Design Brief

Inserting *Ideonella sakaiensis* genes into chassis *Comamonas testosteroni* to create bacterial super-degrader for plastic waste reduction*

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The widespread issue of plastic pollution continues to plague the world, lacking a sustainable and practical solution. Solutions such as recycling and incineration can be effective as a means to dispose of plastic, but they often breed problems like pollution that threaten the environment's health and are generally unfilterable. In response, this project aims to develop an efficient way to degrade polyethylene terephthalate (PET), one of the most common single-use polymers. To explore this possibility, we propose using the bacteria Comamonas testosteroni as a chassis to host genes from Ideonella sakaiensis to produce a super bacterium with an enhanced ability to degrade plastics. The design aims to utilize C. testosteroni's ability to target and break down benzene rings that hold PET together, alongside enzymes PETase and MHETase from I. sakaiensis, which leave monomers that help with plastic degradation. Since C. testosteroni can be grown in standard laboratory conditions, we suggest inserting the PETase and MHETase genes from I. sakaiensis into chassis C. testosteroni via transformation, creating a more efficient plastic-degrading microbe that would help develop a new waste management process for PET without negative environmental impacts.

Keywords: Plastic degradation, *Comamonas testosteroni, Ideonella sakaiensis,* PET

The world produced 459.75 million tons of plastic waste in 2019, nearly a 100 million increase from the year prior, during which 360 million tons of plastic waste had been stockpiled (Ritchie et al., 2023). Most of the plastic waste coming from single-use plastic is a low-effort solution for companies that need durable, cheap, and lightweight packaging, and its utility is exponentially increasing. However, waste accumulation is the primary ramification of using it in excess. Polyethylene terephthalate (PET), the typical plastic of choice for large-scale corporations, is a transparent, lightweight plastic used to make rigid containers like water bottles and take-out containers, typically taking up to 2,500 years to degrade fully while simultaneously leaching harmful chemicals into the environment. As for current solutions, plastic

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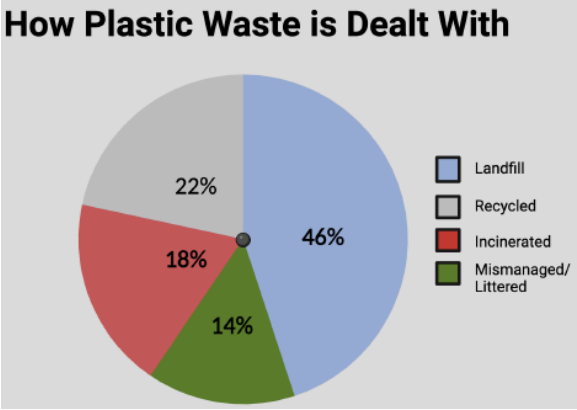

Figure 1: Current methods for managing plastic waste (data from UNEP, 2022).

waste is disposed of via one of three methods: recycling, landfills, or incineration (See Figure 1) (Bauman, 2019). The United States alone produces 40 million tons of plastic waste each year, with 85% of that contributing to landfills (UNEP, 2023).

The current solutions available to this issue need to become more practical and sustainable in the long term; the solutions often induce environmental damage and pose potential risks to public health. Incinerated plastics create harmful gasses that affect our atmosphere and are toxic to breathe. Landfills and littered plastics leach chemicals, assisted by the water cycle, into the soil, groundwater, and nearby bodies of water, which in turn harms plants, animals, and even humans. Large amounts of plastic in the ocean cause physical harm to ocean life, causing irreversible damage to food chains (Fava, 2022).

With the application of newly effective active plastic waste removal methods, changes would be drastic. Reducing the total amount of plastics can mitigate future spills and deter plastic redistribution into the environment. Currently, there are three areas where that overarching negative impact looms: marine life, soil/plant life, and air quality. Over half of all sea turtles and seabirds have ingested plastic (Alberghini, 2022). Several dozen species of fish have high rates of detected microplastics, many of which are regularly consumed by the general population. The average person consumes or inhales anywhere between 74,000-121,000 microplastics a year (Cox, 2019). With a new plastic waste removal system, the 12,000-24,000 tons of plastic consumed annually by marine life can also be effectively reduced (Alberghini, 2022). While some ocean cleanup methods have succeeded, most notably The Ocean Conservancy and The Ocean Cleanup, many of those plastics have not been removed (Fava, 2022). However, ocean impact is not the only issue.

