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# Plastic Biodegradation by *Peseudomonas aeruginosa* UKMCC1011 Using A Modified Winogradsky Column

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**Abstract**

The use of plastic as a packaging has been increased in the market. Plastics was polymers that difficult to degrade in nature which to be a source of environmental damage. This study was conducted to see the potential of *Pseudomonas aeruginosa* UKM1011 and natural bacteria to degrade plastics using modified Winogradsky columns. The natural substrate used as soil, sand, and water were taken from Tasik Kejuruteraan UKM Bangi, Selangor. Modified Winogradsky columns using 600ml mineral bottles filled with 300 gr of soil or sand as a substrate and 300 ml of water mixed with medium salt as nutrients. The degradation process carried out for 60 days with parameters were the measurement of optical density (OD) at 600nm, pH conditions, and percent weight loss every 15 days. The results show *P. aeruginosa* were able to utilize plastics as the carbon source in modified Winogradsky column. The highest plastic degradation was black plastic on the sand substrate by the added *P. Aeruginosa* was 1.6684% followed by $OD_{600}$was 0.599 and pH 8.2. While the high test degradation of yellow plastic on the sand substrate was 1.2302% followed by $OD_{600}$was 0.593 and pH 7.9.

*Keywords*: Biodegradation, Plastic, *Pseudomonas aeruginosa*, Winogradsky

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## 1. Introduction

Plastic is a polymer material that is used in daily activities. Plastic is used mainly for packaging due to its water resistance properties and low price. Increased use of plastics will generate wastes that pollute both the land and water environment. Plastics have properties that are not easily degraded and will accumulate in landfills or buried in the soil. This can affect biological activity in the soil especially microorganism over nematodes.

Plastic wastes caused many problems to the environment especially the inability to degrade efficiently. Biodegradation process can be used as an option because the process is environmentally friendly. Marnika Sharma [1] explained that biodegradation process could be done by microorganisms such as bacteria, fungi, yeast, and algae that occurs aerobically or anaerobically. In this process, it is expected for microorganisms to degrade or break down natural polymers such as lignin, cellulose, and hemicellulose, or synthetic polymers like polyethylene [2].

One method that can be done to detect the process of biodegradation of plastics by using Winogradsky column. Aniyah and Shovitri [3], Badriyah and Shovitri [4], and Fadlilah and Shovitri [5] has successfully conducted research on biodegradable plastics using microorganisms with Winogradsky column. Example of microorganisms that were used is such as *Bacillus*, bacteria from wastewater, and bacteria from soil litter. In that study, the biodegradation process of plastic taken with 3 to 4 months. Bacterial density at 600nm, percent weight loss of plastics, and pH were measured.

Winogradsky column is not a natural environment but mimics the natural environment. The environment and organisms were mixed during the preparation and continued to evolve over time. When sealed and exposed to light, microbial successions will evolve according to the concentration of oxygen, nutrients and light present. Depending on the various concentrations of nutrients and the type of soil or sand used, various bacteria will emerge from time to time. Microorganisms that can be used in plastic biodegradation processes are bacteria that have ability to degrade organic materials such as cellulolytic, lignolytic, lipolytic, amylolytic and capable of lowering plastic polymers [6].

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Research on plastic biodegradation using microorganisms with Winogradsky column method is rarely performed. Therefore, in this study the growth of microorganisms in the modified Winogradsky column during the biodegradation process were monitored for to 60 days. The environmental change like pH in the modified Winogradsky column that support the bacteria growth was also monitored. The success of plastic degradation was evaluated by determining the weight loss of plastic.

## 2. Methodology

### *2.1. Sampling and Preparation of Plastic*

Plastic samples are black and yellow plastic bags bought in the night market area in Hentian Kajang, Selangor Darul Ehsan. The plastic bag was cut in size 3x3 cm. Then surface sterilization using alcohol (70%) for 10 minutes and dried to 15 minutes as modify from Muller [7]. Then the plastic sample was weighed on a digital scale to determine the initial dry weight of the plastic.

