

**Analysis of *Aspergillus oryzae* degradation of commercial agricultural mulch
films composed of poly(butylene adipate-co-terephthalate) and poly(lactic acid)**

Report to the Resource Recovery Fund Board

By

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Abstract

The filamentous fungus, *Aspergillus oryzae*, was inoculated on a fungal minimal media (containing 1% sucrose carbon source) with commercially available biodegradable agricultural mulch films composed of synthetic aliphatic polyesters poly(butylene adipate-co-terephthalate) (PBAT) and poly(lactic acid) (PLA). The vessels were incubated at 21°C for 44 days, during which time trials of oxygen and carbon dioxide concentrations were performed. An assay for enzymatic activity of esterases was performed using a p-nitrophenyl butyrate substrate solution that produced p-nitrophenol via ester hydrolysis that was measured spectrophotometrically. The enzymatic activity of *A. oryzae* in the presence of the mulch films produced three times the amount of p-nitrophenol than in the absence of the mulch films. A comparison of initial and final masses supported the indication of biodegradation, with significant differences between treatment films. Metabolomics analysis showed higher activity towards the mulch films than the media, with significant increases in amino acid, carbohydrate, and fatty acid metabolites.

Keywords: agricultural mulch film, *Aspergillus oryzae*, biodegradation, degradation

1. Introduction

The excessive production and disposal of petroleum-based plastics has had an undesirable effect on the environment, causing dangerous levels of pollution in the atmosphere. Due to their inexpensive production and desired physical-chemical properties, these non-biodegradable synthetic polymers are widely used (Cho et al. 2011). Approximately 140 million tonnes of plastics are produced worldwide every year (Shimao 2001). The production and manufacture of synthetic plastics each require approximately 4% of annual petroleum production. Approximately 50% of synthetic polymers are intended for single use (i.e. packaging, agriculture films; Hopewell et al. 2009) and consequently disposed of.

Agriculture has been revolutionized through the use of plastic film products by providing benefits such as row covers, silage bags, and mulches. The largest volume of agricultural plastics used is as mulch films. 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 tonnes of agricultural plastic wastes are generated on Maritime farms every year. Nova Scotia uses the largest weight of mulch films and row covers in the Maritimes, contributing 30 of the estimated 37 tonnes generated per year (CleanFARMS 2012).

The lifetime of mulch film exceeds the crop cycle duration and therefore it is usually left in the soil where it will only partially degrade, causing management problems. The use of biodegradable plastics can aid in achieving the goal of responsible plastics disposal in agricultural systems. These biodegradable mulch lifetimes are much shorter in soil, but may still leave residual fragments which could interfere with equipment or, if removed and composted, contaminate compost piles. The production cost of biodegradable plastics are higher than recalcitrant synthetic plastics, and the current disposal systems cannot recover or equal the cost of production, making them uneconomical for most manufacturing companies (Maeda et al. 2005).

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). 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 often used method of evaluating polymer degradation in laboratory tests (Shah et al. 2008). 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). This leaves open the possibility of using specific microorganisms to enhance the degradation process of these plastics.

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 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 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 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). Poly(ϵ -caprolactone) (PCL) is another aliphatic polyester that is commercially produced but has limited applications due to the high cost of production and poor mechanical properties. In contrast, poly(butylene succinate) (PBS) (Fig. 1 C) has a low production cost and good mechanical properties, similar to polypropylene (Zhao et al. 2005).

