DissolvBio

Directed Evolution Toward an Unpolluted Future

Problem Statement

In 2017 alone, humans produced over 100 million tons of the most abundant plastic on earth: polyethylene (PE) [1]. Americans use 2.5 million polyethylene bottles every hour [2]. Unfortunately, the massive amount of PE produced has an even more massive environmental toll. An overwhelming 75.5% of this plastic ends up as solid waste in landfills or as a serious environmental pollutant [3]; a recent study estimates that there are over 42.5 million tons of PE polluting oceans alone [4]. 40 million tons of PE is being added to landfills every year [3]. Most problematic of all, PE does not readily degrade. A 1990 study found that samples of PE degraded a mere 5% over a ten year period and that a PE water bottle requires 450-1000 years to degrade fully [5]. Accumulation of PE waste has been connected with severe sealife poisoning, premature human cancer development, and the mass toxification of groundwater [6]. Though the environmental damage caused by PE is well known, the material is useful and cheap therefore its manufacturing shows no signs of slowing; more PE was produced in 2017 than ever before, and experts predict that US production will increase 75% by 2022 [7, 8]. In summary, PE is mass produced, endangers humans and wildlife, and requires millenia to degrade [9]. Plastic pollution will be one of humanity's most enduring legacies on Earth. Accordingly, an effective strategy to reduce PE waste would have global significance.

Existing Solutions

Currently, there is no viable method for dealing with PE waste. One common strategy is large-scale burning, which is the fate of 15% of PE worldwide [3]. However, burning plastic releases toxins into the air that precipitate from the atmosphere, contaminate drinking water, and can be absorbed through the skin [6]. Humans can also be indirectly exposed to toxins created by burning plastics when they consume contaminated food such as fish, meat, or dairy products [10]. Toxic fumes released during incineration increase the risk of respiratory disease, central nervous system damage, kidney failure, and reproductive issues [11]. This process also releases a massive amount of CO_2 into the atmosphere. Overall, mass burning as a means of PE waste disposal is unsustainable, damages the environment, and endangers human health.

Landfills have been the “solution” to PE waste for decades and store 40% of post-consumer PE waste [3]. Unfortunately, landfill managers are struggling to make space for these massive amounts of plastic waste. Landfills with large amounts of plastic threaten to leach contaminants into groundwater. Additionally, there are fewer and fewer active landfills in the United States as voters choose to close these unsightly facilities within their counties. Instead, much plastic waste travels thousands of miles to be deposited into

landfills as far away as Mexico or China [12]. This shipping process adds an additional 350 tons of CO_2 to the already large environmental toll of plastic waste [13].

Another proposed solution to plastic waste accumulation is switching to biodegradable plastics, which entered the market recently with the promise of reducing the amount of PE needed. However, these bioplastics have not been well received because they have compositional flaws that limit their functionality and applications [14]. Furthermore, they emit methane, an incredibly potent greenhouse gas, faster than other types of trash while degrading [15]. Many decades may pass before bioplastics are of high enough quality to enter the mainstream, and their introduction does nothing to reduce the piles of PE already polluting our world.

Lastly, many turn to recycling as a solution to plastic pollution. However, a major limitation of conventional recycling is the high degree of sorting required before plastic waste can be processed. Most PE waste is non-homogenous, meaning that it is mixed with other types of plastic, contaminated with food, or has traces of toxic or non-recyclable waste. When melted during the recycling process, this non-homogenous plastic will phase-separate (like oil and water) and deform when cooled, resulting in structural weaknesses that limit the utility of recycled plastics [16]. To avoid phase-separation, plastics entering a recycling plant must be cleaned to remove contaminants, sorted by plastic type and color, and washed again to remove any labels or adhesives. This process is energetically expensive and time-consuming. Because of these difficulties, 70% of post-consumer PE waste that arrives at recycling plants is rejected due to its low purity. As a result, only 9.5% of all PE produced is recycled [3]. Transporting PE waste to a recycling facility only to return it to a landfill is expensive and releases additional CO_2. Some plastic waste that is rejected from recycling facilities is “downcycled” into lesser quality plastics such as outdoor decking and fencing materials [3]. Though downcycling prolongs the usability of waste plastics, the process renders the new plastic product unrecyclable [17]. The method adds a stepping stone between the trashcan and the dump, yet fails to solve the plastic waste crisis.

