Study on biodegradation of polyethylene by microorganisms isolated from Zhanjiang Mangrove Reserve

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Abstract

Zhanjiang Gaoqiao Mangrove Reserve is one of the largest mangrove reserves in China, and its unique ecosystem makes the mangrove rich in microbial resources. In this study, the sediments from Zhanjiang Gaoqiao Mangrove was used as the samples, and polyethylene (PE) was used as the sole carbon source in the carbon-free medium. The microorganisms in the mangrove sediment were enriched, isolated, purified to obtain PE-degradable bacteria; The species of PE-degrading bacteria were determined by morphological analysis, biochemical identification and molecular identification; The degradation ability of the strain to PE was evaluated by weight loss rate and Fourier transform infrared spectroscopy (FTIR). The obtained results presented three strains of pseudo-degradable bacteria S1-1, S1-2 and S1-3, which belonged to \textit{Pseudomonas aeruginosa}, \textit{Acinetobacter venetianus} and \textit{Klebsiella pneumoniae}, respectively. Among them, S1-3 had the best degradation effect, and the weight loss rate reached 4.25% after 120 days. The weight loss rates of S1-1 and S1-2 were 3.81% and 3.88%, respectively. Under the observation of FTIR, it was found that all three strains could change the internal structure of PE. This study laid a foundation for the verification of PE degradation and provided a reference for the bioremediation of PE pollution in the environment.

Introduction

Mangrove ecosystem is one of the most productive and ecologically influential ecosystems, playing an important role in providing food and habitat for marine and terrestrial organisms and transporting carbon and nutrients to adjacent coastal zones or oceans (Bayen, 2012; Kulkarni et al., 2018). Because of its unique characteristics (such as rich organic carbon), mangrove ecosystem has been identified as an important sink of plastic pollutants in land and sea (Nor & Obbard, 2014). In theory, microorganisms can use many hydrocarbons as carbon sources or plastics as the only carbon source (Chen et al., 2021), but the high molecular weight of plastics limits the catalytic reaction of many enzymes as substrates (Naji et al., 2019). It is reported that mangrove sediments are considered to be a good source of microorganisms capable of degrading Polyethylene (PE) bags and plastic cups (Deng et al., 2021).

The output of PE accounts for about 1/4 of the total plastic output in the world, ranking the first place among the five general resins. At present, the microorganisms that degrade plastics are generally screened in the soil, ocean and the intestines of insects (Huang et al., 2020), mainly bacteria and fungi (Corinaldesi et al., 2021; Zhang et al., 2021). It is found that PE is the main type of plastic in the sediment of Gaoqiao mangrove in Zhanjiang (Chen et al., 2022). PE plastic is one of the plastics that are difficult to degrade and have high yield. Among many plastic pollution treatment methods, the use of microbial degradation is more environmentally friendly, mainly to eliminate environmental pollution and not to cause secondary pollution to the environment (González-Pleiter et al., 2021). In the remediation of PE plastic pollution, there are few studies on microbial degradation remediation.

At present, the reported plastic-degrading bacteria contain is more than 30 genera of bacteria and fungi, including \textit{Bacillus}, \textit{staphylococcus}, \textit{Achromobacter} and so on (Beiras et al., 2021; Rong et al., 2021). The
The purpose of this study is to obtain PE-degrading bacteria, and explore their degradation effect on PE by taking the mangrove sediments in Zhanjiang as samples, and then enriching, separating and purifying the microorganisms in the mangrove sediment with PE as the sole carbon source. The results lay a foundation for the verification of PE degradation and provide a basis for the bioremediation of PE pollution in the environment.

**Materials And Methods**

**Mangrove sediment sampling**

In October 2020, 10 sampling sites were randomly selected from Gaoqiao Town Mangrove Nature Reserve, Lianjiang City, Zhanjiang City, Guangdong Province. Sediment samples with depths of 3 cm and 5 cm were collected by five-point sampling method and packed in clean glass containers for the isolation and identification of plastic-degrading bacteria.