### *2.2. A sampling of Water, Soil and Sand*

Samples of water, soil, and sand are taken from Tasik Kejuruteraan Universiti Kebangsaan Malaysia. Soil and sand samples were taken from lake edge with zigzag pattern of the composite method by three random points of sampling [8]. Each soil and sand sample from each point is stirred evenly on sterile polybags. Soil samples were dug 1 kg using a shovel and put in a ziplock. All the tools used have been sterilized before using.

Water samples were taken on the water flow in the Tasik Kejuruteraan Universiti Kebangsaan Malaysia. The water was filtered by a coarse filter to separate the water with pieces of waste. The filtrate of the screened product was filtered using Whatman grade 1 filter paper with a pore size of 11 µm. The grade filter paper is selected based on bacterial sizes 0.2 to 2.0 µm in diameter and 2-8 µm in length [9].

### *2.3. Breeding and inoculum making of Pseudomonas aeruginosa*

The culture of *Pseudomonas aeruginosa* UKM1011 from nutrient agar (NA) stock is taken with ose which has been sterilized aseptically. Next inoculated on a new NA by scratching the oseas zigzag on the NA surface. Isolates were incubated at the incubator at 37˚C for 24 hours.

The inoculum used was nutrient broth (NB). Inoculation was done by taking bacteria from NA using ose and transferred in NB aseptically. Then incubated using a shaking incubator with agitation speed 120 rpm at 37˚C for 24 hours. The inoculum measured optical density at 600nm ($OD_{600}$).

### *2.4. Preparation of modified Winogradsky column*

The degradation process used modified Winogradsky Column. The column modified using a mineral water bottle that the volume was 600ml. Bottle was filled with 300g of soil or sand sample. Next added Mineral Salt Medium (MSM) was mixed in 300ml water sample. Then insert the plastic pieces until submerged in the substrate soil or sand. Thereafter, 5ml ($OD_{600}$ was 0.557) of Pseudomonas aeruginosa added as a microorganism which could assist the plastic biodegradation process. Covered the bottle cap with a wrap or tape. The degradation process uses this method for 60 days and the measurement of $OD_{600}$, pH, and weight loss of plastic calculated every 15, 30, 45, and 60 days.

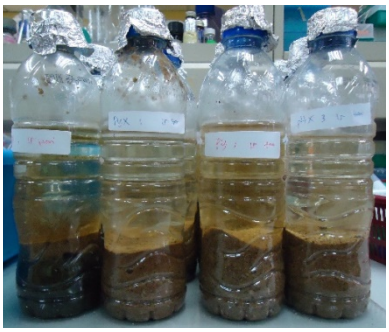

Figure 1. Modified Winogradsky column

### *2.5. Measurement of Optical Density (OD) Microorganism*

Optical density measurements were performed every 15, 30, 45, and 60 days.Measurement of the OD was done by separating the plastic pieces with microorganisms in accordance with the modified method used by Ainiyah and Shovitri [3]. Plastic piece inserted into the harvest falcon bottle and added 30 ml of sterile distilled water. The inoculum was measured with a UV-VIS spectrophotometer at 600nm.

### *2.6. Weight Loss of Plastic*

Measurements of weight loss from plastic biodegradation were performed every 15, 30, 45, and 60 days. Pieces of plastic taken from the Winogradsky column using aseptic tweezers. Then the plastic is sterilized with 70% alcohol and dried. After drying, the plastic weighed on a digital scale. The formula for calculating the percentage of plastic weight loss:

$$Weight\ loss = \frac{wb - wa}{wb} \times 100\%$$

Information:
Wa = Weight after degradation (g)

Wb = weight before degradation (g)


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*2.7. Statistical Analysis*

The data obtained from the study shown in tables and graphs. The results of calculations weight loss of plastic will be statistically analyzed by Analysis of Variance (ANOVA) followed by Duncan's New Multiple Range Test (DNMRT) at the level of 5% of the time biodegradation by microorganisms using modified Winogradsky column.

## 3. Result and Discussion

*3.1. Optical Density (OD) Microorganism*

The process of plastic biodegradation with modified Winogradsky columns expected to occur in aerobic (with oxygen) and anaerobic (without oxygen). In aerobic conditions, microorganisms use oxygen as an electron acceptor. While in anaerobic state, microorganism used others electron acceptors like $NO^{3-}$, S, $CO_2$ and $Fe^{3+}$. The growing medium that used in the biodegradation process is under 1:1 of soil-water and sand-water. Water samples mixed with Mineral Salt Medium (MSM) as nutritional stability. The growing medium was not added of carbon source, so it expected that microorganisms were stimulated to use plastic as a carbon source. According to Alex [10], in a state of lack of carbon resources, microorganisms will defend by forming biofilms on plastics.