Clearly, none of the current methods for dealing with PE waste are sustainable, long-term solutions, and many create additional environmental health issues. A massive societal problem remains unsolved.

Proposed Innovation

Hundreds of contemporary industrial processes are driven by specialized microbes, from the production of cutting-edge medicines to the bioremediation of oil spills [18]. These microbes produce enzymes capable of precise chemical transformations and are significantly more efficient than man-made processes. Specialized enzymes develop and evolve over eons of mutation and natural selection. Within the past two decades, scientists have isolated a few microbes capable of partially degrading polyethylene [19]. For example, members of the genus *Pseudomonas* are able to degrade 15 - 25% of PE over a 30 day period [19]. One isolated strain, *Pseudomonas sp. E4*, degraded 28.6% of solid PE over 40 days using the enzyme alkane hydroxylase, or *AlkB* [20]. By comparison, plastic would be expected to naturally degrade just 0.0013% in the same time period [5]. *AlkB* is theorized to solubilize PE, allowing other bacterial enzymes to access PE’s Carbon-Carbon bonds and

break it down fully [20]. Though *Pseudomonas* is a promising candidate for the destruction of problematic PE waste, little attention is given to these bacteria because their degradation is too slow for industrial application. Unfortunately, evolution takes eons and PE has only been manufactured for 70 years; evolution has lacked sufficient time to hone the ability to efficiently metabolize plastics.

DissolvBio is dissatisfied with complacency. Polyethylene is polluting our planet today; a solution cannot wait . It is with great excitement that we propose a solution.

At the forefront of chemical biology, a method for protein engineering called Directed Evolution has gained much traction in recent years [21]. Directed Evolution takes the key components of evolution and moves them into a laboratory where the process can be sped up to thousands of times its natural rate [22]. Directed Evolution experiments first generate many variants of an enzyme by intentionally introducing mutations. The pool of variant enzymes is then screened for proficiency. Lastly, variants that perform better than the original enzyme are selected to serve as the starting point for the next round, in which the cycle is repeated. Enzyme efficiency is increased incrementally, round after round, and the final enzyme is significantly better than the original.

Dr. Francis Arnold, a UC Berkeley alumna who won the 2018 Nobel Prize in Chemistry for her work on Directed Evolution [23], demonstrated that the process has massive potential when she applied it to a degradative enzyme called *subtilisin-E*. In just four rounds of Directed Evolution, she managed to increase the performance of *subtilisin-E* to 256 times its natural level [23]. Further evidence of the power of Directed Evolution comes from Arnold's 2013 paper in which she showed that the technique could not only be used to improve preexisting enzymes but also to create novel enzymes active in pathways for which no known enzyme exists [24,25]. For example, an enzyme that breaks down hormones has been engineered to instead break down a completely new compound, the difficult-to-degrade industrial chemical propane [26].

We propose to utilize Directed Evolution to develop enzymes that can efficiently degrade PE, reducing the amount of pollution in landfills and ecosystems. With funding from a UC Berkeley research grant, we have transferred *AlkB* from *Pseudomonas sp E4*, the strain reported to be able to degrade PE [20], into common laboratory bacteria. With remaining funds, we will generate mutants of *AlkB* and assess their ability to degrade PE. The best mutant strains will be selected for use in subsequent rounds (demonstrated in Figure 1). Dozens of other Directed Evolution experiments suggest that substantial improvement of PE degrading enzymes is not only possible but likely, indicating that massive environmental impact is within our grasp.

