The Advancement of PET (Polyethylene Terephthalate) and PE Degradation

# Abstract:

The bacteria recently found in Japan in 2016, known as Ideonella Saikenesis, has found to produce an enzyme (PETase) that has the ability to degrade plastic relatively quickly. Due to is thermal lability, it is not currently seen as an economically effective way to reduce plastic pollution. This handicap causes PETase to degrade at temperatures of around 85-90 degrees Celsius. The majority of the plastic pollution in the world is found in areas of southern Asia and the middle east (Ritchie and Roser 2018). If this bacterium were to be implemented into these areas, PETase would denature and the bacterium would likely die. Thus, proving mass efforts to not be effective. Possible remedies to this problem would be to overexpress the ISF6 gene to yield a greater efficiency of plastic degradation and to insert an entire operon (SpoVA) from the *Clostridium botulinum* bacterium that causes increased resistance to heat.

# Introduction:

Polyethylene.Terephthalate.(PET) and polyethylene.(PE) are extremely common and are the most widely produced and used**.**plastics in the world. They are extremely resistant to degradation in nature due to their origins from fossil oil (Liu et. al 2018). PET is used for synthetic fibers, bottle resin, packaging, etc. (Sinha et. al. 2010). The synthetic production of plastic is roughly 50 million tons per year (U.T. Bornscheuer et. al. 2016). Steps have been taken to reduce the amount of PET in the ecosystem, such as recycling and other techniques. These common remedies are often long physical or/and chemical processes that consume lots of energy resources and can emit harmful by‐products. Microorganism‐derived enzymes have been considered as an option to reduce the PET problem, but not many were effective or successful.

Previous studies found that fungal and bacterial thermophilic hydrolases were able to catalyze PET through a process of hydrolysis (Fecker et. al. 2018). These were effective but only at high temperature.

Other enzymes studied are lipase, carboxylesterase, and cutinase (Fecker et. al. 2018). All of these have been found to degrade PET and the most effective was cutinase. Cutinase was most effective at degrading PET at the temperature range of 50–70 °C (Wei 2017). However, its efficiency was diminished when faced with a PET substrate that is highly aggregated. Steps have been taken to help enhance the effectiveness of cutinase but it would be better to conclude it as not being a viable option and to find another enzyme that has the ability to deteriorate the PET at a lower temperature.

Scientist recently discovered an enzyme from the bacteria Ideonella Sakaiensis (previously known as 201-F6) at a bottle recycling plant in Japan in 2016. It is found to have superior degradation properties than the previously discovered variants. Caterpillars have also been found to have excellent results (primarily from the wax moth variety) (Bombelli et. al. 2017). Similar to the bacteria Ideonella sakaiensis, they too have a digestive system that allows them to degrade PE rapidly. This combination of enzymes may lead to a long lasting cure to the worlds plastic pollution.

# PETase

Ideonella Sakaiensis is an aerobic, non-spore-forming, rod-shaped bacterium that was isolated from a microbial congregation of two or more microbial groups that degrades poly(ethylene terephthalate) (PET) collected in Sakai city, Japan (Tanasupawat.et al. 2016). The newly found enzyme, known as PETase, introduces a new method to disposing of PET (polyethylene terephthalate) waste..It has been found.that.PETase is very efficient in the degradation of PET. The bacteria that harbored these PET degrading enzymes were pulled from samples of PET-debris-contaminated samples such as sediment, soil, wastewater and sludge as targets. PETase breaks down PET to mono(2-hydroxyethyl) terephthalic acid (MHET) just like cutinase, but it can be down at room temperature. Despite its varying thermal lability , it is able to degrade much more efficiently than the previously looked at enzymes.

# Conditions and preferred environment

Ideonella Sakaiensis were grown on agar plates and their contents were observed and analyzed. The conditions of growth were in the pH range 5.5–9.0 (preferably around a pH 7–7.5) and at a temperature of 15–42 ºC (optimally at 30–37 ºC) (Tanasupawat et al. 2016). There were eight isoprene units (Q-8). C16 : 0, C1 7 : 0 cyclo, C18 :1ω7c and C12 : 0 2-OH and they are predominantly cellular fatty acids that were considered to be major isoprenoid quinone structures (these are compounds that are composed of a hydrophilic head group and an apolar isoprenoid side chain and are essential to life).