Another problem lies within the effects on soil and the harm plastic waste induces on plant life. Amongst these chemicals, phthalates like bisphenol A (BPA) present negative health repercussions for animals and humans in the environment. Plastics can persist for 20-500 years, meaning the detriments to marine life and soil/plant life are long-lasting. As of March 2022, the United Nations had begun drafting a resolution to address the global plastic waste issues, but the committee is only on its third session with two more planned. There is no resolute solution in the foreseeable future, leaving the drastic effects ever increasing (UNEP, 2023). The U.S. has 10,000 closed landfills and 3,000 active ones. Landfills release methane, a greenhouse gas, that contributes to climate change. The landfills also take up 1,800,000 acres of land in the U.S. that could be used for other purposes. Landfills threaten people's health with smoke, odor, water supply contamination, and bugs (Vasarhelyi, 2021).

When they are incinerated, plastics release various chemicals into the environment, and the byproducts of this process are often toxic. Furthermore, the residue of ash and char that transpires as a result of the burning of plastic causes even more harm to the soil. A bacterium that can decompose this plastic will help reduce plastic waste that plagues the world. (Rosenberg, 2021). The plastic waste is put in the combustion chamber of an incinerator. It can be burned in a process known as waste to energy that can generate electricity, hot water, and heat. Incineration causes air pollutants that can cause heart and lung disease, heavy metals that can cause neurological problems, and toxic chemicals that can lead to cancer (Singla, 2021).

Rajagopalan Vasudevan, a professor of Engineering at Thiagarajar College of Engineering, proposed using plastic waste to make plastic asphalt to pave roads in 2002

(Edel, 2020). Vasudevan reasoned that using plastic waste would be better than banning single-use plastics altogether, which India had planned. By 2020, India had paved numerous roads using Vasudevan's patented plastic asphalt, but other scientists have found an issue with it, believing it releases toxic gasses during production (Caceres, 2023).

Recycling starts with the plastic waste being shredded. Then, the plastic is washed to remove any filthiness. It is then centrifuged and dried to remove any remains. Homogenization then occurs to unify everything to make plastic agglomerate. It then goes through a filament extruder and is often turned into pellets. These pellets can be used to create new products (SINTAC, 2023). The flaw in the recycling process is that a byproduct of recycling residue goes back into the plastic waste. In 2019, 55 Mt was collected for recycling, but only 33 Mt was made into plastic scrap; the other 22 Mt was filtered back into plastic waste (U.N., 2022). Informing people to reduce their waste production often does not work, as they believe they are too small of a contribution to make a difference. The ways of dealing with plastic waste involve converting plastics into different products, but our project proposes degrading the plastic. Our new solution would reduce the overall environmental impact of plastics.

*Comamonas testosteron*i, a pink-pigmented, gram-negative bacteria, was discovered in 1987. *C. testosteroni*, unlike most bacteria, can break down lignin found in plants and certain aromatic substances. *Ideonella sakaiensis,* found in 2016*,* is an aerobic bacterium that can produce enzymes

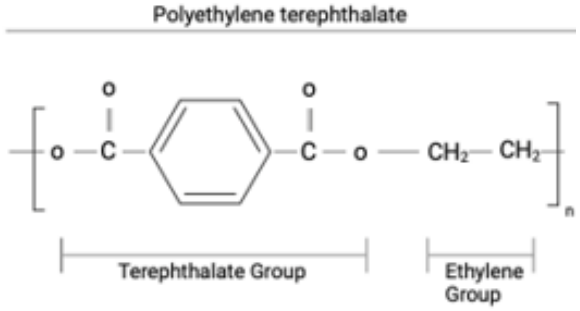

Figure 2. Splitting Polyethylene terephthalate, PET plastic.