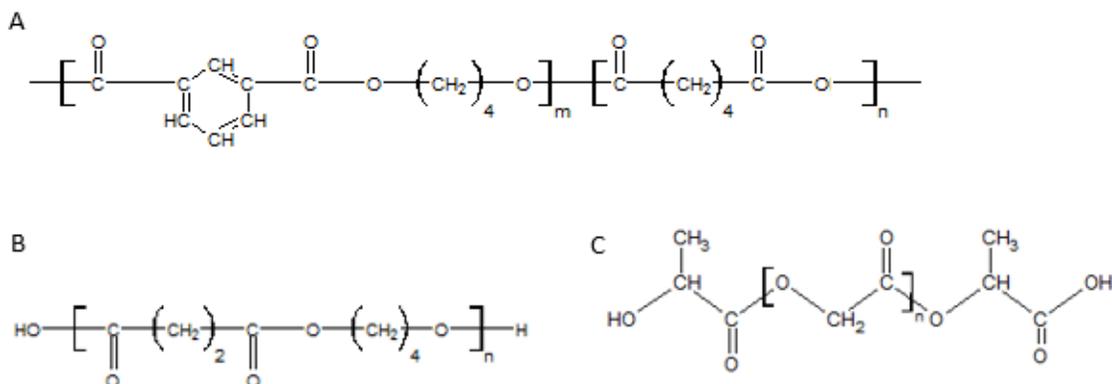


Fig. 1: Monomer structures of PBAT (A), PLA (B), and PBS (C) (Drawn on ChemSketch)

The biodegradation of PBAT, PLA, and a blend was studied by Weng et al. (2013) using soil conditions. The samples were buried 40 cm deep in soil and left to degrade for four months, with a specimen of each being removed in monthly increments. After four months, the PBAT film was merely

residual fragments, the PLA film remaining was small amounts of residual debris, and the blend retained larger fragments. Evaluations were done visually, by scanning electron microscope (SEM), as well as elemental analysis of the carbon, hydrogen and oxygen contents of the films before and after degradation. The elemental analysis showed a decrease in carbon content and an increase in oxygen content, which indicated degradation of the plastic films.

PBS is used as the main component in commercially available biodegradable plastic mulch films. Synthetic mulch films are the most common and highly consumed plastic products on agricultural farm lands and have led to an increase in plastic wastes entering the environment directly. The removal and disposal of the used films is cumbersome and energy consuming. Koitabashi et al. (2012) studied the degradation of biodegradable mulch films in the soil environment by phylloplane fungi isolates from leaf surfaces of gramineous plants. Using a commercially available biodegradable mulch film composed of PBS, PBSA, and PBAT at a weight based ratio of 47:37:17, trials were performed on both sterilized and unsterilized soil. The film was placed on top of the soil, and a liquid culture broth of the isolates was poured on the surface of the film. After 6 days of incubation at 28°C, the film was washed with distilled water, air dried, weighed and evaluated for degradation rate. This experiment was repeated three times. During incubation, gray-colored mycelia thoroughly covered the film on sterilized soil. The weight loss of recovered residual film accounted for 99.8% (SD = 0.11) of the initial weight. The surface of the film on unsterilized soil was only sparsely covered by several fungal colonies of different colors, with an average weight loss of 14.6% (SD = 2.18), indicating that degradation was slower on unsterilized soil (Koitabashi et al., 2012).

A. oryzae is a filamentous fungus that is capable of secreting large amounts of enzymes, making it well suited for biodegradable plastic degradation systems. *A. oryzae* secretes a carboxylic ester hydrolase when grown on such polyesters as fermentation substrates. This enzyme has been identified as cutinase, an esterase, by Maeda et al. (2005). In this study, *A. oryzae* was grown under culture

conditions containing emulsified PBS and emulsified poly(butylene succinate-co-adipate) (PBSA) as the sole carbon source. The media had a translucent appearance due to the added emulsified plastics, so digestion of the PBS and PBSA by *A. oryzae* could be identified by the clearing of the culture. The ester bonds present in the PBS and PBSA structures were enzymatically hydrolyzed by the secreted esterase, which recognized and hydrolyzed the plastics. The catalytic pocket was investigated and found to be divided into carboxylic acid and alcohol recognition sites (Maeda et al., 2005).

To further evaluate the applications of *A. oryzae* in biodegrading aliphatic polyesters, a study to evaluate the actions of the fungus when cultured with commercial agricultural mulch films (composed of PBAT and PLA) was performed.

