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Biphenyl-degrading Bacteria Isolation with Laser Induced Visualized Ejection Separation Technology and Traditional Colony Sorting

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Abstract

In this work, biphenyl was used as carbon source to enrich microorganisms from polychlorinated biphenyls (PCBs)-contaminated paddy soil samples, and the taxonomic structures in both of the soil samples and the fourth-generation enrichments were examined with high-throughput sequencing. Single cells were isolated from the enrichments via single cell sorting technology named Laser Induced Visualized Ejection Separation Technology (LIVEST) and also traditional single colony sorting, and the genera of the isolates were identified using 16S rRNA sequencing. The results from high-throughput sequencing present that enrichment from generation to generation can considerably change the microbial community. Comparing the two sorting methods, the LIVEST is more time-saving and cell-targeted for microbial resource exploration. Based on the further verification of biphenyl degradation, it was found that some strains belonging to genera *Macrococcus*, *Aerococcus* and *Metabacillus* are capable in degrading biphenyl, which have not been reported yet.

Keywords Biphenyl · Bacterial enrichment · Single cell sorting · Single colony sorting · Precise sorter · Degradation verification

Introduction

In the last decades, global industrialization and urbanization have affected all environmental compartments, and environmental issues and ecological safety have been becoming critical restrictions for economic development and human health nowadays. Organic contaminations are rather complex, and a great amount of efforts have been spent on their determination, analyses and elimination (Matamoros et al. 2012; Fidalgo-Used et al. 2007; Wang et al. 2021a, b). Microorganisms play an indispensable role in contaminant detoxification and substance transformation, and microbioremediation has been recognized as an eco-friendly and cost-effective approach for environmental improvement (Wang et al. 2021a, b; Rahman 2020). In particular, some microbiomes possess certain metabolic pathways to catabolize a wide range of organics into CO_2 and H_2O (Kirkwood et al. 2006; Teng and Chen 2019; Wang et al. 2019; Petsas and Vagi 2019). Indigenous microbes adapting to polluted environments enable to decompose local contaminants, so using specific substrate to enrich and isolate functional microbes is effective to obtain contaminant-degrading bacteria from environmental compartments (Li et al. 2000; McRae et al. 2004; Siciliano et al. 2001).

At present, more than 525 thousands of microorganisms have been discovered and identified, while through single-cell and metagenomic sequencing technologies, it is found that most microorganisms still belong to unexplored dark matter (Solden et al. 2016; Cena et al. 2021). In the last decades, many cell-isolation technologies have been developed, such as flow cytometry, laser capture microdissection, limiting dilution, manual cell picking, microfluidics and so on (Zeb et al. 2019; Ishii et al. 2010; Gross et al. 2015). The laser capture microdissection and manual cell picking can procure targeted cells under direct microscopic visualization, while the others are mainly based on collecting droplets

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containing single cells. Depending on the specific isolating requirements, many factors can be considered to select a proper sorting method, such as sample nature and origin, cell integrity and viability, throughput, sample volume, cell purity and isolation efficiency. The LIVEST can present the view of a single cell with a size range of 0.5–20 μm under the microscope, and the cell properties can also be observed with other optical techniques such as fluorescence imaging and Raman spectroscopy (Liang et al. 2022; Li et al. 2022). The cell isolation methods were initially developed for disease diagnosis and biomedical research. In the recent years, however, single cell isolation is also widely employed to discover microbial resources from environmental compartments, especially the ones with specific functions (Kawai et al. 2000; López et al. 2004; Urbanek et al. 2017; Spini et al. 2018).

Even though a certain amount of work has been conducted on cell isolation, there is still a large space to understand microorganism community in the environment. In this work, the LIVEST was applied to isolate biphenyl-degrading strains from paddy soil, and also the traditional single colony sorting method was used as a reference for cell sorting. The microorganisms in the soil were enriched four generations using biphenyl as carbon source, and the two sorting methods were employed to isolate single cells from the enrichment. Furthermore, the genera of the isolates were identified with 16S rRNA gene sequencing, and the variations of the bacterial community in the soil and enrichments were examined with high-throughput sequencing to illustrate the enrichment effects on the community diversity. Consequently, the degradation ability of the isolates were further verified based on their growth with biphenyl.

