A System for Degrading PET in the Environment

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Reviewed on 28 April 2018; Accepted on 19 May 2018; Published on 22 October 2018

Plastic pollution is a major environmental problem that disturbs the health and biodiversity of terrestrial and aquatic ecosystems. As of now, the primary way to handle plastics is mechanical recycling, in which plastics are sorted, melted, and extruded into new products. However, only 9% of the currently existing 8.3 billion metric tons of plastics have been recycled (Parker, 2017). Plastic pollution is a particular concern in oceans because gyres – circulating bodies of water – trap plastic products into growing mounds. Moreover, marine organisms ingest plastic that is floating in the ocean, which subsequently creates health problems for them. Our ultimate goal is the bioremediation of areas where plastic has built up. We plan to treat such areas by genetically engineering the yeast species *Saccharomyces cerevisiae* to break down polyethylene terephthalate (PET) plastic into the environmentally benign molecules ethylene glycol and terephthalate, by using the enzymes PETase and MHETase from *Ideonella sakaiensis.* We will verify that both of our proteins are expressed extracellularly and that our kill switch and limiting system are effective at controlling the growth of *S. cerevisiae.*

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Background

As massive amounts of plastics are produced each year, plastic buildup in oceans and landfill sites is increasingly becoming a major problem for the environment. If steps are not taken to combat this, it could severely and adversely affect life on Earth in a short period of time. We plan to use genetically engineered *Saccharomyces cerevisiae* to produce the enzymes PETase and MHETase, both originally found in the bacterium *Ideonella sakaiensis* (Yoshida et al., 2016). PETase will be used to break down PET plastic into another compound, MHET, which will subsequently be catabolized by MHETase into ethylene glycol and terephthalate.

Systems Level

We plan to use yeast to secrete PETase, an enzyme which breaks down PET into mono (2-hydroxyethyl) terephthalic acid (MHET) plastic polymers. The yeast then hydrolyzes MHET via MHETase into the environmentally benign monomers ethylene glycol and terephthalic acid. To determine if PETase and MHETase are produced and able to break down PET plastic, we will introduce the modified yeast to PET plastic and observe if degradation occurs. In the future, we will add a green fluorescent protein (GFP) gene into our system to reduce the time required

to detect successful expression. For more information on the addition of GFP, please see the "Future steps" section below.

Device Level

We chose to use the yeast strain *S. cerevisiae* as our system chassis because of its ability to extracellularly secrete protein. This ability will allow for the breakdown of PET plastics into organic components directly in an affected environment, so that progression of the treatment can be directly monitored and altered as necessary.

Parts Level

Both DNA sequences begin with the standard constitutive promoter BBa_J63005. Because the quantity of plastic to be degraded may be variable, the amount of protein needed to complete the degradation is also variable. Therefore, by keeping the system 'on' by default, we can produce as much PETase and MHETase as needed. We plan to stop the production of PETase and MHETase after the plastic degradation process is complete by killing the *S. cerevisiae* population with two kill switches. The first kill switch will be encoded within the systems while the second kill switch is external. For more information on the kill switches, please see the Safety section below. We will not use an inducible system due to the difficulty of detecting PET plastic. For the PETase system (Figure 1), the constitutive promoter is followed by the standard ribosome binding site BBa_B0034. The next component of the sequence is PETase (BBa_K2010000), which degrades PET plastic into MHET. We will finish the sequence with a standard terminator (BBa_J63002).

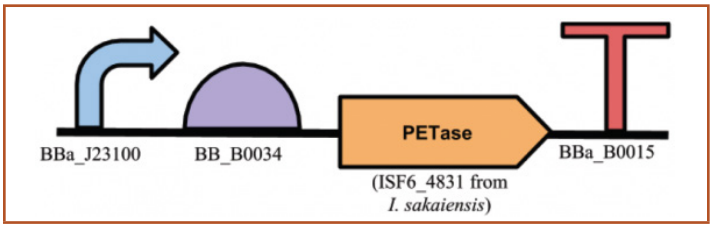

Figure 1. *PETase system.*

We plan to produce a nearly identical, separate system for the production of MHETase (Figure 2). This system will parallel the PETase system, the only difference being that the MHETase coding sequence (CDS) will replace the PETase CDS. The order for the MHETase system will be as follows: constitutive promoter BBa_J63005, ribosome binding site BBa_B0034, the MHETase CDS (BBa_K2110014), and the terminator BBa_J63002.