2. Materials and Methods

2.1 Preliminary Trials

Prior to approval for obtaining *A. oryzae*, work was done with microorganisms isolated from plastics found in compost at the Colchester Balefill. The plastics were placed on two different types of media (Potato Dextrose Agar and Nutrient Agar, to promote growth of fungi and bacteria, respectively) and the colonies were re-isolated to obtain a pure culture of each. The pure colonies were observed under a microscope for tentative identification. *Ascomycetes* (yeast) and *zygomycetes* (microscopic fungi) were found on the Potato Dextrose Agar plates, and both gram-negative and gram-positive bacteria were found on the Nutrient Agar. Using three separate treatments (yeast, fungi, bacteria), pieces of a household biodegradable plastic were submersed in a 250mL mason jar containing liquid broth (Sabouraud Dextrose Broth for yeast, bacteria, and Tryptic Soy Broth for fungi).

2.2 Culturing of A. oryzae

A pure culture of *A. oryzae* was obtained January 31, 2014 from the American Type Culture Collection (ATCC No. 42149) and rehydrated in sterilized water to produce a spore suspension. The suspension was cultured on a fungal minimal media (312 Czapek's Agar), containing 3.0g NaNO₃, 1.0g

K_2HPO_4 , 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl, 0.01g $FeSO_4 \cdot 7H_2O$, 30.0g commercial grade sucrose and 15.0g Agar

No. 1. The cultures were incubated at 21°C and biomass was observed microscopically after 7 days.

2.3 Vessel Inoculation

Biomass from the cultivars was inoculated in 250mL mason jars containing modified minimal media (sucrose reduced to 1% w/w) and commercial mulch films with a known initial mass. Films were obtained from Willeap (black film composed of PBAT, PLA and starch; South Korea) and Indaco (clear film composed of PBAT and PLA; Canada). Control vessels containing only minimal media and inoculated biomass were used to determine the growth of *A. oryzae* in the absence of films. The vessels were incubated at 21°C for 44 days.

2.4 Oxygen and Carbon Dioxide Data Collection

Oxygen and carbon dioxide data was collected during four separate 72 hour time trials during the vessel incubation. Prior to sealing the vessels, they were aerated with high quality atmospheric air that had been oxygen saturated by bubbling through distilled water. A PreSens Oxygen Probe was used to take direct measurements of oxygen concentration through a fiber optic sensor. Headspace extractions were performed to measure carbon dioxide concentrations using gas chromatography. Measurements were taken at 0 hr and 72 hr.

2.5 Enzymatic Activity Assay

The *A. oryzae* spore suspension was added to 250mL Erlenmeyer flasks containing liquid modified minimal media (1% sucrose, no agarose) and Willeap black mulch film with a known initial mass. Control flasks without mulch film were used to determine background enzymatic activity. The flasks were incubated at 21°C on orbital shaker (set at 100rpm) for 14 days. Following the incubation, 2mL liquid media was removed by pipet and filtered through Whatman No. 1 filter paper to remove solids. The extract and media was added to a p-nitrophenyl butyrate substrate solution, a known

esterase substrate that produces p-nitrophenol via enzymatic hydrolysis, which can then be measured spectrophotometrically @ 405nm.

2.6 Initial and Final Mass Comparison

In the vessel and flask incubations, the initial mass of the mulch film was recorded. Following incubation, the films were removed, gently washed with distilled water, allowed to dry, and then massed to obtain a final mass. The initial and final masses were compared.

2.7 Metabolomic Analysis

Indaco mulch film was inoculated in four replicates of the 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 3. Fungal growth from separate cultivars containing solely media was also collected as a Control in replicates of 3. Samples were prepared using a method adopted from Liebeke & Bundy (2012), which consisted of addition of two extraction solutions (1:1 acetonitrile:methanol and 2:2:1 acetonitrile:methanol:water) where the samples were sonicated and centrifuged for each. The supernatants were the nitrogen evaporated in a fume hood, and the internal standards were added (Leucine D-3, Glucose U ¹³C, Myristic Acid, at 1mM 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 μ L sample was injected in splitless mode, following five pre-injection solvent washes (4 μ L acetone), two sample washes (1 μ L) and three sample pumps (1 μ L), followed by five post-injection solvent washes (4 μ L acetone). Helium was the carrier gas, and the instrument was set at a constant flow of 1 mL min⁻¹ (Pressure = 8.2317 psi). The column was an Agilent DB-5MS 5% phenyl 95% methylpolysiloxane (dimensions of 30 m

length \times 0.25 mm i.d. \times 0.25 μ m 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 NIST 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 a 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.

