm and large pellets
6.5	Clear media with biofilm and thick filamentous extensions
6.0	Translucent media with filamentous growth and small pellets
5.5	Opaque media with filamentous growth

The morphology observed in the current study aligns with the characterization of the microscopic and macroscopic morphology of *A. oryzae* by Carlsen et al. (1996). The study described the agglomeration morphology as being a function of pH; pellet formation occurs at pH levels high than 5, whereas the fungus exists as freely dispersed hyphal elements at lower pH values. In the present study, freely dispersed hyphal elements were observed at pH 5.5 (Fig. 2.2), but at higher pH levels biofilm as well as large filamentous pellets were observed (Fig. 2.3, 2.4).

Figure 2.2: Filamentous fungal growth observed visually at pH 5.5

Figure 2.3: Biofilm with small pellet fungal growth observed visually at pH 6.5

Figure 2.4: Biofilm fungal growth observed visually at pH 7.5

Villena et al. (2007) studied the morphological effect of *Aspergillus niger* in submerged cultures and biofilm cultures on enzymatic activity and found that the fungal biofilm was efficient for enzyme production. The visual observations of fungal growth and esterase activities of the current study align with this finding, illustrating that the biofilm growth of *A. oryzae* within the neutral pH range produced greater esterase activity.

2.4.3. Metabolomic Analysis

From the metabolomics analysis, ten metabolites expressed by *A. oryzae* were found to be significantly different between the PBAT/PLA film and media growth substrates (Fig. 2.5).

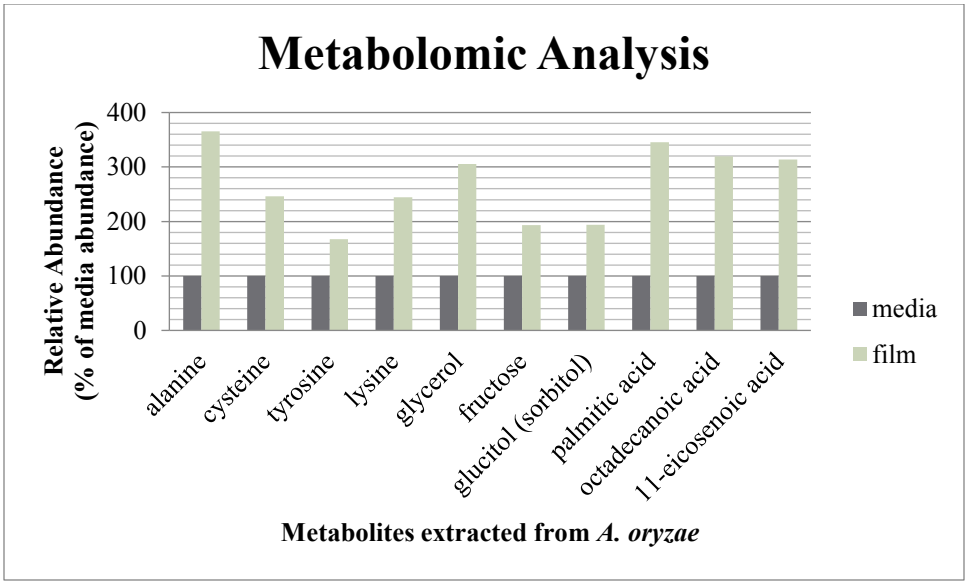


Figure 2.5: Metabolites Extracted from *A. oryzae* in Relative Abundance of Media

The significantly different metabolites can be grouped by their similarities: amino acids (p = 0.004, 0.017, 0.023, 0.004 for alanine, cysteine, tyrosine, lysine); carbohydrates (p = 0.008, 0.011, 0.012 for glycerol, fructose, gluticol); and fatty acids (p = 0.002, 0.002, 0.006 for palmitic acid, octadecanoic acid, 11-eicosenoic acid). The higher levels of these cellular metabolites in the film treatment may indicate higher levels of activity towards the film than towards the media. The increased presence of amino acids may indicate higher levels of protein synthesis, speculated to be associated with enzyme production. The higher levels of carbohydrates could be attributed to accumulation of energy storage compounds. Increased levels of glycerol and fatty acids may mean that triglycerides are being produced to make more cell membranes, hence more activity near the film. Though more than 96% of the genes of *A. oryzae* are uncharacterized, approximately 80% of the characterized genes involved in metabolism are within secondary metabolism (synthesis of bioactive compounds) and extracellular enzymes (Anderson 2014). The profiled metabolites are listed in Table A-19 and the one-way ANOVA for individual metabolites are summarized in Tables A-20 to A-40.

2.5. Conclusions

An active esterase was secreted by *A. oryzae* when in the presence of synthetic polyesters PBAT and PLA. Due to the active enzyme being previously reported as cutinase for polyester

degradation (Maeda et al. 2005), the esterase can be tentatively identified as cutinase. This enzyme can be isolated and further analyzed for its industrial applications.

The change in mass of the plastic films (though not a direct measure of degradation) supports the indication that biodegradation is occurring. The films were qualitatively observed to be more brittle following incubation, indicating a change in physical and chemical composition of the mulch films.

The effect of inducing agents on the biodegradation of PBAT/PLA film by *A. oryzae* exhibited negligible differences in the percent change in mass of the PBAT/PLA films, however, it is not a direct measurement of biodegradation. The enzymatic activity of *A. oryzae* was displayed a more detailed representation of the effect of the inducing agents, where it was evident that yeast extract and olive oil (at both 0.5 and 1.0%) inducing agents showed the greatest production of pNP as a result of esterase activity, and a fifteen-fold increase from that of the positive control. The KH_2PO_4 inducer displayed no esterase activity, having had an inhibiting effect on *A. oryzae*.

The effect of initial pH of the culture media on the biodegradation of PBAT/PLA film by *A. oryzae* showed an increase in percent change in mass with increasing initial pH level, with a 20% decrease in mass at a pH of 7.5. The percent change in mass appeared to plateau at the neutral pH range, indicating some inhibition of degradation at lower pH levels. The change in pH of the culture media provided more insight as to the activity of the fungus, having experienced a decrease in pH from the basic initial culture media, and the largest change in pH in terms of magnitude. The acidic initial culture media experienced a lesser change in pH, though the change was an increase in pH to a more neutral pH, and the initially neutral culture media experienced negligible change in pH. The greater changes in pH can be attributed to increased enzymatic activity, which would align with the findings of the percent change in mass that there was increased degradation with increasing pH.

The enzymatic activity assay further displayed increased degradation activity with increasing pH, as the initial pH treatments in the neutral range produced the greatest amount of pNP. These findings align with the literature as the cutinase enzyme is more active in the neutral range. The

acidic initial pH levels resulted in less degradation as the overall neutral surface charge of the cutinase enzyme was disrupted, and the surface charge changed with changing pH.

Metabolomic analysis showed increased activity towards the PBAT/PLA film when compared to the minimal media, resulting in 10 significantly increased metabolites. The increased presence of amino acids indicates potentially higher levels of protein synthesis, while the higher levels of carbohydrates could be attributed to accumulation of energy storage compounds, and the increased levels of glycerol and fatty acids may mean that triglycerides are being produced to make more cell membranes.

Considering the aforementioned use of these "biodegradable" films being primarily agricultural, their outdoor exposure to UV irradiation and consequently photo-oxidation would render them more susceptible to biodegradation following their useful life. This is important to consider as *A. oryzae* has demonstrated significant biodegradation of these PBAT/PLA films without UV irradiation, prompting the speculation of increased biodegradation following their outdoor application.

Chapter 3: Effect of Pro-Oxidant and Artificial Weathering on Biodegradation of Polyethylene by *Aspergillus oryzae*

3.1. Introduction

Commercial polyethylene (PE), a common component of consumer products, is the largest volume polymer produced globally and has a global production of 90 million tonnes per year (Plastics Europe, 2016). PE is composed of long hydrocarbon chains that are hydrophobic and without any functional groups, making them inaccessible to microorganisms. Synthetic polymers (e.g. PE) have been labeled as recalcitrant, meaning that they are resistant to microbial or enzymatic attack. These plastics are extremely resistant to oxidation and biodegradation because they contain antioxidants and stabilizers.

Pro-oxidants aid in the insertion of oxygen atoms into the polymer chain during photo-oxidation. The most active pro-oxidants are those based on metal combinations capable of yielding two metal ions of similar stability and with an oxidation number differing by one unit, e.g. Mn^{2+}. Thus, the material degrades via a free radical chain reaction involving oxygen from the atmosphere. The primary products are hydro peroxides, which can either thermolyse or photolyse under the catalytic action of a pro-degradant, leading to chain scission and the production of low molecular mass oxidation products, such as carboxylic acids, alcohols, ketones, and low molecular mass hydrocarbons (Konduri et al., 2011).

While PE is easily recycled, products used in agriculture or some industrial conditions can become dirty or contaminated which makes it unacceptable for recycling. Burning of these plastics, a common but illegal disposal method, can release toxic gases and landfilling is an unsustainable option. There is demand for methods to degrade low density polyethylene (LDPE) and high density polyethylene (HDPE) polymers which are low cost and have low environmental impacts. The addition of pro-oxidants and the use of UV irradiation can lead to oxidation and chain scission producing certain functional groups which make the polymer more available to microorganisms. *Aspergillus oryzae* is a filamentous fungus that has been shown to degrade pro-oxidant and UV pretreated PE (Konduri et al., 2011).

Intertape Polymer Group (IPG) is a multi-national company specializing in the development and manufacture of film-based, pressure-sensitive, and water-activated polyethylene and specialized polyolefin films, coated fabrics, tapes, and packaging systems for industrial and retail use. The annual revenue of IPG exceeds $158 million, with facilities across North America, including a manufacturing facility in Truro, Nova Scotia, Canada. IPG specifically is targeting opportunities for end-of-life management of their commercial plastic products, while also looking for innovative solutions within the manufacturing component of their processes. Their long-life film RU88X-6 NovaShield® II with ArmorKote™ is a heavyweight fabric intended for applications requiring UV stability such as membrane structures and alternate daily landfill covers. The single use short-life film R94W NovaThene® Eco-Media SSP is a lightweight, recyclable polyethylene fabric intended for single sheet banner print applications (Robbin Spencer, Intertape Polymer Group, Personal Communication, 2015).

The present research is focused on a fungus, *A. oryzae*, that has demonstrated the ability to degrade synthetic plastics, using the plastic as a source of nutrition for its growth. The application of this fungus in the degradation of recalcitrant plastics was investigated. By addition of pro-oxidants, recalcitrant plastics are made more susceptible to photo-oxidation and subsequently biodegradation. The study investigated the effect of the pro-oxidant on plastic biodegradability after various UV irradiation exposure periods. The present research analyzed the enzymatic activity using spectrophotometry to determine optimal conditions for degradation. Fourier transform infrared analysis was used to investigate changes in the functional groups on the plastic surface. The current research will provide data on the potential of microbial degradation of conventionally used plastics. This research will help inform a new framework for development of degradable plastic and specifications for environmental management of waste plastic products. The outcome of this research may provide some direction for integration of stable fungal spores within the matrix of manufactured plastic for timed degradation of the products.

