INVESTIGATION OF THE BIODEGRADATION OF A SYNTHETIC POLYESTER OF BUTANEDIOL, ADIPIC ACID, AND TEREPHTHALIC ACID BY HYDROLASE-PRODUCING SOIL MICROBES

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Introduction

Ever since their initial production at an industrial scale at the beginning of the 20th century, plastics have revolutionized the materials industry. Their properties such as their malleability, versatility, high chemical resistance and high volume to weight ratio as well as favorable production conditions compared to other materials such as metals have made them an essential base material in almost every major manufacturing industry today.

Worldwide production of polymer materials was estimated to be around 150 million tons in the mid 1990s, with an average yearly consumption of 80-100kg per capita in industrialized countries. [1] An estimated forty percent of this total production is discarded into landfills. [2]

Some of the properties that have made these materials so attractive commercially such as their resilience to degradation, and high volume to weight ratio have not only made plastics a large portion of domestic waste, but also pollutants that will effectively remain in landfills and other waste management facilities for hundreds of years.

Considering the scale of production and consumption of these materials, a multitude of different alternatives are now being studied to deal with these issues. Recycling alternatives, education campaigns, use of alternate or reusable materials, promoting lower consumption of these materials are some examples of solutions being considered.

One of the concrete solutions being put forward to alleviate the pollution burden caused by plastic production is to design new biodegradable polymers targeting specific sectors such as the plastic film and packaging industry producing materials for one time use applications. Growing public awareness and new government policies and initiatives have prompted the plastics industry to research and develop new materials that are readily degraded in the environment. [1]

A number of materials have been developed with varying degrees of success. One of those is a aliphatic-aromatic copolyester of 1,4-butanediol, adipic acid and terephthalic acid. It has been commercialized by BASF of Germany under the trade name Ecoflex™ and by Eastman-Kodak of the United States under the trade name Eastar Bio™. The copolyester id produced from the ramdom polymerization of the monomers of adipic acid / butanediol, and terephthalic acid / butanediol shown in Figure 1 below. The degradation of this copolyester was investigated in compost environments [3] and

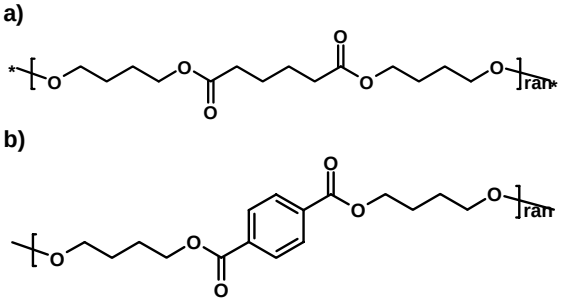

Figure 1: **(a)** Adipic acid and **(b)** terephthalic acid monomers of Ecoflex™ and Eastar Bio™

with compost isolates [4]. Extensive research has been published for experiments done at elevated temperature with *Thermobifida fusca* (previously known as *Thermomonospora fusca*) and a hydrolase isolated from this bacterial strain [4, 5] Results from these studies indicate rapid degradation of the copolyester.

The purpose of this work was to investigate the degradation potential of Ecoflex™ by hydrolase-producing soil microbes. This was achieved by determining the resiliency of the copolyester to microbial attack at ambient temperatures. Screening experiments with pure strains of bacteria, yeasts and fungi were set up to determine the enzymatic pathway used by microbes to breakdown this polymer in the environment. The degradation of Ecoflex™ films was followed by weight loss measurements. Analytical procedures using gas chromatography (GC), and gel permeation chromatography (GPC) were used to identify degradation intermediates and to characterize the polymer films after their exposure to different pure microbial strains.

Methodology

Chemicals

The following chemicals were used and obtained from the suppliers specified: 1,4-butanediol, tetramethylammonium hydroxide (TMAH), trimethylphenylammonium hydroxide (TMPAH), 2,2,4,4,6,8,8- heptamethylnonane, terephthalic acid, myristic acid, pentadecane and glycerol from Sigma-Aldrich; chloroform, ethyl acetate, methanol, diethyl ether, adipic acid, phenolphthalein from Fisher Scientific/Acros Chemicals. All chemicals were reagent grade.