Safety

As per our plan for the use of this system, we intend to release it into the environment. Naturally, this means that it must have extensive safety controls to prevent the escape of genetically modified organisms into the environment. To this end, we propose two independent control mechanisms: a kill switch to permit the instant elimination of the yeast if necessary, and a fail-safe growth limiter to place a hard limit on the number of replications the yeast can undergo.

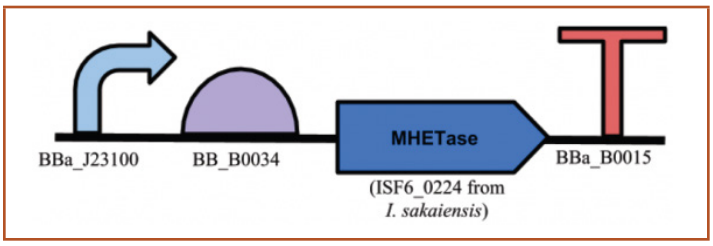

Figure 2. *The MHETase system.*

Kill Switch

Since our yeast cells will be released into the environment, we designed a kill switch: a final safeguard mechanism for shutting down our organism. The kill switch would be activated by the addition of preprotoxins, a special type of toxin molecule that only yeast cells are susceptible to. Preprotoxins kill susceptible cells in a dose-dependent manner either by inducing apoptosis, or via necrotic pathways. Overall, preprotoxins would be the best molecules to use for the kill switch as they would not affect any plants or animals in the environment, just our modified yeast (Reiter et al., 2005).

The cytotoxic effectiveness of preprotoxins stems from their ability to create pores in cell membranes, which eventually results in cell death. Moreover, preprotoxins can bind to two receptor molecules in the *S. cerevisiae* cell wall: the β-1,6-D-glucan receptor and β-1,6-mannoprotein receptor. The endogenous toxins K1 and K28 then kill the yeast cells in a receptor-mediated process (Breinig et al., 2002).

The first process involves the binding of the K1 toxin to the cell wall receptor β-1,6-D-glucan, which facilitates the entry of the toxin into the cytoplasm. Subsequently, the preprotoxin binds to the Kre1p receptor, located on the cytoplasmic side of the plasma membrane. This binding induces the formation of selective ion channels in the membrane that disrupt the membrane function, eventually culminating in cell death.

The second process involves the binding of the preprotoxins to the β-1,6-mannoprotein receptor, which allows the K28 toxin entry into the cell. From there, the K28 variant moves from the cytoplasm to the nucleus, where the toxin shuts down DNA synthesis in the yeast cell. This inevitably results in apoptosis as the prevention of DNA synthesis leads to a lack of DNA repair, which over time fosters the degradation of the cell (Zhang et al., 2006).

Overall, preprotoxins would function as an effective kill switch because they not only degrade yeast cells through two different pathways, but also demonstrate specificity only towards yeast, thus they would be environmentally sound in aquatic environments. Other kill switch possi-

bilities include the addition of ammonium, acetic acid, or other fungicides. However, the addition of any of these compounds into a water body could drastically impact other organisms, as the toxic nature or acidic conditions that could result from the use of these compounds may inhibit these other organisms' metabolic activity. Thus, the use of preprotoxins would be the best way to eradicate the modified yeast cells with minimal environmental damage.

Growth Limiter

As we intend to release our yeast into the environment, there is a significant possibility that our yeast may escape from the intended bioremediation area, even if we attempted to activate the kill switch. Therefore, we will incorporate a fail-safe limit on the number of replications yeast can undergo in the wild. We plan to use the growth limiter developed by the 2014 iGEM team Cooper Union, which operates through the elimination of telomerases, as yeast without telomerases can only undergo a limited number of replications before senescence (Jay et al., 2016).

Yeast with nonfunctional copies of two genes, *EST2* and *RAD52*, cannot extend their telomeres and therefore irreversibly pass into senescence after a limited number of replications (LeBel et al., 2009). However, we still wish to permit indefinite replication in laboratory situations. Therefore, we will destroy the yeasts' native copies of *EST2* and *RAD52* and add them back under the positive control of galactose. Therefore, when the yeast are growing on galactose media in the lab, they will be able to grow indefinitely; however, when released into the wild, they will be prevented from replicating indefinitely.