3.2. Objectives and Hypothesis

The objective of the current study was to evaluate the susceptibility of polyethylene film to degradation by *A. oryzae* after different levels of UV exposure, with or without the presence of

pro-oxidants. The susceptibility to biodegradation following pro-oxidant pretreatment and UV irradiation was assessed by correlating the generation of oxidation products on the surface of the film to the action of pro-oxidant pretreatment and UV irradiation, and the consequent loss of oxidation products following incubation with *A. oryzae.* The degradation was evaluated in terms of mass loss, enzymatic activity, production and consumption of oxidation products, and changes in physical properties of the film. The degradation was described in terms of UV/Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), and colorimetry.

It was hypothesized that when exposed to pro-oxidant pretreatment and UV irradiation would increase susceptibility of the PE film to biodegradation by *A. oryzae*. It was also hypothesized that upon increasing UV irradiation exposure, the generation of oxidation products would increase and thus enhance biodegradation of the PE film.

3.3. Materials and Methods

3.3.1. Cultivation Methods

Fungal Strain

Aspergillus oryzae RIB40 (ATCC-42149) (Machida 2002) was used as the enzyme source for all studies with PE plastics. A pure culture of *A. oryzae* (RIB40) was obtained from the American Type Culture Collection (ATCC No. 42149) and rehydrated in sterilized H_2O to produce a spore suspension. The suspension was cultured on potato dextrose agar (PDA). The cultures were incubated at 21 °C and biomass was observed microscopically after 7 days.

Media Preparation

Potato dextrose agar (PDA) (Difco) was prepared by adding 39.0 g PDA powder per 1 L distilled H_2O and autoclaving at 121 °C. The autoclaved media was poured onto petri dishes in a laminar flow hood and allowed to solidify. PDA was used for preparation of colonies from the rehydrated spore suspension. In addition, 312 Czapek's Dox Agar (CDA) fungal minimal media containing 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 30.0 g commercial grade sucrose, and 15.0 g Agar No. 1 powder per 1 L distilled H_2O and autoclaving at 121 °C (ATCC) was prepared for cultivation of colonies for use in the incubation studies. In the biodegradation assays, 312 Czapek's Dox (CD) broth fungal minimal media containing 3.0 g

$NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 30.0 g commercial grade sucrose per 1 L distilled H_2O and autoclaving at 121 °C was prepared (omitting the sucrose when plastic film was used as a carbon source).

Spore Suspension Preparation

Conidiospores of *A. oryzae* RIB40 were prepared from colonies on CDA plates. The conidiospore suspension (1×10^9 conidiospores in 0.025% Tween 80 and 0.8% NaCl) was inoculated into 100 ml CD broth medium, and the final concentration of conidiospores was 1×10^6 spore/mL (approximated using a Haemocytometer).

Polyethylene Samples

Polyethylene (PE) samples were received from an Intertape Polymer Group manufacturing facility (Truro, Nova Scotia, Canada). Two types of PE film were used in the study: i) long-life film RU88X-6, and ii) short-term single use film R94W. The long-life RU88X-6 film contains a woven HDPE skrim, or a string mesh made of nylon woven into plastic sheets for reinforcement, with a LDPE coating on each side. It is a white film (available in a range of colors) with a thickness of approximately 0.59 mm (± 10%; ASTM D1777) and contains UV stabilizer UV100. The single use short-life R94W contains a woven HDPE skrim with a LDPE coating on each side. It is a white/grey film with a thickness of approximately 0.13 mm (± 10%; ASTM D1777) and contains UV stabilizer UV100 (Robbin Spencer, Intertape Polymer Group, Personal Communication, 2015).

Eleven treatments of each PE film type were analyzed: (1) untreated PE, (2) untreated PE with *A. oryzae*, (3) PE treated with UV irradiation for 500 h and *A.oryzae*, (4) PE treated with UV irradiation for 1000 h and *A. oryzae*, (5) PE treated with UV irradiation for 1500 h and *A. oryzae*, (6) PE treated with UV irradiation for 2000 h and *A. oryzae*, (7) PE treated with manganese stearate and *A. oryzae*, (8) PE treated with manganese stearate, UV irradiation for 500 h, and *A.oryzae*, (9) PE treated with manganese stearate, UV irradiation for 1000 h, and *A.oryzae*, (10) PE treated with manganese stearate, UV irradiation for 1500 h, and *A.oryzae*, and (11) PE treated with manganese stearate, UV irradiation for 2000 h, and *A.oryzae*.

Pro-oxidant pretreatment of polyethylene samples

To study the effect of pro-oxidant exposure on the biodegradability of PE film after different levels of UV exposure, PE film used in the study was chemically pretreated for two days in the pro-oxidant solution, which acts as a photo inducer, before subjecting films to UV irradiation. Maganese stearate was used to study the effect of pro-oxidant pretreatment on the susceptibility to biodegradation of polyethylene. Prior to pretreatment, PE films were cut into pieces (approximately 10 x 10 cm), weighed, disinfected in 70% ethanol for sterilization, and dried in a laminar-flow hood (Konduri et al., 2011).

UV irradiation of polyethylene samples

To stimulate partial photolysis during the natural weathering of polyethylene exposed to sun, chemically pretreated and untreated PE samples were subjected to partial photolysis in a QUV accelerated weathering tester (Q-Lab Model QUV/se). The PE was subjected to a program of alternating cycles of UV light and moisture at controlled, elevated temperatures. This simulated the effects of sunlight using special fluorescent UV lamps, and the effects of dew and rain with condensing humidity and/or water spray.

The QUV exposure irradiance was 1.35 W/nm/m^2 (UVA 340 nm lamps), where one hour of exposure equates to 14.6 hours of accelerated aging. (25 days exposure in the QUV accelerated weathering tester = 365 days accelerated aging). Individual PE films were exposed to UV irradiation representing five different time periods: (i) 0 h, (ii) 500 h (equivalent to 6 months), (iii) 1000 h (equivalent to 12 months), (iv) 1500 h (equivalent to 18 months), and (v) 2000 h (equivalent to 24 months).

Biodegradation Assay

The biodegradation assay was performed in 250-mL Erlenmeyer flasks by adding 100 µL of pure active *A. oryzae* culture into 100 mL of CD broth containing chemically (pro-oxidant, UV irradiation) pretreated and untreated polyethylene samples as a carbon source in separate flasks. The biodegradation assay was performed with positive (*A. oryzae* inoculated CD broth with chemically treated/untreated PE film) and negative (uninoculated CD broth with untreated PE film) controls. The flasks were randomly assigned to locations within the agitator and incubated

at 21 °C at 100 rpm for 30 days with constant agitation. Three replicates were prepared for each pretreated and untreated PE film.

3.3.2. Analytical Methods

Film harvest

After exposure to *A. oryzae* for 30 days, LDPE films were harvested, washed in 70% ethanol to remove as much biomass as possible, dried at 45 °C and massed. Each of the films with and without chemical treatment was compared with the corresponding uncultured material (negative control) as well as with the cultured material.

Determination of Mass Loss

Recovered LDPE films were analyzed for degradation by weight loss before and after microbial treatment using an electronic balance. The percent change in mass of the inoculated LDPE samples is given by the formula:

Percent change in mass = (initial mass – final mass)/initial mass x 100% (Eq. 3.1)

Colorimetry

Color change was measured as an indication of degradation by weathering and by microbial action. A Konica Minolta CR400 colorimeter was used to measure and determine changes in the lightness of the sample (L*) and color coordinates; redness (a*) in the green-red axis and yellowness (b*) in the blue-yellow axis. The color indexes were measured in triplicate on each experimental unit, following different stages of the experiment; prior to pro-oxidant treatment, post pro-oxidant treatment, prior to UV irradiation, post UV irradiation, prior to incubation with *A. oryzae*, and post incubation. The color change is expressed through the differences of the color indexes;

$$\delta L = L^* - L_i^* \; ; \; \delta a = a^* - a_i^* \; ; \; \delta b = b^* - b_i^* \quad \text{(Eq. 3.2)}$$

where *i* indicates the initial value (prior to pro-oxidant treatment, UV irradiation, incubation with *A. oryzae*). From these algebraic increments, the so-called total color change, ΔE, a positive quantity corresponding to a distance in the (L*, a*, b*) space, can be computed;

$$\Delta E = \sqrt{(\delta L^2 + \delta a^2 + \delta b^2)} \quad \text{(Eq. 3.3)}$$

(Darabi et al., 2012)

Enzymatic Activity

Esterase activity was assayed in a substrate solution containing 100mmol Tris-HCl buffer (pH 7.3), 0.03% TritonX-100 and 0.01% p-nitrophenyl butyrate (pNPB) (Verma et al. 2013). Following incubation, 2 mL of liquid media was removed by pipet and filtered through Whatman No. 1 filter paper to remove solids. The extract was then added to the pNPB substrate solution, a known esterase substrate that produces p-nitrophenol (pNP) via enzymatic hydrolysis, which can then be measured spectrophotometrically at 405 nm. The enzyme assay was carried out for 60 minutes (absorbance measured in 10 minute intervals) in triplicate. A control containing only the pNPB substrate solution was assayed with the experimental units to account for any error due to the substrate solution itself.

Standards of pNP were prepared at concentrations of 0.0, 0.84, 1.68, 2.52, 3.36, 4.20, 8.40, 16.80, 25.20, and 42.0 µmol in order to perform a linear regression analysis of pNP concentration versus absorbance at 405 nm.

The linear regression analysis of concentration of pNP (µmol) from absorbance at 405nm was found to significantly contribute to the model ($p < 0.001$) (Table A-1). The regression equation for the concentration of pNP (µmol) from absorbance at 405 nm was found to be $y = 0.6667 + 58.67x$ (Table A-2). The measurements for absorbance at 405 nm were then used to calculate the production of pNP in µmol through the regression equation.

Fourier Transform Infrared Spectroscopy

Infrared spectra of polyethylene samples at different stages of the experiment were obtained with an FTIR Perkin Elmer Spectrum One spectrometer in attenuated total reflectance (ATR) mode with a diamond crystal collecting 32 scans. Each spectrum was obtained within the range of 4000-650 cm^{-1} with a wavelength resolution of 4 cm^{-1}. Changes in the surface structure of the polyethylene following different stages of the experiment were analyzed; prior to pro-oxidant treatment, post pro-oxidant treatment, prior to UV irradiation, post UV irradiation, prior to

incubation with *A. oryzae*, and post incubation. The structural changes in the film surface due to both abiotic and biotic exposures were investigated using FTIR spectroscopy.