2.8 Statistical Analyses

Analysis of results was carried out in Minitab using the Analysis of Variance (One Way and Two Way-General Linear Model) at a 95.00% confidence interval ($\alpha = 0.05$).

3. Results and Discussion

3.1 Preliminary Trials

The preliminary trials were used to determine proper methodology for the proposed research. It was decided to continue the use of the PreSens Oxygen Probe and headspace extractions for oxygen and carbon dioxide data collection. However, it was decided to use solid media instead of liquid media to obtain this data due to the dissolution of oxygen in the headspace into the liquid media affecting the collected data. The solid media also allowed for better visual observations during the incubation.

3.2 Oxygen and Carbon Dioxide Concentrations

A consistent decrease in oxygen concentration was noticed when the data from the four time trials was compiled (Fig. 2). The decrease in concentration of the Control and Indaco vessels was found to be significant ($p = 0.032$ and 0.050 , respectively). The carbon dioxide data was inconsistent though it was expected to show a trend (Fig. 2). The compiled changes in carbon dioxide concentrations were not found to be significant.

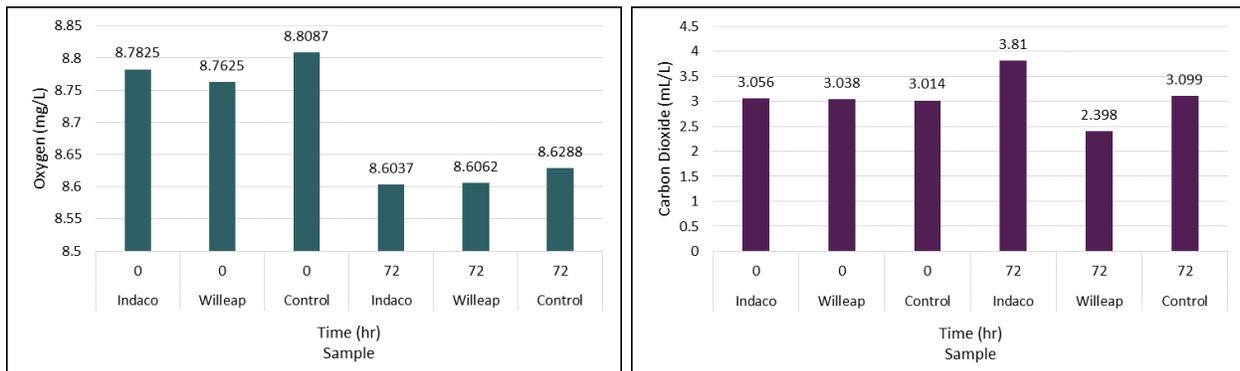


Fig. 2: Oxygen consumption and carbon dioxide evolution by fungal agent when exposed to different compostable plastic materials.

The Indaco and Control treatments showed a general increase of carbon dioxide concentration. The more notable decrease in concentration of the Willeap treatment could be attributed to carbon use for microbial growth. The lack of distinct production in that of the Control treatment could be a result of carbon dioxide dissolving in the media. The compiled data averages showed high standard deviations for carbon dioxide data than for oxygen data (Table 1).

Table 1: Mean oxygen consumption and carbon dioxide evolution over a 72 hour time trial exposure of compostable plastics to <i>A. oryzae</i>.				
	Sample	Time (hr)	Average	Standard Deviation
Oxygen (mg/L)	Control	0	8.8087	0.1368
		72	8.6288	0.1644
	Indaco	0	8.7825	0.1686
		72	8.6037	0.1647
	Willeap	0	8.7625	0.1699
		72	8.6062	0.1556
Carbon Dioxide (mL/L CO₂)	Control	0	3.014	2.52
		72	3.099	3.357
	Indaco	0	3.056	1.768
		72	3.81	4.13
	Willeap	0	3.038	3.274
		72	2.398	2.236

The time trials were examined separately due to the inconsistency of the compiled data.