Polymer Preparation

Polymer films. Ecoflex™ in pellet form was obtained from BASF (Germany). The films were made using a solvent casting technique. All films were made by dissolving three pellets in 3ml of chloroform. The dissolved polymer and solvent was left to stand for 24 hours in 2.5cm diameter aluminum weigh dish. The initial weight of the films was then recorded. The weight of the films ranged between 80mg and 100mg. For the degradation experiments, the films were sterilized with 10% bleach solution in distilled water for a period of 5 minutes and rinsed with sterile distilled water.

Polymer Pellets. Polymer pellets were sterilized using 10% bleach solution in distilled water. The pellets were stirred in a covered beaker for a period of one hour before inoculation.

Derivatization

Adipic acid and terephthalic acid were derivatized to form their respective methyl diesters using TMAH and TMPAH as specified in the procedure described by ASTM designation D 5974 – 96. 2ml sample of ethyl acetate containing myristic acid as the internal standard was blown down and subsequently derivatized.

Microorganisms

All microorganisms were purchased from the American Type Culture Collection (ATCC, United States). They are listed in Table 1. All strains were grown in shake flasks containing their recommended growth media at the recommended temperature. The cultures were subsequently stored at -70^{o}C in a glycerol/media mixture. The cultures were revived and transferred to fresh media before inoculation. Inoculums were incubated for a period not exceeding 7 days.

Degradation Experiments

The Degradation experiments were run on both solid and liquid media.

Solid Media Degradation Tests. For the solid media degradation experiments, microorganisms were incubated at 30^{o}C. Experiments were run for periods of 21, 35 and 50 days and films were weighed before and after their exposure to the microorganisms. Before inoculation, the polymer films were sterilized according to the procedure highlighted above. For solid medium experiments, the microorganisms were incubated on YM agar (DIFCO) or minimal salts media (MSM) and 20g/L agar (DIFCO). MSM contained 4g/L NH_4NO_3, 4g/L KH_2PO_4, 6g/L Na_2HP_4, 0.2g/L $MgSO_4{\cdot}7H_2O$, 0.01g/L $CaCl_2{\cdot}2H_2O$, 0.01g/L $FeSO_4{\cdot}7H_2O$, 0.014g/L Na_2EDTA (Fisher Scientific/Acros Chemicals). Before inoculation, the films were deposited on the agar plates for a period of 24 hours to check for possible contamination. If no growth was observed on the agar plates, they were inoculated with 0.5ml of a pure microbial strain.

Table I: Microorganism List

Name	Origin	Isolated	Type	Selection Criteria
Rhodococcus rhodochrous	ATCC 13808	Soil	Bacteria	produces esterases, degrades plasticisers
Rhodococcus rhodochrous	ATCC 21766	Soil	Bacteria	produces esterases, degrades plasticisers
Rhodococcus sp.	ATCC 29671	Soil	Bacteria	degrades hydrocarbons
Rhodococcus ertythropolis	NCIMB 13064	Soil	Bacteria	degrades halogenated compounds
Rhodococcus ertythropolis	ATCC 4277	Soil	Bacteria	produces cholesterol oxidase
Corynebacteria sp.	ATCC 21744	-	Bacteria	degrades plasticizers
Corynebacteria hydrocarbooxydans	ATCC 21767	Soil	Bacteria	degrades plasticizers
Thermobifida fusca	ATCC 27730	Soil	Bacteria	degrades cellulose
Pseudomonas putida	ATCC12633	-	Bacteria	degrades aromatic acids
Pseudomonas fluorescens	ATCC 13525	-	Bacteria	degrades esters
Pseudomonas acidovorans	-	-	Bacteria	degrades plasticizers
Pseudomonas aeruginosa	ATCC 10145	-	Bacteria	produces lipases
Bacillus subtilis	ATCC 21332	-	Bacteria	produces surfactant
Bacillus subtilis	ATCC 6051	-	Bacteria	test for degradation resistance of latex paint
Bacillus sp.	ATCC 19385	Soil	Bacteria	degrades hydocarbons
Acinetobacter sp.	ATCC 31012	Tar on beach	Bacteria	degrades petroleum, produces esterases
Sphingobium herbicidovorans	ATCC 70029	Soil	Bacteria	degrades herbicides (2,4-D)
Unidentified mixed culture	ATCC 53922	Soil	Bacteria	degrades paint
Aeromonas sp.	ATCC 55641	Soil	Bacteria	degrades aromatic and aliphatic compounds
Streptomyces clavuligerus	ATCC 27064	Soil	Bacteria	degrades plasticizers
Glisomatix murorum	ATCC 16277	Soil	Fungi	degrades cellulose, lignin, xylan
Paecilomyces lilacinus	ATCC 200182	Soil	Fungi	degrades PHA/PHB, PCL
Phanerochaete chrysosporium	ATCC 24725	vitis venifera	Fungi	degrades PHA/PHB, lignin
Aspergillus niger	ATCC 16888	-	Fungi	degrades pulp and paper mill wastewater
Trametes versicolor	ATCC 12679	beech canker	Fungi	degrades cellulose, lignin
Penicillium pinophilum	ATCC 9644	Radio set	Fungi	degrades plastics
Candida bombicola	ATCC	Bumblebee	Yeast	degrades esters
Candida cylindrace	ATCC 14830	Soil	Yeast	produces cholesterol esterases
Candida tropicalis	ATCC 15114	-	Yeast	degrades plasticizers
Candida lipolitica	ATCC 20390	-	Yeast	degrades plasticizers