Carbonyl indices were used as a parameter to monitor the degree of degradation of PE film. The carbonyl groups with peaks ranging from 1630cm^{-1} to 1840cm^{-1} were observed at each stage of exposure. The carbonyl, ketone carbonyl, ester carbonyl, and vinyl bond indices for PE were calculated using the following formulas:

Carbonyl bond index $$CI = \frac{absorption\ at\ 1710cm^{-1}}{absorption\ at\ 1465cm^{-1}}$$ (Eq. 3.4)

Ketone carbonyl bond index $$KI = \frac{absorption\ at\ 1715cm^{-1}}{absorption\ at\ 1465cm^{-1}}$$ (Eq. 3.5)

Ester carbonyl bond index $$ECI = \frac{absorption\ at\ 1740cm^{-1}}{absorption\ at\ 1465cm^{-1}}$$ (Eq. 3.6)

Vinyl bond index $$VBI = \frac{absorption\ at\ 1640cm^{-1}}{absorption\ at\ 1465cm^{-1}}$$ (Eq. 3.7)

where the intensity of absorption at 1710, 1715, 1740, 1640, and 1465 cm^{-1} corresponds to carbonyl, ketone carbonyl, ester carbonyl, vinyl bond, and methylene bonds, respectively (Albertsson et al., 1987; Sudhakar et al., 2007; Fotopoulou and Karapanagiot, 2015).

3.3.3. Statistical Analysis

Assumptions

To ensure the validity of a statistical test, the assumptions of normality, constant variance, and independence must be considered and met. If the assumptions are not met, the appropriate transformations were applied to the individual datasets, and the results were back transformed.

Factorial Design Analysis

Factorial designs involve the study of the effects of two or more factors, wherein each complete trial or replicate of the experiment, all possible combinations of the levels of factors are investigated. The effect of a factor is the change in response produced by a change in the level of a factor. These primary factors of interest in the experiment are known as the main effects. When

the difference in response between the levels of one main effect is not the same at all levels of the other main effect, there is an interaction between the factors.

The analysis of variance general linear model (ANOVA GLM) procedure was used to analyze both the main effects and interaction effects using statistical software (Minitab®). Sources of variation included variation within the treatment, variation among interactions, and the error associated with the main effects and interactions. The sum of squares addressing the overall variability of the data, degrees of freedom and mean square values were found for each main effect and interaction effect as well as the error. An F-test was then employed to determine the resulting p-value for each of the three main effects, three two-way interactions, and one three-way interaction. If the p-value was less than the level of significance and the alternative hypothesis was accepted, then the interactions or main effects were further analyzed for inhomogeneity of means using pairwise multiple means comparison. Tukey's test is a conservative test that uses pairs of means to determine significant differences and form letter groupings while controlling experiment-wise error rate. Duncan's and SNK tests are moderately conservative tests based on pairs of means to form letter groupings. Fisher's least significant difference (LSD) test is the least conservative test and is based on pairs of means to form letter groupings.

The magnitude of experimental error should be used to determine which pairwise comparison to employ in multiple means comparison. This can be evaluated by using the coefficient of variation ($\mathrm{CV} = \frac{S}{\bar{y}}$) or by considering the nature of the experiment. If the coefficient of variation is less than 10% (low experimental error), then Tukey's Honestly Significant Difference (HSD) method would be appropriate. If the coefficient of variation is greater than 10% (moderate to high experimental error), the Fisher's Least Significant Difference (LSD) method would be appropriate. It is also important to consider the numerical size of the values; if the values are small, the coefficient of variation would be inflated due to a small $\bar{y}$ as the denominator.

The significance level was set at 95% ($\alpha = 0.05$). The datasets were analyzed using Minitab® 18 Statistical Software.

Factorial Design Analysis of Percent Change in Mass

The experiment was a 2x2x5 three-factor factorial with three replications (n = 3). There were two levels of film (R94W, RU88X-6), two levels of manganese stereate pro-oxidant treatment (+, -) and five levels of QUV exposure (0 h, 500 h, 1000 h, 1500 h, 2000 h). The factors were fixed. The ANOVA GLM procedure was used to analyze both the main effects and interaction effects of the film, pro-oxidant, and QUV exposure on the response, percent change in mass.

The assumptions of normality and constant variance were satisfied when a cube-root transformation was applied to the response values. The assumption of independence was assumed through randomization. The ANOVA GLM procedure was then carried out using Minitab® 18 Statistical Software on the transformed data (Table A-19). The untransformed data is presented below.

Factorial Design Analysis of ΔE Colorimetry

The experiment was a 2x2x2x5 four-factor factorial with three replications (n = 3). There were two levels of film (R94W, RU88X-6), two levels of manganese stereate pro-oxidant treatment (+, -), two levels of *A. oryzae* exposure (+, -), and five levels of QUV exposure (0 h, 500 h, 1000 h, 1500 h, 2000 h). The factors were fixed. The ANOVA GLM procedure was used to analyze both the main effects and interaction effects of the film, pro-oxidant, QUV exposure, and *A. oryzae* exposure on the response, ΔE.

The assumptions of normality and constant variance were satisfied when a square-root transformation was applied to the response variables. The assumption of independence was assumed through randomization. The ANOVA GLM procedure was then carried out using Minitab® 18 Statistical Software on the transformed data (Table A-21). Because the four-way interaction of Film*Mn*QUV*Ao was not significant ($p > 0.05$), the GLM was reduced to three-way interactions, allowing the degrees of freedom associated with the four-way interaction to be moved to the error. The untransformed data is presented below.

Linear Regression Model

Linear regression is used to understand and model linear relationships between two or more quantitative variables. The linear regression model is a linear equation that describes the

relationship between a dependent variable and one or more independent variables. The linear regression model with one independent variable was used to describe the relationship between absorbance at wavelength 405 nm and the concentration of pNP in µmol by means of pNP standards. The linear regression model is described as $y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$ where the parameter β_0 is the intercept of the plane, β_1 is the partial regression coefficient that measures the expected unit change in y per unit change in x_1, and ε is the error terms associated with the model and is assumed to follow a normal distribution with a mean of 0 and a variance of σ^2 ($\varepsilon_i \sim NID(0, \sigma^2)$), with a constant variance, and be independent of one another (Montgomery, 2013).

Repeated Measures Analysis

Repeated measures are used when the same response variable is measured on the same experimental unit on two or more occasions. This repetition can be either temporal (different points in time) or spatial (different points in space). Repeated measures analysis is common in biological and agricultural research when studying growth/decay curves, or examining the long term effect of treatments. However, repeated measures violates the assumption of independence of the error terms. This is because time, which is included in the model as a factor, cannot be randomized. As measurements are taken, subsequent measurements will depend on the previous measurement. The consequence of this violation is the same as that of violating constant variance; the mean squares error (MSE) will be inflated, causing the F-value to be deflated, and subsequently causing the p-value to be inflated. This violation will result in difficulty detecting significant differences between treatment effects.

The proc mixed method in SAS® employs univariate analysis using the most appropriate covariance structure. Identifying the most appropriate covariance structure is a key step in the proc mixed method, as the covariance structure embodies the type of relationship of the values over time. The most common temporal covariance structures are: i) Autoregressive order 1 (AR(1)), when the correlation between time points is ρ, which is constant, both ρ and σ^2 stay constant, but is not appropriate for unequally spaced time points; ii) Compound symmetry (CS), when a group of time points depends on another group of time points and/or there is different dependence within different groups of time points; and iii) Unstructured (UN), when there is no pattern to ρ, but variance is constant (verified with transformation). To determine the most appropriate covariance structure, the model should be run in SAS with several possible

covariance structures. The structure that gives the smallest absolute value for Akaike's Information Criterion (AIC) or Schwarz's Bayesian Criterion (SBC) (depending on statistical religion) is the most appropriate structure. Both AIC and SBC are model-fit criteria, which are basically log values that penalize for the number of parameters estimated. Note that while SBC imposes a heavier penalty than AIC, it is less commonly used by statisticians.

Repeated measures analysis was carried out on the enzymatic activity data collected from the pNP assay using the proc mixed procedure in SAS® 9.3. In order to determine the appropriate covariance structure, the model was run for each AR(1), CS, and UN covariance structures. The models were evaluated based on the convergence criteria being met, the number of iterations (*i*), the AIC value, and the assumptions of normality and constant variance being met.

The response variables for all treatments at Time = 0 was omitted. Due to the large number of data points being equal to zero, the normality and constant variance were skewed. The assumptions of normality and constant variance were satisfied when a cube-root transformation was applied to the response variables. The autoregressive order 1 (AR(1)) covariance structure was found to be the most suitable, having the lowest |AIC| and meeting convergence criteria (Table A-43). The compound symmetry (CS) covariance structure was also found to be suitable, having an equal |AIC| and meeting convergence criteria, however the AR(1) covariance structure was selected due to the time points being equally spaced. The proc mixed procedure was then carried out using SAS® on the transformed data. The untransformed data is presented below.

3.4. Results and Discussion

3.4.1. Percent Change in Mass

The ANOVA GLM is summarized in Table A-40. A significant three-way interaction between the type of film, pro-oxidant pretreatment, and QUV exposure was determined for the percent change in mass of the film following incubation with *A. oryzae* (Film*Mn* QUV) ($p = 0.003$). The coefficient of variation (Table A-41) was used to determine the appropriate multiple means comparison method. A moderate coefficient of variation (10-30%) suggested the use of Fisher's LSD method.

Table 3.1: Grouping Information of Means of Percent Change in Mass (%) of RU88X-6 and R94W PE Films (± Standard Deviation) Exposed to *A. oryzae* with and without Mn Pro-Oxidant Exposure at Five Different QUV Exposures Using the Fisher's LSD Method and 95% Confidence for Three-Way Interaction of Film*Mn*QUV (N = 3)

QUV	RU88X-6		R94W	
(hours)	Mn (+)	Mn (-)	Mn (+)	Mn (-)
0	$0.05 \pm 0.04_{Ba}$	$0.75 \pm 0.94_{Aa}$	$0.64 \pm 0.28_{Aa}$	$0.16 \pm 0.24_{Ba}$
500	$0.07 \pm 0.04_{Ba}$	$0.05 \pm 0.02_{Bb}$	$0.49 \pm 0.22_{Aa}$	$0.87 \pm 1.01_{Aa}$
1000	$0.11 \pm 0.01_{Ba}$	$0.10 \pm 0.05_{Bb}$	$0.50 \pm 0.23_{Aa}$	$0.15 \pm 0.03_{ABa}$
1500	$0.10 \pm 0.01_{BCa}$	$0.07 \pm 0.02_{Cb}$	$0.40 \pm 0.22_{ABa}$	$0.43 \pm 0.16_{Aa}$
2000	$0.08 \pm 0.03_{Ba}$	$0.12 \pm 0.03_{ABb}$	$0.42 \pm 0.10_{Aa}$	$0.24 \pm 0.13_{Aa}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns).

For the long-life RU88X-6 film, there were not any significant differences in percent change in mass between pro-oxidant exposures for each QUV exposure (with the exception of 0 hours QUV exposure). This is another notable finding as it was hypothesized that the pro-oxidant exposure would generate more oxidized functional groups upon QUV exposure that would then be degraded by the fungal strain. However, the significant difference between percent change in mass for pro-oxidant exposure at 0 hours QUV exposure is supportive of the oxidizing action of the pro-oxidant, even in the absence of artificial weathering. Within the pro-oxidant exposure treatment, QUV exposure at 1000 hours (equal to 12 months outdoor exposure) resulted in the highest percent change in mass, followed by QUV exposure at 1500 hours (equal to 18 months outdoor exposure), though not significantly different from that of the remaining QUV exposures. This could be indicative of a plateau of oxidized functional groups being generated by the QUV exposure.