Oxygen consumption over 72 hours in Time Trial 1 (Fig. 3 A) showed a significant difference between treatments over time (interaction Time*Sample, $p = 0.014$) (Table 2). The Indaco, Willeap, and Control treatments showed a significant decrease in oxygen concentration over time ($p = 0.001$, 0.004 and 0.005 , respectively), though the change in carbon dioxide concentration over time was found to be insignificant for all three treatments. It is evident, however, that the Control and Indaco treatments showed an increase of carbon dioxide concentration whereas the Willeap treatment experienced a

decrease in carbon dioxide during Time Trial 1 (Fig. 3 A).

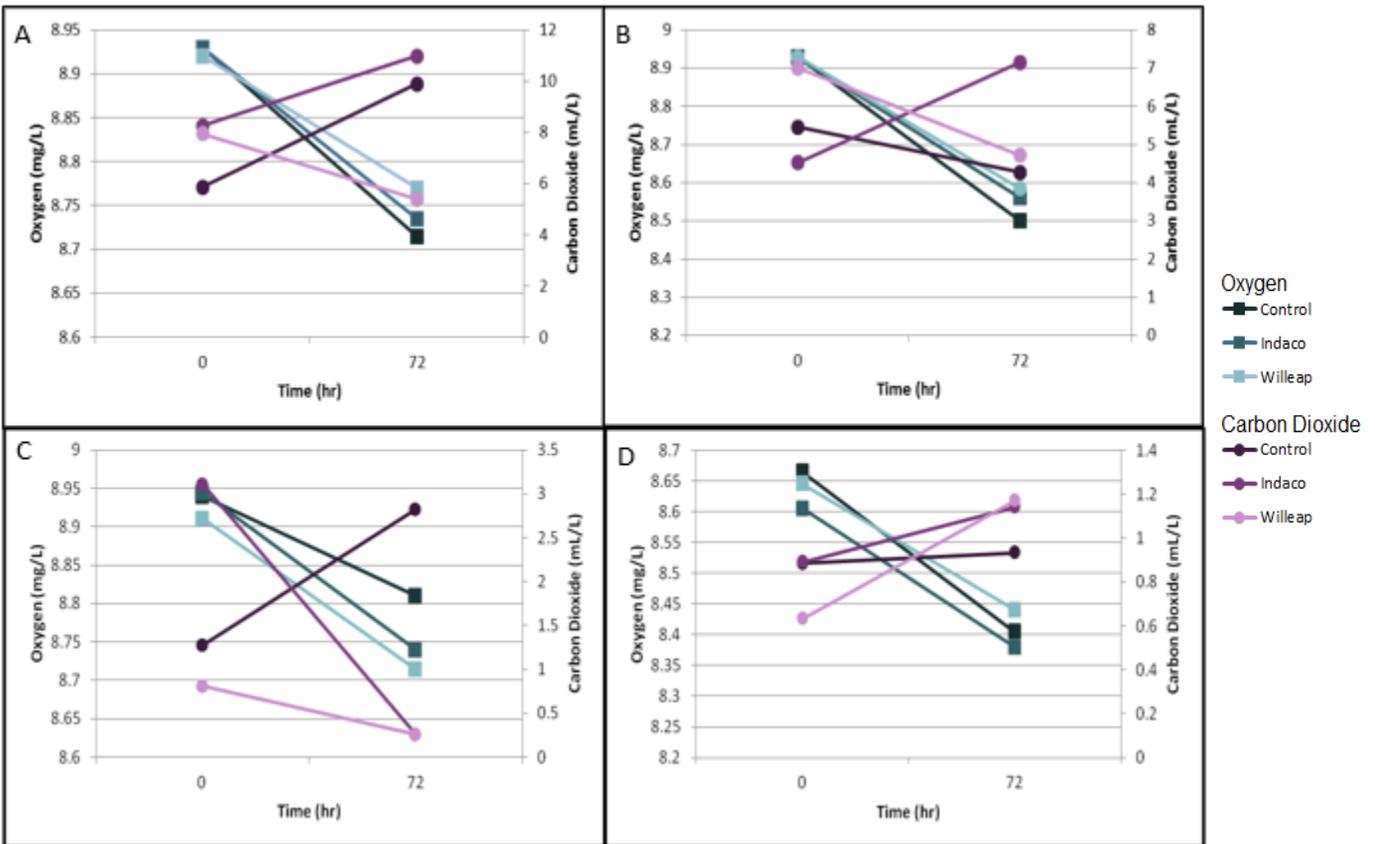


Fig. 3: Oxygen consumption and carbon dioxide evolution from fungal plates over several sampling periods including Time Trial 1 (A), Time Trial 2 (B), Time Trial 3 (C), and Time Trial 4 (D) with different formulations of compostable plastics, Indaco and Willeap.