**Reagent and solution configuration**

Na$_2$B$_4$O$_7$, CuSO$_4$, CaCl$_2$, ZnSO$_4$, H$_2$SO$_4$, CaCO$_3$, FeSO$_4$, MgSO$_4$, MnSO$_4$ tryptone yeast extract, NaCl, HCl and Agar are all domestic pure reagents for analysis; Low density PE is purchased from Alfa Aesar Co., Ltd for analysis; TIANamp Bacteria DNA Kit (TIANGEN), Green Taq Mix (Novozan), DL2000 DNA Marker (Takara); Ordinary plastic fresh-keeping bag (PE), purchased from the supermarket of Guangdong Ocean University; Glutaraldehyde, absolute ethanol, C$_{12}$H$_{25}$SO$_4$Na, NaCl, KCl, KH$_2$PO$_4$, Na$_2$HPO$_4$ and HCl are all domestic pure reagents for analysis.

PBS buffer: Dissolve 8 g NaCl, 1.44 g Na$_2$HPO$_4$, 0.27 g KH$_2$PO$_4$ and 0.2 g KCl in 800 mL distilled water, adjust pH solution to 7.4 with HCl, finally add distilled water to 1 L, sterilize 20 min at 121 ℃, store in 4-guarantee refrigerator, set aside.

2% sodium dodecyl sulfate (SDS): 20 g C$_{12}$H$_{25}$SO$_4$Na was dissolved in 1 L distilled water and sterilized for 20 min at 121 ℃ for 0.1 MPa. It was stored in a 4 ℃ refrigerator.

**The medium used for the test**

**Liquid medium**

1. MM30 liquid medium (g/L): 1 g (NH$_4$)$_2$SO$_4$ 3 g KH$_2$PO$_4$ 6 g Na$_2$PO$_4$ 0.5 mL Trace metal salt solution, pH is 7.1; Mixture 44 solution (mg/100 mL): 17.7 mg Na$_2$B$_4$O$_7$, 39.2 mg CuSO$_4$, 20.1 mg CaCl$_2$, 1095 mg ZnSO$_4$ 250 mg EDTA, 100 μL and 150 μL concentrated H$_2$SO$_4$ were added to prevent precipitation; Trace metal salt solution (mg/100 mL): 0.5 mg EDTA, 1 mg CaCO$_3$, 0.5 mg FeSO$_4$, 10 mg MgSO$_4$, 10 mg MnSO$_4$, 10 mL Mixture 44.

2. LB liquid medium (g/L): 10 g Tryptone, 5 g Yeast extract, 10 g NaCl, pH is 7.0.
Solid medium

18% Agar was added to the liquid medium, sterilized by high pressure steam (121 °C, 0.1 MPa) for 20 min, then poured into a petri dish and set aside after cooling and solidification.

Screening of PE-degrading microorganisms

PE preprocessing

The PE powder used in the experiment was pretreated by putting it in a sterile petri dish, irradiating it under ultraviolet light for 12 hours, and turning it every 30 min. For PE film treatment, the plastic fresh-keeping bag was cut to the size of 3×5 cm, and then irradiated under ultraviolet light for 12 hours, flipped every 30 min. In order to verify the asepsis, the powder and film irradiated under ultraviolet lamp were inoculated in solid medium and cultured in a constant temperature incubator at 30 °C for 3 days. If there is no colony growth around the powder and film, it is considered aseptic.

Enrichment and purification of PE plastic degrading bacteria

Put 10 g of sediment sample into a flask and add 90 mL aseptic distilled water, shake fully for 15 min, take 1 mL supernatant, add it to a 50 mL flask, add 30 mL MM30 and 0.2g PE powder, shake in a constant temperature concussion incubator at 30 °C for 180 r/min, put 1 mL bacterial liquid into new MM30 and PE every 7 days, and passage for 7 times. Finally, 10 μL bacterial solution was diluted to 10-4 with aseptic distilled water, coated on MM30 solid medium, and evenly sprinkled with PE powder. The colonies with different shapes, sizes and colors were selected and purified repeatedly on MM30 solid medium after incubation in a constant temperature incubator at 30 °C for 48 h. Finally, the single colony was inoculated in LB broth medium for 12 ~ 24 hours, then 25% glycerol was added and stored in the refrigerator at -80 °C.

