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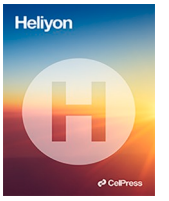

Research article

Isolation of a polyethylene-degrading bacterium, *Acinetobacter guillouiae*, using a novel screening method based on a redox indicator

Hong Rae Kim, Chaerin Lee, Hyeyoung Shin, Jongwon Kim, Mija Jeong, Donggeon Choi *

Department of Research and Development, Repla Inc., Suwon 16679, Republic of Korea

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ABSTRACT

Plastic, a polymer synthesized from petrochemicals, is used worldwide. However, natural degradation of plastic is difficult, causing environmental pollution, with microplastics posing a serious threat to human health. In this study, we aimed to use a new screening method based on the oxidation-reduction indicator, 2,6-dichlorophenolindophenol, to isolate a polyethylene-degrading bacterium, *Acinetobacter guillouiae*, from insect larvae. Plastic-degrading strains are identified by the color change in the redox indicator from blue to colorless as plastic metabolism occurs. Polyethylene biodegradation by *A. guillouiae* was verified through weight loss, surface erosion, physiological evidence, and chemical changes on the plastic surface. In addition, we analyzed the characteristics of hydrocarbon metabolism in polyethylene-degrading bacteria. Results suggested that alkane hydroxylation and alcohol dehydrogenation were key steps in polyethylene degradation. This novel screening method will pave the way for high-throughput screening of polyethylene-degrading microorganisms and extending its application to other types of plastics may potentially address plastic pollution.

1. Introduction

Plastics, which are synthetic polymers made from petrochemicals, have been widely used in modern society. Approximately 380 million tons of plastic waste are generated annually [1]. Most plastic waste is disposed through incineration and landfills, as they exhibit low natural degradation. However, landfills contaminate groundwater and soil, while incineration causes air pollution producing carcinogens such as dioxin [2]. In addition, plastics dumped in the ocean threaten marine ecosystems [3,4]. Plastics exposed to ultraviolet (UV) light and seawater become microplastics [5], which are ingested by organisms, causing various diseases.

One method of solving the plastic-waste problem is to use insect larvae that digest and degrade plastic in a short period of time.

Abbreviations: SEM, scanning electron microscope; CFU, colony forming unit; FT-IR, Fourier–transform infrared; XPS, X-ray photoelectron spectroscopy; UV, ultraviolet; PE, polyethylene; PVC, polyvinyl chloride; GPC, gel permeation chromatography; FT-NMR, Fourier–transform nuclear magnetic resonance; DCPIP, 2,6-dichlorophenolindophenol; LB, Luria-Bertani; LCFBM, liquid carbon free basal medium; OD, optical density; ATR FT-IR, attenuated total reflection Fourier–transform infrared spectroscopy.

* Corresponding author. Department of Research and Development, Repla Inc., 237, Yeongtong-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16679, Republic of Korea.

E-mail address: dgchoi@repla.co.kr (D. Choi).

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Mealworms (*Tenebrio molitor* larvae) [6,7], waxworms (*Galleria mellonella* larvae) [8,9], and superworms (*Zophobas atratus* larvae) [10,11,12] degrade polystyrene, polyethylene (PE), and polyvinyl chloride (PVC). Furthermore, this degradation ability of insects decreased after antibiotic treatment, suggesting that gut microorganisms play an important role in plastic degradation [13].

In addition, metabolic pathways of microorganisms involved in the plastic degradation has been analyzed [14,15,16]. Plastics are derived from petroleum compounds, with carbon-based linear backbone structures, similar to those of lipid compounds. The alkane backbone structure of plastics can be hydroxylated by alkane monooxygenase to produce alcohol, which is converted to aldehyde by alcohol dehydrogenase, this aldehyde is converted to fatty acid by aldehyde dehydrogenase, and then the fatty acid is degraded via the lipid metabolic pathway (Fig. 1) [17,18]. A recent proteomics study demonstrated that the expression levels of enzymes involved in alkane and lipid metabolisms are upregulated in microorganisms when plastic is the only available carbon source. This finding supports the previous research on the plastic degradation pathway [14,15].

Pseudomonas sp., is one of the most well-known plastic-degrading microorganism. It is capable of degrading various types of plastics, such as PVC [19,20], polyurethane [21,22], and poly (3-hydroxybutyrate) [23]. Specifically, *Pseudomonas aeruginosa* is an effective PE-degrading microorganism [24,25].

The plastic-waste problem is garnering great attention from researchers. However, plastic biodegradation is difficult to study because the microorganisms involved cannot be rapidly screened and their degradation rates cannot be easily compared. In previous studies, bacterial mixtures were incubated for 60 days in minimal medium with plastic as the sole carbon source to screen for plastic-degrading candidates [26]. Attempts were made to spray plastic onto solid plates to reduce the screening time [27]. Among the candidates isolated from the initial screening, plastic-degrading microorganisms were extensively verified by analyzing the degradation of plastic films using various physical and chemical analytical methods [4,28], such as scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FT–IR) spectroscopy, Fourier transform nuclear magnetic resonance (FT–NMR) spectroscopy, and gel permeation chromatography (GPC). The incubation period required to degrade plastic samples was typically 30–60 days, and extensive resources were required to analyze the plastic biodegradation ability of bacteria [26,29].

In the process of screening for strains that degrade lipid pollutants, the metabolic capacity of strains is visually and rapidly analyzed using 2,6-dichlorophenolindophenol (DCPIP), an oxidation–reduction indicator. DCPIP is blue in color in its oxidized state and colorless in its reduced state. Its absorbance decreases as reducing molecules are synthesized by the metabolic processes of microorganisms. Due to its convenience for measuring metabolic capacity, DCPIP has been widely used in hydrocarbon metabolism research [30,31,32]. To the best of our knowledge, no previous study has reported using DCPIP for analyzing plastic biodegradation. Since plastics are synthesized from petrochemicals and are hydrocarbon compounds, DCPIP can be potentially applied to plastic research fields. Furthermore, the plastic-degrading metabolic pathway in a single bacterial strain was previously analyzed using proteomics and metabolomics [15]. However, there has been no detailed investigation on the polyethylene degradation ability of bacteria by involving step-by-step analysis of the metabolic pathway through experimental evaluation of the representative PE degradation intermediates.