# Other Solutions to PETase shortcomings:

Ideonella sakaiensis enzyme can be an extremely effective solution to this worldwide problem. Its main shortcoming is its relatively low thermal stability (Son. Et. al. 2019). This undesirable quality may hinder its progress into the commercial market for widespread use. Measures are being taken to remedy this issue, such as the development of a better rational protein engineering strategy.

With the chemical structure of this enzyme known, researchers are finding a way to develop a better method to alter its structure via this strategy. One in particular that may yield high success is the IsPETaseS121E/D186H/R280A variant. Structural differences lie in its ability to stabilize the β6-β7 connecting loop and extended subsite IIc. The following revision yielded a melting temperature increase of 8.81 °C and PET degradation activity was enhanced by 14-fold at 40 °C when compared to its wild type (IsPETase).

ISF6 is the gene involved in the degradation and assimilation of the plastic poly(ethylene terephthalate) (PET). This gene allows Ideonella sakaiensis to use PET as a major energy and carbon source for further growth and dvelopment. It also likely works together alongside MHETase to depolymerize PET (Yoshida et. al. 2016).

The reaction further catalyzes the hydrolysis of PET to produce mono(2-hydroxyethyl) terephthalate (MHET) as a major product (Austin et. al. 2018). There is also a depolymerization of another semiaromatic polyester called poly(ethylene-2,5-furandicarboxylate) (PEF). PEF is a new and is a bioderived PET replacement with improved gas barrier properties. MHET is different from PETase in that it doesn’t degrade aliphatic polyesters like polylactic acid (PLA) and polybutylene succinate (PBS). It is also able to hydrolyze bis(hydroxyethyl) terephthalate (BHET) to produce MHET without any decomposition.

This information is extremely important because by overexpressing this gene. The rate at which plastic will degrade could be significantly amplified. This extreme increase could result in the degradation of plastics much sooner and faster in countries that are periodically exposed to warmer temperatures over 85 degrees Celsius. There will likely still be a risk that this enzyme (PETase) will still denature at these temperatures but will be much more efficient if this modification occurred.

Previous research mentioned that one of the ways to increase Ideonella saikenesis effectiveness in damp soild and aquatic conditions could be from inserting DNA from Azotobacter sp. (ATCC 4412) into the Ideonella’s plasmid (Widyastuti 2018). This brought up the notion that the generation would make them better able to survive in soil and aquatic areas or where any plastic waste is located. Azotobacter also has many similarities to Ideonella. They are both negative-gram bacteria, do not produce endospore and they both mobilize with flagella. This may suggest that they are much more likely to have a smooth transformation.

Increasing thermal lability could be very difficult considering that there are not many bacterium’s that maintain stability at 100 degrees Fahrenheit. However, there is a toxic bacterium Clostridium botulinum that has been found to contain sporogenes (spoVA2) that allow for higher temperature viability while not contributing to toxicity. These particular sporogenes could be implemented into Ideonella Saikenesis and be used to increase the thermal liability of PETase.

# Methods:

Cell Growth:

Once the Ideonella Saikenesis sample has been acquired. Grow four cultures of cells as a for a control, ISF6 insertion, spoVA2 sporogene insertion, and both . Using this culture, dilute the cells into a 10ml culture at approximately OD600= 0.1 and grow the cells until they reach an OD600 = 0.4 to 0.6 (OD = Optical density). This process should take about two to three hours

Primer Design:

Primer design was centered around the insertion mutations of the ISF6 gene and the spoVA2 sporogenes . A database of Ideonella Saikenesis genes and function was used to determine what gene would be targeted and also had the genetic sequence for it as well.. Targeted sequences included both the promoter/terminator sequence. This included a sequence that was 40 base pairs before the start (promoter sequence) of it and 40 base pairs (inhibition sequence) after.

PCR and Gel Electrophoresis:

The gel electrophoresis will allow the DNA to be analyzed once it becomes denatured. This is done so, that the new segments can be amplified with the correct changes being made for it.

The PCR (92 °C/120 s) reaction was started using the following: DNA template, Diluted Primers, 2X Master Mix, and water. They would be pipetted in this order into a PCR tube: 28 μl water (for 100 ul final volume), 2 μl DNA template (2 ng/ul prepared plasmid), 10 μl forward Primer (4uM), 10 μl Reverse Primer (4uM), 50 μl Master Mix (2X). Cooling is crucial to prevent denaturing thus the PCR reaction was stored into a freezer with a temp of -20 degrees Celsius.