For the short-life R94W film, QUV exposure at 500 hours (equal to 6 months outdoor exposure) without pro-oxidant treatment experienced the highest percent change in mass, though it is not significantly different from the other R94W film treatments. Within the pro-oxidant exposure treatment, QUV exposure at 0 hours (equal to no outdoor exposure) resulted in the highest percent change in mass, followed by QUV exposure at 1000 hours and 500 hours (equal to 12 months and 6 months outdoor exposure, respectively). Though these percent changes in mass

were not significantly different than the percent changes in mass of the 1500 hours and 2000 hours QUV exposures (equal to 18 months and 24 months outdoor exposure, respectively), it is interesting that the lesser QUV exposures experienced higher percent change in mass. This may be indicative of the film's susceptibility itself to the pro-oxidant exposure and fungal exposure. It could also be indicative of a lack of oxidized functional groups produced as a result of QUV exposure following pro-oxidant exposure.

When comparing the data of both types of films, it is evident that the short-term single use film (R94W) resulted in a higher percent change in mass than the long-life film (RU88X-6), indicating that this film may be more susceptible to biodegradation by *A. oryzae*. This can be attributed to the thickness of the film, as the RU88X-6 film is four times as thick as the R94W film. It is also evident that within the R94W films, those treated with the manganese pro-oxidant pretreatment (Mn +) prior to QUV exposure resulted in a higher percent change in mass. This supports the hypothesis that a pro-oxidant pretreatment will cause a film to be more susceptible to photodegradation and, consequently, biodegradation. Though mass loss is not a direct measure of degradation, it supports the indication of biodegradation via enzymatic degradation of the film. Thus, in order to select what treatment combination to use, other factors such as the application of the film, production time/cost, and labor should be considered.

3.4.2. Colorimetry

The ANOVA GLM is summarized in Table A-42. Three significant three-way interactions between i) the type of film, pro-oxidant pretreatment, and fungal exposure (Film*Mn*Ao) ($p = 0.026$), ii) the type of film, QUV exposure, and fungal exposure (Film*QUV*Ao) ($p = 0.007$), and iii) pro-oxidant pretreatment, QUV exposure, and fungal exposure (Mn*QUV*Ao) ($p = 0.024$) were determined for the change in colorimetry (ΔE). The coefficient of variation (Table A-44) was used to determine the appropriate multiple means comparison method. A moderate coefficient of variation (10-30%) suggested the use of Fisher's LSD method. IPG considers a ΔE value that is greater than or equal to 2 to be a significant change in the colorimetry of the PE film.

Table 3.2: Grouping Information of Means of ΔE of RU88X-6 and R94W PE Films (± Standard Deviation) Exposed and Unexposed to *A. oryzae* with and without Mn Pro-Oxidant Exposure Using the Fisher's LSD Method and 95% Confidence for Three-Way Interaction Film*Mn*Ao For All Levels of QUV Exposure (N = 15)

	RU88X-6		**R94W**	
Ao	**Mn (+)**	**Mn (-)**	**Mn (+)**	**Mn (-)**
+	$1.75 \pm 0.86_{Aa}$	$1.50 \pm 0.79_{ABa}$	$1.28 \pm 0.62_{Ba}$	$1.27 \pm 0.51_{Ba}$
-	$0.70 \pm 0.31_{Ab}$	$0.19 \pm 0.07_{Bb}$	$0.41 \pm 0.16_{Ab}$	$0.41 \pm 0.28_{ABb}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns).

From Fisher's LSD method (Table 3.2), it is evident that two treatment combinations of the three-way interaction of film, pro-oxidant pretreatment, and incubation with *A. oryzae* give the highest change in colorimetry (ΔE) and are not significantly different from one another for all QUV exposures. It is clear that the long-life film (RU88X-6) experienced the highest change in colorimetry (ΔE) following incubation with *A. oryzae*, with and without pro-oxidant pretreatment. These changes can be attributed to yellowing through weathering (QUV and pro-oxidant incurred), as well as surface changes caused by fungal biodegradation.

The results show that for both types of film, with and without pro-oxidant pretreatment, and regardless of QUV exposure, the incubation with *A. oryzae* resulted in a higher change in colorimetry (ΔE) than those not incubated with the fungal strain, indicating that incubation with *A. oryzae* yields changes in the surface chemistry of the film.

Table 3.3: Grouping Information of Means of ΔE of RU88X-6 and R94W PE Films (± Standard Deviation) Exposed and Unexposed to *A. oryzae* at Five Different QUV Exposures Using the Fisher's LSD Method and 95% Confidence for Three-Way Interaction Film*QUV*Ao For All Levels of Mn Pro-Oxidant Exposure (N = 6)

QUV	RU88X-6		R94W	
(hours)	Ao (+)	Ao (-)	Ao (+)	Ao (-)
0	$0.70 \pm 0.14_{Ab}$	$0.56 \pm 0.48_{ABab}$	$0.72 \pm 0.16_{Ab}$	$0.18 \pm 0.08_{Bb}$
500	$1.53 \pm 0.60_{Aa}$	$0.56 \pm 0.48_{BCab}$	$0.67 \pm 0.18_{Bb}$	$0.24 \pm 0.14_{Cab}$
1000	$1.80 \pm 1.02_{Aa}$	$0.45 \pm 0.24_{Bab}$	$1.60 \pm 0.36_{Aa}$	$0.49 \pm 0.11_{Bab}$
1500	$2.03 \pm 0.69_{Aa}$	$0.26 \pm 0.09_{Cb}$	$1.92 \pm 0.25_{Aa}$	$0.58 \pm 0.18_{Ba}$
2000	$2.08 \pm 0.73_{Aa}$	$0.39 \pm 0.26_{Bab}$	$1.48 \pm 0.26_{Aa}$	$0.56 \pm 0.24_{Bab}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns).

From Fisher's LSD method (Table 3.3), it is evident that seven treatment combinations of the three-way interaction of film, QUV exposure, and incubation with *A. oryzae* give the highest change in colorimetry (ΔE) and are not significantly different from one another for both treated and untreated with pro-oxidant. It is important to note that within these seven treatment combinations, that for both types of film, with and without pro-oxidant pretreatment, the incubation with *A. oryzae* resulted in a higher change in colorimetry (ΔE) than those not incubated with the fungal strain. These seven treatment combinations also include the longer time frames of QUV exposure, and do not include any films that were not exposed to UV irradiation. It was also noted that for both the R94W and RU88X-6 films, with increasing UV irradiation (regardless of pro-oxidant pretreatment) there was an increase in the value of ΔE following incubation with *A. oryzae,* whereas without incubation, the increasing UV irradiation produced inconsistent values of ΔE for RU88X-6. This aligns with the literature that photo-oxidation is the initial step, causing changes in the surface of the film through yellowing, and making the films more susceptible to further changes in the surface by *A. oryzae*.

Table 3.4: Grouping Information of Means of ΔE of PE Films (± Standard Deviation) Exposed and Unexposed to *A. oryzae* with and without Mn Pro-Oxidant Exposure at Five Different QUV Exposures Using the Fisher's LSD Method and 95% Confidence for Three-Way Interaction Mn*QUV*Ao For All Levels of Film Type (N = 6)

QUV	Mn (+)		Mn (-)	
(hours)	Ao (+)	Ao (-)	Ao (+)	Ao (-)
0	$0.69 \pm 0.11_{Ac}$	$0.61 \pm 0.43_{Aa}$	$0.73 \pm 0.18_{Ad}$	$0.13 \pm 0.03_{Bc}$
500	$1.10 \pm 0.68_{Ab}$	$0.61 \pm 0.43_{Ba}$	$1.10 \pm 0.61_{Acd}$	$0.19 \pm 0.15_{Cbc}$
1000	$2.12 \pm 0.76_{Aa}$	$0.58 \pm 0.13_{CDa}$	$1.27 \pm 0.42_{Bbc}$	$0.36 \pm 0.14_{Dab}$
1500	$2.00 \pm 0.71_{Aa}$	$0.46 \pm 0.15_{Ba}$	$1.94 \pm 0.19_{Aa}$	$0.38 \pm 0.28_{Bab}$
2000	$1.67 \pm 0.39_{Aa}$	$0.51 \pm 0.20_{Ba}$	$1.89 \pm 0.79_{Aa}$	$0.45 \pm 0.32_{Ba}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns).

From Fisher's LSD method (Table 3.4), it is evident that five treatment combinations of the three-way interaction of pro-oxidant pretreatment, QUV exposure, and incubation with *A. oryzae* give the highest change in colorimetry (ΔE) and are not significantly different from one another for both film types. Of these five treatment combinations, there is a minimum QUV exposure of 1000 hours (equivalent to 12 months weathering), with pro-oxidant pretreatment, and there is exposure to *A. oryzae*. The effects of pro-oxidant pretreatment, UV irradiation, and incubation with *A. oryzae* showed a synergistic effect with increased UV exposure.

The two types of films used in this study (RU88X-6 and R94W) are both composed of a HDPE skrim and a LDPE coating, but with differences in thickness. The films are both composed of a white coating, making the changes in the colorimetry a useful measurement to determine the surface effects of weathering, with and without pro-oxidant exposure, and fungal exposure.

3.4.3. Enzymatic Activity

The proc mixed method with the unstructured covariance structure was run with the transformed response values of enzymatic activity. The type three test of effects is summarized in Table A-45. The 3-way interaction between Film*Mn*QUV was found to be significant ($p < 0.0001$). The single four-way interaction was not found to be significant ($p > 0.05$).

The main effects of time, type of film, and QUV exposure were all found to be significant ($p < 0.05$). However, the main effect of pro-oxidant pretreatment was not found to be significant ($p > 0.05$), although its interaction with other main effects was significant. A larger production of μmol pNP over time indicates increased esterase activity as a result of the hydrolysis of pNPB. The increased esterase activity indicates an increased production of esterase enzymes by *A. oryzae*.

Table 3.5: Grouping Information of Means of pNP Production (µmol) of *A. oryzae* (± Standard Deviation) with and without Mn Pro-Oxidant Exposure at Five Different QUV Exposures Using the Fisher's LSD Method and 95% Confidence for Three-Way Interaction Film*Mn*QUV (N = 3)

QUV	RU88X-6		R94W	
(hours)	Mn (+)	Mn (-)	Mn (+)	Mn (-)
0	$2.19 \pm 1.28_{Aab}$	$1.19 \pm 0.52_{Bc}$	$1.22 \pm 0.36_{Be}$	$1.12 \pm 0.38_{Bc}$
500	$2.97 \pm 1.12_{Ba}$	$2.46 \pm 0.54_{Ba}$	$6.62 \pm 5.26_{Ad}$	$1.48 \pm 0.37_{Cc}$
1000	$1.46 \pm 0.69_{Cb}$	$1.60 \pm 0.54_{Cabc}$	$19.60 \pm 14.19_{Ba}$	$34.32 \pm 13.66_{Aa}$
1500	$1.51 \pm 0.40_{Bb}$	$1.42 \pm 0.56_{Bbc}$	$13.86 \pm 9.23_{Ab}$	$13.40 \pm 8.20_{Ab}$
2000	$1.52 \pm 0.47_{Cb}$	$1.31 \pm 0.42_{Cbc}$	$8.13 \pm 4.93_{Bc}$	$11.74 \pm 5.50_{Ab}$

Means that do not share a letter are significantly different (upper case across rows, lower case within columns).