In this study, we proposed introducing an intermediate screening process that can easily identify a strain wherein plastic is the carbon source from among the candidates isolated from the bacterial mixture sample (soil, intestinal solution, etc.). We used, for the first time, the oxidation-reduction indicator, DCPIP, for screening plastic-degrading bacteria. Six microorganisms isolated from insect larvae were analyzed, and two of them degraded plastic. These two candidates were, *P. aeruginosa*, a well-known plastic-degrading bacterium, and *A. guillouiae*., which has not yet been reported as a plastic degrader. In addition, we measured the metabolic capacities of these bacteria with respect to the hydrocarbon metabolic pathway (which is thought to be the metabolic pathway for plastic biodegradation) and analyzed its correlation with the plastic-degradation ability of the bacteria.

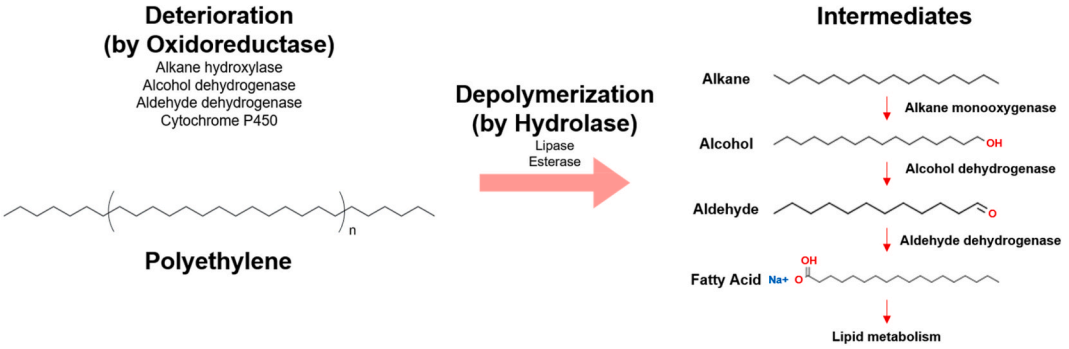

Fig. 1. Schematic diagram of polyethylene biodegradation by microorganisms. The first step in the biodegradation of polyethylene involves the deterioration by oxidoreductase. Enzymes such as alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase and cytochrome P450 are known to be involved in this process. In the second step, depolymerization occurs through the action of hydrolases such as esterase and lipase. As a result, long-chain hydrocarbon intermediates are formed and hydrocarbons can be classified into four types: alkane, alcohol, aldehyde and fatty acid.

2. Experimental

2.1. Isolating plastic-degrading bacteria from insect larvae

Six candidate plastic degraders were isolated from two different species of insect larvae, mealworms and superworms, purchased from online market (S-WORM, Republic of Korea) and bred with PE as the sole carbon and energy source through enrichment of their gut microbiome. Each worm was starved for 48 h to eliminate residual carbon sources in the gut prior to plastic feeding. After the starvation period, worms (n = 50) were placed in a growth chamber with 2 g of PE supplied for 14 days at 25 °C. To isolate plastic-degrading bacteria, the intestines of the worms were extracted (n = 10), minced with a scalpel, and resuspended in 0.9% saline solution. Intestinal solutions were centrifuged at 800 rpm for 5 min to isolate the intestinal epithelial cells. Supernatants were diluted with saline to 1/100 concentration and spread on the Luria-Bertani (LB) agar plate. Intestinal isolates were incubated at 25 °C for >24 h until colonies appeared. After incubation, each individual colony was transferred into LB broth medium for enrichment. The isolated single colonies were identified using the universal primers 27-F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492-R (5′-GGTTACCTTGTTACGACTT-3′) for 16S rRNA sequencing.

2.2. Identifying PE-degrading bacteria using screening method

The metabolic capacity of PE-degrading bacteria was determined using the oxidation-reduction indicator, DCPIP. Its colorimetric change, measured at a wavelength of 600 nm, directly indicated the metabolism of plastic components. To screen for plastic-degrading bacteria, candidates were selected based on bacterial cultivation in liquid carbon free basal medium (LCFBM) as minimal medium with 8 g/L of PE (Mw ~4,000 and Mn ~1,700, Sigma-Aldrich, Burlington, MA, USA) and 10 mg/L of DCPIP (Sigma-Aldrich, Burlington, MA, USA) as the sole carbon source and electron acceptor, respectively. The medium (pH 6.8) consisted of 0.7 g KH_2PO_4, 0.7 g K_2HPO_4, 0.7 g $MgSO_4{\cdot}7H_2O$, 1 g NH_4NO_3, 0.005 g NaCl, and 0.002 g $FeSO_4{\cdot}7H_2O$ per 1 L, as described previously [10], with 1 mL of 100-fold trace mineral stock solution (0.3 g H_3BO_3, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.03 g $MnCl_2{\cdot}4H_2O$, 0.03 $NaMoO_4{\cdot}2H_2O$, 0.02 g $NiCl_2{\cdot}6H_2O$, and 0.01 g $CuSO_4{\cdot}5H_2O$ per 1 L). The medium and PE were sterilized in an autoclave and UV-C sterilizer, respectively, before use. Each bacterial cell was precultured in LB broth for 24 h under aerobic conditions until a cell density with optical density (OD)600 = 1 (approximately 10^{8-9} cells/mL) was reached. Cell pellets were collected by centrifugation (13,000 rpm and 4 °C) and washed twice with 0.9% sterile saline solution to remove nutrients retained from the LB broth for inoculum preparation. Finally, the washed pellets were resuspended in 1 mL of LCFBM broth. After washing, we measured the OD of the bacteria and inoculated them at a concentration of 1/100 based on the OD = 1.

The experiments were performed with one experimental and two control groups. Control 1, containing only DCPIP in LCFBM, was used to monitor the decrease in OD of DCPIP. In control 2, bacteria were cultured in LCFBM and DCPIP without a carbon source to monitor the effect of bacteria on the DCPIP. In the experimental group, bacteria were cultured in LCFBM and DCPIP with PE as the carbon source to determine their metabolic capacity. Both the control and experimental groups were identically set for the six candidate bacterial strains. The absorbance (OD) of DCPIP was measured at 600 nm every 24 h, from days 1–7 inoculation. To measure the absorbance of DCPIP, the cultured cells were centrifuged (13,000 rpm for 5 min) and the supernatant was transferred to a plastic cuvette. OD was measured at a wavelength of 600 nm using a spectrophotometer (Spectronic 200, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.3. Evaluating plastic biodegradation

2.3.1. Weight loss measurement

To evaluate the biodegradation of PE, the dry weight of the plastic was measured after cultivation of 8 g/L of powdered PE (Mw ~4,000 and Mn ~1,700, Sigma-Aldrich, Burlington, MA, USA) with 25 mL working volume of LCFBM in 50 mL conical tubes. The inoculum cells were prepared as described previously (Section 2.2). After cultivation, the plastics were collected using a cell strainer (pore size: 40 μm, SPL, Republic of Korea) and treated with 2% SDS overnight to remove microorganisms attached to the plastic surface [33]. Then, the plastics were thoroughly washed twice with deionized (DI) water and oven-dried at 60 °C for 24 h. Weight loss of the plastic was measured at 7, 14, and 28 days of incubation. Experiments were performed in triplicate to obtain the standard deviation of the mean values.