The Indaco and Willeap treatments showed a greater decrease in oxygen concentration than the Control treatment in Time Trial 2 (Fig. 3 B). All treatments showed a significant decrease in oxygen concentration over time ($p = 0.003, 0.000, 0.019$ for Indaco, Willeap, Control respectively). The results show that the Control and Willeap treatments experienced a decrease in carbon dioxide concentration, whereas the Indaco treatment showed a significant increase in carbon dioxide concentration ($p = 0.001$) during Time Trial 2 (Fig. 3 B). The carbon dioxide concentrations were found to be significantly different between treatments over time (interaction $p = 0.004$).

Indaco and Willeap treatments experienced a greater decrease in oxygen concentration than the Control in Time Trial 3, though only Indaco showed a significant decrease ($p = 0.037$) (Fig. 3 C). The

carbon dioxide concentrations were found to be significantly different between treatments over time (interaction $p = 0.000$). The results show that the Indaco and Willeap treatments experienced a significant decrease in carbon dioxide concentration ($p = 0.001$ and 0.000 , respectively), whereas the Control showed an increase in carbon dioxide during Time Trial 3 (Fig. 3 C). Oxygen concentration over 72 hours in Time Trial 4 (Fig. 3 D) did not show a significant difference between treatments. Only the Control treatment experienced a significant decrease in oxygen concentration ($p = 0.026$). However, the Willeap treatment showed a significant increase carbon dioxide concentration ($p = 0.036$) in Time Trial 4 while Indaco and Control treatments increased only slightly (Fig. 3 D).

Table 2: P-Values of Two Way ANOVA for Conc. vs Time*Sample (Interaction)			
	Time Trial	P-Value	R ² Value
Oxygen	1	0.014	0.9882
	2	0.312	0.9674
	3	0.483	0.8102
	4	0.820	0.7804
Carbon Dioxide	1	0.340	0.0107
	2	0.004	0.3009
	3	0.000	0.7442
	4	0.226	0.3225

3.3 Enzymatic Activity Assay

A standard curve (Fig. 4) and regression equation of p-nitrophenol concentration were obtained from the absorbance values of working standards measured at 405nm:

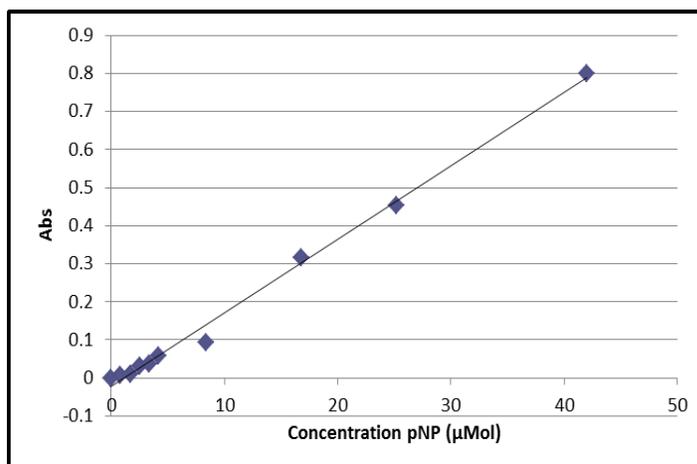


Fig. 4: Standard curve of concentration p-nitrophenol vs absorbance

$$\mu\text{Mol} = 0.012 + 67.8(\text{Abs}) \quad [\text{Eq. 1}]$$

The absorbance of the samples (Treatment and Control) was measured in 5 minute intervals over a 45 minute period. The p-nitrophenyl butyrate substrate solution was used as a reference prior to each of the interval readings. The esterase activity of the Treatment flasks was evidently higher and expressed a more linear production of p-nitrophenol than that of the Control flasks (Fig. 5). There was a lag in p-nitrophenol production in the Control flasks.