Gel electrophoresis was used to determine whether or not the PCR reaction was successful. Thus acknowledging that the mutations were applied to the gene. To begin this process, The PCR mixture was mixed with a buffer (6x loading buffer mixed with 5 ul of distilled water). This buffer should increase the overall density of the mixture and cause an even dispersion when it is placed into the wells of the gel. The buffer also contains a dye for a better visualization of the DNA fragments.

After the mixture has been performed, the samples are placed into the wells of the agarose gel, noting what was in each mixture. Caution must be used as to not puncture the bottom of the gel. Place a ladder in the gel to help determine what the base pair length is. Allow the system to run and the DNA will begin to separate. Once complete, you may put the gel onto a UV light. Note what the base pairs of the ladder are equal to and compare it to the target DNA.

Transformation:

The transformation is started using the Ideonella cells that were used in culture, 5 µl of Carrier DNA (Salmon Sperm 10mg/ml) was added and 30 ul PCR generated DNA (amplicon). The cells were mixed by gently tapping them. Next, 250 µl of PEG/TE/LiAc was added. Mix by pipette. The reaction can then be incubated for 30 minutes at 30°C. This reaction will then be heat shocked by putting it in the water bath at 42°C for 15 minutes.

The cells are centrifuged at 5000 RPM for 30 seconds to remove any pellets. The supernatant can then be gently removed and care must be taken to not disturb the pellet that was resting on the tube.

After that, the cell pellet was rinsed with 100ul of 1M Sorbitol and suspended. Next, all cells were then plated onto the media (YPD for deletion and Khan for overexpression, and overexpression+deletion). The mixture of cells were dispersed in the petri dishes by inserting glass bead and the dish was shaken. The plate was then placed upside down in a 30°C incubator for 24 hours.

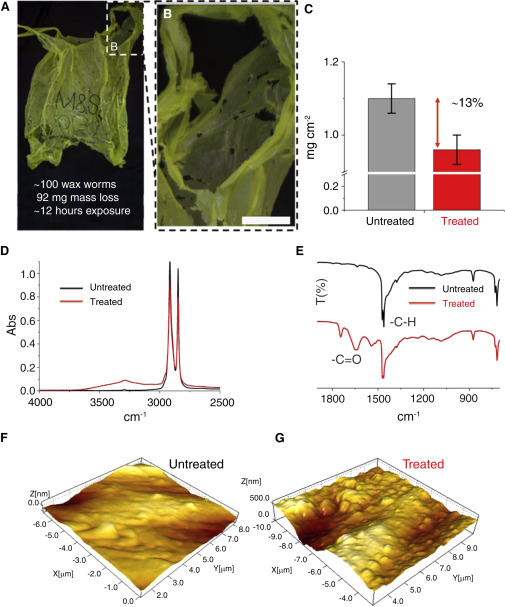
The control can be then be compared to those being tested and any improvements may be recorded. The cultures that underwent any modifications would be expected to perform much well when degrading plastic in high heat or other undesirable environments.

Discussion:

The reasoning why PETase is so effective at room temperature simply lies in its disulfide bond on its active site. This will reduce hydrolase activity and destabilize the catalytic triad (Han et. al. 2017). Altering the DNA that allows this enzyme to resist degradation at higher temperatures does help increase its The structures of these enzymes contained in the PETase, as well as the one in the bacterium Penicillium Simplicissimum, could be further analyzed and applied to a wide use of applications.

This means it may be used for other nondegradable pollutants such as glass for example. The discovery, as well as future developments may cause the overall human impact on the environment to drastically decrease, thus giving the fate of many species that are impacted a greater chance of survival.

# Future Directions

Another study investigated additional methods including nitric acid treatment and the Penicillium Simplicissimum, and wax worms (Bombelli et. al. 2017). Moderate degradation of PET was observed by the nitric acid treatment and the Penicillium Simplicissimum yielded less than stellar results. The wax worms on the other hand (Bombelli et. al. 2017) were extremely effective.

Holes began to appear in the bags after 40 minutes of direct contact with the worms (Fig. 1). Indicating that the worms were eating at the PET contained in the plastic. The worms can degrade the bags extremely quickly, but little is known as to what digestive enzyme gives them the capability to do this.

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