2.3.2. CFU counting

To determine whether the bacteria proliferated using PE as a carbon source, we measured the density of the viable cells by calculating colony forming unit (CFU). The abiotic and biotic controls were set with only PE in LCFBM, and only bacteria in LCFBM, respectively, to determine bacterial proliferation in minimal medium without carbon sources. The experimental group was prepared with inoculated bacteria and 8 g/L of PE in 25 mL of LCFBM. Bacterial suspensions collected on 0, 7, and 14 days were serially diluted. Briefly, 1 mL of each solution was taken and mixed well with 0.9% saline solution. This solution (1 mL) was serially diluted up to seven times. A total of 100 μL of each solution was spread on an LB agar plate and incubated for 24 h until a colony appeared. CFU/mL were calculated by counting the number of colonies obtained from the serially diluted solutions.

2.3.3. SEM imaging

PE films were used to investigate its physical and chemical changes. The surface characteristics of the PE films were examined by a

field emission SEM (Merlin compact, Zeiss, Germany). The average molecular weight (24,000 Da) and density (0.93 g/cm^3) of PE films (0.2 mm thickness) were cut into 2 × 2 cm pieces, and 8 g/L of the films were incubated with bacteria for seven days. After seven days of cultivation, the incubated films were collected and rinsed with DI water. The films were fixed with a carbon tape and coated with platinum to obtain a thickness of 5 nm. Biofilm formation and corrosion on the film surface were examined using SEM images to evaluate the biodegradation ability of the candidate plastic degraders.

2.3.4. Evaluating PE biodegradation using XPS

XPS (K-Alpha, Thermo Electron, Waltham, Massachusetts, USA) was used to analyze the elemental composition on the surface of the PE films. Samples were fixed with a carbon tape and measured in the energy range of 276–300 eV for C1s scan, and 0–1,350 eV for survey scan.

2.3.5. ATR FT–IR spectroscopy

To identify changes in the chemical structures of the PE films, an ATR FT-IR spectrometer (iS50, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to observe changes in the functional group. The FT-IR analysis was performed based on single-bounce attenuated total reflection spectroscopy with wavenumbers ranging from 400 cm^{-1} to 4,400 cm^{-1}.

2.3.6. Changes in hydrophilicity of the PE surface

The change in surface hydrophilicity due to degradation of the plastic by *A. guillouiae* was measured by contact angle analysis. The PE films were treated with 2% SDS and washed with DI water. To prepare the film samples, both the edges of the PE films were fixed on a glass slide with cellophane tape, and approximately 15 μL of water was applied to the surface using a contact angle analyzer (Phoenix 300, SEO, Republic of Korea). The left and right contact angles were measured simultaneously, and the average value was calculated.

2.4. Analyzing hydrocarbon metabolism

For analyzing the ability of putative plastic degraders to metabolize hydrocarbons, changes in DCPIP absorbance were monitored while the six strains were cultured with four types of hydrocarbons as carbon sources. Since PE metabolism occurs in the order of alkane, alcohol, aldehyde, fatty acid, followed by lipid metabolism such as β-oxidation, substances that can be utilized by cells were selected. Hexadecane, 1-hexadecanol, dodecyl aldehyde, and sodium stearate (Sigma-Aldrich, Burlington, MA, USA) were used as representatives of alkane, alcohol, aldehyde, and fatty acid, respectively (Table S1). These substances have 12–18 carbon chains and can be degraded by lipid metabolism. Experiments were performed with 0.25% (w/v) of each substrate except sodium stearate (0.1%). Bacteria were cultured in LCFBM with DCPIP with each type of hydrocarbon (alkane, alcohol, aldehyde, and fatty acid) as a carbon source. Each cell type was cultured in LB broth for 24 h and inoculated after three washes with 0.9% saline. The DCPIP absorbance of the cultured solution was measured using 1 mL of the centrifuged supernatant. Samples were measured at a wavelength of 600 nm every 24 h for five days.

2.5. Statistical analyses

All experiments were carried out in triplicate. Means of results were compared using Student's *t*-test to indicate statistically significant differences between control and experimental groups for DCPIP and weight loss measurements and CFU. The significance level for all analyses was set at $P = 0.05$. Statistical analysis was performed using SigmaPlot software (version 15.0).

3. Results

3.1. Identification of effective PE-degrading bacteria, A. guillouiae, using a new screening method

The bacterial strains isolated from the insect intestines were identified through 16S rRNA sequencing (Table 1). The six strains identified were *Klebsiella oxytoca, Enterobacter hormaechei*, *Escherichia fergusonii*, *Mixta theicola*, *P. aeruginosa*, and *A. guillouiae*. We screened for plastic-degrading bacteria among these strains. Controls 1, 2, and 3 and the experimental group (DCPIP + PE + bacteria) were evaluated. There was no statistical difference ($p > 0.05$) between controls 1 and 2, indicating that LDPE had no effect on DCPIP. To analyze the relative absorbance reduction with respect to the metabolic capacity of bacteria in minimal broth, DCPIP absorbance

Table 1
Characteristics of bacteria isolated from insect larvae.

Highest-homology strain	Maximum identity (%)	Insect larvae	Size (μm)	Shape
Klebsiella oxytoca	99	Mealworm	0.6–1.0 × 1.2–3.0	Rod-shaped
Enterobacter hormaechei	99	Mealworm	0.3–1.0 × 0.6–6.0	Rod-shaped
Escherichia fergusonii	99	Mealworm	1.5 × 2	Ovoid-shaped
Mixta theicola	100	Mealworm	1–5 x 0.5–1.0	Rod-shaped
Pseudomonas aeruginosa	99	Superworm	1.0 × 2.0	Rod-shaped
Acinetobacter guillouiae	99	Superworm	0.9–1.6 × 1.5–2.5	Ovoid-shaped

ratio was calculated by dividing the absorbance ratio of the experimental group by that of the control group 3. The DCPIP absorbance ratio of 1.00 indicated the same absorbance in the experimental group compared to that of the control group 3, indicating that the bacteria were unable to metabolize plastic.

