Investigating the Effectiveness of Bacteria and its Synergistic Effect on Biodegradation of Plastic

Kevin Ong Gheng Seong, He Yue, Hu Buyan

Group 1-31

Abstract

Increasing global production and consumption of plastic is generating huge amounts of plastic waste. Hence, it is becoming increasingly important to research for alternative ways to manage such waste. Polyethylene terephthalate (PET), a type of plastic, is this project's main focus since it is highly resistant to natural degradation as well as its widespread use in Singapore. This project will investigate the effectiveness of *Bacillus subtilis* and *Pseudomonas putida* on the biodegradation of PET and whether any synergistic effect is shown by the two bacterial species in the rate of biodegradation via colony count test, measurement of carbon dioxide (carbon dioxide) concentration, measurement of decrease in dry weight of PET immersed in bacterial culture, surface changes in PET and spectroscopic analysis. Results from the colony count test, measurement of carbon dioxide concentration, indicate that the bacteria were able to utilise PET as a carbon source, observed decrease in dry weight of PET immersed in bacterial culture, surface changes in PET and spectroscopic analysis have demonstrated that both *B. subtilis* and *P. putida* possess the ability to degrade PET and display a synergistic effect. This implies that exposing PET waste to *B. subtilis* and *P. putida* cultures are potential alternative methods to accelerate the biodegradation of PET in landfills.

Introduction

In 1950 the world produced only 2 million tonnes of plastic per year. By 2015, annual production had increased nearly 200-fold, reaching 381 million tonnes. This is roughly equivalent to the mass of two-thirds of the world population. Over the period from 1950 to 2015, cumulative production reached 7.8 billion tonnes of plastic — more than one tonne of plastic for every person alive today (Ritchie, 2018). According to Parker (2018), 91% of plastics are not recycled and 40% of plastics are used for packaging, there is thus an urgent need to find a natural way to biodegrade plastics so as to minimise the impact of plastic pollution on the environment. Bacteria are known for their metabolic diversity and high adaptability. *Ideonella sakaiensis*, is one such bacteria which is able to degrade PET, since it secretes PETase, an enzyme to catalyze the hydrolysis of PET to monomeric mono-2-hydroxyethyl terephthalate (MHET), and further secretes another enzyme MHETase to cleave Mono-terephthalic acid, to ethylene glycol and terephthalic acid (Shosuke *et al*., 2016). Kale, Deshmukh, Dudhare, and Patil (2015) had also noted that a mixture of bacteria

from the *Microbacterium* and *Pseudomonas* genus caused significant surficial degradation of Low Density Polyethylene (LDPE) film and change in bulk structural characteristics, as well as 30% and 20% weight loss/month for polythene bag after exposure to liquid culture of *Bacillus amylolyticus* and *Bacillus subtilis*. Muhonja, Makonde, Magoma, and Imbuga (2018) conducted an Fourier Transform Infra-Red spectroscopy (FTIR) analysis, which revealed the appearance of new functional groups attributed to hydrocarbon degradation after LDPE sheets were incubated with bacteria and fungi, thus indicating biodegradation. These reasons provide compelling evidence that there may be other evolved metabolic pathways that can degrade other types of plastic like PET, which is of particular concern since it is strong, durable, chemically and thermally stable and highly resistant to natural degradation (Kint & Muñoz-Guerra, 1999). Additionally, 467 million PET bottles are used each year in Singapore according to the Straits Times. However, most research papers focus on LDPE, as it is a more easily degradable kind of plastic. Hence more research can be conducted on PET, to investigate if *B. subtilis* and *P. putida* possess the ability to degrade this material and be used in landfills to accelerate biodegradation.

Objectives and Hypotheses

The objectives of this project were to investigate the effectiveness of *B. subtilis* and *P. putida* on the biodegradation of polyethylene terephthalate (PET), and whether any synergistic effect was shown by a mixture of bacterial species in the rate of biodegradation. We hypothesise that *B. subtilis* and *P. putida* show varying degrees of biodegradation and synergistic effect in biodegradation of polyethylene terephthalate (PET).

Methods & Experimental Procedures

Growth of precultures of bacteria

Bacillus subtilis (ATCC19659) and *Pseudomonas putida* (ATCC31800) were inoculated into 10 ml LB broth and grown overnight at 30°C in a shaking incubator. The absorbance of each bacterial culture at 600 nm was standardised at 0.8.

