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Conversion of Polyethylenes into Fungal Secondary Metabolites

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Abstract: Waste plastics represent major environmental and economic burdens due to their ubiquity, slow breakdown rates, and inadequacy of current recycling routes. Polyethylenes are particularly problematic, because they lack robust recycling approaches despite being the most abundant plastics in use today. We report a novel chemical and biological approach for the rapid conversion of polyethylenes into structurally complex and pharmacologically active compounds. We present conditions for aerobic, catalytic digestion of polyethylenes collected from post-consumer and oceanic waste streams, creating carboxylic diacids that can then be used as a carbon source by the fungus *Aspergillus nidulans.* As a proof of principle, we have engineered strains of *A. nidulans* to synthesize the fungal secondary metabolites asperbenzaldehyde, citreoviridin, and mutilin when grown on these digestion products. This hybrid approach considerably expands the range of products to which polyethylenes can be upcycled.

Introduction

Plastic production is currently accelerating at a rate faster than any other material on the planet,[1,2] and is estimated to reach a global production volume of 1.1 billion tons annually by 2040. Only 9% of plastics were recycled as of 2015.[3] Millions of tons of plastics, in the form of trillions of plastic particles,[4] leak from waste management systems into the environment, posing increasing threats to our food supply and ecosystems.[5] Generally, polyesters are frequently recycled (ca. 30% of polyethylene terephthalate (PET)), unlike polyolefins (ca. 6% of low-density polyethylene (LDPE)).[2,3] Due to their robust microstructures and excellent physicochemical properties, polyethylenes have been utilized to deliver countless improvements to quality of life and health. Polyethylenes are, thus, likely to remain ubiquitous in society. To minimize the environmental hazards they present, we must reclaim value embedded in these materials by developing viable upcycling approaches for them.

The same physicochemical properties that make polyethylenes useful also hinder their degradation and recycling. Further exacerbating this problem are additives that necessarily accompany any post-consumer waste stream, e.g. colorings and plasticizers. Unlike polyesters and nylons, the chemical methods known to recycle or remanufacture polyethylenes are limited. Some forcing methods (e.g. refluxing nitric acid, deep UV radiation) are known to cleave polyethylenes, the former to give carboxylic acids.[6–8] This type of strategy was exemplified in an oxidative process in which O_2 and nitric oxide (NO) were shown to cleave polyethylenes to carboxylic acids, nitrates, and other oxygenates at 170 °C and 40 atm with 65% total yield.[9]

Separately, oxidant-free, catalytic approaches are emerging for polyethylene upcycling, including alkane metathesis, hydrogenolysis, and related pathways to convert polyethylenes to light alkanes.[10,11] While these have modest yields and require energy-intensive conditions, they avoid the potential uncontrolled reactions that can result from heating organics with O_2. Still, oxidative conditions have the important advantage of tolerance to impurities associated with post-consumer polymer waste. These impurities, especially salts, are a particular concern in samples recovered from the oceans or recycling centers.

Chemical approaches to polyethylene degradation generate a diverse distribution of products because there are no functional handles in their pure hydrocarbon structures to direct a catalyst to where the polymer should be cleaved. We've shown, by contrast, that thermoset epoxies and fiber-reinforced polymers (FRPs) can be upcycled selectively,

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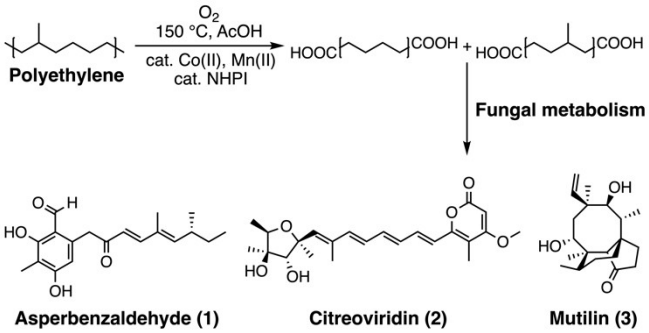
owing to the special chemistry of their linking nitrogen atoms.[12–14] In polyethylenes, the diversity of products arising from cleavage either limits the value of these products or creates a challenge of separating them. Thus, there is growing interest in employing biological systems to break down plastics.