After seven days of incubation, the DCPIP absorbance ratio of each strain was 1.01, 1.01, 0.99, 1.01, 0.89, and 0.63 for *K. oxytoca*, *E. hormaechei, E. fergusonii, M. theicola, P. aeruginosa*, and *A. guillouiae*, respectively (Fig. 2A, Fig. 2B). Among them, *K. oxytoca*, *E. hormaechei, E. fergusonii* and *M. theicola* showed no statistically significant difference in DCPIP absorbance ratio up to seven days. The strains that showed statistical significance ($p < 0.05$) were *P. aeruginosa* and *A. guillouiae.* Both of these showed a constant decrease in DCPIP absorbance ratio with time. For *P. aeruginosa*, the DCPIP absorbance ratio decreased to 0.90 and 0.89 after five and seven days, respectively, which was statistically significant ($p < 0.05$). *A. guillouiae* showed decreasing DCPIP absorbance ratio in the order of 0.90, 0.78, 0.68, 0.62, and 0.63 after 1, 2, 3, 5, and 7 days, respectively, and all the values were statistically significant ($p < 0.05$) (Fig. 2C).

This result was visually confirmed using the DCPIP-based screening method. Fig. 2D shows the samples from controls 1, 2, and 3 and the experimental group (DCPIP, LDPE, and *A. guillouiae*) after seven days of culture. In Controls 1, 2, and 3, no color change was observed, that is, the color remained blue. However, when both *A. guillouiae* and LDPE were added, the transparency increased due to the decrease in absorbance caused by the reduction of DCPIP.

3.2. Evaluation of PE biodegradation by A. guillouiae

The evidence of PE biodegradation, physical surface corrosion and biofilm formation by *A. guillouiae* was investigated through SEM imaging. Image of the pristine plastic film showed no damage or bacteria on the plastic surface. The PE films incubated with *A. guillouiae* for seven days showed signs of corrosion on the surface. Moreover, the image showed that *A. guillouiae* had actively proliferated on the plastic surface (Fig. 3A).

The proliferation of the strains using PE as the sole carbon and energy source was evaluated by calculating CFU/mL. The initial

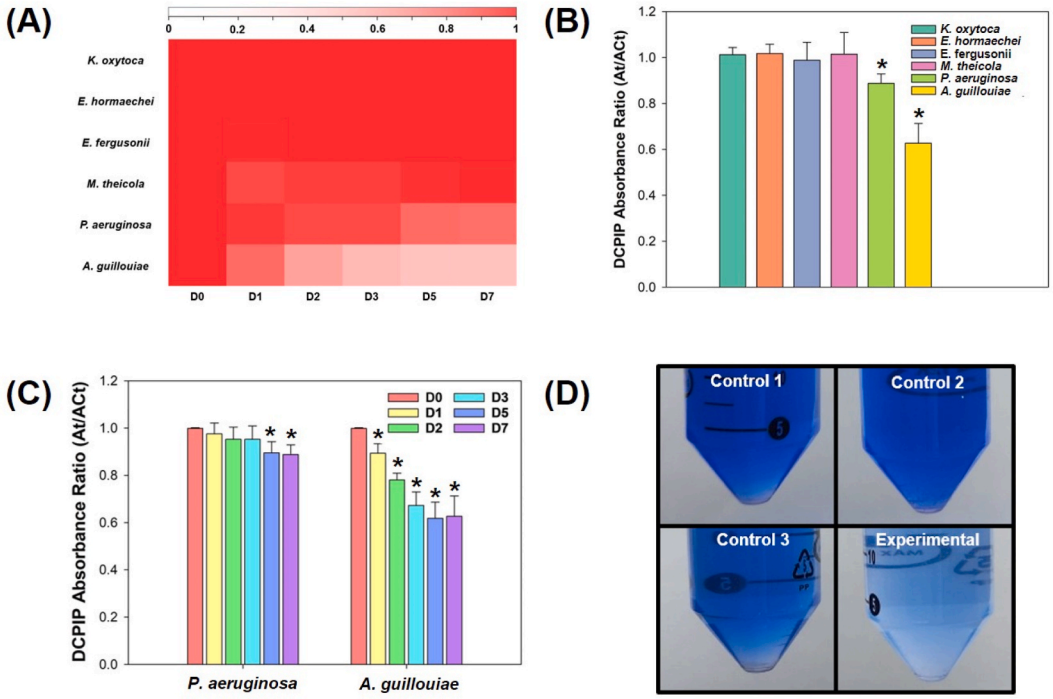

Fig. 2. Use of DCPIP indicator to analyze the characteristics of candidate plastic degraders using polyethylene (PE) as the sole carbon source. (A) The heat map shows the degradation of PE, with the colorimetric changes of DCPIP. The DCPIP values of the experimental group, which included the six strains of bacteria cultured with PE, were normalized to those of the control group, which included the six bacterial strains cultured without PE. (B) The DCPIP absorbance ratio of the six strains depends on their ability to degrade PE after 7 days. At and ACt represent the absorbance values of the experimental and control groups at that time, respectively. The asterisk on the bar indicates the statistically significant difference in the absorbance value ($p < 0.05$) between the experimental and control groups analyzed by Student's *t*-test. (C) The bar graph indicates the degree of PE-degradation ability of *P. aeruginosa* and A. guillouiae at specific time points from Day 0–7 after inoculation. (D) The color of the medium containing the DCPIP indicator indicates the metabolic ability of the bacteria, which can be visually distinguished based on the extent of decolorization. Each image represents minimal medium + DCPIP (Control 1), minimal medium + DCPIP + PE (Control 2), minimal medium + DCPIP + A. guillouiae (Control 3), and minimal medium + DCPIP + PE + A. guillouiae (Experimental). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