Bacteria are uniquely suited for PE breakdown because they can cope with differences in plastic purity, size, and chemical composition much more efficiently than mechanical or chemical processes [28-30]. They achieve this by changing their gene expression and activity to best suit their surroundings, contrasted with chemical processes that have little to no tolerance for variability [28]. Additionally, bacterial breakdown is a safer option than chemical breakdown because toxic chemicals are neither required as catalysts nor produced as waste [31].

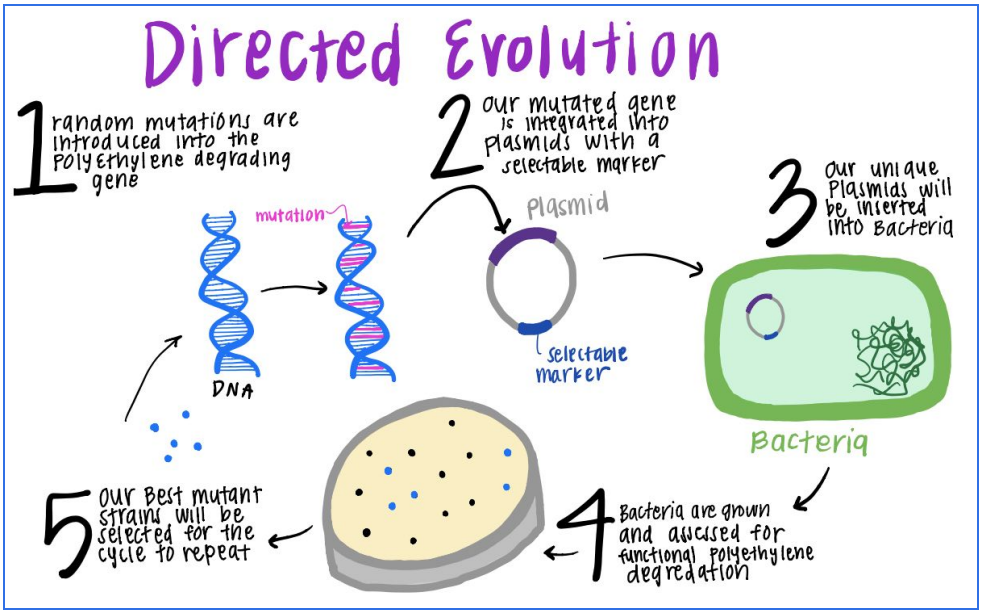


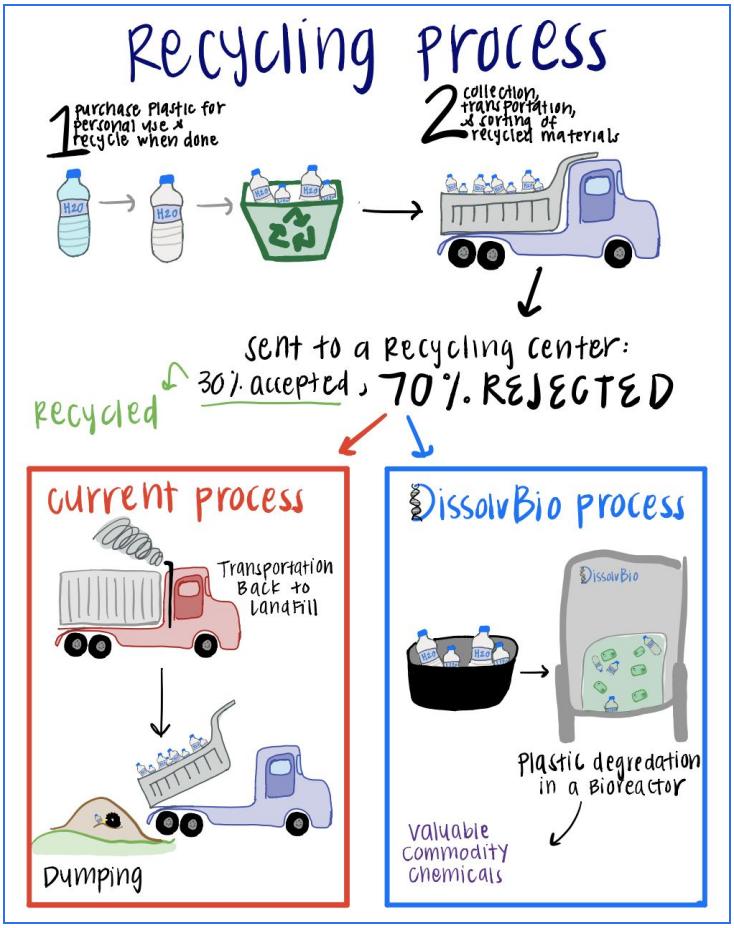
Figure 1: Our process for Directed Evolution with the PE degrading enzyme, AlkB, is based on the tried-and-tested method used by molecular biologists around the world.