Over the past two decades, several groups have made tremendous progress toward the biological upcycling of plastic degradation products. The discovery of enzymes capable of depolymerizing PET has inspired extensive interest in both PET degradation and upcycling.[15–18] Separately, whole-cell biocatalysts have also been employed to reclaim value embedded in PET. Several groups have demonstrated the microbe-facilitated generation of polyhydroxyalkanoates (PHAs) or related products from plastic-derived substrates.[19–21] Others have shown that PET-derived PHAs can be converted to both alkenoic acids and hydrocarbon fuels.[22] One group utilized *E. coli* to convert PET-derived terephthalic acid into diverse aromatics, including gallic acid and catechol.[23] Others have engineered *E. coli* to upcycle terephthalic acid into vanillin.[24]

In contrast to PET, however, fewer biological upcycling approaches have been developed for polyolefins such as LDPE and HDPE. Recently, one group utilized *Pseudomonas putida* to biologically upcycle HDPE, in addition to other plastics, to PHAs and β-ketoadipate.[25] While several groups have investigated the chemical[26–28] and biological degradation of these polymers,[29–33] approaches to biologically valorize these polymers are limited.

We aimed to exploit fungi, which produce products worth billions of dollars each year[34] to biologically upcycle polyethylenes. Their biosynthetic products include medically valuable secondary metabolites (SMs) including antibiotics, the cholesterol-lowering statins, immunosuppressants, and antifungals.[35] Because they have been reported to use diacids as carbon sources,[36,37] we sought to generate structurally diverse and pharmacologically active SMs directly from polyethylene-derived substrates.

We show here that post-consumer polyethylenes can be rapidly degraded to generate substrates that are suitable for upgrading by fungal metabolism. As a proof of principle, we demonstrate that these plastic-derived substrates can be used to produce the diverse SMs asperbenzaldehyde, citreoviridin, and mutilin in useful yields (Scheme 1). We also demonstrate robust genetic engineering strategies that permit the expression of biosynthetic gene clusters (BGCs) from many different organisms. Thus, in principle, this method expands the catalogue of products to which polyethylenes can be upcycled to thousands of SMs.



Scheme 1. The upcycling of polyethylenes to SMs. Polyethylenes are chemically degraded using metal catalysts and pressurized oxygen to generate a distribution of diacids, which are metabolized by fungi to rapidly produce structurally diverse SMs.

Results and Discussion

Optimization of Polyethylene Digestion

By adapting conditions for the conversion of cyclohexane to adipic acid,[38] we were able to optimize an initial system for polymer cleavage. Using O_2 consumption and ^{1}H NMR integration for indicative signals as our characterization handles (Figure S1), we eventually found conditions based on cobalt and manganese salts and a phthalamide-based NO source[9] that give useful oxidative cleavage results (Table S1). The distribution of α,ω-diacid products that are produced by the oxidative chemistry was further quantified by GCMS (see Supporting Information, Table S2 and Figures S2–4).

We observed that re-charging our reactor with additional O_2 did not restart the polymer cleavage reaction and hypothesized that *N*-hydroxyphthalimide (NHPI) serves as a source of NO, which is vented from the reactor headspace upon O_2 recharge. We see rapid hydrolysis of NHPI to phthalic acid upon reaction initiation. We further observed that our metallic catalysts lost reactivity in the recharge process (Table S3, entries 4–6), and that a better result was obtained when metal salts were added portion-wise along with O_2 and NHPI at recharge (compare entries 6–7). Under conditions optimized for full polymer conversion to relatively small diacid products (Figure S5), we observed 86 wt % mass recovery (entry 8) from a 5 g sample of clean polymer. Note that branched products were not tabulated in this yield, because they could not be unambiguously identified by identity to an authentic sample. Further, addition of oxygen to the polymer adds weight, so the molar yield of carbon atoms was 52 % to the named products.