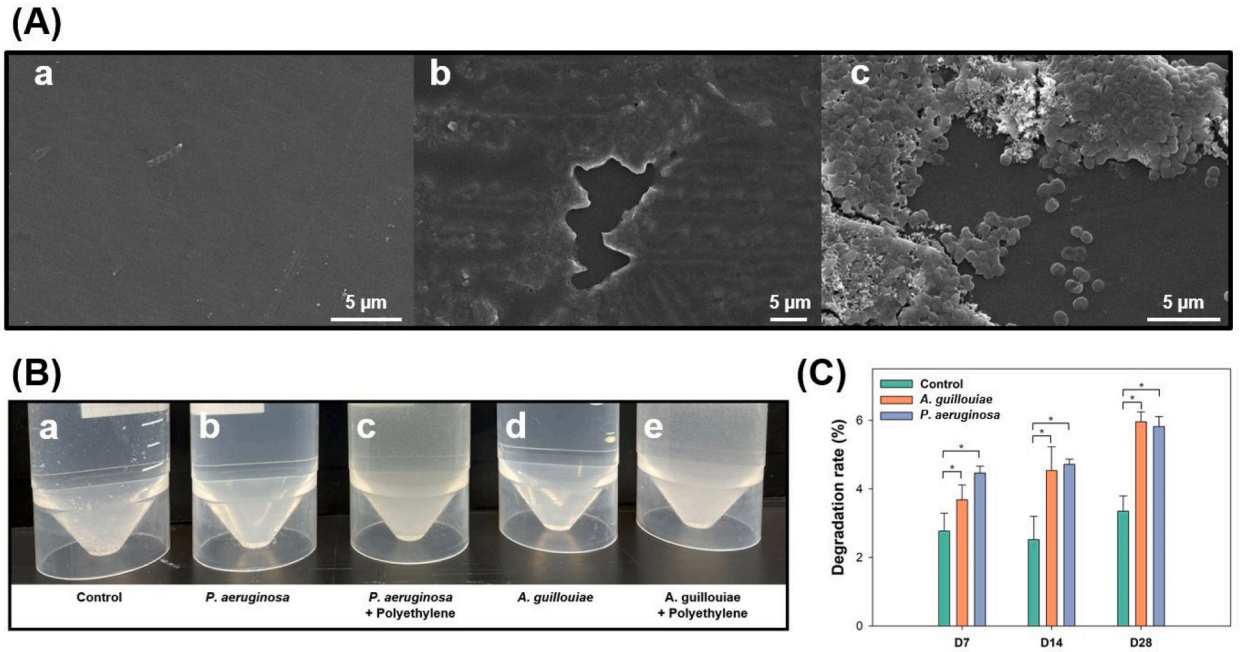

Fig. 3. Characteristics of biodegraded polyethylene (PE). (A) SEM images showing the micro-characteristics of the surface of PE films, where a: pristine PE film; b: corrosion by biodegradation; c: A. *guillouiae* proliferated and formed a biofilm on the PE film surface. (B) Turbidity of the minimal medium containing PE inoculated with bacterial cells, indicating the metabolic ability of the two strains to use PE as the sole carbon source; a: minimal medium + PE, b: minimal medium + *P. aeruginosa*, c: minimal medium + PE + *P. aeruginosa*, d: minimal medium + A. guillouiae, e: minimal medium + PE + A. guillouiae (C) PE degradation rate of both *P. aeruginosa* and A. guillouiae compared with that of the abiotic group (control; without bacteria) after 7, 14, and 28 days of incubation. The asterisk indicates the statistically significant difference ($p < 0.05$) in PE degradation rate between the experimental and control groups analyzed by Student's *t*-test.

inoculation concentration of *A. guillouiae* was 3.80E + 06 CFU/mL. In the control group, the concentration decreased to 4.27E + 05 CFU/mL ($p < 0.05$) after seven days of incubation, and bacterial death was observed. Thus, the strain did not survive in the minimal medium without a carbon source. In contrast, a 14-fold increase in CFU/mL of *A. guillouiae* ($p < 0.05$) was observed in the experimental group. For *P. aeruginosa* the initial inoculation concentration was 2.67E + 06 CFU/mL, which increased to 5.00E + 06 CFU/mL in the minimal medium, without statistical significance, and increased to 5.57E + 07 CFU/mL ($p < 0.05$) after the incubation with PE (Table 2). In contrast to *A. guillouiae, P. aeruginosa* survived and maintained its population in the minimal medium, although it did not proliferate. During the incubation period, turbidity of the medium increased due to the proliferation of bacteria. However, no change was observed when the cells were incubated in minimal medium, but when PE was added, the bacteria proliferated using plastic as a carbon source (Fig. 3B).

The biodegradation of plastic by *A. guillouiae* was evaluated by measuring the reduction in the mass of plastic. When *A. guillouiae* was cultured with PE as the sole carbon source, and the mass of PE decreased by 3.68%, 4.54%, and 5.96% after 7, 14, and 28 days, respectively. The weight loss ratio measured in the control group was 2.77%, 2.52%, and 3.35% after 7, 14, and 28 days, respectively, with statistically significant differences in results observed between the control and the experimental groups ($p < 0.05$). The results obtained for *P. aeruginosa* were compared with those obtained for *A. guillouiae.* The mass reduction rate obtained for the group containing *P. aeruginosa* was 4.47%, 4.72%, and 5.82% after 7, 14, and 28 days, respectively (Fig. 3C).

XPS analysis was performed to investigate the changes in chemical structure induced by biodegradation on the surface of PE. Results showed that the composition ratio of oxygen increased after incubation with *A. guillouiae* (Fig. 4A). In the C1s scan, a peak at about 283.8 eV, representing the C–C bond, was observed for the control PE sample. Peaks at 285.8 eV and 287.3 eV, representing ether bond and carboxyl group, respectively, appeared after incubation with *A. guillouiae* (Fig. 4B).

FT-IR analysis was used to compare the results obtained. Compared with those of the control PE film, carbonyl absorption at 1,700

Table 2
Viable cell numbers according to colony-forming unit after 7 day-cultivation on PE medium.