Determination of number of colony forming units and rate of carbon dioxide release

The plastic was cut into strips of 5cm X 1cm. For the test set-up, 0.1 ml of bacterial culture was added to 19.9 ml of 0.1% peptone and 0.5% sodium chloride with 1% (w/v) plastic as carbon source. For the negative control, 0.1 ml bacterial culture was added to 19.9 ml 0.1% peptone and 0.5% sodium chloride only. Five replicates each of the test and negative control set-ups were prepared. The cultures were incubated at 30°C for 2 days. Using a carbon dioxide probe, the concentration of carbon dioxide in the tubes was measured. After serial

dilution with normal saline, 0.1 ml of the bacteria was spread on LB agar plates. The plates were incubated at 30°C for 1 day and the number of colonies of bacteria was determined.

Decrease in dry weight and surface changes in PET

Preweighed pieces of the plastic (5 cm x 1 cm) were cut and added to bacterial cultures in the test set-up. No bacteria were added in the negative control set-up. At weekly intervals, the plastic was removed, dried and weighed. It was then added to a fresh bacterial culture. At the end of 6 weeks, the plastic surface was analysed for pits and holes with scanning electron microscopy (SEM).

$$\text{Weight loss (\%)} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100$$

Spectroscopic analysis

The changes in the polymer bonds due to biodegradation are determined using the FTIR-ATR spectrophotometer. The plastic films exposed to all the bacteria were analyzed at regular intervals in Magna-IR 560 Spectrophotometer (Nicolet) and FTIR spectra of the films were obtained by OMINIC. E.S·P.5.0 software. Carbonyl index (CI) was used to measure the degree of biodegradation as its value depends on the degraded carbonyl bond. The lower the carbonyl index, the greater the extent of degradation. CI was obtained by the formula:

$$\text{Absorption} = 2 - \log_{10}(\text{Transmittance/\%})$$

$$\text{Carbonyl Index (CI)} = \frac{\text{Absorption at } 1740\text{cm}^{-1} \text{ (the maximum of carbonyl peak)}}{\text{Absorption at } 1460\ \text{cm}^{-1}\text{(the maximum of carbonyl peak)}}$$

Results

Colony Count Test

The Mann-Whitney U test p value for both bacteria was 0.01208, indicating a significant difference between the control and test. More colonies obtained in the test set-up compared to the negative control set-up, as shown in the bar graphs (Figure 1) and images of colony count plates (Figure 2). This suggests that the bacteria could utilise PET as a carbon source, resulting in higher carbon dioxide concentration and higher rate of growth.