Materials and methods

Biphenyl-degrading bacteria enrichment

The soil sample used for cell isolation was collected from a paddy field in Taizhou city in China, and the field was polluted with PCBs due to its close distribution to an informal electrical and electronic waste (e-waste) recycling site. Taizhou is one of the largest recycling areas for almost 40 years in China, and the local soil has been polluted by the emissions from e-waste dismantling process (Chen et al. 2014). The soil sample was taken from the surface 20 cm of the field with pH of 5.7 and 4.5% content of organic matter. Each of the samples was divided into two parts, one part was naturally dried (DS sample) and another part was stored in a sealed plastic bag to keep it fresh (FS sample). For the biphenyl-degrading bacteria enrichment, 5 g of each of the two types of soil samples was added to 100 mL sterilized inorganic salt solution with biphenyl as its carbon source. The first-generation enrichment samples were cultured at 30 °C with 180 r/min until 7 days, and then 5 mL of suspension from each enrichment samples was inoculated to 100 mL sterilized inorganic salt solution for the second-generation enrichment. The enrichment processes were repeated until the fourth generation whose suspension was then used for cell sorting. The enrichments from fresh soil has three replicates from generation to generation and the replicates in the fourth generation were named FSE1, FSE2 and FSE3; the dry soil has one enrichment from generation to generation and the fourth generation was named DSE.

The inorganic salt solution contains the following components per liter: 0.01 g $CaCl_2$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 1 g KH_2PO_4, 1 g NaCl, 3 g $K_2HPO_4 \cdot 3H_2O$, and 1 mL trace salt solution containing 4 mg H_3BO_4, 4 mg MoO_3, 4 mg $MnSO_4 \cdot 5H_2O$, 4 mg $CoCl_2 \cdot 6H_2O$, 20 mg $CuSO_4 \cdot 5H_2O$, 28 mg $ZnSO_4 \cdot 5H_2O$. Biphenyl solution was added to conical flasks until its solvent volatilized, then 100 mL sterilized inorganic salt solution was added and the nominal biphenyl concentration is 300 mg/L. The variations of the biphenyl concentration in the system were not measured, since the only aim of the enrichment process was to obtain bacteria that can survive with biphenyl as the carbon source.

Single cell isolation

The single cell was sorted using the instrument named Single Cell Precision Sorter (HOOKE PRECI SCS, China) based on LIVEST laser ejection. During ejection, the laser pulse acted on the cell-coated chip to push away a cell and its surrounding microenvironment into a cell receiver containing 4 μL inorganic salt solution. The pulse did not directly act on cells, which avoided cell damage and improved cell survivability. The LIVEST can isolate cells with the size of 0.5–20 μm, so it is capable in sorting various kinds of cells including archaea and eukaryotic cells. Due to the visible ejection, the method is able to avoid abiotic interferences. In addition, this technology can automatically recognize cells by setting parameters such as cell length, cell length-diameter ratio, area and so on, while here we selected cells based on their coccus or rod features with 50× amplification (Fig. 1).

The enriched culture was washed three times and resuspended with saline, the suspension was diluted to around 10^6 cell/mL. Afterwards, glycerol with 4% content was added to the dilution with the volume ratio of 1:1 and mixed together for cell protection from sorting. A drop of the mixture was coated onto a sorting chip which was then loaded onto PRECI SCS system for cell sorting. The collected cells were then transferred to culture tubes with the media of

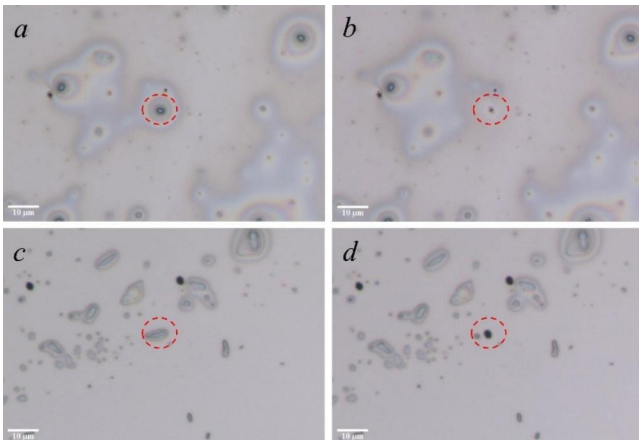

Fig. 1 Overview on cell layer and targeted cell selection. The coccus (fig. a) and rod cells (fig. c) labeled with red circles are the targeted cells for ejection, the black points in fig. b and d labeled with red circle are the location where the targeted cells were ejected with laser

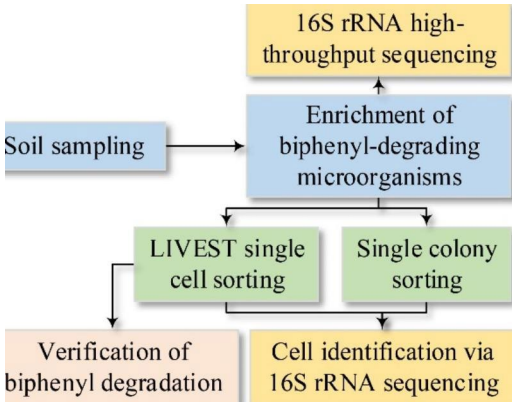

Fig. 2 Experimental process of this study

inorganic salt solution and biphenyl for genus identification and storage.