Liquid medium experiments. For liquid medium experiments, the microorganisms were grown in 500ml shake flasks containing 100ml of liquid media. The flasks were sterilized in an autoclave for 15 minutes with saturated steam at 121°C. These degradation experiments were also run at 30°C for a period of 21 days and 35 days. Shake flasks were incubated in a shaker (New Brunswick Scientific) at 200rpm. For liquid medium experiments, microorganisms were grown either on nutrient broth (DIFCO) or YM broth (DIFCO). The polymer films were sterilized according to the procedure highlighted above and the flasks placed in the shakers for a period of 24 hours. If no growth was observed, the flasks were then inoculated with 2ml of a pure microbial strain.

Batch Reactor

A 2L New Brunswick Scientific Batch reactor was used with 1.5L of MSM, 4g/L glucose and 0.1g/L yeast extract (DIFCO, United States). The reactor was run at room temperature (22°C) for 30-day period. The reactor was sterilized with saturated steam at 121°C for 1 hour. The sterilized polymer pellets (serilized according to procedure mentioned above) were inserted inside the reactor 15 minutes after it was taken out of the autoclave. Once the media reached room temperature, the glucose and a 10ml inoculum of *Bacillus subtilis* ATCC 21332 was injected the reactor. The runs were stopped after 30 days.

Gas chromatography

A Varian CP-3800 gas chromatograph with an FID detector and Sil 5CB 15mx0.53mm column (Varian, St-Laurent, Qc.) with the following settings was used: injector temperature 250°C, initial column temperature 40°C, a 2min hold time, with 10°C ramp rate to a final temperature of 300°C, and the detector temperature was set at 300°C. For shake flasks experiments, the entire contents of the flask was extracted once with 10ml of organic solvent. The organic solvent used was ethyl acetate or chloroform depending on the nature of the analysis. For the batch reactor runs, 20ml samples were extracted with 10 ml of ethyl acetate and subsequently derivatized. A gas chromatograph/mass spectrophotometer GC/MS was used to identify the metabolites (Thermo Quest model TRACE GC 2000 / Finnigan POLARIS, equipped with a RTX-5 MS column (Resteck) with 0.25mm internal diameter).

Results and Discussion

The first experiments were set up to assess the biodegradation potential of Ecoflex™. This screening stage was done by exposing Ecolfex™ films to a variety of microorganisms in order to identify organisms that could readily degrade the copolyester. Table 1 lists the microorganisms used and their degradation properties.

The organisms were selected according to their ability to degrade similar compounds, such as esters or polyesters, other polymers, their ability to produce hydrolases, their use as material integrity testers, or their known ability to degrade hydrocarbons.

Results of experiments indicate that all microbes show minimal to no degradation after 21 days of incubation. Furthermore, no significant increase in degradation is obtained by increasing the exposure time to 35 days. This was probably due to the pH shift in the absence of any buffer in the growth medium. The organisms showing the most degradation were *Paecilomyces lilacinus* grown on YM agar, *Bacillus subtilis* ATCC 21332, *Penicillium pinophilum* and *Bacillus subtilis* ATCC 6051 respectively. Partial results of the screening are shown in Figure 2. Note that none of the microorganisms tested was able to degrade more than 5% of the original weight of the films.