Strain	Medium	Mean (CFU/mL)
A. guillouiae	LCFBM	4.27E + 05
A. guillouiae	LCFBM + PE	5.63E + 07
P. aeruginosa	LCFBM	5.00E + 06
P. aeruginosa	LCFBM + PE	5.57E + 07

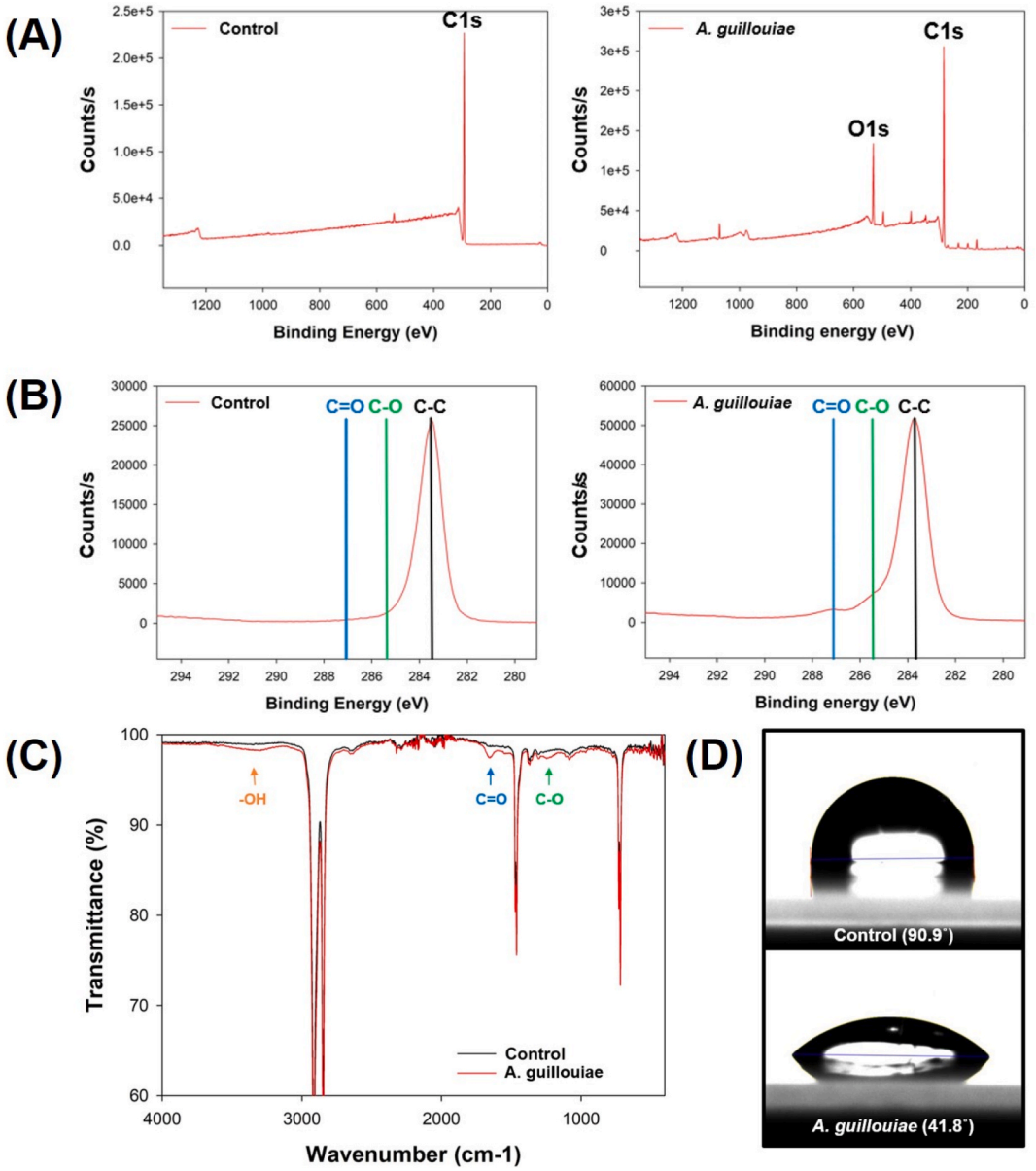

Fig. 4. Changes in chemical properties of the polyethylene (PE) film (A) Element analysis of the surface of PE film, using X-ray photoelectron spectroscopy, shows the composition ratio of oxygen and its increase when the PE film was incubated with A. guillouiae (B) The binding energy graph shows the atomic changes after biodegradation. The distinct changes in the peaks of C–O bond (285.8 eV) and C═O bond (287.3 eV) are indicated by green and blue lines, respectively. (C) Attenuated Total Reflection Fourier Transform Infrared analysis of PE degraded by A. guillouiae shows the difference in functional group between a pristine film (control) and biodegraded PE film. The distinct peaks of transmittance (%) values are at 1,200 cm^{-1} (C–O stretching), 1,700 cm^{-1} (C═O stretching), and 3,300 cm^{-1} (O–H stretching). (D) The contact angle images show the change of hydrophilicity on the surface of the PE film after biodegradation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cm^{-1}, C–O stretching absorption at 1,200 cm^{-1}, and O–H stretching absorption at 3,300 cm^{-1} were detected for the PE film biodegraded by *A. guillouiae* (Fig. 4C).

Oxidation of the plastic increases the hydrophilicity of the surface, which results in accelerated biofilm formation and biodegradation by microorganisms. To investigate the hydrophilicity of PE, the contact angle of water droplets on a PE film surface was measured. The average contact angle was 90.9° for the control PE, while it was 41.8° when the PE was exposed to *A. guillouiae* (Fig. 4D).

3.3. Analysis of hydrocarbon metabolism

To elucidate the metabolism of plastic intermediates (metabolic sources) within the microbial cell, various hydrocarbons corresponding to the intermediates in the fatty acid metabolic pathway (alkane to fatty acids) were used. DCPIP was used to measure the ability of the isolated strains to metabolize the hydrocarbons. The metabolic capacities of the strains for each hydrocarbon were expressed as a heat map. Data showed that aldehyde and fatty acid metabolism was observed in most of the strains, and the overall metabolic capacity decreased for alcohol and alkane. In addition, strains that used the products of the previous step tended to utilize all the metabolites of the metabolic pathway. The only exception was *E. fergusonii*, which metabolized only aldehydes (Fig. 5).

K. oxytoca metabolized aldehydes and fatty acids. *E. hormaechei* and *M. theicola* metabolized alcohol, aldehyde, and fatty acids, but alcohol was rarely metabolized. *P. aeruginosa* and *A. guillouiae* metabolized all the compounds from alkane to fatty acid in the fatty acid metabolic pathway (Table 3).

Considering the metabolic rate of the strains for alkane and alcohol metabolism, *K. oxytoca*, *E. hormaechei, E. fergusonii* and *M. theicola* showed no decrease in DCPIP absorbance ratio, within five days. *P. aeruginosa* and *A. guillouiae* were the only two strains that metabolized alkanes (Fig. S1A). Within five days, DCPIP was completely reduced when alcohol was used as the metabolic source for the culture of *A. guillouiae.* In contrast, the DCPIP absorbance ratio for the culture of *P. aeruginosa* remained at up to 0.56 (Fig. S1B).