Single colony sorting

For single colony sorting, the enriched culture was diluted to proper concentration and then spreaded onto inorganic salt agar plates supplementing biphenyl as carbon source on the agar surface. The spreaded plates were cultured at 30 ℃ for 72 h and then individual colonies were selected to identify their genera.

Sequencing and genus identification

DNA was extracted from the expanded culture of the single cell and the fourth-generation enrichment using DNeasy Blood & Tissue Kit, and the extraction from the fresh and dry soil samples used DNeasy PowerMax Soil Kit (QIAGEN) following their corresponding operation instructions. The extracted DNA from single cell culture was subjected to PCR amplification with the universal primers 27 F and 1492R, which is prepared for 16S rRNA sequencing analysis. The extracted DNA from the soil samples and the fourth-generation enrichment samples was amplified at V3 and V4 region with the primers 341 F and 806R, which is prepared for 16S rRNA high-throughput sequencing. Both of the two sequencings were analyzed in Sangon Biotech Co., Ltd. Shanghai, China. The whole experiment process in this study is presented in Fig. 2.

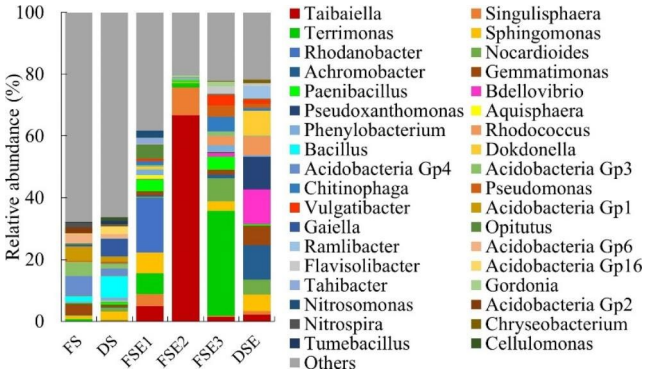

Fig. 3 The relative abundance of genera in different samples (FS: fresh paddy soil; DS: dry paddy soil; FSE1 to FSE3: the fourth generation of bacterial enrichment from fresh paddy soil; DSE: the fourth generation of bacterial enrichment from dry paddy soil.)

Results and discussion

Taxonomic variation

The taxonomic structures in both FS and DS samples are rather diverse and there is no obvious variation in the genera abundance between these two types of soil (Fig. 3). After enrichment with biphenyl, however, the genus diversity has noticeable decreases in the four samples FSE1, FSE2, FSE3 and DSE compared to FS and DS, respectively. Meanwhile, there are considerable differences in the taxonomic structures among the enriched samples, even though FSE1, FSE2 and FSE3 are cultured from one soil sample and treated identically. The different patterns might result from species competition, which is continually enlarged from generation to generation. Therefore, both the external conditions and the genera structure can, to some extent, influence individual species survival.

In the four enriched samples, the main genera with relative abundance above 10% are *Taibaiella*, *Terrimonas*, *Rhodanobacter*, *Achromobacter*, *Bdellovibrio*, *Pseudoxanthomonas*, while they have less than 1% in both FS and DS samples. It has been found that some of the genera in environmental compartments can be enriched using biphenyl or PCBs as carbon source, such as *Rhodanobacter,*