The growth of microorganisms (biofilms) can be measured by cell concentration or cell density. In this project, bacterial growth was observed by measuring OD at 600nm with spectrophotometer UV-VIS. $OD_{600}$ measurements used this method based on the turbidity of the solution in the growth medium. Turbidity can be called optical density because of the absorption of light at certain wavelengths. When microorganisms increased, there was increased in turbidity of the substrate growth.

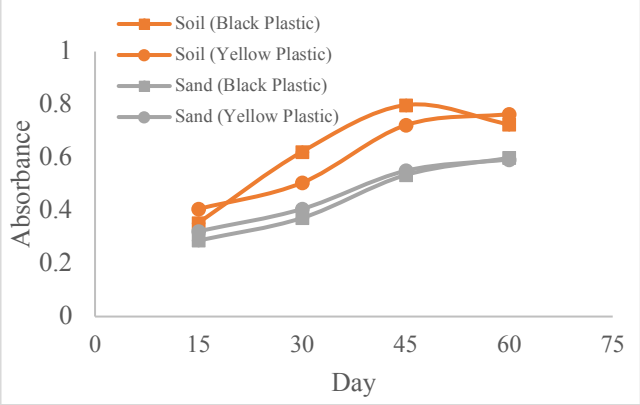


Figure 2. Optical density by *P. aeruginosa*.

In the sand substrate, the OD for black plastic has increased from 0.287 ± 0.031 to 0.599 ± 0.028 for 60 days of biodegradation. Yellow plastic also increased OD from 15 days to 60 days, amounted 0.322 ± 0.067 to 0.593 ± 0.049. The highest OD was obtained on black plastic on 60 days of observation (Figure 2).The results of ANOVA and DNMRT showed that there was significant difference ($p>0.05$) between OD with biodegradation time on soil and sand substrate in modification of Winogradsky column (Table 1).

Bacteria are unicellular microorganism that widespread in nature, such as in soil, sand and water. The number of bacteria depends on the circumstances. For example, the number of bacteria in the soil depends on the type and level of soil fertility [11]. In the plastic biodegradation process with modified Winogradsky columns, suspected that bacteria were able to grow and survive for 60 days. This showed that the increased in the number of $OD_{600}$ during the biodegradation time (Figure2). It seen that the OD in the soil substrate has increased from 15 days to 60 days for both black and yellow plastic. The same thing happens in sand substrate, which shows increased OD. Increased of OD can be assumed that bacteria were capable of splitting and capable of using plastic as a carbon source. In soil substrate for yellow plastic at 30 days biodegradation process had higher OD than other days. This may be due to the type of soil that was clay attached to the plastic, thus affecting the turbidity of the water density solution for the measurement of OD using a spectrophotometer.

*Pseudomonas aeruginosa* was the easy to grow in various culture substrate because the nutritional requirement was very simple. These bacteria can grow in distilled water and will grow well with the presence of elements N and C [12]. Therefore *P. aeruginosa* UKMCC1011 can grow on soil and sand substrate in a modified Winogradsky column, which is seen from the increase in $OD_{600}$ over 60 days of biodegradation. Soil and sand substrate increased $OD_{600}$ for black plastic and for yellow plastic. There was decreased of OD from 45 days on the soil medium for black plastic. This may indicate that the bacteria at the end of stationary phase. The end of the stationary phase was reduction in the availability of nutrients or a build up of toxic metabolic outcomes that will result in reduced cell [13].