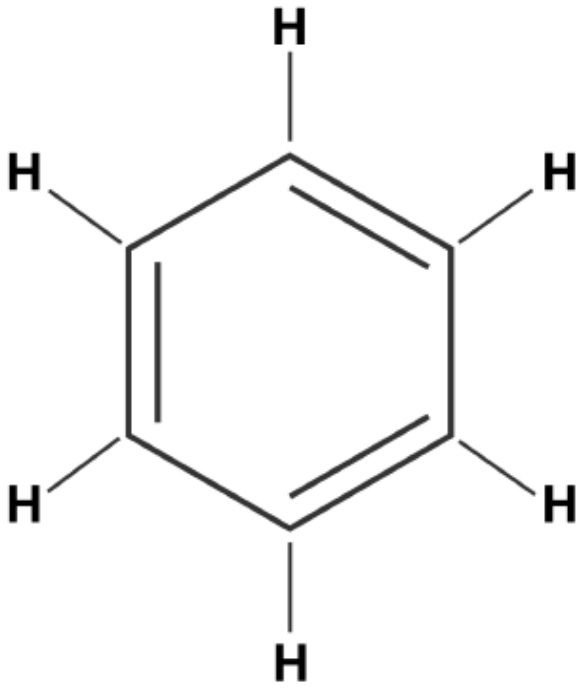

Figure 3. Benzene ring's molecular structure.

capable of decomposing PET plastic (see Figure 2). Due to its discovery in plastic waste disposal sites, interest has been raised regarding the use of bacteria in plastic degradation. *I. sakaiensis* utilizes powerful enzymes, PETase and MHETase, to break apart PET plastic into separate parts, most notably by targeting the carbon-oxygen bonds within the plastic. This process splits PET into a Terephthalate group and an ethylene group. The Terephthalate group is mono-2-hydroxyethyl terephthalate, known as terephthalic acid. While the ethylene group is ethylene glycol. Both monomers pose no threat to the environment (Yoshida, 2016). While *I. sakaiensis* produces enzymes that target carbon-oxygen bonds, *C. testosteroni* targets carbon from the benzene rings that hold PET together. These benzene rings are cyclic, aromatic, highly stable building blocks (see Figure 3). This property allows the plastic to maintain its rigid, transparent form. *C. testosteroni* can break down these rings for energy, splitting the long chain of polymers that make up the plastic. *C. testosteroni* can make an ideal chassis for plastic degradation due to its ability to break down these benzene rings. Furthermore, because these plastics will be actively decomposed into a non-harmful end product,

fewer plastics will be present to plague the environment. Decomposition is more beneficial than simply removing or reusing plastic due to the risk of reintroducing plastics to the environment.

Systems level

Two of the enzymes *I. sakaiensis* produce are PETase and MHETase. These enzymes degrade PET plastic into MHET and other monomers. MHET is further broken down into terephthalic acid and ethylene glycol. In this reaction, *I. sakaiensis* can utilize PET for energy. *C. testosteroni* is a similar bacterium, which can break down aromatic compounds within PET and use them for energy. With the genes that create plastic-degrading enzymes in *I. sakaiensis*, *C. testosteroni* will be able to degrade plastic more efficiently. The plasmid pET-21b(+) has proven to be an effective vector in transferring the genes that create PETase and MHETase in *I. sakaiensis* 201-F6, the strain with the most promise for the degradation of PET (Austin et al., 2018). This modification equips *C. testosteroni* with the enzymatic machinery needed to break down PET quickly. Our bacteria will be an environmentally friendly way to get rid of the sizable part of PET plastic polluting factories and the environment. By applying our genetically engineered bacteria to media containing PET plastics, runoff of plastic into the environment will be reduced at the source.

Device level

The enzymes PETase and MHETase, found in *I. sakaiensis*, break down plastic through hydrolysis. This process involves a water molecule cleaving one or more chemical bonds that hold the polymers together, effectively breaking down the plastic into its constituent parts. This process plays a crucial role in our project as it breaks the chains of PET into smaller, more microbially manageable fragments.

After inserting genetic material from *I. sakaiensis* 201-F6 into a plasmid, the plasmid will be transformed into our chassis, *C. testosteroni* KF-1, through electroporation. Electroporation involves permeabilizing the cell membrane in an electric field, which then creates temporary pores in the cell membrane, allowing easy incorporation of genetic material. This method has been highly successful in the *Comamonas* genus, particularly on the *C. testosteroni* KF-1 strain (Wilkes et al.; L., 2023). By subjecting *C. testosteroni* to a short, high-voltage external electric field, our plasmid can cross the cell membrane, facilitating the uptake of genetic material (see Figure 4). The plasmid pET-21b(+) contains a gene for resistance to ampicillin, which will be used as a selector for transformed bacteria. As *C. testosteroni* is killed by ampicillin, properly transformed bacteria will survive ampicillin, whereas a non-transformed bacterium will not.