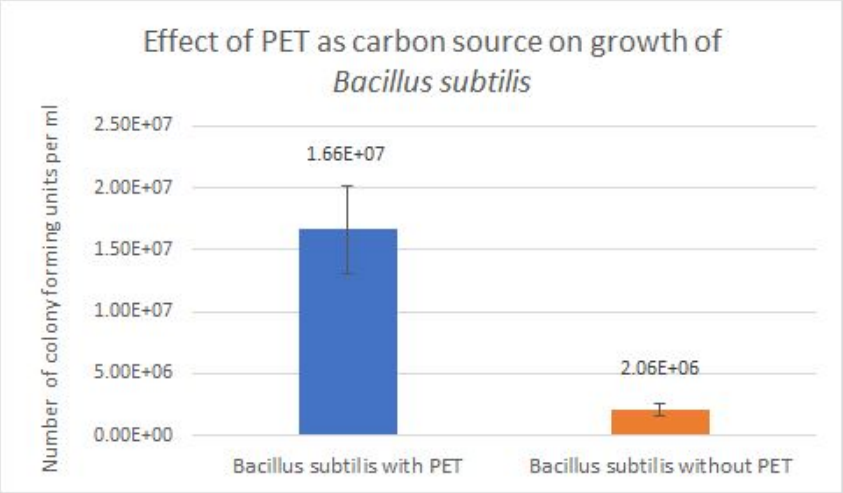

Fig. 1: Effect of PET on the growth of *B. subtilis*

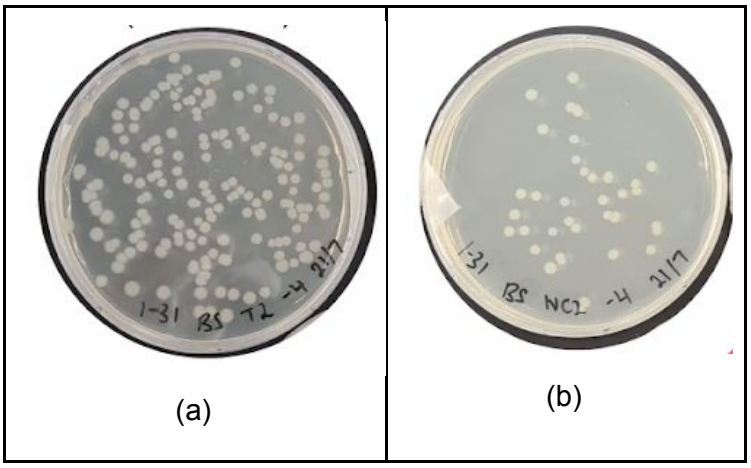

Fig. 2: Colony count plates showing (a) *B. subtilis* with PET strip (test), (b) *B. subtilis* without PET strip (negative control)

Carbon dioxide concentration test

Carbon dioxide concentration released by the test set-up (with PET) was higher than that of the negative control set-up (without PET) (Fig 3), indicating higher rate of respiration and growth of bacteria exposed to PET strips. The Mann-Whitney U test was performed for *B. subtilis*, *P. putida* and mixed culture. The p value for all of the set-ups was 0.00512, indicating a significant difference between the control and test. The concentration of carbon dioxide released from the mixed culture was higher than that for individual bacteria cultures, suggesting a synergistic effect.

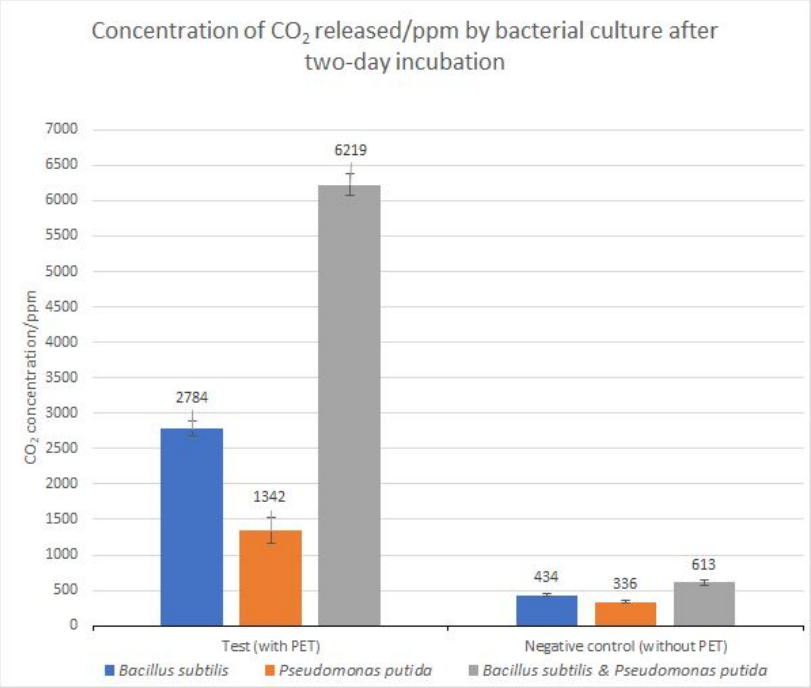

Fig. 3: Effect of PET on the concentration of carbon dioxide released from bacteria.

Decrease in dry mass

A decrease in dry weight can be observed for PET strips immersed in *B. subtilis*, *P. putida* and mixed cultures, while no decrease in dry weight was seen for PET strips in the negative control set-up (without bacterial culture). The percentage decrease in dry weight for *B. subtilis, P. putida*, the mixed culture and negative control set-up was 21.4%, 16.7%, 20.2% and 0% respectively (Fig 5). The Mann-Whitney U test was performed for all of the set-ups and the p value was 0.01208 for *B. subtilis* and 0.00512 for *P. putida* and the mixed culture, indicating significant difference between the initial and final dry weights. The p value was 0.9442 for the negative control set-up, indicating no significant difference between the initial and final dry weights.