Table 1. Optical density by *P. aeruginosa*.

| Day | Optical Density* | | | |
|---|---|---|---|---|
| | Soil (Black Plastic) | Soil (Yellow Plastic) | Sand (Black Plastic) | Sand (Yellow Plastic) |
| 15 | $0.354 \pm 0.050^{b}$ | $0.406 \pm 0.096^{b}$ | $0.287 \pm 0.031^{b}$ | $0.322 \pm 0.067^{b}$ |
| 30 | $0.622 \pm 0.262^{ab}$ | $0.505 \pm 0.078^{ab}$ | $0.372 \pm 0.067^{b}$ | $0.406 \pm 0.085^{b}$ |
| 45 | $0.798 \pm 0.330^{ab}$ | $0.722 \pm 0.295^{ab}$ | $0.534 \pm 0.057^{a}$ | $0.550 \pm 0.056^{a}$ |
| 60 | $0.725 \pm 0.427^{a}$ | $0.764 \pm 0.042^{a}$ | $0.599 \pm 0.028^{a}$ | $0.593 \pm 0.049^{a}$ |

*) The average value of Optical Density from three replications. The rank of the same alphabet on one column states not to differ significantly at the 5% confident level ($p \leq 0.05$) based on the DNMRT test.

*3.2. pH Conditions*

Growth was the process of increasing the size or substance or the mass of an organism.For example, a macro creature said to grow as it grows taller, bigger or heavier. In single-celled organisms more growth was defined as the growth of colonies, i.e. the increase in the number of colonies, the size of the colony or the mass of microbes in the colony more and more [12]. One of the major factors in bacterial growth is the value of hydrogen ions (pH). During the growth of microorganisms, the pH concentration in the substrate affects proteins (enzymes and transport systems) contained in the cell membrane. The structure of the protein will change when the pH in the substrate changed. Microorganisms have enzymes that function perfectly at a certain pH. When pH drift occurs, the growth and metabolism of the organism stop. Bacteria require optimum pH (6.5-7.5) to grow optimally. The minimum and maximum pH values for growth of most bacterial species are 4 and 9 [11].

Table 2. pH conditions by added *P. aeruginosa*.

| Day | pH Conditions* | | | |
|---|---|---|---|---|
| | Soil (Black Plastic) | Soil (Yellow Plastic) | Sand (Black Plastic) | Sand (Yellow Plastic) |
| 15 | $6.489 \pm 0.021^{b}$ | $6.417 \pm 0.011^{b}$ | $6.593 \pm 0.096^{b}$ | $6.645 \pm 0.189^{b}$ |
| 30 | $7.196 \pm 0.472^{a}$ | $6.977 \pm 0.270^{a}$ | $7.011 \pm 0.463^{b}$ | $6.981 \pm 0.352^{b}$ |
| 45 | $7.578 \pm 0.156^{a}$ | $7.218 \pm 0.210^{a}$ | $6.947 \pm 0.497^{b}$ | $7.281 \pm 0.538^{ab}$ |
| 60 | $7.336 \pm 0.125^{a}$ | $7.228 \pm 0.114^{a}$ | $8.263 \pm 1.009^{a}$ | $7.930 \pm 0.581^{a}$ |

*) The average value of pH conditions from three replications. The rank of the same alphabet on one column states not to differ significantly at the 5% confident level ($p \leq 0.05$) based on the DNMRT test.

In the biodegradation result using the Winogradsky column modification, it was found that pH conditions ranged between 6 and 7. In Table 2 shows that the pH in soil substrate was 7.336 for black plastic and 7.228 for yellow plastic at 60 days of biodegradation. Tortora, Funke and Christine [9] suggest that bacteria can grow at a pH of about 7, because it is the right pH ie not very acidic or alkaline. Most bacteria cannot grow too alkaline. Basically, none of microorganism can grow well at pH more than 7 and very rarely bacteria were found at pH below four because many bacteria produce acidic or alkaline metabolic products.

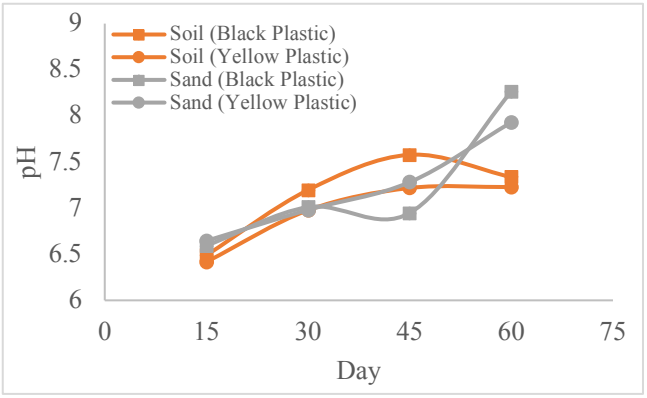


Figure 3. pH conditions by added *P. aeruginosa*.