Once our organism is engineered and rigorously tested, our vision is to use large bioreactors to break down PE at an industrial scale. Thousands of biotechnology processes take place in bioreactors [32]; these stainless steel chambers range from 1 liter to 20,000 liters, depending on their application, and are designed to maximize bacterial growth (Figure 2). We will first degrade PE in a 5L bioreactor, which will test the industrial viability of our method and demonstrate proof of concept. With the 5L model, we will optimize our method and scale up to larger reactors.

Figure 2: An example of an industrial bioreactor.

The PE waste-stream we intend to focus on is post-consumer PE rejected from recycling plants. PE rejected from recycling plants presents a concentrated waste-stream, the removal of which would reduce plastic waste without interfering with PE recycling [33]. Our vision is a system of large-scale degradation plants located close to recycling plants that can accept PE waste destined for landfills (Figure 3); this will minimize the amount of time, money, and CO_2 required to ship rejected plastics to our facility.

We believe this vision has the potential to be either a private or public entity, but we aim to establish the technology prior to constructing a full-fledged business model as many factors remain unknown. A cost that guides private viability is that of large scale bioreactors. A 5000L bioreactor costs approximately $75k to operate per 2-week cycle [34]. To make bacterial plastic degradation economically feasible at scale, we have explored metabolically engineering our bacteria to produce valuable organic compounds such as biofuels or pharmaceuticals [35, 36]. We have identified suitable candidate compounds but their production would be further explored after our first year. The proposed method has market potential, however the ability to degrade plastic alone would be monumental and is therefore our primary goal.



UC Berkeley is uniquely situated to be a launchpad for our project because of its close partnerships with the Joint Bioenergy Institute, Lawrence Berkeley National Laboratory, and the Energy Biosciences Institute, academic centers which explore the degradation and valorization of recalcitrant waste. Our team has made personal relationships with researchers at these institutions, many of whom have already provided invaluable guidance.

Figure 3: The current process for managing waste rejected from recycling plants juxtaposed with the DissolvBio process.

Potential Difficulties

We firmly believe that recognizing weaknesses is a necessity for success. DissolvBio has consulted with multiple experts to identify difficulties we may encounter going forward.

With this in mind, we acknowledge that our target enzyme, *AlkB*, may not be the only enzyme necessary to degrade plastics, as cellular metabolic pathways are intertwined and complex [38]. We have used published scientific literature to identify a few alternative targets ranging from peroxidases to cytochromes that might be able to perform the necessary chemistry. Additionally, should we lack success with our primary method of mutagenesis, we have explored fallback methods of generating mutants such as Evolvr, in which a CRISPR element is incorporated into the bacteria and continuously generates targeted mutations [39]. All things considered, we plan to concentrate our funds and efforts on our primary target unless it fails.