Its highly tolerant O_2-based conditions give this method the critical and distinguishing feature of tolerance of post-consumer wastes. We demonstrate that feature here with four examples (Figure 1 & Table S4). Note that plasticizers and branched fragments from this LDPE film were omitted from the product accounting, although a large portion of these products are likely suitable for fungal digestion. A plastic grocery bag was homogenized into diacids of length C4–C12 with 34 wt % mass recovery, with an additional 2 % of longer diacids (Table S4, entry 1). The balance of material comprises branched diacids derived from polymer branches, which are likely suitable for fungal metabolism. The bag must also contain colorings and plasticizers, which we account as non-products. A plastic milk jug and laboratory squeeze bottle (entries 2–3) were homogenized, respectively in 63 and 54 wt % mass recovery. These gave a distribution of products generally of higher mass than the grocery bag as shown in

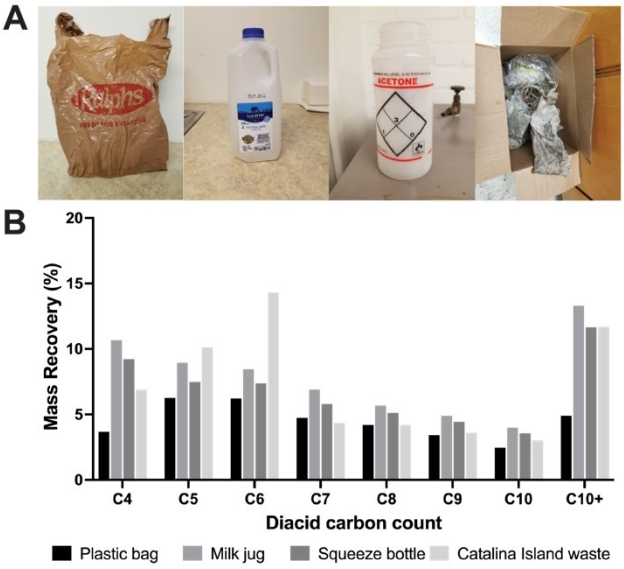


Figure 1. Post-consumer plastics degraded in this study. A) From left to right: LDPE plastic grocery bag, HDPE milk jug, LDPE laboratory squeeze bottle, Pacific gyre waste collected from Santa Catalina Island, CA. B) The distribution of diacid products after post-consumer polyethylene waste degradation using our optimized reaction.

Figure 1B. The higher and lower recoveries are explained by the difference of HDPE versus LDPE: the HDPE milk jug does not have polymer branches that are omitted from the recovery calculation.

Fungal Upgrading of Polyethylene Digestion Products

Fungi represent attractive candidates for diacid upgrading due to their robust growth capabilities, inexpensive cultivation requirements, engineerable metabolic pathways, and potential to synthesize metabolites with potent and diverse bioactivities. Short chain diacids, however, have been reported to inhibit fungal growth.[39] We confirmed that C4–C8 (studied individually, Figure S6) were toxic to the model filamentous fungus *A. nidulans* (strain FGSC A4) even when glucose was present as a carbon source. We found, however, that *A. nidulans* utilizes C10 and C12 diacids as sole carbon sources (Figure S7) without signs of toxicity. We thus devised a system to separate polyethylene digestion products of ≥ 10 carbons from those < 10 carbons. A series of pH-controlled liquid-liquid extractions permitted the rapid separation of C10+ diacids from light diacids and metal salts (Figures S8–9).

In a representative example (vide supra), 27 wt % of ocean-sourced polyethylenes were converted to diacids that were discretely identifiable using authentic standards. It should be noted that light diacids are not waste products. They may be used in large-market applications such as in the synthesis of PBCx, a biodegradable plastic emerging in agricultural applications.[40] Our data also suggest that these light diacids possess antifungal properties (Figure S6) that may be exploited.

For attempts to produce SMs from polyethylene-derived diacids, the heavy diacid extract was added to liquid minimal media at a concentration of $10\,g\,L^{-1}$. Liquid cultures were inoculated with fungal strains and incubated for several days (see Supporting Information for a full extraction protocol, culture conditions, and media recipes). SMs were analyzed and quantified from culture extracts via HPLC-DAD and HPLC-DAD-MS.