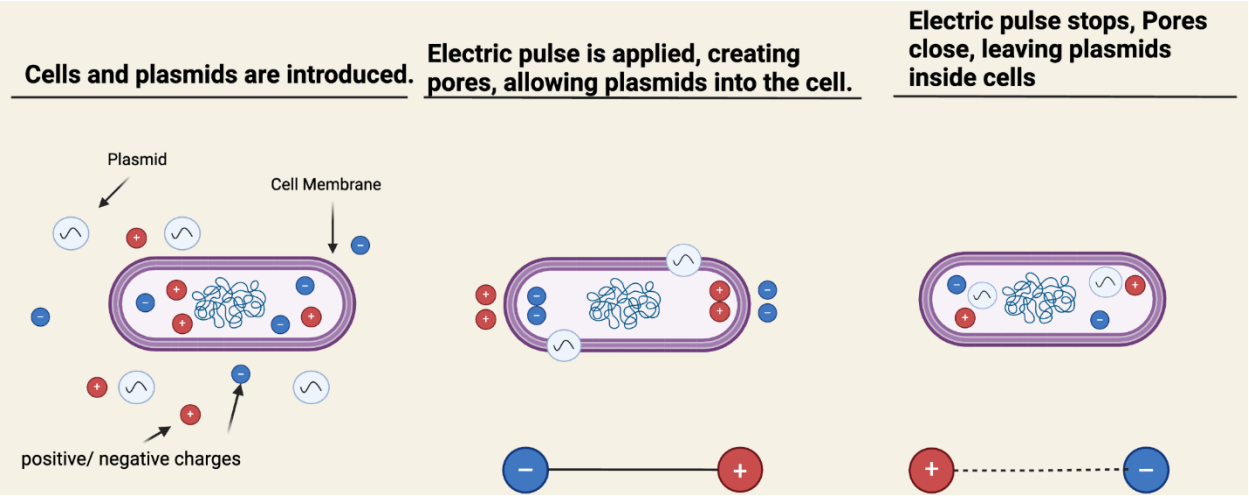

Figure 4. Process of Electroporation.