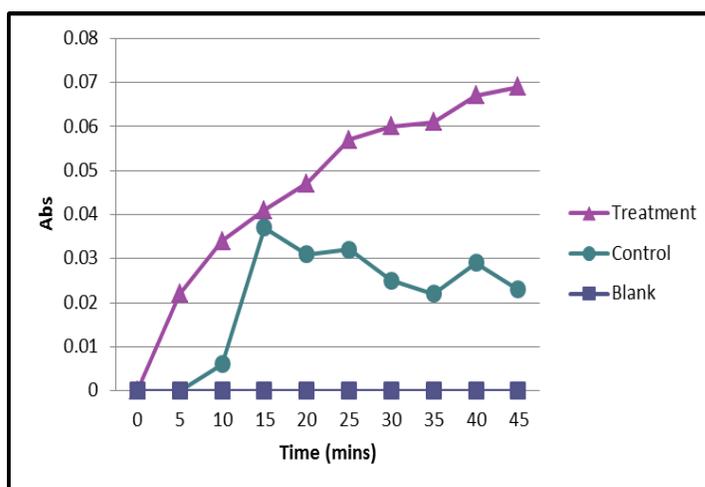


Fig. 5: Absorbance @ 405nm of samples over 45 minutes

It was evident that more esterase enzymes were secreted by *A. oryzae* in the presence of the Willeap mulch film. The esterase activity of the Treatment flasks produced 2.98 times the amount of p-nitrophenol when compared to that of the Control flasks (Table 3).

Table 3: Initial and Final p-Nitrophenol Concentrations (μMol)		
	Initial	Final
Blank (pNPB)	0.000	0.000
Control	0.000	1.571
Treatment	0.000	4.690

3.4 Initial and Final Mass Analysis

Though not a direct measure of biodegradation, the loss in mass of the mulch film supports the indication of biodegradation via hydrolysis of the ester bonds.

Of the vessel incubation (44 days), Willeap film showed the greatest change in mass, being significantly greater than that of the Indaco film ($p = 0.014$) (Table 4). This could be attributed to the presence of starch in the mulch film.

During the cutinase assay incubation (15 days), Willeap film showed an even greater significant change in mass than either film in vessel incubation ($p = 0.006$). This could be explained by the smaller film size as well as increased exposure of the film surface to the active enzyme due to the media being liquid.

Table 4: Mulch Film Mass Analysis					
	$m_i - m_f$ (g)	Sample Comparison	P-Value	R_2 Value	Tukey's Grouping
Indaco (vessel)	0.00403	Vessel Incubation	0.014	0.6648	Indaco A
Willeap (vessel)	0.01282				Willeap B
Willeap (flask)	0.01857	Vessel and Flask Incubation	0.006	0.7163	Indaco B Willeap (vessel) AB Willeap (flask) A

Indaco vessels replicates 3 and 4 were contaminated and omitted from the average, though the contaminating microorganism actually caused a greater loss of film mass (Fig. 6). This microorganism will be investigated further.