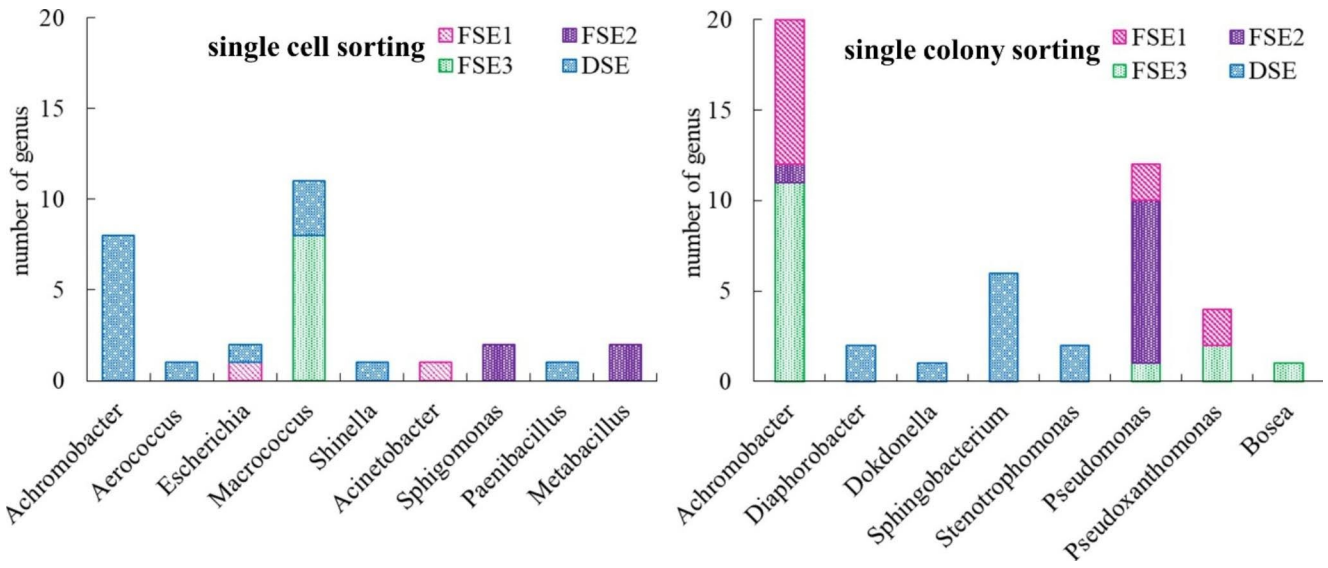

Fig. 4 The number of isolates from single cell sorting (left figure) and single colony sorting (right figure). The y axis is the number of isolates in different genera (shown in x axis) isolated from samples FSE1, FSE2, FSE3 and DSE

Achromobacter, *Bdellovibrio*, *Pseudoxanthomonas* (Uhlik et al. 2012; Luo et al. 2008; Macedo et al. 2005; Koubek et al. 2013). Intriguingly, *Taibaiella* and *Terrimonas* have the highest abundance in the community FSE2 and FSE3, respectively, hence it can be suspected that there are potential biphenyl-degrading species from the genera, even though there is no literatures reported about their degradability of biphenyl. In contrast, the genera, *Bacillus*, *Acidobacteria* Gp4 and *Gaiella* have the abundance above 5% in FS or DS samples, but after enrichment they have less than 0.5% in the four enriched samples, suggesting that bacterial community and/or the culture might not be favorable for their survival.

Single cell and single colony sorting

The isolates from single cell and single colony sorting are distributed in different genera (Fig. 4). The genera *Achromobacter*, *Aerococcus*, *Escherichia*, *Macrococcus*, *Shinella*, *Acinetobacter*, *Sphigomonas*, *Paenibacillus* and *metabacillus* were obtained from single cell sorting, while the genera *Achromobacter*, *Diaphorobacter*, *Dokdonella*, *Sphingobacterium*, *Stenotrophomonas*, *Psedomonas*, *Pseudoxanthomonas* and *Bosea* were isolated from single colony sorting. Only strains from genus *Achromobacter* were sorted from both of the two methods. These significant differences in the genus patterns might result from two reasons. One is that the two sorting methods used different sorting culture media, which could select different genera. Another one is that before single colony sorting the enrichments were stored in − 80 °C, which could also change the taxonomic structures of the enrichments.

Most of the genera have been reported to be capable in catabolizing biphenyl, including *Achromobacter sp.* (Hong et al. 2009), *Acinetobacter sp.* (Asturias et al. 1994), *Paenibacillus sp.*(Sakai et al. 2005), *Sphigomonas yanoikuyae* (Kim and Zylstra 1999), *Sphigomonas wittichii* (Seah et al. 2007), *Stenotrophomonas sp.* (Ganesh-Kumar et al. 2010) *Stenotrophomonas maltophilia* (Elufisan et al. 2019), *Pseudomonas pseudoalcaligene* (Taira et al. 1992), *Pseudomonas putida* (Hayase et al. 1990) and *Bosea sp.* (Egorova et al. 2021). However, the others have not been reported to use biphenyl as carbon source, including *Aerococcus*, *Escherichia*, *Macrococcus*, *Shinella*, *Sphigomonas*, *Metabacillus*, *Diaphorobacter*, *Dokdonella*, *Pseudoxanthomonas*.