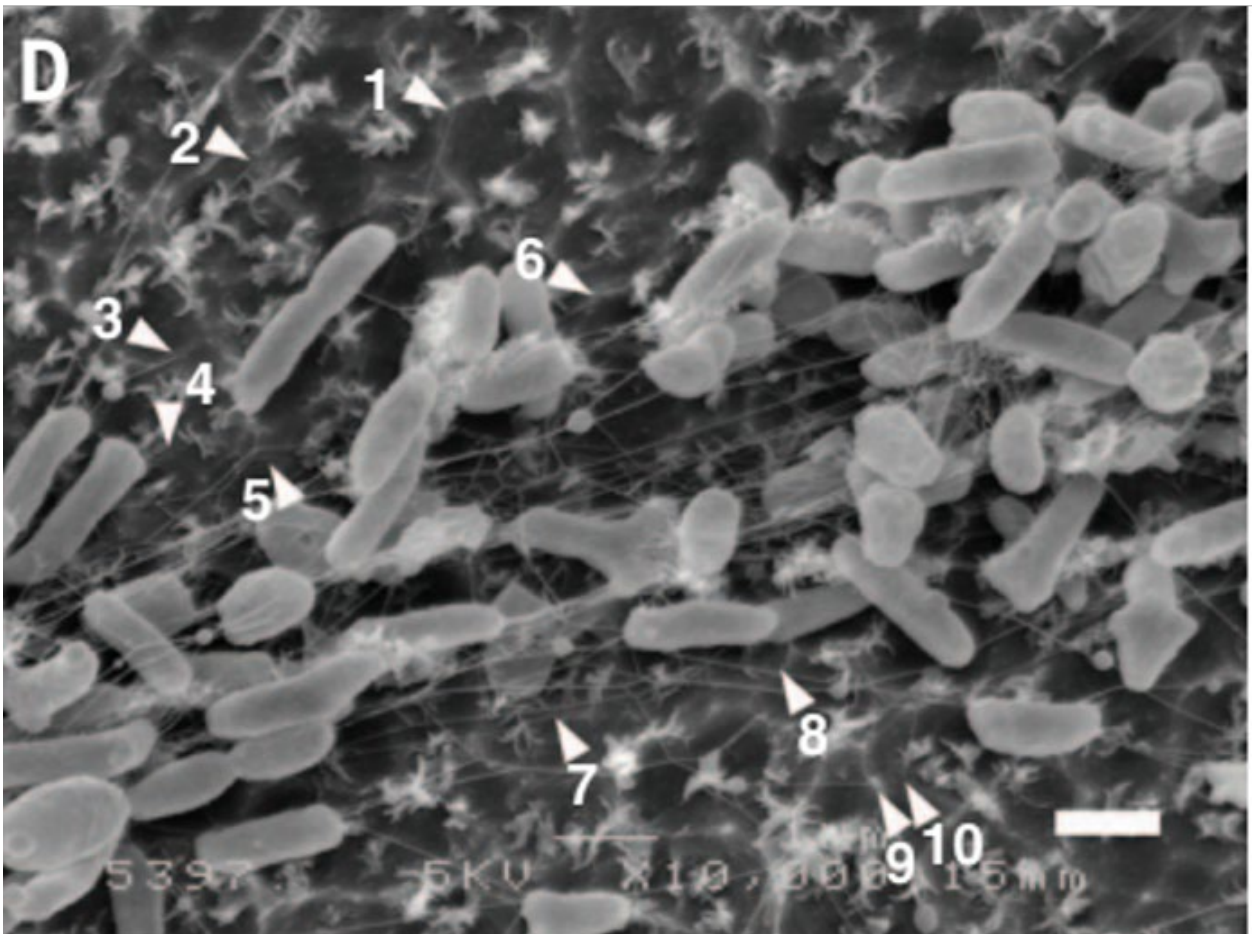

Figure 5: I. sakaiensis on PET film after 60 hours. (Shosuke Yoshida et al, 2016).

Parts level

The pathway through which *C. testosteroni digests* PET plastic is very similar to the pathway through which it breaks down lignins and other aromatic compounds. These similarities exist because of *C. testosteroni's* ability to dismantle complex benzene rings that exist in many polymers. These rings are hexagonal structures that are made of six carbon atoms, consisting of alternating single and double bonds that require the disruption of specific enzymes to be disassembled. When the *C. testosteroni* starts to degrade the rings, it employs specialized enzymes, like dioxygenases and oxygenases, to provide the ring with oxygen. Then, the benzene ring undergoes cleavage, which breaks the ring down into multiple smaller molecules. This capability makes it easier for the bacterium to utilize aromatic compounds as sources of carbon and energy, thus assisting in the breakdown of synthetic polymers like PET.

I. sakaiensis degrades bacteria very differently. First, *I. sakaiensis* attaches itself to PET using its flagellum. After it is tightly attached, the process of degradation begins. *I. sakaiensis* is able to grow and more tightly attach its appendages to the PET (see Figure 5).

The pathways of *C. testosteroni* are essential in the degradation process. As a starting point, *C. testosteroni* recognizes lignin compounds, or plastics, as a potential carbon source, using its surface proteins to adhere to the substrates. The bacteria then has to travel through a metabolic pathway to decompose the compounds; however, the 2-3 meta and ortho pathways are unavailable as protein expression for a few of the genes are blocked. Fortunately, the 4-5 meta pathway is fully available, containing all of the Prnd

genes from AB to F. Missing in the other two are the PraA and PraH genes in the 2-3 pathway, as well as the PcaG gene in the ortho pathway. Once the molecules are degraded into metabolized compounds, the intermediates can be incorporated into a central metabolic pathway such as TCA (tricarboxylic acid) or glycolysis. These pathways allow the bacterium to extract nutrients from the degraded plastic products fully, thus enhancing its degrading abilities (Wilkes et al., 2023).

The *C. testosteroni* strain KF-1 is proven to be relatively simple and effective when grown on aromatic substances, as compared to succinates hosted by the 2-3 meta and ortho pathways, where its growth is generally inefficient. In both of those pathways, the potential genes that would complete the pathway have not been fully identified, and thus, cannot be used. Instead, in the KF-1 genome specifically, the presence of certain genes suggests it uses the 4-5 meta pathway to break down PCA into pyruvate and oxaloacetate without losing carbon. The ideal temperature for growing the KF-1 strain is exactly 30 degrees Celsius, which would optimize growth and metabolic activity, however through the pathway, *C. testosteroni* generates NADPH, which helps in reducing power.