Initial attempts to elicit SM production from various wild-type fungal strains resulted in only small amounts of SMs as detected via HPLC-DAD-MS. We consequently genetically engineered *A. nidulans* to overexpress SM biosynthetic genes or biosynthetic gene clusters (BGCs) and this proved effective, allowing robust and efficient SM production.

In order to determine the versatility of this system, we attempted to engineer fungal strains to produce various SMs using several BGC activation/expression approaches (Table S5). The SM used as a readout for the first of these systems was asperbenzaldehyde, a major polyketide intermediate in asperfuranone biosynthesis.[41] Asperbenzaldehyde and its derivatives disassemble tau filaments, inhibit lipoxygenases, and inhibit the interactions of the oncogenic RNA-binding proteins HuR and Musashi-1 with their target mRNAs.[42–44] We chose to target a biosynthetic intermediate because it can serve as a discovery platform that can easily be synthetically modified.

We developed three strains with different systems for driving asperbenzaldehyde production: LO2955, LO8355, and LO10050. All molecular genetic modifications were executed using previously described fusion PCR-based construct generation and transformation protocols.[45] In strain LO2955, the *afoD* gene was deleted, blocking asperfuranone biosynthesis such that asperbenzaldehyde, its biosynthetic precursor, accumulates. Further, the promoter of the *afoA* gene that encodes the transcription factor (AfoA) that drives expression of the asperfuranone BGC was replaced with the *alcA* promoter (*alcA*(p)), which is highly inducible with a variety of alcohols and ketones, including methyl ethyl ketone.[46] To increase expression of AfoA, we next replaced the promoter of the *alcR* gene with the strong, constitutive *gpdA* promoter[47] in LO2955, creating strain LO8355. The *alcR* sequence encodes a transcription factor that drives expression of *alcA*.[48]

In addition, we developed a new, strong constitutive promoter system that employs a positive feedback loop (Figure 2) and incorporated it in strain LO10050. This system requires no induction and should drive strong expression on any carbon source, whereas the AlcA system is repressed by a number of sugars including glucose. The positive feedback system is designed to drive very high levels of transcription. In addition to the new promoter system and the deletion of *afoD*, LO10050 also carries deletions of the entire sterigmatocystin BGC (genes AN7804-AN7825) and the emericellamide BGC (genes AN2545-AN2549). Deletion of these highly expressed BGCs increases the pool of SM precursors, which are then free to feed into asperbenzaldehyde biosynthesis.

Yields of each strain grown in liquid lactose minimal media (LMM) were quantified via HPLC-DAD (Figure S10). Each strain gave substantial yields but yields from LO10050