Penicillium pinophilum showed a significant difference in degradation depending on the nature of the media used. A two fold increase in degradation was exhibited when P. pinophilum was grown on MSM agar with no co-substrate compared to growth on YM agar. Furthermore, both *P. lilacinus* and *P. pinophilum* showed lower degradation rates when grown on liquid YM than on solid media (YM agar).

It is interesting to note the difference in the degradation exhibited by both strains of *Bacillus subtilis*. This difference may be explained by the fact that strain 21332 produces a surfactant, and strain 6051 does not. The presence of surfactant may therefore enhance the degradation of the polyester. However, further studies need to be conducted to determine the exact nature of surfactant enhancement, such as increase in solubilization or emulsification to name a few. [6-8]

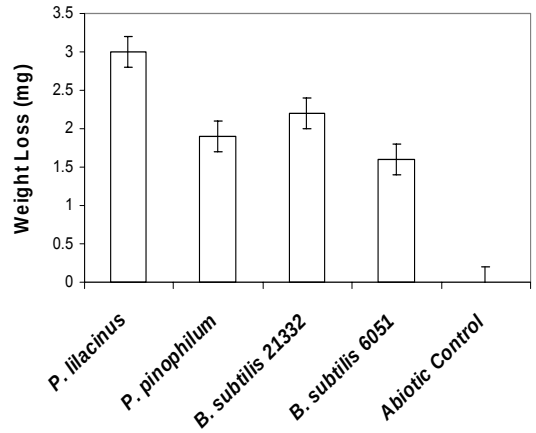

Figure 2: Weight loss of films after 21 days of incubation at 30°C with the pure microbial strain indicated. Note that the results shown for *P. lilacinus* and *P. pinophilum* are with YM agar and for *B. subtilis* in nutrient broth.

It is important to note that the screening experiments were performed in rich microbiological media. It has been shown that the availability of water soluble substrate may in fact slow the degradation process by inhibiting the production of enzymes used in the degradation of other carbon sources.[9, 10] In addition, no inducer was used as a co-substrate which may explain some of the discrepancy between these degradation experiments and those run with compost isolates[4, 5].
White rot fungi were also used in this study. The ligninolytic enzymes produced by these organisms have been shown to degrade a wide range of substrates. [11] These organisms are widely used to test materials for their resiliency to microbial attack. Surprisingly, the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* were unable to significantly breakdown the polymer films under the given test conditions, both in the presence an absence of a co-substrate.

In the second part of experimentation, a batch reactor was set up to identify any metabolites produced from the degradation of the copolyester. The experiments were run in MSM to facilitate the identification of any degradation intermediates. Figure 3 is a typical chromatogram of a derivatized sample taken from the reactor after several days of exposure. Using GC-MS, two of the peaks were identified as the acids forming the copolymer, adipic acid, terephthalic acid and an unknown metabolite.

Further experimentation has shown that *B. subtilis* is incapable of mineralizing adipic acid and terephthalic acid. Therefore these metabolites slowly accumulate in the reactor with time.

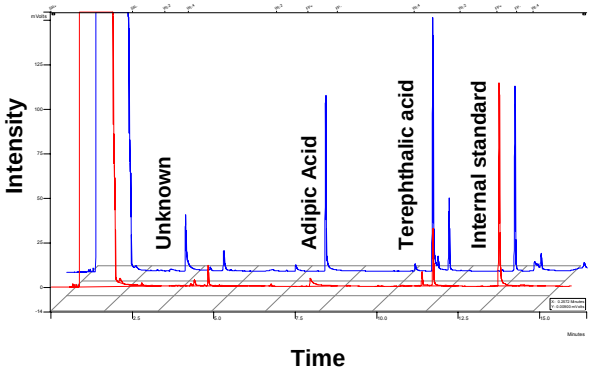

Figure 3: GC chromatograms of two batch reactor samples extracted with ethyl acetate. Both runs were inoculated with *B. subtilis* ATCC 21332. The chromatogram in the forefront is from a reactor run without Ecolfex™. The chromatogram in the back is from a sample taken after 10 days, showing the release of metabolites in solution.

Figure 4 illustrates the concentration of adipic acid in solution as it is released in the media with the breakdown of the copolymer by *Bacillus subtilis*. The abiotic control also shown in Figure 4 is evidence that some abiotic degradation of the polymer is occurring. However, the abiotic degradation is significantly less than in the presence of *B. subtilis*. Note that the two acid monomers were released in such low concentrations that they did not affect the pH of the growth medium.