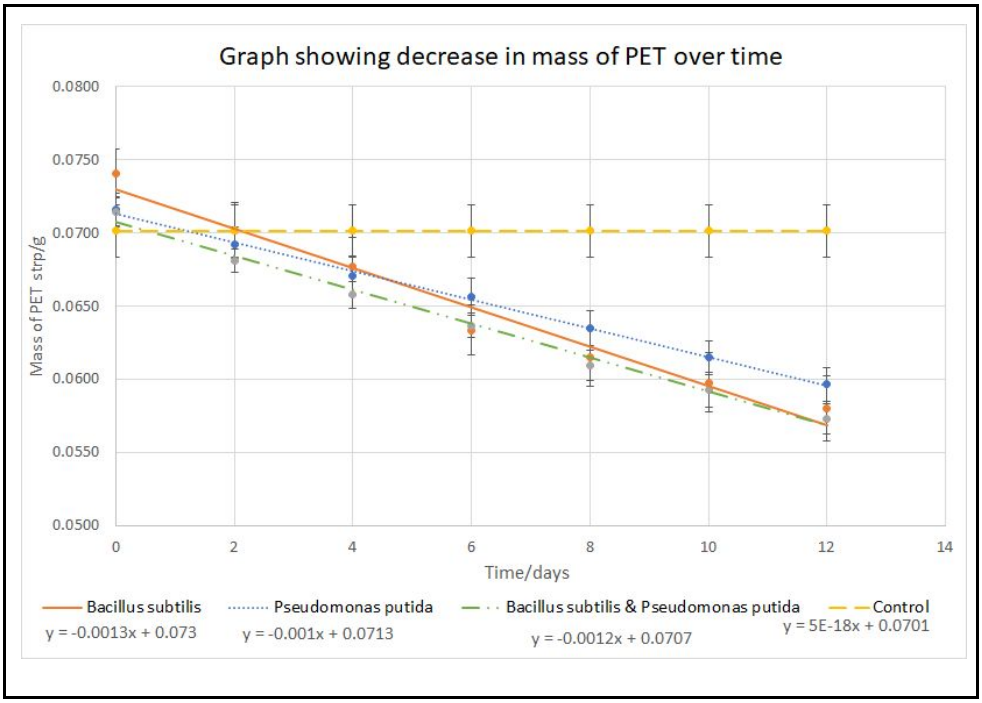

Fig 4: Graph showing a decrease in mass of PET in presence of bacteria.

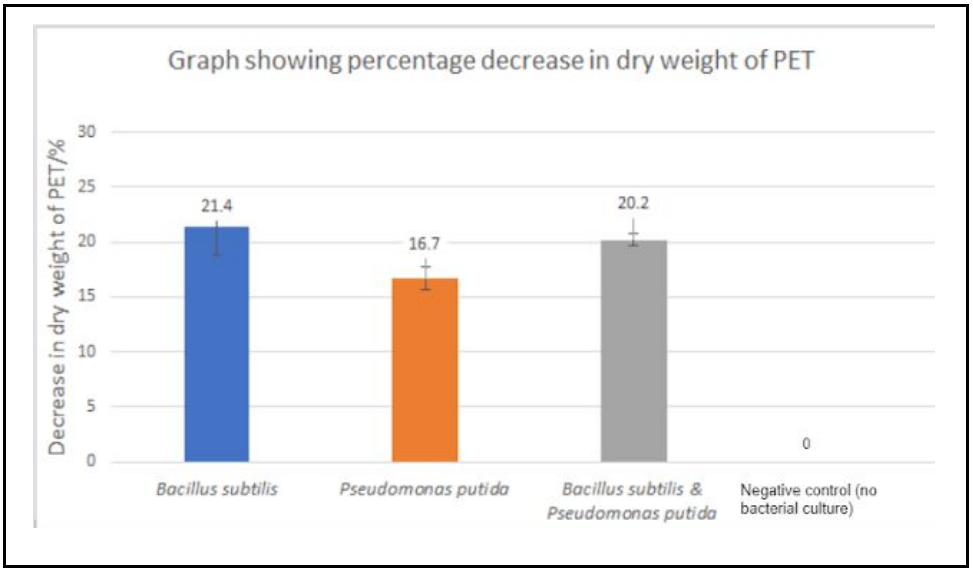

Fig. 5: Bar graphs showing the percentage decrease in dry mass of PET strip.