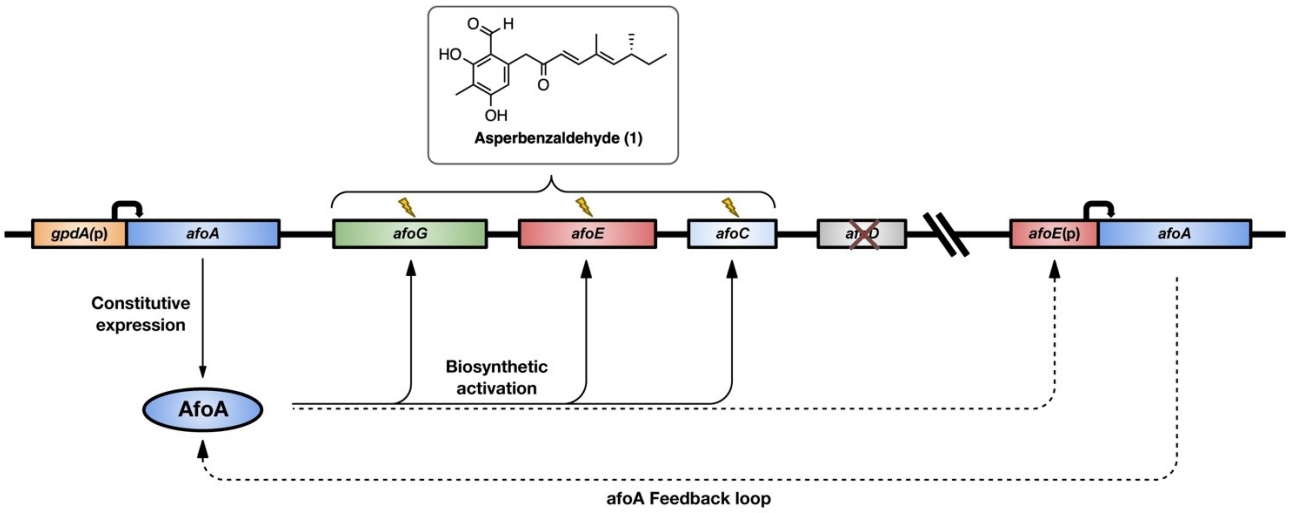


Figure 2. Overview of the novel promoter system driving production of asperbenzaldehyde in strain LO10050. The constitutive promoter *gpdA*(p) drives expression of *afoA*, encoding the AfoA transcription factor. AfoA binds to the promoter regions of genes *afoG*, *afoE*, and *afoC* within the asperbenzaldehyde BGC, leading to their expression and subsequent asperbenzaldehyde production. AfoA also binds to the *afoE* promoter (*afoE*(p)) controlling a second copy of *afoA* inserted elsewhere in the genome, driving additional AfoA production. This results in a positive feedback loop that generates high levels of both AfoA and asperbenzaldehyde. Note that *afoD* is deleted, halting conversion of asperbenzaldehyde to downstream metabolites. Other genes responsible for conversion of further downstream products to asperfuranone, the final product of the pathway, are not shown.

were the highest (4.3 $g\,L^{-1}$ from 15 $g\,L^{-1}$ lactose, or 29 % mass conversion of lactose to asperbenzaldehyde). We therefore selected this strain to assay for asperbenzaldehyde production on polyethylene digest.

To determine the general utility of the system, we also attempted to express the diterpene antibiotic platform mutilin from the basidiomycete *Clitopilus passeckerianus* and the F1-ATPase β-subunit inhibitor citreoviridin from *A. terreus* var. *aureus*.[49] Mutilin is an intermediate in the biosynthetic pathway for pleuromutilin, which binds to the peptidyl transferase center of the bacterial ribosome, thus halting protein synthesis.[50] Mutilin is therefore an attractive platform for medicinal discovery efforts toward overcoming bacterial antibiotic resistance. Further, basidiomycetes are phylogenetically distant from ascomycetes such as *A. nidulans* and the ability to produce mutilin would indicate that this system works for BGCs from very diverse fungi. Citreoviridin is a potent mycotoxin that uncompetitively and noncompetitively inhibits ATP hydrolysis and ATP synthesis, respectively, by binding to the β-subunit of F1-ATPase.[51] Compounds in this class of mycotoxins have been investigated for the treatment of cancer.[52] In total, four genes from *A. terreus* var. *aureus* and five genes from *C. passeckerianus* were transferred into an *A. nidulans* recipient strain and placed under control of *alcA*(p) to generate robust producers of citreoviridin and mutilin, respectively.

Engineered fungal strains were incubated in liquid minimal media supplemented with 10 $g\,L^{-1}$ polyethylene digest extracts (PMM, polyethylene minimal media). Culture media and/or mycelia were extracted with appropriate organic solvents (see Materials and Methods), which were then analyzed via HPLC-DAD or HPLC-DAD-MS (Figure 3). Standard curves were generated (Figures S11–13) using purified standards to quantify SM yields in PMM relative to glucose minimal media (GMM) or minimal media controls. SMs were purified from polyethylene digest cultures and confirmed via ^{1}H NMR (Figures S15–17) and tandem MS (Figures S18–20).

Our results indicate that engineered fungal strains can efficiently produce useful quantities of each target SM in under one week. Interestingly, microscopic examination of LO10050 when cultured in liquid PMM revealed initial stunted growth relative to GMM controls (Figure S21). However, we observed ample hyphal growth after 48 hours and abundant asperbenzaldehyde crystals after 72 hours of incubation in PMM, which is consistent with our findings regarding asperbenzaldehyde titers.