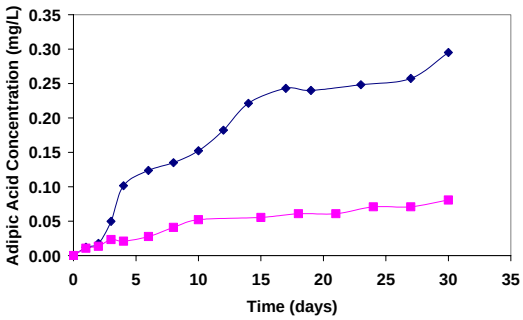

Figure 4: Plot of adipic acid concentration vs. time in a batch reactor for an (■) abiotic run and a run with (♦) *B. subtilis*.

Figure 4 indicates that the degradation may be growth-associated as we see a rapid release of adipic acid in the early stages of the degradation run, as the bacteria multiply. The release of adipic acid then seems to reach a plateau at around 15 days. This may correspond to the organism's death phase. Nevertheless, there is still a measurable release of adipic acid in solution after this initial 15 day period indicating that the hydrolase is still active.

Figure 5 is an illustration of the effect of a co-subtrate addition on the biodegradation of Ecoflex™ by *B. subtilis* 21332. Glucose and fresh media was added as the rate of adipic acid released in solution started to plateau. Note that the dip in the concentration of adipic acid can be explained by the addition of fresh MSM. The plot shows that the addition of glucose introduces a lag in the release of adipic acid. This is followed by an increase in the rate of adipic acid release. The rate at which adipic acid is released in solution is significantly greater than without substrate addition. This may be due to the increase in viable biomass in the reactor, compared with the run shown in Figure 4. This seems to suggest that degradation may depend on the activity of the enzyme or the organism rather that solely on the growth of the organism.

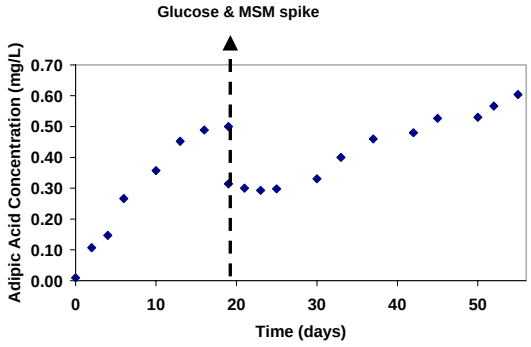

Figure 5: Plot of adipic acid concentration vs. time for a batch reactor run. After 19 days, 300ml of fresh MSM media and 1g of glucose was added.

Finally, after being incubated with pure strains of microorganisms, Ecoflex™ films were dissolved in chloroform and these samples were analyzed using gel permeation chromatography (GPC). The results for films incubated with *Bacillus subtilis* and *Paecilomyces lilacinus* are shown in Table 2. These results seem to indicate that more than one mechanism may be involved in the breakdown of the copolyester. The polymer films incubated with *P. lilacinus* show a much more drastic change in the molecular weight distribution as compared to the abiotic sample. This is in sharp contrast to the results obtained for *B. subtilis*, with which the molecular weight distribution remains essentially unchanged. This would indicate that more than one enzymatic pathway is involved in the degradation of Ecoflex™. The degradation with *P. lilacinus* appears to be internal whereas the degradation exhibited by *B. subtilis* appears to be restricted to the ends of the polymeric chains.

Table 2: Preliminary GPC Results for one untreated pellet, one abiotic film, one film exposed to P. lilacinus and one film exposed to B. subtilis 21332.

	Untreated Pellets	Exposed Films		
		Abiotic	*B. subtilis*	*P. lilacinus*
Number Average Molecular Weight	67400	65600	64800	32300
Weight Average Molecular Weight	177700	170100	177000	152300
Polydispersity	2.64	2.59	2.73	4.72

Conclusions

From the screening experiments, it can be concluded that the degradation of Ecoflex is a slow process. There is no strong evidence to conclude that this process is growth associated. Enzyme or active biomass concentration is a more likely responsible to enhance biodegradation. Finally, preliminary GPC results indicate that more than one degradation pathway may exist to breakdown the polymer.

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