Surface changes in PET

More pits and holes were observed for PET strips in the test set-ups (with bacterial culture) than those immersed in the negative-control set-up (without bacterial culture) (Fig 6). This suggests that the presence of *B. subtilis* and *P. putida* caused physical changes to PET.

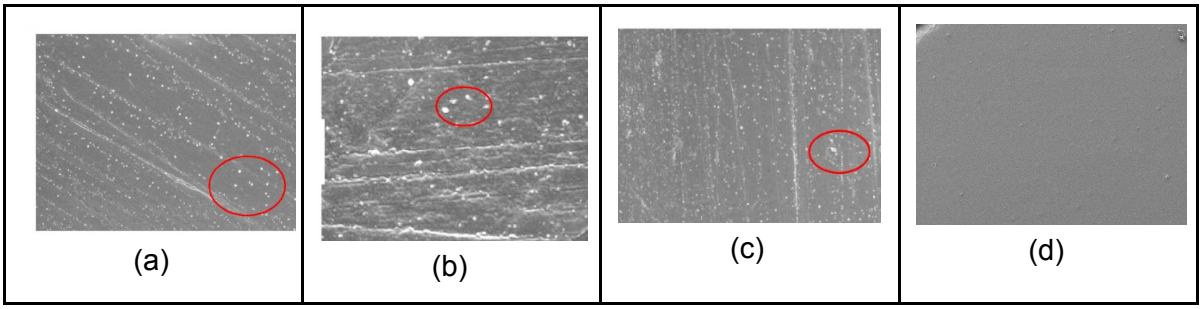

Fig. 6: SEM images of PET strips immersed in (a) *B. subtilis* culture, (b) *P. putida* culture, (c) mixed culture and (d) peptone solution (negative control). Pits and holes were observed for (a), (b) and (c) (circled regions)

Spectroscopic analysis

Transmittance values detected at wavenumber range 1740cm and 1460cm corresponding to C=O bonds and C-H_2 bonds present in the chemical structure of PET were used in the calculation of the carbonyl index (circled in green). The FTIR spectra of the PET strips immersed in peptone solution (negative control), *B. subtilis*, *P. putida*, *B. subtilis & P. putida* cultures are shown in Figures 7, 8, 9 and 10 respectively. The carbonyl index of the PET strips after 6-week incubation is shown in Fig 11. The percentage decrease in carbonyl index with respect to the negative control for *B. subtilis*, *P. putida*, *B. subtilis & P. putida* cultures was 43.1%, 33.3% and 53.9% respectively. The lowest carbonyl index and largest percentage decrease from the negative control were observed for the PET immersed in the mixed culture, indicating a synergistic effect since a larger decrease in the carbonyl index indicates greater biodegradation of PET.

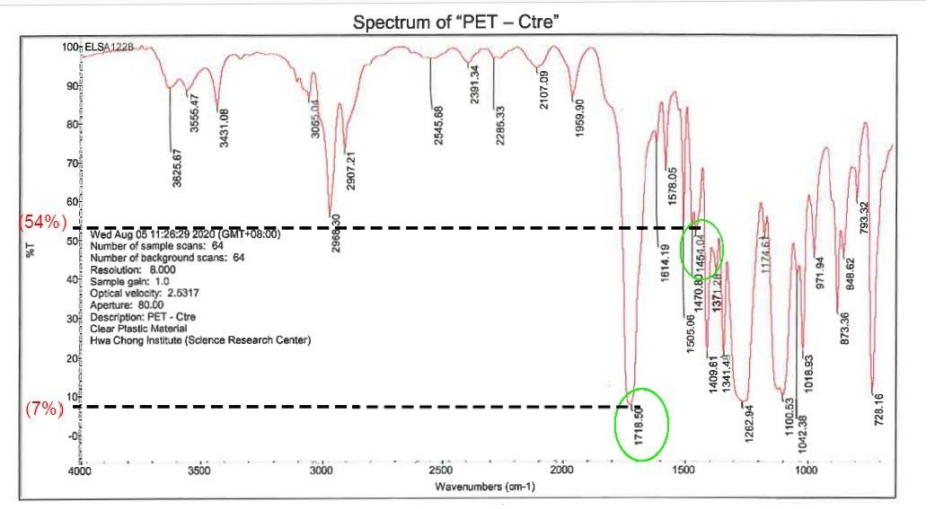

Figure 7: FTIR spectrum of negative control PET strip immersed in 0.1% peptone, 5% sodium chloride solution.