Future Experimentation

Unfortunately, we were unable to test our systems in a laboratory setting because both PETase and MHETase BioBrick parts were out of stock in the iGEM parts registry. If we could acquire the aforementioned coding sequences necessary to complete both systems, we would perform DNA extraction and purification using standard procedures, such as a minipreparation and spin column purification.

Our first goal is to test the efficacy of this system, and if it is able to degrade PET. Future experiments will aim to study the rate at which our system can degrade PET, and the optimal aquatic environmental conditions for yeast protein expression, including pH.

Environment Optimization

The optimal pH for PET film hydrolysis is 9 while the pH of freshwater lakes is 6.5–8.5, so PETase activity could potentially be suboptimal in a freshwater lake. Tests for the optimum temperature for our system must also be conducted, as well as determining the minimum operating temperatures. While the optimal temperature for yeast performance is 37°C, the optimal temperature of PET hydrolysis is 40°C. Both of these conditions are likely to be different from the temperature of a body of water, which my fluctuate as a result of seasonal changes and vary by geographical location.

Proof of Concept

A possible configuration to test for the successful production of PETase and MHETase is the use of a reporter protein that consecutively follows the protein of choice via a constitutive promoter (see Figures 3). Green fluorescent protein (GFP) is commonly used as a reporter protein to indicate that an initial gene has been correctly expressed. If a GFP gene is inserted properly into another organism, it will be able to act as a visual tag to show the expression of other genes. However, it is important to note that there is a possibility of PETase being expressed but GFP failing to be expressed and vice versa. Nevertheless, correct assembly of this system will typically allow GFP to function effectively as a reporter protein: GFP should only be allowed to be properly expressed if the first protein (PETase or MHETase) is expressed as well. Alternatively, tests such as SDS-PAGE or Western Blotting may be used to validate the presence of the proteins without altering the current system plans (Figures 1 and 2).

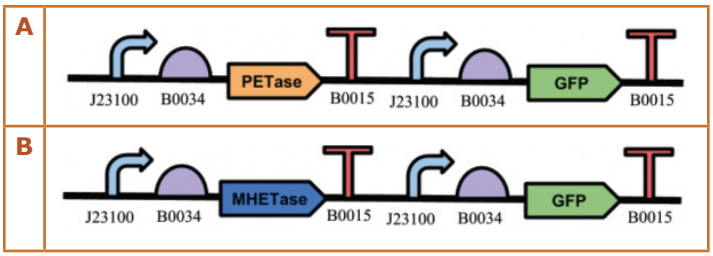

Figure 3. *A) PETase with GFP indicator. B) MHETase with GFP indicator.*

Fortuitously, testing the actual breakdown of PET, our end goal, is quite feasible, so a GFP indicator or other tests may be unnecessary. We hope to test for the successful expression of PETase and MHETase by letting the modified yeast "digest" very thin PET plastic. Using a scanning electron microscope, catabolism of the PET plastic can be verified if increasing ruggedness and holes are observed in the surface of the PET plastic (Tianjin iGEM team 2016). However, the additional use of a GFP indicator is worth consideration as it may act as a quicker indicator of protein expression than plastic surface degradation.

Efficiency Testing

The following experiments would be vital for maximizing the efficiency of our system.

We would firstly need to develop the time frame needed for protein expression, as well as determine the exact rate of plastic degradation for a variety of sizes of PET plastic sheets. An additional experiment could be the concentration of protein needed to break down PET. Our system must also be tested for degradation rates with

different densities of PET plastic.

Another area for future exploration is the addition of tags to boost transcription efficiency. Extensive testing has been previously conducted by the Harvard 2016 iGEM team and others on this subject, but PETase and MHETase modified with transcription tags have often been unsuccessful in transcription and translation. Further research into boosting promoter-binding affinity that results in improved rates of protein expression is also required. This will be necessary to determine if our system is (or can be made to be) competitive with current mechanical collection and recycling methods.