Safety

Safety considerations are crucial when harnessing the plastic-degrading capabilities of *C. testosteroni.* The Federal Institute for Occupational Safety and Health (Bundesanstalt et al. und Arbeitsmedizin or BAuA), a German organization, designated the specific strain KF-1 of *C. testosteroni* as Biosafety level 1, confirming that the KF-1 strain is not likely to cause any harm to a healthy person (BAuA, 2015). However, although certain strains of *C. testosteroni* have been labeled as Biosafety level 2 by BAuA when used in genetic engineering, no update has been made to the Biosafety level of KF-1 when used in genetic engineering, which makes KF-1 an ideal strain for bioengineering (DSMZ, n.d.). Furthermore, *C. testosteroni* exhibits low virulence and infrequent associations with acute appendicitis; though infections are rare, they typically respond well to antibiotic treatment. Amongst the 29 patients with *C. testosteroni* related infections and no underlying causes, all survived. Among the four casualties where *C. testosteroni* was detected in a polymicrobial infection, all individuals had former underlying conditions.

Conversely, those without such complications experienced smooth recoveries aided by antibiotic treatments (Bayhan, 2013). Given that *C. testosteroni* will undergo genetic engineering to enhance its plastic degradation capabilities, caution is necessary during its implementation. However, using genetic engineering and proper precaution, *C. testosteroni* can be made safer.

Consequently, rigorous testing in controlled laboratory environments will address some unforeseen changes, such as increased growth rates, ensuring the safety of working with genetically engineered bacteria. Introducing the genetically engineered bacteria to a non-lab environment is also vital, as it will mimic environments the bacteria may frequent. While plastic degrading processes using *C. testosteroni* and *I. sakaiensis* would take place in a controlled lab-like environment, the monomers, Terephthalic acid, and Ethylene Glycol, created as a byproduct post-degradation are benign to the environment (Yoshida, 2016).

Although friendly to the environment, terephthalic acid (TPA) may pose mild amounts of harm when exposed to humans. TPA can irritate the eyes, skin, throat, lungs, and nose, disrupting breathing, which leads to shortness of breath, coughing, and wheezing. Also, repeated unprotected exposure to TPA can lead to kidney problems (N.J. Health, 2000). The NFPA Health Rating for TPA is 1, meaning that it is slightly hazardous or that, under emergency conditions, it can cause significant irritation to humans (U.S. National Library of Medicine, 2004). The ACGIH recommends that during an 8-hour shift, the airborne exposure to TPA should be limited to 10 mg/m3 (N.J. Health, 2000). We will keep to this limit for anyone who handles the TPA to ensure their safety. Other safety measures will be put in place, such as proper respirators and ventilation. Specialized air filtration will

allow for constant uniformity in the ambient environment. These precautions will allow for work with TPA without irritation or hazardous conditions.

Similarly, ethylene glycol poses mild harm when exposed to humans. When exposed to the skin, it can cause irritation, and when inhaled in large amounts, it can irritate the upper respiratory tract and mucous membranes. Inhaled concentrations higher than 80 ppm will lead to a cough and respiratory discomfort that is intolerable. When the eyes are exposed to ethylene glycol vapor, it can irritate. However, when exposed to liquid Ethylene Glycol, there could additionally be swelling to the cornea, eyelid, conjunctiva, and iris, as well as cornea or conjunctiva injury. When ingested, there can be effects ranging from mild, such as drowsiness and slurred speech, to severe, such as acute kidney failure and absence of urine excretion that will cause a build-up of toxic chemicals and chemical imbalances in the bloodstream and death (CDC, 2021). Ethylene glycol can cause reproductive harm and may be a teratogen, as it was identified to be one in animals. To deal with these potentially harmful effects, we will handle ethylene glycol with the utmost precision and care. ACGIH recommends that the air exposure level does not exceed 39 ppm, which we will take care to ensure that no worker reaches over that level (N.J. Health, 2016). This low exposure can be reached using sufficient ventilation and proper PPE amongst workers.