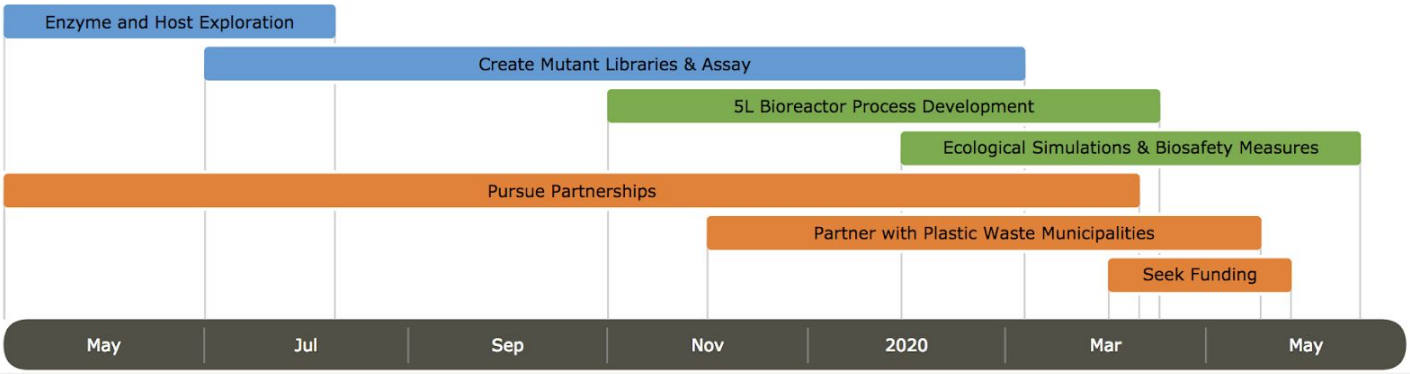
Additionally, we recognize that scaling bioreactor size is a challenge that requires careful planning in order to improve yield while preserving safety, stability, and sterility. Standard operating procedures have been developed for sterility control and inoculum development and can be integrated easily at smaller scales for our 5L first year goal [40]. Scaling to larger bioreactor volumes introduces new challenges relating to process drift and uniformity. We hope to prepare for scaling challenges preemptively by fine-tuning controls at smaller scales, optimizing yield, and meeting regulatory standards.

We also recognize that any metabolic or genetic engineering project faces societal concerns about safety, specifically about genetically altered organisms escaping from the laboratory. To address this, we will join the thousands of labs across the United States working with modified bacteria and enforcing rigorous protocols designed to prevent escape [41]. Our final organism will be engineered to be auxotrophic, meaning that it is unable to synthesize a critical nutrient [41]. This nutrient will be supplied in their growth media so that they propagate without issue in our bioreactors, but will die quickly outside of this controlled setting. Additionally, we will incorporate genetic failsafes that make lab bacteria's DNA incompatible with natural settings, further lowering the chance of escape [42]. Above all, we will never release our bacteria outside the laboratory unless extensive testing has been done, government permits are secured, and a bioethics advisory board approves the decision.

Lastly, biodegrading plastic with living bacteria would involve converting some percentage of the polyethylene into respired CO_2 [43]. This is an issue that will not be resolved within the early stages of our project, yet we have compiled a list of potential solutions. High concentrations of CO_2 improve plant growth and many large industrial facilities have begun to capture CO_2 with adjacent greenhouses to reduce emissions into the atmosphere [44]. Other industries have outfitted their facilities with carbon capture machinery [45]. We could also engineer our plastic-degrading bacteria to produce biofuels, and in doing so divert some carbon away from CO_2 and into beneficial products [35].

Timeline

DissolvBio plans to pursue the timeline outlined below in order to meet our goals for the first year of research. We plan to further test AlkB and related enzymes in various hosts, demonstrate improvement of the enzyme using Directed Evolution, and lastly demonstrate industrial viability by degrading plastic in a 5L bioreactor.



Budget

We received a Sponsored Projects for Undergraduate Research (SPUR) grant from UC Berkeley after our proposal was approved by a panel of biologists. The funds have been used to synthesize *AlkB* and begin degradation experiments in several different bacterial hosts. We also received additional funding from UC Berkeley's Regents and Chancellor's

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Research Fellowship. Funding from the Big Ideas Contest would be used to continue ongoing experiments, perform several rounds of Directed Evolution, and test the scalability of microbial biodegradation in a 5L bioreactor. A detailed budget can be found below.