In addition, we must prove that we can extracellularly express both proteins. This is crucial for the actual implementation of our system for bioremediation, so that the system can operate freely in the environment until it is no longer desired, at which point the kill switch or inhibitory mechanism will be triggered. Yeasts have a wide array of secretory expression tags, so we plan on performing extensive testing to identify the tag that allows for the maximum expression rates of PETase and MHETase.

Control Testing

As any organism genetically engineered for eventual release into the environment requires extremely robust controls, we will need to complete extensive testing of both of our control systems for the modified yeast: the kill switch and the growth limiter.

For the growth limiter, we will need to validate three things. Firstly, if we successfully add *EXT2* and *RAD52* back to our growth-limited yeast, the yeast will then grow indefinitely in the lab. Secondly, the yeast must pass into senescence after an appropriate period of time. Thirdly, we must validate that this control remains evolutionarily stable. The first two requirements are easy to verify by simply growing the yeast for a period of time with and without galactose, and comparing the duration of time before yeast populations significantly decline. However, verifying evolutionary stability over a long period of time and for large population sizes of yeast will be difficult, especially as our growth limiter relies on DNA damage. Therefore, our validation plan is to grow up a very large population of yeast, and see if any are able to inactivate the growth control. If they are, we will add in a second inducible promoter under the control of a different transcription factor, so that a double mutation is required to cause control failure. Once the growth limiter can pass this test, we will find a completely different way of preventing telomere lengthening, place it under inducible control, and subject it to the same tests so that we will have two independent growth limiters to ensure safety in the final system.

Discussion

Though our team has yet to test this system, other synthetic biology teams have proven successful in the expression and verification of the catabolic properties of PETase and MHETase on PET plastics (for example, see the 2016 Tianjin iGEM team's PLASTERMINATOR page at: https://2016.igem.org/Team:Tianjin). In addition, several iGEMs have constructed the PETase and MHETase nucleotide sequences into BioBrick parts, some even including secretion systems for the proteins (http://2016.igem.org/Team:Harvard_BioDesign). However, none of these BioBrick parts for PETase and MHETase are currently in stock or demonstrate reproducible fidelity. Thus, there is great capacity for outreach and collaborative possibilities. Our final option is the manual synthesis of these genes according to NCBI or UniProt sequences. Nonetheless, we plan on the eventual construction of our system in order to test its feasibility and efficacy.

Our previous design for the PETase and MHETase systems combined both PETase and MHETase genes in the coding sequence region (CDS) of the system (Figure 4A). However, it came to our attention that there is a high likelihood of the protein being misfolded if the encoding sequence is too long. Thus, we decided to separate the combined genes into two separate CDS systems. Initially, we had planned on implementing both the PETase and MHETase systems into one strand of nucleotides (Figure 4B). After further feedback from peers, we revised the system so that the PETase and MHETase systems are entirely independent on separate strands. This is simply due to the increased efficiency and agency over the functioning model. With separate strands, the amounts of PETase and MHETase can be more specifically monitored and tailored to suit the treatment area. In addition, this will allow for more efficient protein degradation, as we will first release PETase only, allowing the enzyme to fully metabolize PET into MHET, before proceeding with the addition of MHETase. Thus, no incomplete or "transition" metabolites between PET and MHET should be present in the treated area with the proposed system (see Figures 4).

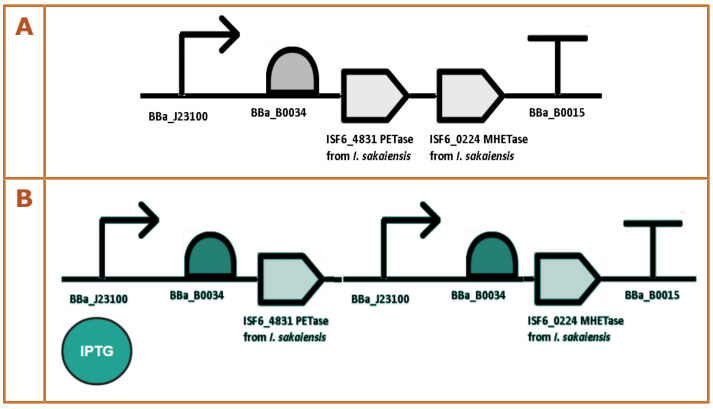

Figure 4. *Models 1 (A) and 2 (B).*

A future possibility for our design systems is the mass production of these two proteins by biotechnology companies. If this were the case, the proteins could be expressed in a more rapidly growing chassis, such as *E. coli*, then extracted and purified using standard means,

such as detergents and spin columns. This could allow for a greater ability to upscale the production of PETase and MHETase. In addition, there would be finer control over the quantities of PETase and MHETase produced, allowing for more precise treatment of polluted areas. This would also eliminate the risk of genetically modified organisms escaping, as well as reducing the toxic potential of any chemicals we might use as a kill switch for the constitutive system.