Strain identification

Gram staining kit was used for staining identification and Hukai microbial biochemical identification tube (070010 bacterial biochemical identification tube) was used for biochemical identification. Gram staining was used following the listed steps: (1) fixing: absorbing 10 μL bacteria on the slide, adding aseptic distilled water, diluting coating, and then fixing by alcohol flame; (2) primary dyeing: adding crystal purple dye, 1 min, rinsing with distilled water; (3) mordant dyeing: adding Gram iodine solution, 1 min, rinsing with distilled water; (4) decolorization: dripping 95% decolorized alcohol for 30 s, rinsing with distilled water. (5) re-staining: drop of Gaza yellow fuhong dye solution, 1 min, rinse with distilled water; (6) microscopic examination: after the glass slides were dried, the staining was observed under the microscope, Gram-positive bacteria were purple and Gram-negative bacteria were red.

Gene sequencing and phylogenetic tree analysis
In order to further determine the species and genus of the isolated bacteria, PCR amplification and sequencing were used to further identify the isolated bacteria at the molecular level. The isolated strains were cultured in LB broth medium for 36 ~ 48 h. DNA was extracted by bacterial DNA extraction kit and amplified by PCR with 515F and 907R primers (Table 1, Table 2 and Table 3). After the PCR product was obtained, 1% agarose gel electrophoresis was performed, and the rest was sent to Guangzhou Tianyi Biotechnology Co., Ltd for sequencing. The homology of the sequences was compared in the BLAST data of NCBI. Finally, the phylogenetic tree was constructed by using the software MEGA 7.0 for analysis.

PE weight loss rate detection

After activating the degradation bacteria and adjusting OD600 to 1, 4 mL solution and 36 mL MM30 medium were inoculated in a 50 mL wide-mouth conical flask, and then 4 portions of PE membrane were exposed for 120 days. One portion of PE membrane was taken out every 30 days. To remove the bacteria on the membrane surface, the specific steps are carried out as follows: (1) rinse with PBS buffer to remove excess culture medium and bacteria; (2) soak with 2% sodium dodecyl sulfate (SDS) solution for 2 hours; (3) rinse with warm distilled water to remove excess SDS; (4) 2% glutaraldehyde fixed solution for 2 hours; (5) 50% ethanol ultrasound twice, 30 min each time; (6) 75% ethanol overnight. (7) 100% anhydrous ethanol ultrasonic 3 times, 30 min each time. (8) after drying to constant weight, weigh with 3 repeats in each group, and calculate the corresponding weight loss rate, weight loss rate = (original weight-degraded weight) / original weight × 100%.

Detection by PE Fourier transform infrared spectrometer

After activating the degrading bacteria and adjusting OD600 to 1, 4 mL solution and 36 mL MM30 medium were inoculated in a 50 mL wide-mouth conical flask, and then put into 0.2 g PE powder. In addition, the blank control group was treated with 4 mL saline and 36 mL MM30 medium, and then added 0.2 g PE powder. After continuous culture in 180 r/min incubator at 30 ℃ for 120 days, a certain amount of MM30 medium was added every 10 days to maintain the final reaction system at 40 mL, with 3 repeats in each group. After the bacteria were removed according to the previous method and dried, the Fourier transform infrared spectrometer was used to detect the changes of functional groups on the surface of PE in the spectral range of 675 ~ 4000 cm⁻¹.

Results And Analysis

Isolation and purification of PE-degrading bacteria

32 single colonies were obtained by repeated purification and inoculated on solid MM30 medium with PE as the sole carbon source. After continuous culture for 24 hours, three colonies with the best growth were selected and named as S1-1, S1-2 and S1-3, respectively. On LB solid medium, all the three strains formed visible colonies after 24 hours (Fig. 1). S1-1: the colony is blue-green, different size, flat and moist, irregular edge, umbrella-shaped extension; S1-2: the colony is round, tiny, smooth surface, neat edge; S1-3: the colony is white and round, smooth surface, neat edge.
Identification of PE-degrading bacteria

Three strains of PE-degrading bacteria were identified by Gram staining (Fig. 2) and biochemical identification (Table 4). It was found that all three strains were gram-negative bacteria.

Phylogenetic tree analysis of strains

The sequencing sequences of three strains were compared in the BLAST of NCBI. According to the principle of selecting reference sequence, some homologous sequences with similarity between 98% and 100% were selected, and the phylogenetic tree was constructed by ortho-position merging method in distance method using MEGA 7.0 software. According to the developmental tree, S1-1 (Fig. 3-1) was identified as *Pseudomonas aeruginosa*, S1-2 (Fig. 3-2) was identified as *Acinetobucter venetianus*, and S1-3 (Fig. 3-3) was identified as *Klebsiella pneumoniae*.