From Fisher's LSD (Table 3.5), it is evident that the short-life single use R94W film without pro-oxidant pretreatment and exposed to QUV at 1000 hours (equal to 12 months outdoor exposure) resulted in the highest production of µmol pNP and is significantly different from all other treatments, for all time points of the enzyme activity assay. The R94W film appears to be more susceptible to biodegradation by *A. oryzae*, which can be attributed to its thickness (less than that of the long-life RU88X-6 film). However, it is interesting that the pro-oxidant untreated film yielded a large production of µmol pNP, as it was hypothesized that the pro-oxidant and UV exposure would have a synergistic effect with the biodegradation of the film. With the pro-oxidant treated R94W film, the highest production of µmol pNP occurred at the QUV exposure of 1000 hours (equal to 12 months outdoor exposure) as well, indicating that there may be a plateau of weathering exposure at which the photo oxidation is inhibited until the oxidized groups are removed from the surface of the plastic. For both untreated and pretreated R94W films, the increasing QUV exposures resulted in decreased enzymatic activity by *A. oryzae*, further supporting the indication of a plateau.

Within the long-life RU88X-6 film, for both pro-oxidant treated and untreated, the highest production of µmol pNP occurred at the QUV exposure of 500 hours (equal to 6 months outdoor exposure), for all time points during the enzyme activity assay. This is notable as the RU88X-6

film was not expected to be susceptible to biodegradation without increased chemical transformation to the surface of the film due to it being a long-life film intended for outdoor use.

3.4.4. FTIR Analysis of PE Film Surface

Structural changes in the surface of the PE films following pro-oxidant pretreatment, UV irradiation, and subsequent incubation with *A. oryzae* were investigated using FTIR.

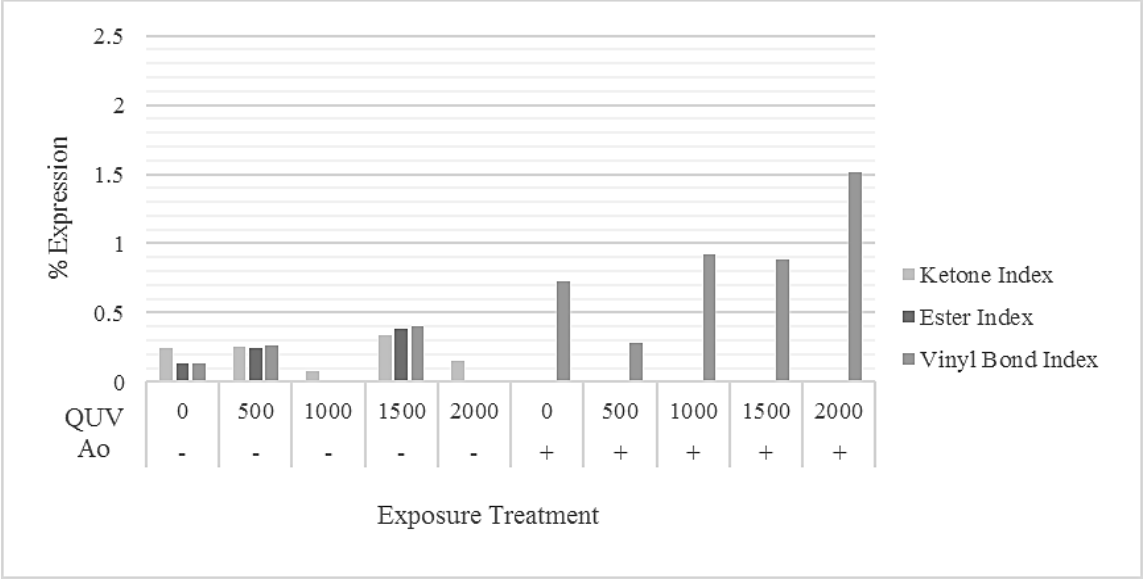


Figure 3.1: FTIR Indices of RU88X-6 PE Film Without Mn Pro-Oxidant Exposure Prior to (-) and Post (+) *A. oryzae* Exposure at Five Different QUV Exposures

The long-life RU88X-6 film showed varying increases in ketone and ester carbonyl groups upon exposure to UV irradiation without pro-oxidant pretreatment (Fig. 3.1). The inconsistencies may be attributed to the UV stabilizers used in the production of the film and the lack of pro-oxidant pretreatment prior to UV irradiation. The largest production of carbonyl groups was observed at 1500h QUV exposure (equal to 18 months outdoor exposure), indicating that there may be a plateau in carbonyl group production on the surface of the film upon UV exposure. Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus.

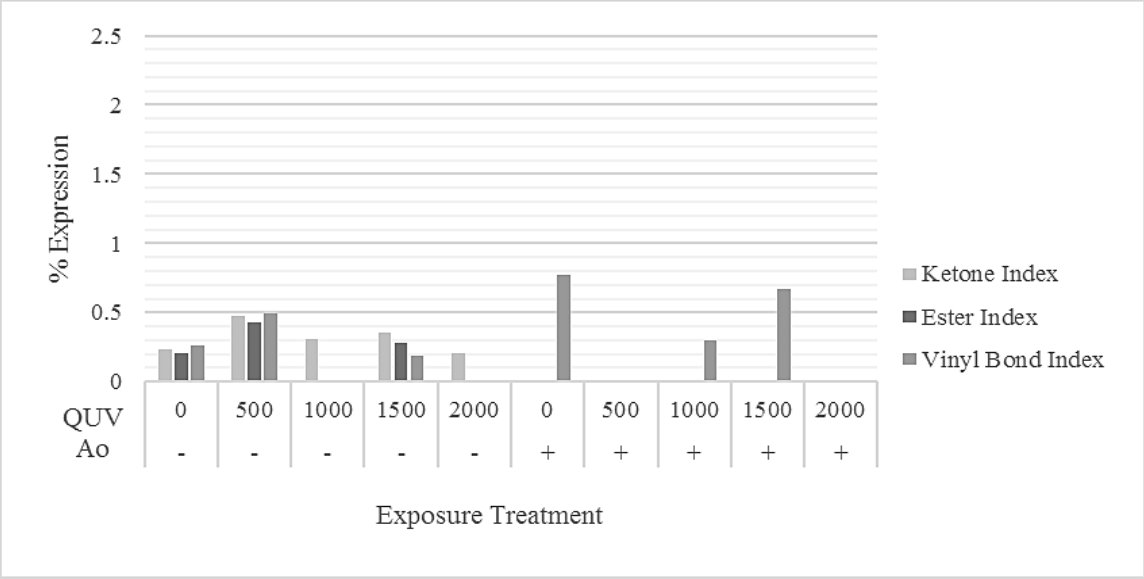


Figure 3.2: FTIR Indices of RU88X-6 PE Film With Mn Pro-Oxidant Exposure Prior to (-) and Post (+) *A. oryzae* Exposure at Five Different QUV Exposures

The long-life RU88X-6 film showed an increased production of ketone and ester carbonyl groups upon exposure to UV irradiation and pro-oxidant pretreatment, though experienced similar variation between QUV exposure periods (Fig. 3.2). These inconsistencies may be attributed to the UV stabilizers used in the production of the film and their reaction with both pro-oxidant pretreatment and UV irradiation. The largest production of carbonyl groups was observed at 500 hours QUV exposure (equal to 6 months outdoor exposure), which could be attributed to an earlier plateau in carbonyl group production due to the increased photo-oxidation as a result of the action of the pro-oxidant pretreatment. Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus.

When comparing the effect of pro-oxidant pretreatment on carbonyl group production, it is evident that pro-oxidant pretreatment caused an initial increase in carbonyl group production with and without UV irradiation (0 hours QUV exposure and 500 hours QUV exposure). The production of carbonyl groups without UV irradiation and with pro-oxidant pre-treatment can be attributed to oxidation reactions caused by atmospheric oxygen in the absence of UV irradiation. At 1500 hours QUV exposure (equal to 18 months outdoor exposure), the RU88X-6 film without pro-oxidant pretreatment exhibited a larger production of carbonyl groups than that of the film with pro-oxidant pretreatment. This could be a result of a carbonyl group production saturation

that occurred at an earlier stage of UV irradiation when the film was exposed to the pro-oxidant pretreatment.

Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus. This indicates the breakdown of the polymer chain and the presence of oxidation products following UV irradiation, with or without pro-oxidant pretreatment, that were subsequently consumed by *A. oryzae.* The presence of vinyl (double) bonds following incubation with *A. oryzae* are a result of Norrish Type II mechanism, in which the chain scisson leads to the formation of double bonds (Sudhakar et al., 2007).

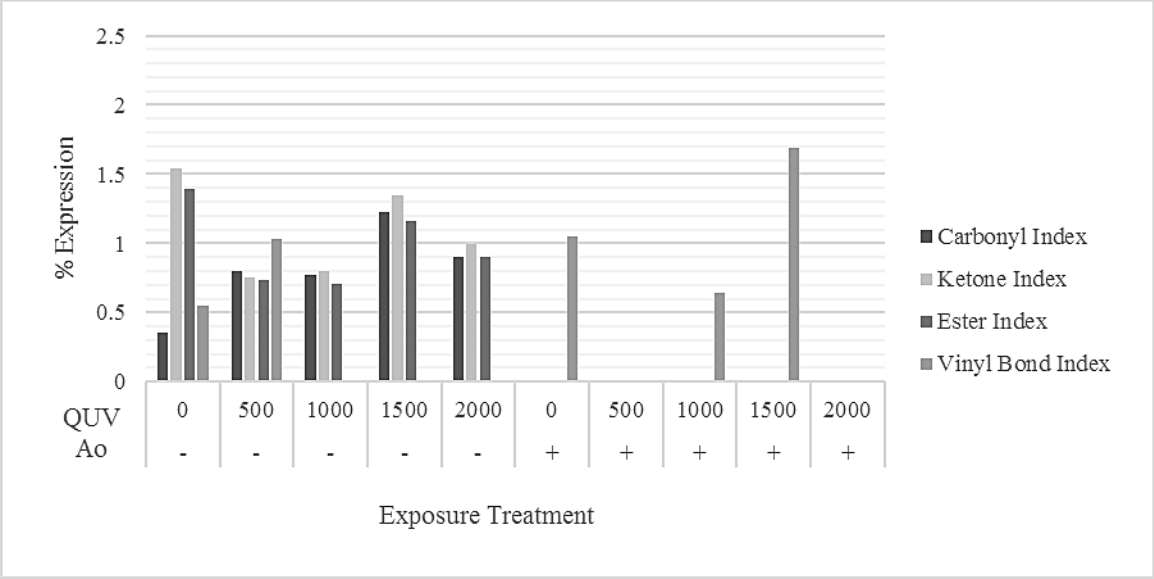


Figure 3.3: FTIR Indices of R94W PE Film Without Mn Pro-Oxidant Exposure Prior to (-) and Post (+) *A. oryzae* Exposure at Five Different QUV Exposures

The single use short-life R94W film experienced a large production of carbonyl, ketone, and ester groups following UV irradiation, without pro-oxidant pretreatment (Fig. 3.3). The increased production of these functional groups without UV irradiation (0 hours QUV exposure) is an interesting finding as without pro-oxidant pretreatment, increased oxidation via atmospheric oxygen is unexpected. However, the short-life intended use of the film indicated that it should experience weathering upon outdoor exposure over a short period of time. Following UV irradiation, the production of the carbonyl, ketone, and ester functional groups is fairly consistent, with the greatest production of these functional groups occurring at 1500 hours QUV exposure (equal to 18 months outdoor exposure). These slight inconsistencies may be attributed to the UV stabilizers used in the production of the film and the lack of pro-oxidant pretreatment prior to UV irradiation. Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus.