A second variant of the system could instead use an inducible promoter. However, there is no currently existing promoter that can selectively and accurately detect PET plastic. There is a further risk of environmental toxicity from the added transcription factor, as well as the additional cost of producing the transcription factor.

A significant area of concern is the toxicity of the kill switch systems. For example, preprotoxins are an excellent kill switch because they selectively induce apoptosis in yeast cells. However, further research needs to be conducted on the presence and ecological niche of naturally occurring yeasts in aquatic environments to ensure as little species displacement and ecosystem disruption as possible. Ideally, a less malignant or more selective chemical kill switch will be discovered.

In addition, it is unknown whether a mutation for shorter telomeres in a yeast plasmid could conjugate and spread to other non-target organisms. In such a case, it may be best to perform plastic degradation in a closed, controlled environment away from aquatic ecosystems.

Finally, there is some concern over the environmental toxicity of ethylene glycol and terephthalate, and their effects on aquatic life forms and water quality. Although the Environmental Protection Agency (EPA) has not classified ethylene glycol as a carcinogen, it has been shown to be fetotoxic and linked to detrimental kidney and liver effects in rodents (EPA ethylene glycol hazard summary). This could be a concern if the byproducts were to be ingested by aquatic organisms, potentially manifesting in the biomagnification of harmful toxins in humans from eating seafood.

Acknowledgements

We would like to thank Dr. Natalie Kuldell for her time and expertise, as well as the opportunity to present our design ideas and receive feedback from our peers in BioBuilder at the Final Presentation Session in LabCentral. We would also like to thank our mentor, Anne Burkhardt, for her advice and suggestions.

References

Ajo-Franklin, C. Part:BBa_J63005: Experience [Internet] IGEM: Register of Standard Biological Parts. Cambridge (MA): The International Genetically Engineered Machine Competition. 2006 Oct 11 [cited 2018 Apr 18]. Available from: https://bit.ly/2Ir8dcp.

Breinig, F, Tipper, DJ, Schmitt, MJ. Kre1p, the plasma membrane receptor for the yeast K1 viral toxin. Cell 2002 Feb 8; 108(3):395–405.

Coghlan, A. Bacteria found to eat PET plastics could help do the recycling. [Internet] New Scientist. 2016 March 10 [Cited 2018 Apr 18]. Available from: https://bit.ly/21rmAOY.

Fondriest.com (n.d.) pH of Water [Internet]. Fundamentals of environmental measurements. Fondriest Environmental Inc [Cited 2018 Apr 18]. Available from: https://bit.ly/2PMfNBB.

Parker, L. A whopping 91% of plastic isn't recycled. [Internet] National Geographic. 2017 July 19 [Cited 2018 Apr 18]. Available from: https://bit.ly/2z2oCCI.

Reiter, J, Eva H, Frank M, Schmitt, MJ. Viral killer toxins induce caspase-mediated apoptosis in yeast. J Cell Biol. 2005 Jan 21;168(3):353.

Ribeiro, GF, Côrte-Real, M, Johansson, B. Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. Mol Biol Cell. 2006 Aug 9;17(10).

UniProt. A0A0K8P6T7 (PETH_IDESA) [Internet] (n.d.) [Cited 2018 Apr 18]. Available from: https://bit.ly/2y2oXC3.

Yoshida, S, Kazumi H, Toshihiko T, Ikuo T, Hironao Y, Yasuhito M, et al. A bacterium that degrades and assimilates poly(ethylene terephthalate). Science 2016 Mar 11;351(6278):1154–96.

Zhang, NN, Dudgeon, DD, Paliwal, S, Levchenko, A, Grote, E, Cunningham, KW. Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. Mol Biol Cell. 2016 May 31; 17(8).