We will monitor the airborne concentrations of TPA and ethylene glycol to ensure it remains at safe levels. There will be emergency eyewash and shower stations readily available, and workers will shower after each shift. The workers will wear personal protective equipment that ethylene glycol cannot permeate. They will use nitrile gloves and protective clothing from CSM and T.K., which are recommended for use with ethylene glycol. These will be cleaned onsite by a trained team who has experience cleaning contaminated clothing, and they will be cleaned each day. Workers will be trained in using respirators and will be equipped with a respirator that is full facepiece powered-air purifying, which will be NIOSH-approved. Employees will use face shields and indirect vent goggles to ensure extra protection (N.J. Health, 2016). There will also be local exhaust ventilation to protect the workers from breathing in TPA.

Discussions

While both *I. sakaiensis* and *C. testosteroni* have shown success in breaking down plastics, this success was merely in a laboratory environment, not in a real-world situation (Yoshida, 2016). PET's will arrive at recycling facilities likely with synthetic adhesives from various labels. This could potentially pose an issue to the degradation process. We are unsure how *C. testosteroni* and and *I. sakaiensis* will handle the synthetic adhesives in its degradation. We are not sure in general, whether *C. testosteroni* and *I. sakaiensis* are able to break down other polymers, including other types of plastics (Alexander, 2023). Due to the associated risks of employing bacteria in recycling facilities, personal protective equipment (PPE) is mandated for workers' safety. These items include gloves, aprons, long sleeves, and other necessary protective gear, as workers often interact with dirt and other contaminants. Although plastic in treatment facilities undergoes processing before disposal, it is crucial to recognize that the majority of plastic waste is held outside treatment facilities, typically in landfills, which leak into the environment. While our "super degrader" may not be able to be utilized outside of a facility, removing the total amount of plastic waste stalls environmental waste leakage (UNEP, 2023).

This product would not necessarily be limited to only plastic. While *I. sakaiensis* has only been tested to degrade PET plastic, the way it uses hydrolysis to split PET may extend beyond just plastic. *C. testosteroni* already shows promise in degrading aromatic compounds, such as synthetic laundry detergent (Wilkes et al.; L., 2023). PETase has its limitations; most notability is due to its low thermal stability; however, it still opens up many options when discussing degradation (Son et al., 2019).