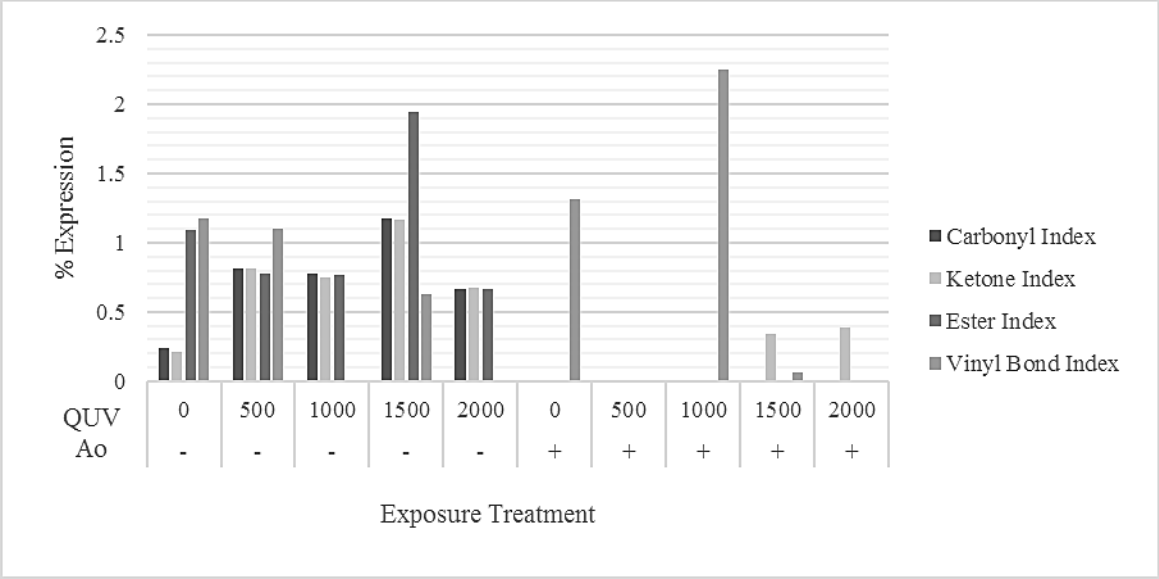


Figure 3.4: FTIR Indices of R94W PE Film With Mn Pro-Oxidant Exposure Prior to (-) and Post (+) *A. oryzae* Exposure at Five Different QUV Exposures

The single use short-life R94W film experienced a large production of carbonyl, ketone, and ester groups following UV irradiation and pro-oxidant pretreatment (Fig. 3.4). The production of these functional groups without UV irradiation (0 hours QUV exposure) and with pro-oxidant pretreatment was expected as Kounty et al. (2006) observed that pro-oxidants can induce oxidation of PE even in the absence of light. Following UV irradiation, the production of the carbonyl, ketone, and ester functional groups is fairly consistent, with the highest production of these functional groups occurring at 1500 hours QUV exposure (equal to 18 months outdoor exposure), which may be indicative of a carbonyl group production maximum on the surface of the film. It was noted that there was residual ketone functional group following incubation with *A. oryzae.* These slight inconsistencies may be attributed to the UV stabilizers used in the production of the film, as well as the type of film, as it is a thin, single use film, which is more susceptible to changes in surface chemistry. Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus.

When comparing the effect of pro-oxidant pretreatment on carbonyl group production, there is increased variation on production of these functional groups with pro-oxidant pretreatment. The increased production of carbonyl functional groups without UV irradiation (0 hours QUV exposure) and without pro-oxidant pretreatment (Fig. 3.3) compared to that with pro-oxidant

pretreatment (Fig. 3.4) is an interesting result as the pro-oxidant pretreatment was expected to increase the production of these functional groups, with and without UV irradiation.

Following incubation with *A. oryzae*, it is evident that these ketone and ester carbonyl groups were degraded by the fungus. This indicates the breakdown of the polymer chain and the presence of oxidation products following UV irradiation, with or without pro-oxidant pretreatment, that were subsequently consumed by *A. oryzae.* The presence of vinyl (double) bonds following incubation with *A. oryzae* are a result of Norrish Type II mechanism, in which the chain scisson leads to the formation of double bonds (Sudhakar et al. 2007).

3.5. Conclusions

Commercially important plastics are resistant to photo-oxidation and biodegradation because they contain antioxidants and stabilizers, thus leading to a potentially immense environmental pollution problem. With abiotic pretreatment by pro-oxidants and QUV exposure (simulating outdoor exposure using UV irradiation), the material experienced chemical transformation that made it more susceptible to microbial attack.

The change in mass of the plastic films (though not a direct measure of degradation) supports the indication that biodegradation is occurring. The percent change in mass of the R94W film was found to be greater than that of the RU88X-6 film for both respective untreated and pro-oxidant treated samples, at all respective QUV exposures, omitting pro-oxidant untreated at 0 hours QUV exposure, which resulted in the greatest percent change in mass (Table 3.1). The increased percent change in mass for the R94W films can be attributed to the thickness of the film, as the RU88X-6 film is four times as thick as the R94W film, and thus its susceptibility to degradation is incrased. Otherwise, the RU88X-6 film did not experience any significant changes in mass between untreated and pro-oxidant pretreated at all other QUV exposures. The R94W films that were pretreated with pro-oxidant experienced a greater percent change in mass than those without pro-oxidant pretreatment, indicating that the pro-oxidant pretreatment increased the susceptibility of the film to biodegradation. The films were qualitatively observed to be more brittle following incubation, indicating a change in physical and chemical composition of the PE films.

Color change is measured as an indication of plastic degradation by abiotic and biotic factors. The effects of pro-oxidant pretreatment and incubation with *A. oryzae* (regardless of QUV exposure) showed significant differences in the values of ΔE as a result of incubation for both the R94W and RU88X-6 films, indicating a large change in the surface chemistry of the film following incubation with the fungus (Table 3.2). When looking at the effects of UV irradiation and incubation with *A. oryzae* (regardless of pro-oxidant pretreatment) on colorimetry, it was noted that for both the R94W and RU88X-6 films that with increasing UV irradiation, there was an increase in the value of ΔE following incubation with *A. oryzae,* whereas without incubation, the increasing UV irradiation produced inconsistent values of ΔE for RU88X-6 (Table 3.3). The effects of pro-oxidant pretreatment, UV irradiation, and incubation with *A. oryzae* showed a synergistic effect with increased UV exposure. The greatest value of ΔE was obtained at 1000 hours QUV exposure, with pro-oxidant pretreatment and incubation with *A. oryzae* (Table 3.4). These results demonstrate the effects of changes in surface chemistry on the colorimetry of the films.

The enzymatic activity of *A. oryzae* gives more insight on the effect of these abiotic treatments on the degradation by the fungus itself. The greatest enzymatic activity for the short-life single use R94W film occurred without pro-oxidant pretreatment at 1000 hours QUV exposure (equal to 12 months outdoor exposure), for all time points of the enzyme activity assay, indicating that this film is susceptible to biodegradation after outdoor exposure even without the use of pro-oxidant pretreatment (Table 3.5). When pretreated with pro-oxidant, the R94W film yielded the greatest enzymatic activity at 1000 hours QUV exposure as well, indicating that there may be a plateau of weathering exposure at which the photo oxidation is inhibited until the oxidized groups are removed from the surface of the plastic. For both untreated and pretreated R94W films, the increasing QUV exposures resulted in decreased enzymatic activity by *A. oryzae*, further supporting the indication of a plateau. For the long-life RU88X-6 film, both pro-oxidant untreated and pretreated films showed the greatest enzymatic activity by *A. oryzae* at 500 hours QUV exposure (equal to 6 months outdoor exposure) which is interesting as this film was expected to be recalcitrant to biodegradation without abiotic treatment as it is intended for long term outdoor use.

FTIR analysis of the film surface provides information on the chemical changes to the functional groups on the surface of the film with abiotic and biotic treatment. Both RU88X-6 and R94W films showed production of carbonyl, ketone, and ester functional groups on the surface of the film, with and without pro-oxidant pretreatment, at all QUV exposures. The long-life RU88X-6 film showed decreased carbonyl functional group production when compared to that of the short-life single use R94W film, which can be attributed to the thickness of the films as well as their compositions for their intended use.

When comparing the effect of pro-oxidant pretreatment on carbonyl group production in the RU88X-6 film, an initial increased carbonyl group production is observed with pro-oxidant pretreatment at 0 and 500 hours QUV exposure (0 and 6 months outdoor exposure, respectively) (Fig. 3.1 and 3.2). The production of carbonyl groups without UV irradiation and with pro-oxidant pre-treatment can be attributed to oxidation reactions cause by atmospheric oxygen in the absence of UV irradiation. The maximum production of carbonyl functional groups for pro-oxidant untreated films was observed at 1500 hours QUV exposure (equal to 18 months outdoor exposure), which may translate to a saturation of carbonyl functional groups on the surface of the film.

The short-life single use R94W film displayed a large production of carbonyl, ketone, and ester functional groups at 0 hours QUV exposure (equal to no outdoor exposure), without pro-oxidant pretreatment (Fig. 3.3). This finding could be attributed to the short-life intended use of the film itself, as it should experience weathering upon outdoor exposure over a short period of time. The production of these functional groups was consistent for all QUV exposures, indicating that this film had experienced oxidized functional group saturation within a short period of outdoor exposure, allowing it to be more susceptible to biodegradation, even without pro-oxidant pretreatment. With pro-oxidant pretreatment, the greatest production of these functional groups was observed at 1500 hours QUV exposure (equal to 18 months outdoor exposure), which could be a result of the pro-oxidant increasing the point of saturation of oxidized functional groups on the surface of the plastic (Fig. 3.4). However, when comparing the effect of pro-oxidant pretreatment, the production of these functional groups was consistent for all other QUV exposures with and without pretreatment. This observation speaks to the increased biodegradability of the short-life film itself.