Expenditures

Item Description	Number	Price per Unit	Total Cost
powdered polyethylene	5	$50.00	$250.00
(NH4)2SO4 (for culture media)	1	$71.60	$71.60
MgSO4·7H2O (for culture media)	1	$41.30	$41.30
FeSO4·7H2O (for culture media)	1	$51.80	$51.80
Na2MoO4·2H2O (for culture media)	1	$90.00	$90.00
Na2WO4·2H2O (for culture media)	1	$157.00	$157.00
MnSO4 (for culture media)	1	$59.30	$59.30
K2HPO4 (for culture media)	1	$48.30	$48.30
Agilent GeneMorph II error-prone PCR kit	3	$513.00	$1,539.00
Pyrex Round-bottomed culture tubes	500	$2.50	$1,250.00
Thermo Scientific™ 0.2 mL PCR Tubes	5	$230.00	$1,150.00
5L Bioreactor	1	$4,350.00	$4,350.00
pBAD Plasmid	1	$400.00	$400.00
Pippette Tips	10	$150.00	$1,500.00
Gene Synthisis	1	$300.00	$300.00
Total			$11,258.30
Tax			8.00%
Shipping			12.00%
		Project Total:	**$12,715.92**

Revenue

Source	Amount
Big Ideas Contest	$10,000.00
Regents and Chancellors Scholarship	$1,000.00
UC Berkeley Microbial Biology Research Grant	$2,000.00
Total	$13,000.00

Measuring Success

For our first year of implementation, we have three primary goals. First, we hope to demonstrate the ability to degrade plastic using engineered bacteria. Using funding acquired from SPUR and Regents' research grants, we have synthesized *AlkB*, incorporated it into a bacterial host, and begun our initial degradation experiments, which are currently being incubated in UC Berkeley professor Steven Lindow's laboratory. We plan to test multiple other bacterial hosts and conditions. When one of these experiments yields significant degradation, we will consider this major goal successfully reached.

Second, we intend to perform several rounds of Directed Evolution on *AlkB* and carefully quantify the improvement in enzyme performance. Though all literature we examined indicates that *AlkB* can be improved with Directed Evolution, demonstrating this in the laboratory will be a huge proof of concept that will help us form partnerships and acquire seed funding. Observing improvement in enzyme performance, even if initially small, will be a major indicator of viability and future success.

Lastly, we plan to demonstrate feasibility at scale by degrading plastic in a 5L bioreactor. The ability to degrade PE in industrial machinery instead of a test tube will be a significant indicator of success. We would consider any amount of degradation at scale to be a huge milestone.

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Team Bios

Ryan Kenneally is a fourth-year at UC Berkeley double majoring in Microbial Biology and Genetics. He specializes in cell biology and ecology. He works at the Lawrence Berkeley National Laboratory. After graduation, he plans to pursue a PhD in Molecular Biology.

Hannah Grossman is a fourth-year at UC Berkeley double majoring in Molecular Environmental Biology and Computer Science. She loves the intersection of data science, environmental science, and public health. She aims to pursue a masters in bioinformatics after graduation.

Jason Hou is a fourth-year double major in EECS and Bioengineering at UC Berkeley. Jason has worked on several projects exploring the use of novel hardware instruments to understand environmental and human biology at both the Maharbiz and Ke Xu Labs.

Will Sharpless is a third-year double majoring in Microbial Biology and Applied Mathematics at UC Berkeley. He has a profound fascination in synthetic biology. Will has worked in both the Keasling and Arkin labs to pursue applications of genetic engineering for manufacturing and environmental control.

Polyethylene waste is a huge problem that impacts humans and wildlife all across the globe. Though, this problem has no current solution, DissolvBio intents to use Directed Evolution to change this reality, moving us toward a less polluted future. Thank you for your time, and thank you for considering DissolvBio.

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Patrick Sorensen, PhD
Robin Johnson, JBEI Director of Commercialization
Ryan Protzko, PhD and founder of ZestBio
Steven Lindow, PhD and Professor of PMB

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