Analysis of degradation effect

Weightlessness rate

After 120 days of degradation (Fig. 4), it was found that S1-3 had the highest degradation efficiency, reaching 3.97% at the time of 60 days, but with the extension of time, the increase rate of the degradation effect became slow, with only 4.06% and 4.25% at the time of 90 days and 120 days. The weight loss rates of S1-1 and S1-2 are rising steadily, and the weight loss rates of S1-1 and S1-2 are almost the same. At the time of 60 days, the weight loss rates of S1-1 and S1-2 are 2.08% and 2.51%, respectively, and the two strains are 3.81% and 3.88% at the time of 90 days, respectively.

Detection and Analysis of PE Fourier transform Infrared Spectrometer

Measuring the change of surface functional groups of PE is an important way to prove whether it is degraded or not. Fig. 5 shows the infrared spectrum of PE before and after microbial degradation. In the infrared spectrum, there are obvious absorption peaks at 2920 cm\(^{-1}\), 2853 cm\(^{-1}\), 1466 cm\(^{-1}\), 1373 cm\(^{-1}\) and 721 cm\(^{-1}\), in which the stretching vibration of Cl-H in alkane-CH\(_2\) is at 2920 cm\(^{-1}\) and 2853 cm\(^{-1}\), and the in-plane bending vibration of alkane-CH\(_2\) at 1466 cm\(^{-1}\). The strong absorption peak at 721 cm\(^{-1}\) is the olefin C-H rocking vibration, which fully shows that the material is a typical PE structure. In the infrared spectrum after microbial degradation, compared with the undegraded control group, a new absorption peak appeared at 3600-3300 cm\(^{-1}\), where the absorption peak was caused by the stretching vibration of O-H, indicating that hydroxyl was produced on the surface of PE, and there was a strong C-O stretching vibration at 1720 cm\(^{-1}\), indicating that there was a certain oxidation on the surface of PE. In the vibration of alkane-CH\(_2\) at 1466 cm\(^{-1}\), the number treated by degrading bacteria decreased obviously, indicating the decrease of alkane-CH\(_2\) group. In summary, the three strains can change the internal structure of PE, decompose PE into small molecular weight polymers, reduce its molecular weight and achieve degradation.
Discussion

PE has the most output among the five general-purpose plastics, with a wide range of applications, high yield. PE are extremely difficult to degrade (Zilius et al., 2020). The use of microbial degradable plastics has gradually become one of the mainstream research fields of plastic degradation because of its mild degradation conditions, no pollution of degradation products and no effect on soil structure and ecological environment (Shah et al., 2008; Zurier & Goddard, 2021). In this study, 32 single colonies were isolated by using PE as the sole carbon source. Through the comparison of plate growth, three strains which could metabolize with PE as the sole carbon source were selected, which were named S1-1, S1-2 and S1-3 respectively. Further morphological analysis, Gram staining identification, biochemical identification and phylogenetic tree analysis showed that S1-1, S1-2 and S1-3 belonged to Pseudomonas aeruginosa, Acinetobacter venetianus and Klebsiella pneumoniae, respectively. At present, many scholars have found that most of Pseudomonas and Acinetobacter bacteria can degrade different components of plastics (Zhou et al., 2021), and Klebsiella has also been proved to be able to degrade some persistent organic pollutants (Geng et al., 2021). However, at present, only 1% of the colonies can be artificially isolated and cultured, and there are still a large number of unculturable bacteria in the mangrove ecosystem (Rodríguez-Narvaez et al., 2021), including plastic-degradable microorganisms. Therefore, the isolation and culture of plastic-degrading bacteria still need to be further improved.