For both RU88X-6 and R94W films, pro-oxidant pretreated and untreated, and at all QUV exposures, the carbonyl functional groups were evidently degraded by the fungus following its incubation. This is observed through the disappearance of these functional groups, and the emergence of vinyl (double) bonds following incubation with *A. oryzae* as a result of chain scisson that lead to the formation of these double bonds. The inconsistencies of the FTIR analyses may be attributed to the targeted analysis of specific functional groups on the spectra, where there may be other significant increases and decreases in non-targeted functional groups.

Chapter 4: Conclusions

The current study focused on the ability of the filamentous fungus, *A. oryzae*, to degrade recalcitrant conventional polyethylene plastics and biodegradable poly(butylene adipate-co-terephthalate)/poly(lactic acid) blend plastics.

The susceptibility of PBAT/PLA films to biodegradation by *A. oryzae* was assessed with the addition of enzyme inducing agents and by changes in the initial pH of the culture medium. The change in mass of the PBAT/PLA films and the increased enzymatic activity of the fungus supported the indication that biodegradation was occurring with the addition of enzyme inducing agents. It was observed that the addition if yeast extract and olive oil as individual inducing agents resulted in increased enzyme activity by *A. oryzae*, increasing esterase activity by fifteen-fold.

The effect of initial pH of the culture media exhibited an increase in mass change with increasing initial pH, with a plateau in mass change at the neutral pH range and a 20% decrease in mass at a pH of 7.5. The change in the pH of the culture media following the incubation period provided insight as to the activity of the fungus, experiencing a large decrease in pH from the more basic initial culture media and a small increase in pH from the acidic initial culture media, indicating a shift towards a more neutral pH in both basic and acidic initial conditions. The large decrease in the basic initial culture media was attributed to increased enzymatic activity as it aligns with the findings of increased mass change with increasing initial pH. It was also observed through the enzymatic activity assay that the degradation activity of the fungus increased with increasing initial pH, exhibiting the greatest enzymatic activity towards the initially neutral culture media. These findings of degradation activity as a result of initial pH align with the literature as the active enzyme, cutinase, is more active in the neutral range.

The metabolomic analysis of *A, oryzae* when exposed to the PBAT/PLA film showed increased production of metabolites compared to exposure to the minimal media. This increase in metabolites, such as amino acids, carbohydrates, and glycerol and fatty acids further indicate the utilization of the PBAT/PLA film as a source of nutrition via tis degradation.

The susceptibility of HDPE/LDPE films to biodegradation by *A. oryzae* was assessed following pro-oxidant pretreatment and UV irradiation treatments. The long-life RU88X-6 film and the short-life single use R94W film were subjected to pro-oxidant pretreatment and five different UV irradiation exposure treatments. The percent change in mass of the R94W film was found to be greater than that of the RU88X-6 film for both respective untreated and pro-oxidant treated samples, at all respective QUV exposures, omitting pro-oxidant untreated at 0 hours QUV exposure, which resulted in the greatest change in mass. Otherwise, the RU88X-6 film did not experience any significant changes in mass between untreated and pro-oxidant pretreated at all other QUV exposures, and the R94W film experienced inconsistent changes in mass between pro-oxidant treatments and QUV exposures.

Colorimetry measurements (ΔE) indicated a significant change in the color if the surface of the RU88X-6 and R94W films as a result of incubation with *A. oryzae*, which can be attributed to changes in the surface chemistry of the films as well as biomass of the fungus itself. With increasing QUV exposure followed by fungal exposure, there was an increase in the value of ΔE, indicating a synergistic effect of QUV exposure and fungal exposure on the colorimetry of the film. The addition of pro-oxidant pretreatment only increased the value of ΔE, resulting in a larger change in colorimetry when the film was exposed to pro-oxidant, QUV exposure, and incubation with *A. oryzae*.

The greatest enzymatic activity of *A. oryzae* was observed for the short-life single use R94W film when pretreated with pro-oxidant and with 1000 hours of QUV exposure. This information indicates that this film is susceptible to biodegradation following outdoor exposure even without the use of pro-oxidant pretreatment, and also that there may be a plateau of weathering exposure at which the photo oxidation is inhibited until the oxidized groups are removed from the surface of the plastic.

The changes in the functional groups on the surface of the films was observed through FTIR. For both RU88X-6 and R94W films, with and without pro-oxidant pretreatment, production of carbonyl, ketone, and ester functional groups was observed at all QUV exposures. The generation of these functional groups as a result of oxidation (whether catalyzed by pro-oxidants and UV irradiation or just atmospheric) are an indication of abiotic degradation and chemical

transformation of the film surface. The presence of these functional groups also indicate susceptibility to degradation by microorganisms, as the degradation of these functional groups was observed following incubation with *A. oryzae*. The production of vinyl bonds following fungal exposure are indicative of chain scisson cause by the degradation action of the fungus.

For the past three decades, worldwide research has focused on the biodegradation of plastics in order to remediate the large volumes of recalcitrant waste the human race has created. The investigation of degradation of these plastics is of importance to the protection of the environment and the solid waste management system. Society demands a cleaner environment, for both environmental and aesthetic reasons. Pieces of plastic littering the ground are not a pretty sight and recycling programs can only recover so much, making it important to properly and efficiently manage wastes.

Future studies must focus on not only the degradation of the conventional plastic materials currently in circulation, but on the production and degradation of bio-based plastic materials. Bio-based plastics comprised of renewable resources present a compromise for the future of plastics in a world moving towards zero plastic waste and renewable resources.

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Appendix

Table A-1: Regression ANOVA for µmol pNP versus Absorbance

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Regression	1	1683.98	1683.98	3234.34	<0.001
Error	8	4.17	0.52		
Total	9	1688.15			

Table A-2: R-Square, Mean Square (S), and Regression Equation for µmol pNP versus Absorbance

R-Square	S	Regression Equation
99.8	0.72157	y = 0.6667 + 58.67x

Table A-3: Paired T-Test for Initial Mass - Final Mass of Inducing Agent Effect

	N	Mean (g)	StDev (±)
Initial Mass (g)	18	0.07563	0.01214
Final Mass (g)	18	0.06604	0.01243
Difference	18	0.009594	0.004209

Table A-4: Confidence Interval, T-Value, and P-Value for Paired T-Test of Inducing Agent Effect on Percent Change in Mass

95% Confidence Interval	T-Value	P-Value
(0.007501, 0.011688)	9.67	<0.001

Table A-5: One-Way ANOVA for Percent Change in Mass versus Inducing Agent Treatment

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	5	316.4	63.28	1.76	0.195
Error	12	431.1	35.93		
Total	17	747.5			

Table A-6: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Inducing Agent Effect on Percent Change in Mass

R-Square	Coefficient of Variation	S	$\bar{y}$
42.33	46.2371	5.99386	12.9633

Table A-7: Evaluation of Transformations, Covariance Structures for Repeated Measures Analysis of Inducer Effect

Covariance Structure	λ	Convergence	*i*	AIC	NPP	Resids vs Fits
AR(1)	1	Y	1	277.3	X	X
CS	1	Y	1	277.3	X	X
UN	1	Y	1	278.4	X	X
AR(1)	½	Y	1	91.2	X	X
CS	½	Y	1	91.2	X	X
UN	½	Y	1	95.8	X	X
AR(1)	⅓	Y	1	16.9	Y	Y
CS	⅓	Y	1	16.9	Y	Y
UN	⅓	Y	1	19.6	Y	Y

Table A-8: Type 3 Test of Fixed Effects for Repeated Measures Analysis of Inducer Effect on pNP Production (µmol)

Effect	Num DF	Den DF	F-Value	P-Value
Time	3	48	22.83	<0.0001
Inducer	5	48	171.88	<0.0001
Time*Inducer	15	48	3.44	0.0006

Table A-9: Paired T-Test for Initial Mass - Final Mass of Initial pH Treatment Effect

	N	Mean	StDev (±)
Initial Mass (g)	18	0.06819	0.01050
Final Mass (g)	18	0.05927	0.01074
Difference	18	0.008928	0.003216

Table A-10: Confidence Interval, T-Value, and P-Value for Paired T-Test of Initial pH Treatment Effect on Percent Change in Mass

95% Confidence Interval	T-Value	P-Value
(0.007329, 0.010527)	11.78	<0.001

Table A-11: One-Way ANOVA for Percent Change in Mass versus Initial pH Treatment Effect

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	5	425.69	85.138	19.34	<0.001
Error	12	52.84	4.403		
Total	17	478.53			

Table A-12: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Initial pH Treatment Effect on Percent Change in Mass

R-Square	Coefficient of Variation	S	$\bar{y}$
88.96	15.703	2.09833	13.3625

Table A-13: Paired T-Test for Initial pH - Final pH of Initial pH Treatment Effect

	N	Mean	StDev (±)
Initial pH	18	0.06819	0.01050
Final pH	18	0.05927	0.01074
Difference	18	0.008928	0.003216

Table A-14: Confidence Interval, T-Value and P-Value for Paired T-Test of Initial pH Treatment Effect on ΔpH

95% Confidence Interval	T-Value	P-Value
(0.007329, 0.010527)	11.78	<0.001

Table A-15: One-Way ANOVA for ΔpH versus Initial pH Treatment Effect

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	6	0.46378	0.077297	18.16	<0.001
Error	14	0.05960	0.004257		
Total	20	0.52338			

Table A-16: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Initial pH Treatment Effect on ΔpH

R^2	Coefficient of Variation	S	$\bar{y}$
88.61	26.349	0.0652468	0.24762

Table A-17: Evaluation of Transformations, Covariance Structures for Repeated Measures Analysis of Initial pH Treatment Effect

Covariance Structure	λ	Convergence	*i*	AIC	NPP	Resids vs Fits
AR(1)	1	Y	1	107.9	Y	X
CS	1	Y	1	107.9	Y	X
UN	1	Y	1	88.5	Y	X
AR(1)	½	Y	1	7.0	Y	Y
CS	½	Y	1	7.0	Y	Y
UN	½	Y	1	-0.3	Y	Y

Table A-18: Type 3 Test of Fixed Effects for Repeated Measures Analysis of Initial pH Treatment Effect on pNP Production (μmol)

Effect	Num DF	Den DF	F-Value	P-Value
Time	3	56	9.98	<0.0001
pH	6	56	3.14	0.0051
Time*pH	18	56	0.75	0.8183