These yields are in contrast to other metabolic engineering efforts; while high-yielding strains have been reported following extensive engineering,[53] ample SM production typically requires much larger quantities of carbon source(s) to achieve comparable yields.[54–56] It is also noteworthy that our yields were obtained from shake flasks with minimal optimization. Alteration of other culture parameters known to influence fermentation titers (e.g. culture length, media components, etc.) should permit significantly higher yields. Use of the strong constitutive promoter system may increase production of citreoviridin and mutilin and codon optimization may further increase mutilin production.

We further note that it was not necessary to employ metabolic engineering strategies to confer the ability to metabolize polymer-derived diacids to the fungi; rather, simple extraction protocols selectively isolated diacids suitable for fungal metabolism. Finally, it is quite likely that polyethylene degradation products can be used as a carbon source in the production of other SMs. The BGCs that we

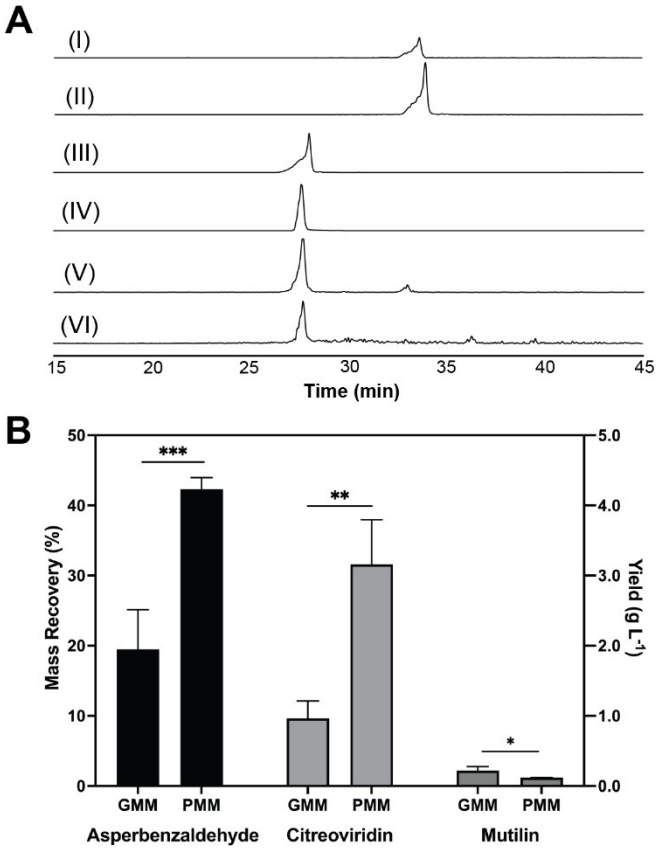


Figure 3. A) Paired extracted ion chromatograms generated via HPLC-DAD-MS. Asperbenzaldehyde production in (I) GMM and (II) PMM; citreoviridin production in (III) GMM and (IV) PMM; mutilin production in (V) GMM and (VI) PMM. Intensities are normalized for metabolites in each condition. B) SM yields produced by engineered fungal strains when grown in PMM and GMM liquid media. Bars represent means ± SD ($n = 3$). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$.

have expressed are from diverse fungi and the approaches we have developed should permit the expression of BGCs from many sources. Furthermore, it should be possible to produce other fungal fermentation products such as organic acids and proteins using the same approach. The combination of the catalytic degradation of polyethylenes with genetic engineering of filamentous fungi represents a promising strategy to plastic upcycling.

Conclusion

We have presented a method to rapidly upcycle post-consumer polyethylenes into structurally diverse and medically useful SMs. We degrade these polyethylenes using oxidative catalysis to generate a distribution of diacids. These diacids are rapidly isolated and upgraded by engineered strains of *A. nidulans* to synthesize bioactive SMs. Taken together, this two-step process dramatically expands the catalogue of products to which polyethylenes can be upcycled to thousands of SMs.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Biosynthesis · Catalysis · Polyethylenes · Synthetic Biology · Upcycling

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