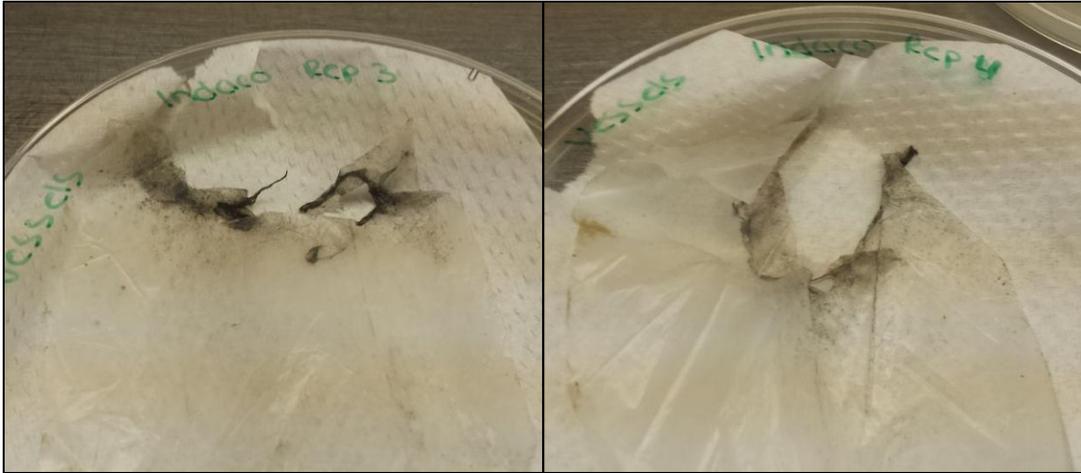


Fig. 6: Indaco Rep 3 and Indaco Rep 4 interesting observations

3.5 Metabolomic Analysis

From the metabolomics analysis, 10 metabolites expressed by *A. oryzae* were found to be significantly different between the film and media growth substrates (Fig. 7).

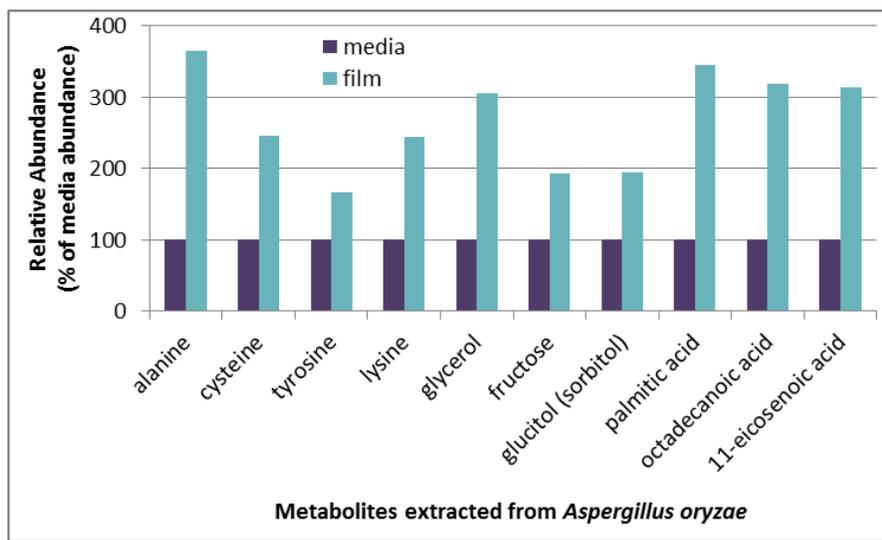


Fig. 7: Metabolites extracted from *A. oryzae* in relative abundance of media

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 indicate higher levels of activity towards the film than towards the media. The increased presence of amino acids indicate higher levels of protein synthesis, speculated to be 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.

4. Conclusion

The general trend of decrease in oxygen concentration is hypothesized to be consumption for respiration, though the carbon dioxide concentrations do not correspond with respiration. The production of carbon dioxide is hypothesized to be microbial respiration, whereas the decrease of carbon dioxide could be attributed to microbial fixation, which is supported by the observation of fungal growth on the plastics in the vessels (Fig.).



Fig. 8: Willeap vessel fungal growth Day 1 (left) and Day 29 (right)

The decrease and low increases could also be a result of carbon dioxide being dissolved in the media (as in the Control treatment, due to the limited carbon in the media and lack of an alternate carbon source) or leaking from the system. Another speculation is that carbon dioxide emissions were reduced due to the formation of calcium carbonate by denitrification through fungal metabolism (Houa et al., 2011). This is possible due to the presence of nitrate in the media and small amounts of calcium in the Agar No. 1 powder.

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 also noted to be more brittle following incubation, indicating a change in surface composition of the mulch films.

Metabolomic analysis showed increased activity towards the Indaco mulch film when compared to the minimal media, resulting in ten significantly increased metabolites. The increased presence of amino acids indicate higher levels of protein synthesis, 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.

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