Table A-19: Metabolites profiled

Name	Retention Time (minutes)	Characteristic Ions						
Ethylene glycol	6.056	147	191	103	148			
Ethylene glycol butyl ether	7.263	75	119	103	175			
Alanine	7.42035	116	147	190				
Pyruvic acid	8.266	147	232	204	130	247	59	
Glycine1	8.52	102	147	204	176			
Valine	9.171	144	218	145	100	147	246	
Urea	9.545	147	189	171	87			
Leucine	9.803075	158	159	147	232			
Glycerine (glycerol)	9.80976	147	205	117	133			
2-methyl succinic acid	9.855	147	261	148	75			
Phosphate	9.94	299	314	300	301	133	211	283
Isoleucine	10.228	158	218	159	147	241		
Proline	10.309	142	216	143	147	232	260	
Butanedioic acid (Succinic acid)	10.38276	147	247	129				
Pyrrole-2-carboxylic acid	10.516	240	166	255	147	241		
Methyl succinic acid	10.67	147	261	217	232	186		
Fumaric (2-butenedioic) acid	10.991	245	147	246	75	143		
Serine	11.116	204	218	147	100	158		
Lactate (lactic acid)	11.201195	147	117	191	219			
Threonine	11.448	218	117	219	147	291		
Glutaric (pentanedioic) acid	11.717	147	261	258	129	204		
Beta-alanine	12.029	174	248	147	290	86	100	
Malic acid	12.503815	147	233	245	335			
Glutamine	12.574	156	155	245	147	347		
2-oxosuccinic acid	12.755	147	233	245	133	189	335	
Methionine	12.81419	176	128	147				
Gamma-aminobutyric acid	12.969855	174	304	147	142			
1,2,3,4-tetraoxybutane	12.987	217	205	103	147	307		
Aspartic acid	13.155	232	218	147	100	202	188	
Cysteine (monomer)	13.19046	220	218	100				
5-oxo-proline	13.205	156	147	157	258	230		
Tartaric acid	13.9	147	292	219	189	133		
Phenylalanine	14.037545	218	192	147				
Beta-hydroxy-beta-methylglucaric acid	14.15	147	247	115	231	273	363	
Asparagine	14.983	116	231	132	188	147		

Putrescine	15.263765	174	175	214				
Suberic (octanedioic) acid	15.27	303	187	169	147	129	117	217
Arabitol	15.48	217	147	103	205	307	319	
Trans-aconitic acid	15.801	147	229	375	285	211	133	156
Phosphoric acid	15.945	357	299	315	147	211	387	285
Acetylglucosamine	16.008	147	219	243	117	319	171	257
Arginine (as ornithine)	16.016305	142	348	128				
Cadaverine	16.26747	174	175	375				
Propane-tricarboxylic acid	16.565	273	147	347	363	375	274	211
Histidine	17.007595	154	254	182	218			
Mannitol	17.21101	319	205	147	217			
Fructose	17.240615	307	217	103	147			
Alloxanoic acid	17.262	331	147	188	431	100	243	
Tyrosine	17.26449	218	219	280	179			
Epinephrine	17.414425	116	355	147	117			
Mannose peak 1	17.695195	319	205	147				
Glucitol	17.772	319	205	147	217	103	157	229
Glucose	17.77828	319	205	147	218			
Glucuronolactone	17.838445	319	147	220	129			
Galactose	17.945405	319	205	147	217			
Mannose peak 2	17.95782	319	205	147				
Lysine	18.10107	174	317	156				
1,6-anhydro-beta-d-glucose	18.147	205	147	189	117	285	375	103
Hexadecanoic acid	18.904	313	117	129	132	145	201	269
Heptadecanoic acid	19.12865	117	327	132	145			
Inositol	19.311	305	217	147	318	191	265	432
N-acetylglucosamine	19.433295	205	319	147	103			
Spermidine	19.59469	116	129	144	100			
Tryptophan	19.767545	202	291	203	218			
d-glucopyranosiduronic acid	19.83	147	327	117	411	129	132	299
Spermine	20.11994	174	200	146	201			
Cysteine (dimer)	20.3415	218	146	411				
Cystathreonine	20.371	218	128	147	278	160	292	342
9,12-octadecadienoic acid	20.424	337	129	81	262	95	117	
Oleic acid	20.483	339	117	129	75	145	410	155
Octadecanoic acid	20.712	341	117	75	132	129	145	342
Arachidonic acid	21.01	117	91	79	106			
d-turanose	21.244	361	85	217	99	169	127	271
11-eicosenoic acid	21.434975	367	129	73	338			
Serotonin	21.69187	174	290	449				
Adenosine	22.97539	230	236	245	540			

Maltose peak 1	24.492885	204	191	361	217
Maltose peak 2	24.630405	204	191	361	217
Cholesterol	26.548045	329	368	458	353

Table A-20: One-Way ANOVA for Alanine vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	12000689	12000689	15.44	0.004
Error	8	6216309	777039		
Total	9	18216998			

Table A-21: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Alanine

R^2	Coefficient of Variation	S	$\bar{y}$
65.88%	45.887	881.498	1921

Table A-22: One-Way ANOVA for Cysteine vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	43.874	43.874	9.00	0.017
Error	8	3.8.997	4.875		
Total	9	82.872			

Table A-23: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Cysteine

R^2	Coefficient of Variation	S	$\bar{y}$
52.94%	45.514	2.20787	4.853

Table A-24: One-Way ANOVA for Tyrosine vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	923236	923236	7.84	0.023
Error	8	942377	117797		
Total	9	1865613			

Table A-25: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Tyrosine

R^2	Coefficient of Variation	S	$\bar{y}$
49.49%	28.459	343.216	1206

Table A-26: One-Way ANOVA for Lysine vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	630.10	630.10	15.61	0.004
Error	8	322.84	40.36		
Total	9	952.94			

Table A-27: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Lysine

R^2	Coefficient of Variation	S	$\bar{y}$
66.12%	33.540	6.35257	18.94

Table A-28: One-Way ANOVA for Glycerol vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	170816	170816	12.18	0.008
Error	8	112198	14025		
Total	9	23014			

Table A-29: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Glycerol

R^2	Coefficient of Variation	S	$\bar{y}$
60.36%	45.902	118.426	258.0

Table A-30: One-Way ANOVA for Fructose vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	4159176	4159176	10.77	0.011
Error	8	3089729	386216		
Total	9	7248905			

Table A-31: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Fructose

R^2	Coefficient of Variation	S	$\bar{y}$
57.38%	30.659	621.463	2027

Table A-32: One-Way ANOVA for Glucitol vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	97484892	97484892	10.53	0.012
Error	8	74094747	9261843		
Total	9	171579639			

Table A-33: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Glucitol

R^2	Coefficient of Variation	S	$\bar{y}$
56.82%	31.191	3043.33	9757

Table A-34: One-Way ANOVA for Palmitic Acid vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	22193	22193	19.28	0.002
Error	8	9209	1151		
Total	9	31402			

Table A-35: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Palmitic Acid

R^2	Coefficient of Variation	S	$\bar{y}$
66.12%	39.683	33.9288	85.5

Table A-36: One-Way ANOVA for Octodecanoic Acid vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	24675	24675	19.50	0.002
Error	8	10124	1266		
Total	9	34800			

Table A-37: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on Octodecanoic Acid

R^2	Coefficient of Variation	S	$\bar{y}$
70.91%	37.636	35.5743	95.0

Table A-38: One-Way ANOVA for 11-Eicosenoic Acid vs Location (Plastic or Media)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	1	56.092	56.092	13.43	0.006
Error	8	33.423	4.178		
Total	9	89.515			

Table A-39: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA of Location Effect on 11-Eicosenoic Acid

R^2	Coefficient of Variation	S	$\bar{y}$
62.66%	44.913	2.04398	4.551

Table A-40: The Analysis of Variance Table for the Three-Factor Factorial, Fixed Effects Model of Percent Change in Mass

Source of Variation	DF	Sum of Squares	Mean Square	F Value	P Value
Film	1	0.81313	0.813131	27.86	<0.001
Mn	1	0.01397	0.13968	0.48	0.493
QUV	4	0.01157	0.002891	0.10	0.982
Film*Mn	1	0.19168	0.191675	6.57	0.014
Film*QUV	4	0.25071	0.062676	2.15	0.093
Mn*QUV	4	0.05541	0.013853	0.47	0.754
Film*Mn*QUV	4	0.55213	0.138031	4.73	0.003
Error	40	1.16756	0.029189		
Total	59	3.05614			

Table A-41: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA GLM of Percent Change in Mass

R^2	Coefficient of Variation	S	$\bar{y}$
61.80	29.467	0.170848	0.5798

Table A-42: The Analysis of Variance Table for the Three-Factor Factorial, Fixed Effects Model of Colorimetry (ΔE)

Source of Variation	DF	Sum of Squares	Mean Square	F-Value	P-Value
Film	1	0.14386	0.14386	6.03	0.016
Mn	1	0.4748	0.47478	19.90	<0.001
QUV	4	1.8985	0.47463	19.90	<0.001
Ao	1	9.0021	9.00215	377.38	<0.001
Film*Mn	1	0.3899	0.38987	16.34	<0.001
Film*QUV	4	0.6064	0.15161	6.36	<0.001
Film*Ao	1	0.1192	0.11917	5.00	0.028
Mn*QUV	4	0.2441	0.06103	2.56	0.044
Mn*Ao	1	0.1889	0.18885	7.92	0.006
QUV*Ao	44	0.8623	0.21557	9.04	<0.001
Film*Mn*QUV	4	0.1067	0.02668	1.12	0.353
Film*Mn*Ao	1	0.1227	0.12269	5.14	0.026
Film*QUV*Ao	4	0.3630	0.09076	3.80	0.007
Mn*QUV*Ao	4	0.2832	0.07080	2.97	0.024
Error	84	2.0038	0.02385		
Lack-of-Fit	4	0.1721	0.04303	1.88	0.122
Pure Error	80	1.8316	0.02290		
Total	119	16.8094			

Table A-43: R-Square, Coefficient of Variation, Mean Square (S), and Mean ($\bar{y}$) for ANOVA GLM of Colorimetery (ΔE)

R-Square	Coefficient of Variation	S	$\bar{y}$
88.08	17.27	0.154448	0.8941

Table A-44: Evaluation of Transformations, Covariance Structures for Repeated Measures Analysis of Pro-Oxidant and QUV Effect

Covariance Structure	λ	Convergence	*i*	AIC	NPP	Resids vs Fits
AR(1)	1	Y	1	1649.4	X	X
CS	1	Y	1	1649.4	X	X
UN	1	Y	1	1599.5	X	X
AR(1)	½	Y	1	615.6	Y	X
CS	½	Y	1	615.6	Y	X
UN	½	Y	1	631.0	Y	X
AR(1)	⅓	Y	1	219.9	Y	Y
CS	⅓	Y	1	219.9	Y	Y
UN	⅓	Y	1	248.1	Y	Y

Table A-45: Type 3 Test of Fixed Effects for Repeated Measures Analysis of Pro-Oxidant and QUV Effect on pNP Production (μmol)

Effect	Num DF	Den DF	F-Value	P-Value
Time	5	252	39.84	<.0001
Film	1	252	592.25	<.0001
Time*Film	5	252	11.54	<.0001
Mn	1	252	0.10	0.7572
Time*Mn	5	252	0.03	0.9996
Film*Mn	1	252	4.47	0.0354
Time*Film*Mn	5	252	0.12	0.9875
QUV	4	252	98.24	<.0001
Time*QUV	20	252	1.24	0.2254
Film*QUV	4	252	127.19	<.0001
Time*Film*QUV	20	252	1.21	0.2428
Mn*QUV	4	252	14.59	<.0001
Time*Mn*QUV	20	252	0.14	1.0000
Film*Mn*QUV	4	252	8.91	<.0001
Time*Film*Mn*QUV	20	252	0.18	1.0000