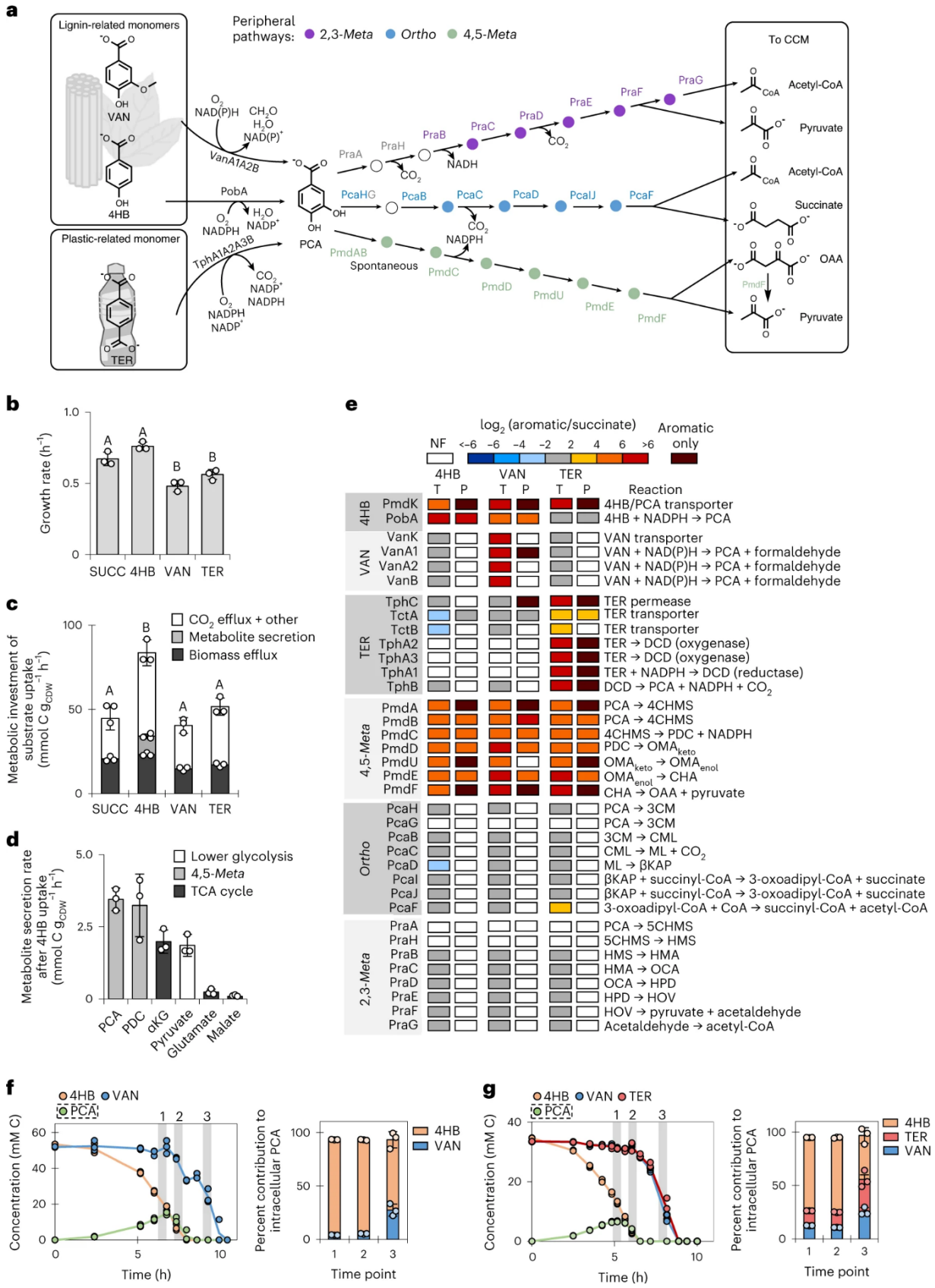

Figure 6: C. testosteroni degradation pathways (4-5 meta availability). (Wilkes et al.; L., 2023)

Next steps

Constructing plasmid pET-21b(+), along with culturing *C. testosteroni* and *I. sakaiensis,* will be crucial next steps. Transforming *C. testosteroni* will also be extremely important, as the functionality of PETase and MHETase genes in *C. testosteroni* has yet to be tested. While data suggests the plasmid pET-21b(+) effectively carries genes from *I. sakaiensis* 201-F6, further testing may be required (Austin et al., 2018). After our design is assembled using electroporation and grown on standard bacteriological media, several tests will be performed. Some next steps in this process would be conducting baseline experiments to gauge a starting point of degradation for both *I. sakaiensis* and *C. testosteroni*. After our bacterial colonies are prepared, they would be applied to our testing site, PET plastic film. This film will be far superior for testing as compared to previous methods because it is processed to remove any contaminants that exist within other commercial PET products. These preparations will allow us to determine the bacteria's performance based purely on their ability to degrade PET plastic. Data would be collected periodically to compare the initial bacteria to the genetically modified "super degrader," accounting for all variables. The bioengineered bacteria will display different degradation rates for hard and soft PET films for degradation so that tests can be conducted on both (Yoshida, 2016).

Afterward, we would examine the speed at which the degradation occurs, as well as any other possible observations that may be relevant to our research. It would be imperative to test on unprocessed plastics, which may contain contaminants and lead to a different result. Processed plastic would be the best for establishing a baseline, but because plastic degrading would occur in a recycling facility, further tests would need to be made with less refined options. While current research is limited to PET plastic, in theory, any plastic that contains aromatic compounds like benzene rings or ester bonds can be broken down by PETase, and MHETase may be able to be degraded. Testing on types of plastics that share these features, such as Acrylonitrile Butadiene Styrene, Polyethylene, Polypropylene, Polycarbonate, Polyvinyl Chloride, or Nylon, may expand the possibilities of plastic degradation using our genetically engineered bacteria.

Author contributions

J.W. and A.G. came up with the original idea. B.C. formatted the citations. A.B. wrote the acknowledgments. A.B. and A.G. wrote and researched for the safety section. All authors worked in tandem with both writing and researching on the background section. A.G. and J.V. took the lead in writing the system, device, and parts. J.V., A.B., and J.W. worked on the next steps, and J.V. and A.G. worked on the discussion. A.G., J.W., and J.V. worked on creating some of the images, A.B. worked on securing copyright for images, and B.C. created the citations. B.C., J.W., and A.G. starred in the video, with J.V. doing the animations and A.B. filming.

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