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Microorganisms have an unequal permeability of the cytoplasmic membrane thus affecting the microbial tolerance to the pH of the environment. There is an assumption that microbes were able to stabilize pH efficiently, but the fact proves that the pH of the environment affects the pH of microbial cells. A decrease in the pH of the microbe is more effective when the environment is acidified with organic acids. To perform metabolism well, microbial require optimal pH of enzyme activity [15]. If the pH environment is not suitable for optimal enzyme activity, then microorganisms cannot metabolize properly. As result, microbes cannot grow optimally. Based on pH, microbes are grouped into acididyl groups (well-grown microbes in acidic pH), neutral (well-grown microbes in neutral pH) and alkalifyl (well-grown microbes at alkaline pH). The pH range for the growth of each microbial group varies greatly. Some microbes able to grow in a wide pH range. In general, the optimum growth of microbial occurs at pH 7 and can grow well in the range of pH 5 until 8.

### *3.3. Weight Loss of Plastic*

Plastics are polymer compounds formed from the polymerization of small molecules (monomers) of hydrocarbons forming long chains with rigid structures. Plastic is a synthetic compound of petroleum (especially short chain hydrocarbons) prepared by the polymerization reaction of the same small molecules (monomers), thus forming long and rigid chains. Plastics that have long-chain carbon bonds and have a high degree of stability, cannot be completely broken down by microorganisms [16]. To degrade the polymer in the soil, the degradation rate depends on soil conditions such as temperature, moisture content, aeration, pH and number of microorganisms.

Especially for biodegradable polymers in soil, the level in biodegradation that occurs depends on soil conditions such as temperature, moisture content (size of water concentration), aeration rate (size of oxygen concentration), acidity (size of acid concentration) and the concentration of microorganisms themselves. Low temperatures greatly inhibit degradation in the soil [17]. Groundwater levels are also important to support hydrolytic degradation. Aeration supports oxidative degradation and aeration rate determines whether aerobic or anaerobic biotic degradation or both-takes place.

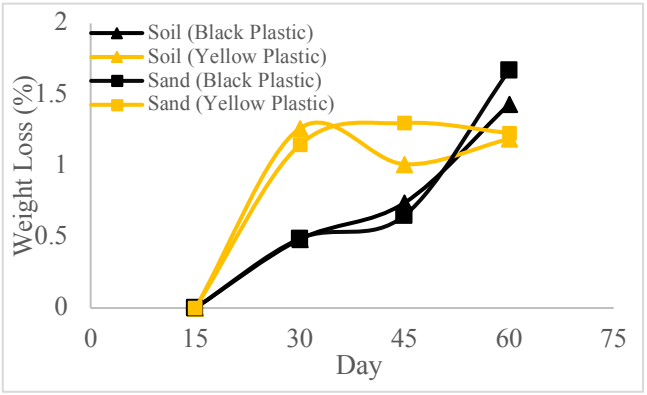


Figure 4. Weight loss of plastic by *P. aeruginosa*.

The results of a plastic biodegradation study using a modification of Winogradsky columns show that plastic polymers can served as carbon sources in terms of reduced percent weight loss of plastics. The highest degradation result occurred in black plastic with the addition of *Pseudomonas aeruginosa* on soil substrate that was equal to 1.4306% for 60 days (Table 3). In Table 4.5 also shows the highest weight loss on soil substrate for yellow plastic that is equal to 0.8503%. Allegedly, the microorganisms were derived from groundwater samples was able to thrive in the column modification Winogradsky. Although there were many bacteria that thrive in oxygen-free environment, there were many more that use oxygen. Biotic degradation also requires that soils may become active microbes. The rate of biotic degradation can be reduced to almost zero in a sterile environment, or when the concentration of microorganisms was very low or even if the material was not completely biodegradable [18].