Some studies have shown that the biodegradation of PE is mainly divided into four stages (Schouw et al., 2016): first, microorganisms gather on the surface of PE for attachment and growth; secondly, microorganisms secrete some enzymes and carry out biological oxidation or hydrolysis to promote the long hydrocarbon chain of PE breaking into short chains (Niu et al., 2021); then the polymerization on the short chain breaks again, and finally forms fatty acids. Finally, the fatty acids are oxidized by β or ω oxidation, and finally decomposed into water, carbon dioxide and some humus (Wang et al., 2020). In this experiment, the degradation ability of three strains to PE was studied. From the weight loss rate of the strain, it was found that the weight loss rates of S1-1, S1-2 and S1-3 were 3.81%, 3.88% and 4.25%, respectively. It can be seen that compared with other reported results that the weight loss rate of the degrading bacteria isolated from mangrove sediments with PE for 40 days was 1.60% (Auta et al., 2017), and that of the strains isolated from ocean waters with PE for 30 days was 1.50% (Harshvardhan & Jha, 2013), some of the strains we screened had higher degradation rates and enriched the strain resources of PE plastic degrading bacteria. However, although the weight loss rate is increasing in terms of time distribution, the increase of weight loss rate is different in the case of the same interval. It is speculated that in pure culture, different strains secrete different enzymes and their functions are not consistent, which leads to the inconsistent weight loss rate of PE in different time periods.

PE plastics are copolymerized from ethylene and a small number of α-olefins, and their chemical structure is stable, so it is difficult to degrade. During the initial degradation, PE plastics will introduce polar functional groups under the action of foreign microorganisms, thus increasing the structural instability of PE plastics, which mainly include carbonyl groups (-CO-), ether bonds (C-O-C), carboxyl groups (-COO-), hydroxyl groups (-OH) and so on (Chen et al., 2018). Under the FTIR detection, it was
found that the film had a smaller hydroxyl absorption peak at 3600 – 3300 cm$^{-1}$ after the treatment of three strains of bacteria, and the absorption peak increased compared with the blank control group, indicating that hydroxyl groups increased, while hydroxyl groups could enhance the hydrophilicity of plastics, resulting in the breaking of long chains of PE and the formation of low molecular weight compounds (Muhonja et al., 2018). The vibration of alkane-CH$_2$ at 1466 cm$^{-1}$ also shows that the peak value decreases obviously after microbial degradation, indicating the decrease of alkane-CH$_2$ group. There is a strong Czochralski stretching vibration at 1720 cm$^{-1}$, indicating that there is a certain oxidation on the surface of low-density PE materials, indicating that microorganisms can change the internal structure of PE, decompose PE into low molecular weight polymers, reduce its molecular weight and achieve degradation (Buchholz et al., 2022).

**Conclusion**

In this study, plastic-degrading bacteria were screened with PE as the sole carbon source, and three strains with better degradation effect were obtained, which were S1-1 *Pseudomonas aeruginosa*, S1-2 *Acinetobacter venetianus* and S1-3 *Acinetobacter venetianus* respectively. Among them, S1-3 *Klebsiella pneumoniae* had the best degradation effect, and the weight loss rate reached 4.25% after 120 days. The weight loss rates of S1-1 *Pseudomonas aeruginosa* and S1-2 *Acinetobacter venetianus* were 3.81% and 3.88%, respectively. Under the observation of FTIR, it was found that the three strains could change the internal structure of PE and achieve degradation. The study laid a certain foundation for the verification of PE degradation, enriched the strain resources of PE plastic degradation bacteria, and provided a theoretical basis for the bioremediation of PE pollution in the environment.

**Declarations**

**Acknowledgements**

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**Authors' contributions**

Conceptualization, Methodology, Data curation, Supervision, Funding Acquisition, Writing - review & editing.

All authors discussed, reviewed and approved the final report.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy protection.

**Competing interest**

The authors declared that they have no competing interests.

**Fundings**

Contract grant numbers GDOE2019A52; 2020SFJD001; 2016A050502062.

**References**


Tables

Table 1 **Primer sequences of target genes**
Table 2 The adding system

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Table 3 PCR amplification procedure

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Table 4 Biochemical identification results
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**Figures**

**Figure 1**

The status on LB solid medium
Figure 2

Gram staining (1000X)
Figure 3

3-1 The evolutionary tree of S1-1 strain 3-2 The evolutionary tree of S1-2 strain 3-3 The evolutionary tree of S1-3 strain
Figure 4

Changes of weight loss rate of strains in different time periods
Figure 5

Infrared spectrum of PE before and after microbial degradation. (a) FTIR spectrum of PE after S1-1 degradation. (b) FTIR spectrum of PE after S1-2 degradation. (c) FTIR spectrum of PE after S1-3 degradation. (d) FTIR spectrum of non degraded PE

Supplementary Files

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