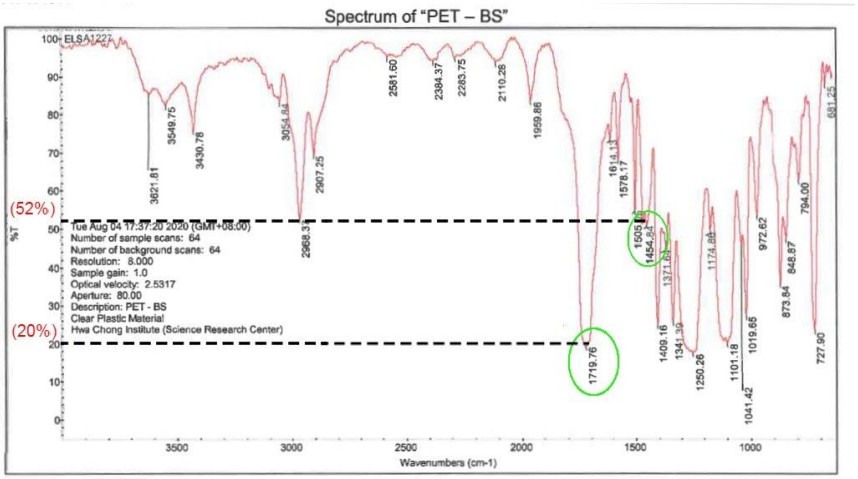

Figure 8: FTIR spectrum of PET strip immersed in *B. subtilis* culture

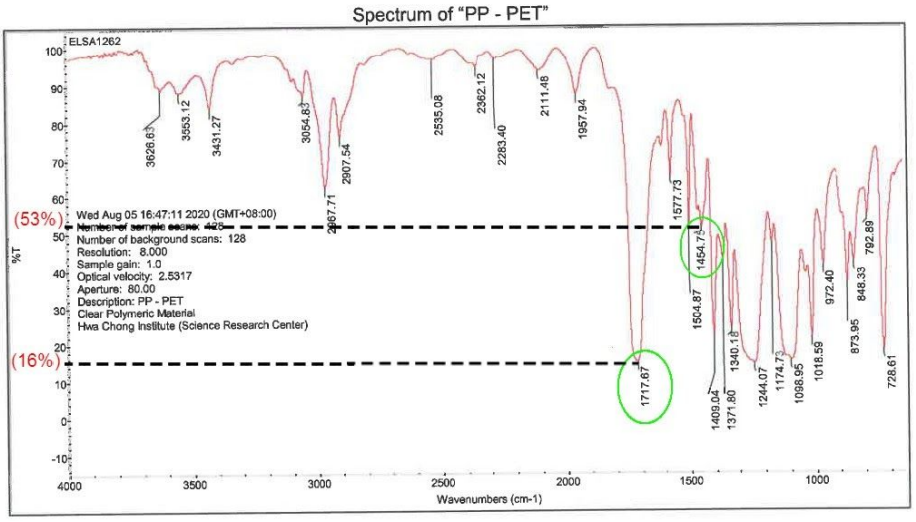

Figure 9: FTIR spectrum of PET strip immersed in *P. putida* culture

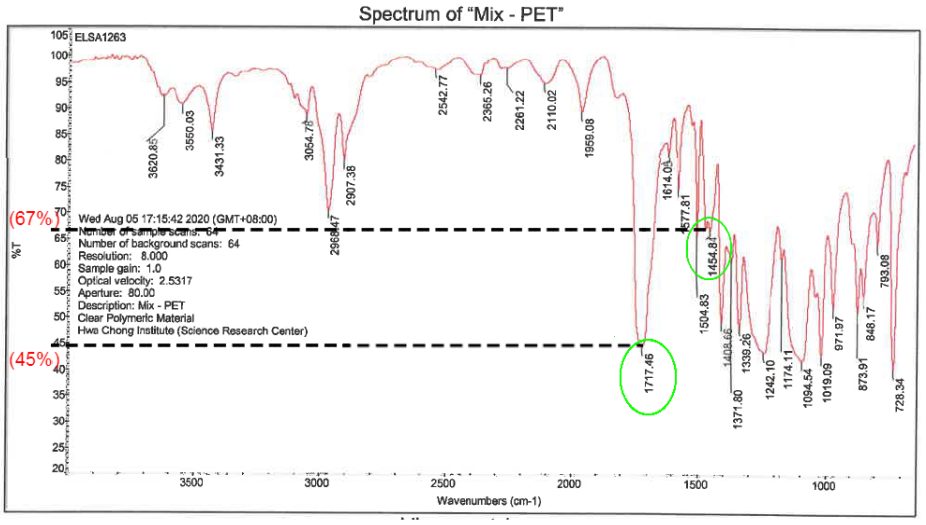

Figure 10: FTIR spectrum of PET strip immersed in *B. subtilis* & *P. putida* mixed culture.

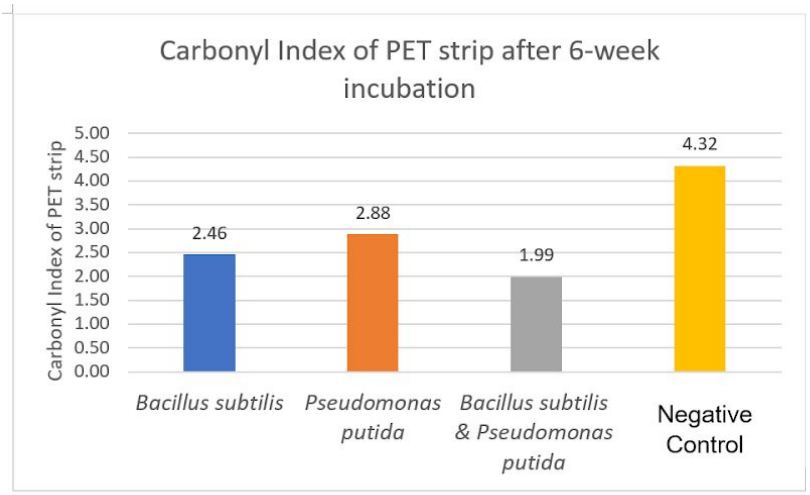

Figure 11: Carbonyl index of PET strips after 6-week incubation in *B. subtilis*, *P. putida*, *B. subtilis & P. putida* cultures and peptone solution (negative control)

Discussion

Weight loss of the PET strip can be attributed to the breakdown of carbon backbone due to enzymatic degradation by these bacteria (Kyaw, Champakalakshmi, Sakharkar, Lim & Sakharkar, 2012). More significant weight loss of PET strip exposed to *B. subtilis* could be due to the secretion of biosurfactants, which increase the surface area of hydrophobic water-insoluble substances like PET, thus increasing the surface area exposed to enzymes for degradation (Vimala & Mathew, 2015). On the other hand, *P. putida* is not known to produce such biosurfactants.

Conclusion

Colony count and carbon dioxide tests have demonstrated that *Bacillus subtilis* and *Pseudomonas putida* were able to utilise PET as a carbon source. Mass loss of PET strip, presence of pits and holes in SEM images. *B. subtilis* and *P. putida* possess enzymes that induce physical changes to PET strips. Lower carbonyl index for PET strips immersed in bacterial culture with respect to negative control (no bacterial culture) have demonstrated degradation of PET strip. The carbon dioxide concentration test and FTIR analysis have also demonstrated that *B. subtilis* and *P. putida* display synergistic effect in biodegradation of PET.

Limitations of this research include carbon dioxide concentration measured being lower than that of true value due to carbon dioxide its diffusion into the surroundings when removing test-tube cap and inserting the probe. The experiment was done in a laboratory setting and

not conducted in the natural environment, thus not accounting for changes in environmental factors such as UV radiation, humidity and temperature.

As an application, *B. subtilis* and *P. putida* culture can be used to accelerate the biodegradation of PET in landfills. The PET strips can be pretreated to allow better attachment of microorganisms on the polymer surface, thus improving biodegradation.This would reduce plastic waste, thus reducing the need for landfills. For further work, the experiment can be conducted with consideration of change in environmental factors (outdoor setting), as well as test the ability to degrade PET on a wider variety of bacteria. The tensile strength of the PET strips can also be tested after incubation.

References

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