This study showed that the weight loss of plastics with the addition of *Pseudomonas aeruginosa* and without bacterial addition was a significant difference (P≥0,05) between the biodegradation time variation of each microorganism on soil substrate on both black and yellow plastic. The difference in weight loss is due to each microorganism has a different gene. The gene is a unit of DNA or RNA molecule of a certain minimum length that carries information about the amino acid sequence of a protein [15]. Microorganisms with the same genus and the same species have different constituent genes and will produce different enzyme activity [19].


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Table 3. Weight loss of plastic by *P. aeruginosa*.

| Day | Weight Loss (%)* | | | |
|---|---|---|---|---|
| | Soil (Black Plastic) | Soil (Yellow Plastic) | Sand (Black Plastic) | Sand (Yellow Plastic) |
| 15 | $0.0000 \pm 0.000^{b}$ | $0.0000 \pm 0.000^{a}$ | $0.0000 \pm 0.000^{b}$ | $0.0000 \pm 0.000^{a}$ |
| 30 | $0.4829 \pm 0.432^{b}$ | $1.2626 \pm 1.150^{a}$ | $0.4876 \pm 0.844^{b}$ | $1.1476 \pm 1.421^{a}$ |
| 45 | $0.7390 \pm 0.643^{ab}$ | $1.0108 \pm 0.879^{a}$ | $0.6503 \pm 0.628^{b}$ | $1.2997 \pm 1.167^{a}$ |
| 60 | $1.4306 \pm 0.374^{a}$ | $1.1882 \pm 0.201^{a}$ | $1.6684 \pm 0.192^{a}$ | $1.2302 \pm 0.646^{a}$ |

*) The average value of weight loss of plastic from three replications. The rank of the same alphabet on one column states not to differ significantly at the 5% confident level ($p \leq 0.05$) based on the DNMRT test.

The percentage of weight loss of plastic was low after 60 days of biodegradation process. Plastic bags on the market are classified as harmful recycled plastic because of excessive dye substances. Plastics designed with a lot of variations in substances or properties that can tolerate heat, hard, relative, and others. Colored plastic bags and polystyrene-based plastic packaging made of scrap plastic whose history of use was unclear through an unacceptable recycling process. The process of recycling in the manufacture of plastics also use certain chemicals [20]. The manufacture of plastic containers uses lead stabilizers (Pb), cadmium (Cd), and white tin (Sn) to prevent damage as well as ester phthalate and adipate ester compounds to flex. Addition of additive and recycling process increases the saturation level of the molecule, so that microorganisms are difficult to degrade. The weight loss was low in yellow plastic was 1.1882% for 60 days biodegradation (Table 3). It can be assumed that microorganisms not able to transport polymers directly through the outer membrane of cells into their cells so that biochemical processes are required which play a role in breaking long and water-soluble polymer molecules, so they can enter the cells. This process was called depolymerization where the polymer was depolymerized or breaks down into smaller monomers before it can be absorbed and degraded in the cell microorganisms. There were two active enzymes involved in polymer biodegradation ie extracellular enzymes and intracellular depolymerases. Extracellular and intracellular enzymes that play a role in depolymerization actively trigger the biological degradation process of polymers [6].

Although the abiotic component of the soil was less supportive of the plastic degradation process, degradation can still be accomplished. However, the degradation process that occurs was not as perfect and optimal if the degradation support components themselves were not available yet. Even if the process cannot available because the degradation support component was not fulfilled, then there was a new alternative to decompose plastic waste by a mixture of microorganisms, namely microbes belonging to the genus *Aspergillus, Sphingomonas, Pseudomonas* that have ability to decipher polyethylene successfully [21, 22]. The microbes were conditioned by the exact chemical composition, acidity, substrate and microbial mixture that can synthesize polymers [23]. This condition made the plastic break down faster within a month. The action of microbes on plastic polymers was important to break down non-living substrates in the environment, then resulting in good management of plastic waste in the future.

## 4. Conclusion

The biodegradation process indicates the presence of *P. aeruginosa* can survive in the modification of Winogradsky columns. *P. Aeruginosa* UKMCC1011 were able to utilize plastics as carbon sources in the modification of Winogradsky columns, as demonstrated by the increase of optical density ($OD_{600}$) for 60 days of the biodegradation process. This study shows that black plastic in sand and soil substrate were successfully degraded than yellow plastic by added *P. aeruginosa*, which accompanied by an increase in $OD_{600}$ and pH conditions.

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