4. Discussion

4.1. Screening of plastic-degrading bacteria using an oxidation–reduction indicator

To the best of our knowledge, we have, for the first time, used the oxidation-reduction indicator, DCPIP, which is used to analyze hydrocarbon metabolism, for developing a rapid and simple screening method for plastic-degrading bacteria. The strain with plastic degrading ability was identified based on the decrease in DCPIP absorbance. Results showed that DCPIP, which is usually used to analyze hydrocarbon and lipid metabolism [30], has the potential to be used in the field of plastic biodegradation research. Among the six strains isolated from insect larvae, two strains showed the ability to metabolize plastic within five days. The DCPIP absorbance ratio after seven days was similar to that after five days, and the bacteria appeared to have reached the steady state. Therefore, using this method, seven days would be the ideal time period for selecting putative plastic degraders. One of the strains identified was *P. aeruginosa*, which is a well-known plastic-degrading bacterium [10,18]. The fact that *P. aeruginosa* was screened using our method,

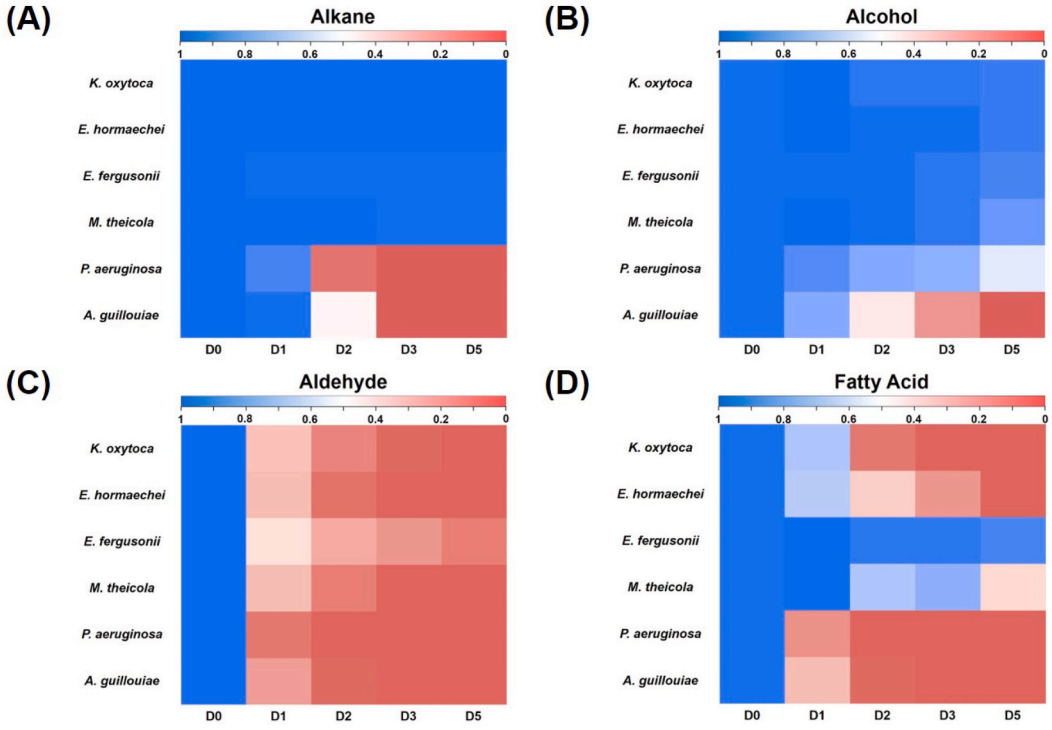

Fig. 5. Hydrocarbon metabolic capacity of the candidate plastic degraders. Heat map showing the DCPIP absorbance ratio of the six candidate plastic degraders for different hydrocarbon sources from Day 0–5. The color changes from blue to red represent the metabolic capacity of the plastic degraders for the different hydrocarbon sources. (A): Hexadecane, (B): 1-Hexadecanol, (C): Dodecyl aldehyde, (D): Na-stearate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Metabolic capability of putative plastic degraders on various hydrocarbons.

Strain	Hexadecane	1-Hexadecanol	Dodecyl aldehyde	Na-stearate
K. oxytoca	–	–	+++	+++
E. hormaechei	–	+	+++	++
E. fergusonii	–	–	++	–
M. theicola	–	+	+++	++
P. aeruginosa	+++	++	+++	+++
A. guillouiae	+++	+++	+++	+++

-: No metabolic capacity, +: Statistically significant but very slow response, ++: Ability observed clearly with naked eyes, but DCPIP absorbance ratio did not decrease to 0, +++: DCPIP absorbance ratio decreased to 0 within 5 days.

also demonstrates the effectiveness of the DCPIP-based screening method for plastic-degrading bacteria.

This screening method allows researchers to quickly and easily identify the plastic degrading ability of bacteria than the methods used in previous studies [30,32]. Methods used in previous studies required 30–60 days to identify the plastic-degrading bacteria. Various traditional analyses, such as GPC, SEM imaging, and weight loss measurements, are used to evaluate bacterial biodegradation of plastic. Additionally, chemical analyses, such as FT-IR, XPS, and FT-NMR, were used to detect changes in the chemical structure and functional groups of the plastic biodegraded by bacteria. While these methods were necessary to confirm the plastic-degrading ability of microorganisms, they are not suitable for screening due to the time and resources required.

In contrast, our method identified plastic-degrading bacteria through the color changes that could be observed with the naked eye (Fig. 2D). Therefore, putative plastic degraders can be easily screened using this method. Furthermore, the effectiveness of these degraders depends on their degradation rate and can be preferentially identified. A limitation of this method is that it cannot be used to screen plastic-degrading microorganisms from a bacterial mixture, such as environmental samples. Environmental samples contain microbial populations that are distributed across communities. While this screening method indicated the metabolic capacity of plastic through color, it does not select strains for degradation. Thus, when applying this method to environmental samples, it is only possible to determine the plastic degradation ability at the community level. However, this method has the advantage of being able to rapidly determine the degradation ability of a single isolated species. Therefore, by using this method as a secondary screening process, candidates screened in the initial screening step can be filtered out. In a high-throughput application, putative degraders can be easily screened by culturing a single strain in a 96-well plate, with the screening medium, and observing the decolorization with the naked eye (Fig. S2).