The LIVEST technology can present a visible cell shape, which provide more information for single cell sorting, especially some strains with special morphology could have more opportunity to be discovered. Compared to the traditional single colony sorting, the LIVEST process does not change the culture medium during sorting, which somewhat avoids genera losses resulting from the inadaptation of different culture medium. In addition, this technology is able to sort cells labeled with fluorescence, isotope and graphene quantum dots for studying cell characteristics such as antibiotic resistance, iron reduction, contaminant degradation (Liang et al. 2022; Gan et al. 2021; Luo et al. 2021; Wang et al. 2020; Li et al. 2022). Meanwhile, combining with genome amplification, microorganisms in situ isolated with the LIVEST was identified and verified for specific functions such as lipid production (Zhao et al. 2022) and also for the investigation of microbial community (Yang et al. 2021). Besides, based on fluorescence lifetime imaging microscopy, the LIVEST has been applied to distinguish

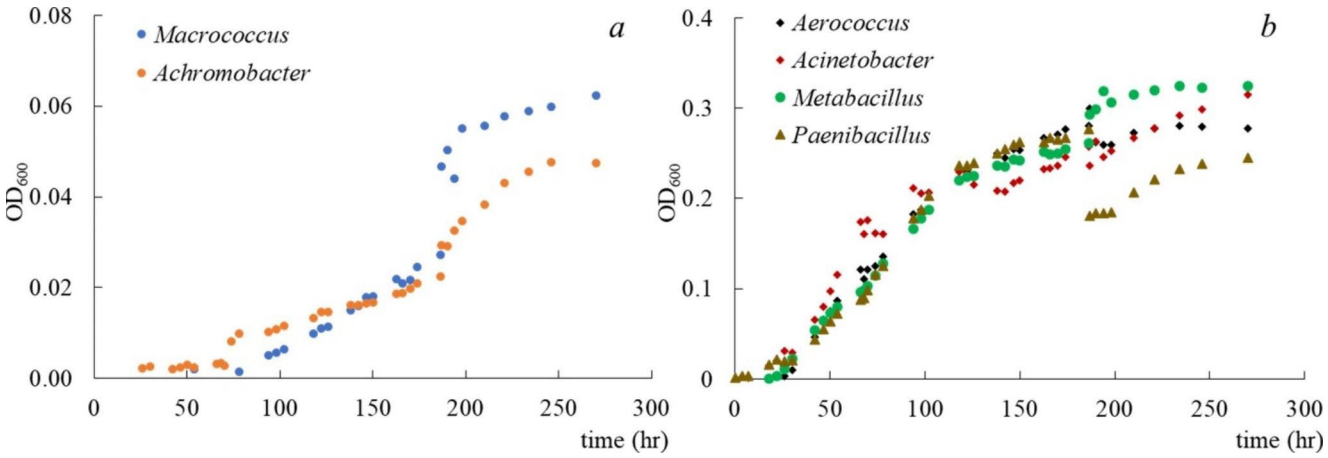

Fig. 5 Growth of the isolated strains with biphenyl as the carbon source, the growth is quantified with OD_{600} (y axis), the culture duration is 270 h (x axis). The figure *a* shows the two slow-growing strains belonging to genera *Macrococcus* and *Achromobacter*, and figure *b* presents the four fast-growing strains belonging to *Aerococcus*, *Acinetobacter*, *Metabacillus*, *Paenibacillus*

and isolate young and aged *Sccharomyces* cells for the improvement of fermentation efficiency (Kong et al. 2022). Therefore, the LIVEST has a great potential to be combined with other methods for the further study and application on single-cell levels.

Verification of biphenyl degradation

Six of the isolated strains were further selected as examples to test their growth with biphenyl as carbon source, the strains belong to genera *Macrococcus*, *Achromobacter*, *Aerococcus*, *Acinetobacter*, *Metabacillus* and *Paenibacillus*. The strains of *Macrococcus* and *Achromobacter* grow rather slowly, while the other four have relatively fast growth (Fig. 5). There is no report on the degradation of biphenyl by genera *Macrococcus*, *Aerococcus* and *Metabacillus* yet, while the strains isolated in this work can use biphenyl to grow, suggesting that some species of the three genera possess the ability to degrade biphenyl.

Conclusions

Microbial community was significantly varied during bacterial enrichment with biphenyl, suggesting that microbiome is rather sensitive to surroundings. A certain number of strains can be obtained from soil samples using both of the LIVEST single cell sorting and single colony sorting, while the genera of the isolates are rather different. Bacterial enrichment with specific substrate is an effective approach to discover some functional microorganisms, and the LIVEST is more time-saving and cell-targeted for single cell sorting.

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Declarations

Competing interests The authors declare that the research was conducted in the absence of any potential conflict of interest.

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