4.2. PE-degrading bacterium, *A. guillouiae*

Acinetobacter sp. is known to degrade oil similarly to *Pseudomonas* sp [34,35,36]. It is suggested that the reason for its PE biodegradation ability is the similarity in the backbone structure between lipids and PE. We investigated PE degradation by *A. guillouiae* using our novel screening method. SEM was used to study the biofilm formation of *A. guillouiae* and its consequent corrosion on the plastic surface. Results indicated that *A. guillouiae* proliferated using PE as the sole carbon source, providing evidence for plastic degradation.

The chemical structure change induced by PE biodegradation was analyzed by XPS and FT-IR. Increased composition ratio of oxygen indicated that PE was oxidized by *A. guillouiae.* Absorption for carbonyl group, ether bond, and hydroxyl group were observed using FT-IR, and the results were consistent with those of XPS analysis. In the C1s scan, the biodegraded plastic showed C–O and C═O bonds, suggesting that PE with stable C–C bond can be depolymerized by microbial enzymes, such as esterase [37,38]. We also investigated the surface hydrophilicity of PE films. The contact angle decreased for the biodegraded PE film, indicating increased hydrophilicity on the plastic surface. A hydrophilic surface facilitates biofilm formation on the surface and promotes plastic degradation [33].

Further evidence of plastic biodegradation was provided by the CFU count of *A. guillouiae* and the weight loss measurement of PE. The increase in the number of CFU confirmed that the strain efficiently used PE to proliferate, and the reduction in the mass of PE also indicated microbial degradation. In addition, there was no statistical difference in the degradation rate between *A. guillouiae* and *P. aeruginosa.* This suggests that the newly isolated plastic-degrading microorganism, *A. guillouiae* has a degradation ability comparable to that of the previously well-known plastic-degrading microorganism, *P. aeruginosa* [18,39].

Furthermore, the ability of *A. guillouiae*, isolated using our screening method, to degrade plastic was confirmed by analyzing the strain, using previously described methods. This indicates that the screening method is effective. The degradation rate of PE was relatively high for *P. aeruginosa* after seven days, while the DCPIP absorbance ratio decreased by 11.1% and 37.1% for *P. aeruginosa* and *A. guillouiae,* respectively, after seven days. These results suggest that the reduction in DCPIP absorbance is not proportional to the plastic weight loss induced by microbial biodegradation. Therefore, the screening method is suitable for selecting putative plastic degraders, but not for comparing the efficiency of strains.

4.3. Hydrocarbon metabolic capacity and plastic biodegradation

We attempted to investigate the underlying reasons for the plastic-degrading ability of *A. guillouiae* and *P. aeruginosa* by analyzing their hydrocarbon-metabolism abilities. The PE backbone is known to be similar to that of the alkane chain, which is gradually

oxidized to alcohol, aldehyde, fatty acid and finally degraded to CO_2 via the lipid-metabolism pathway. Therefore, the ability to metabolize alkane, alcohol, aldehyde, and fatty acid was examined individually for the six strains, and the correlation between hydrocarbon metabolism and plastic-degradation ability was analyzed.

Results suggested that the strains that used the oxidized metabolites closer to fatty acid in the fatty acid metabolic pathway were more effective. Only *A. guillouiae* and *P. aeruginosa* were able to utilize alkane for further metabolism, and thus involved in plastic degradation. This result is consistent with the genetic data on the KEGG website (http://www.genome.jp/kegg/). *A. guillouiae* and *P. aeruginosa* have all the genes encoding the enzymes involved in the metabolic reactions from alkanes to fatty acids in the fatty acid metabolic pathway (Table S2).

A possible explanation for this result could be that the bacteria need to metabolize alkane to utilize PE as a carbon source [40]. Thus, screening of plastic-degrading microorganisms can be effectively performed by checking the genetic database and narrowing down the candidate strains that can metabolize alkanes (Fig. S3). Most of the analyzed strains effectively utilized aldehyde and fatty acid, but alkane and alcohol were only utilized by *A. guillouiae* and *P. aeruginosa.* These results suggested that the transition from alkane to aldehyde (via alkane hydroxylation and alcohol dehydrogenation) may be an important step in the metabolism of plastics. Moreover, this result supports the findings of previous studies that PE is degraded via the lipid-metabolism pathway after the stepwise oxidation of the alkane. Together, these results suggest that oxidation of the alkane chain may be key to the effective degradation of plastics. Currently, studies on PE oxidation using enzymes, such as P450 [41,42], laccase [43,44], and alkane monooxygenase [25,45] are being carried out. Our results further support the need for these studies.

5. Conclusions

In this study, we isolated a PE-degrading bacterium, *A. guillouiae*, using a novel screening method based on the oxidation-reduction indicator, DCPIP. DCPIP was used for the first time to screen plastic-degrading strains and can be potentially applied to various types of plastic. The biodegradation of PE by *A. guillouiae* was analyzed by SEM, FT-IR, XPS, and contact angle analysis, CFU count and weight loss measurement to verify our screening method.

In addition, we investigated the characteristics of hydrocarbon metabolism in PE-degrading bacteria. Among the bacteria studied, *P. aeruginosa*., a well-known plastic-degrading bacterium, and *A. guillouiae* screened in this study, were the only bacteria capable of metabolizing each hydrocarbon from alkane to fatty acid in the hydrocarbon metabolic pathway. The results of this study support the existing theory that PE is degraded by stepwise alkane oxidation and lipid metabolism. Our results provide a deeper insight into bacterial-PE degradation, suggesting that alkane oxidation and alcohol dehydrogenation may be the key steps involved in PE degradation.

Our method can be used as a rapid screening system to narrow down the putative degraders among candidates isolated from environmental samples. In addition, it can be used to screen strains previously deposited in culture collections. By searching the genomic database, microorganisms with genes encoding alkane hydroxylase and alcohol dehydrogenase can be identified, and putative degraders can be screened using our screening method.

Our results pave the way for rapid screening of plastic-degrading microorganisms from various environmental samples or from strains previously reported as degraders of organic pollutants. This novel method can be used to develop high-throughput plastic-degrading microorganism-screening methods to address the plastic-waste problem.

Further studies are needed to investigate the relationships between plastic-degradation rate and decreased absorbance to develop this screening method into a degradation rate-measurement method. Additional research is required for exploring methods to enhance biodegradation efficiency through bioengineering of bacterial alkane and alcohol metabolism, as well as optimize the culture conditions of *A. guillouiae* for potential industrial applications.

Author contribution statement

Hong Rae Kim: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Chaerin Lee: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hyeyoung Shin: Performed the experiments.

Jongwon Kim: Performed the experiments.

Mija Jeong: Performed the experiments.

Donggeon Choi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appear to have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15731.

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