Preparation and Evaluation of a Composite Filler Micro-Embedded with *Pseudomonas putida* for the Biodegradation of Toluene

**Preparation of composite filler with high toluene removal efficiency**

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The main objective of this study was to evaluate the performance of a self-developed filler micro-embedded with *Pseudomonas putida* (*P. putida*) for toluene removal in a biofilter under various loading rates. The results show that the biofilter could reach 85% removal efficiency (RE) on the eighth day and remain above 90% RE when the empty bed residence time (EBRT) was 18 s and the inlet loading was not higher than 41.4 g m$^{-3}$ h$^{-1}$. Moreover, the biofilter could tolerate substantial transient shock loadings. After two shut-down experiments, the removal efficiency could be restored to above 80% after a recovery period of three days and six days, respectively. Sequence analysis of the 16S rRNA gene of fillers in four operating periods revealed that the highly efficient bacterial colonies in fillers mainly included *Firmicutes*, *Actinobacteria* and *Proteobacteria* and that the abundance of *Bacteroidetes* increased significantly during the re-start period.

1. Introduction

The massive discharge of volatile organic compounds (VOCs) has a great negative impact on the environment (1). Toluene is a common pollutant in VOCs and is produced in a large number of industrial activities, such as chemical refining and dye processing. Toluene stimulates skin and mucosa, and when it reaches a certain high concentration, it also causes paralysis of the human nervous system. Compared with photocatalysis and chemical oxidation, using a biofilter to remove VOCs is more economical and environmentally friendly (2). More important is that it does not produce secondary pollution. The key element to ensure the removal capacity of the biofilter is the preparation of the filler. As a carrier for the transfer of pollutants, the filler can provide a suitable growth environment for microorganisms (3).

Micro-embedding technology is a method which uses physical or chemical methods to keep microorganisms in a defined space, ensuring microorganisms with high activity. The principle of using micro-embedding technology to degrade VOCs is to use a hollow porous membrane to intercept microorganisms inside the filler. The pore size of the hollow porous membrane is smaller than that of microbial cells, so that microorganisms can be embedded. The VOCs can enter the interior of the embedded carrier freely due to the small particle size, and the degradation products can flow out of the carrier through the pore size (4, 5).

A large number of studies have been carried out on different types of fillers. Chen *et al.* (6) used a two-layer biofilter filled with new mixed packing materials to remove hydrogen sulfide gas. Dumont *et al.* (7) prepared a nutritional slow-release filler (UP20) to biodegrade H$_2$S. In the above studies, the fillers were not embedded with microorganisms. The concentration of microorganisms in the filler was small, and the removal efficiency of the biofilter was low in the start-up phase, resulting in a longer...
2.1 Preparation of Filler

The composite filler was mainly composed of polyvinyl alcohol, sodium alginate, polypropylene fibre, decomposed plants, calcium carbonate and activated carbon. First, polyvinyl alcohol and sodium alginate, as the embedding and protective agents, were heated, dissolved and cooled to 35°C. Then polypropylene fibre as the skeleton, decomposed plants as nutrients and calcium carbonate as the pH buffer were added into the liquid agent, respectively. Additionally, activated carbon and $P.\ putida$ BRJC1032 (screening from the activated sludge) were mixed with above agents to increase the physical adsorption capacity and biodegradation capacity of toluene. After that, the mixtures were stirred in a container for 15 min and extruded to spherical particles. Finally, these particles were cross-linked in boric acid-calcium chloride solution and dried at room temperature for 24 h. The composite filler micro-embedded $P.\ putida$ for toluene biodegradation. Existing problems with biofilters packed with fillers include bed clogging, low biomass concentration and pressure drops. These problems become more prominent when the biofilter is operated under high VOC loading rates or long-term operation (11). For example, Ryu et al. (12) found that the benzene removal efficiency of a well-designed biofilter decreased from greater than 90% to approximately 75% after 27 days of operation due to clogging caused by the excess growth of biomass. The main objective of this study was to evaluate the performance of a self-developed filler micro-embedded with $P.\ putida$ for toluene removal under various inlet loading rates. The variations in start-up period, pressure drop, biomass concentration and tolerance to transient shock loading were monitored throughout the experiments. Special attention was paid to the analysis of the microbial community attached to these fillers and to monitoring the evolution of the microbial community in various periods.

2.2 Experimental Setup

The experimental system used in this experiment is shown in Figure 1. Three biofilters were constructed with transparent organic glass pipes. Each biofilter consisted of three modules (each module is 105 mm in inner diameter and 500 mm in height), and all of them were filled with 300 mm composite fillers. A sampling port was set in the top of each module. Toluene gas was prepared by mixing fresh air with pure toluene in a mix chamber, and then introduced into the bottom of each biofilter through the three models in sequence. Three biofilters, namely biofilter 1 (BF1), biofilter 2 (BF2) and biofilter 3 (BF3), were used in this experiment to evaluate the start-up performance. BF1 was packed with the composite filler micro-embedded with $P.\ putida$, and both BF2 and BF3 were packed with the sterilised fillers without any microorganisms. However, the nutrient solution used for BF2 at the start-up period was mixed with the $P.\ putida$ suspension and the microbial concentration of the suspension was the same as that of the $P.\ putida$ suspension added in the preparation of the composite filler in BF1. Specially, nutrient solution (0.11 $K_2HPO_4$, 0.04 $KH_2PO_4$, 0.025 $NH_4Cl$, 0.067 $MgSO_4$, 0.036 $CaCl_2$, 0.25 $FeCl_3$, 0.03 $MnSO_4$, 0.04 $ZnSO_4$, 0.03 $(NH_4)_2MoO_4$·4$H_2O$; unit: g l$^{-1}$; adjusted to pH = 7.0 with NaOH) for microorganism growth was sprayed into the filler bed from the top of three biofilters throughout the experiment. The nutrient solution was intermittently sprayed onto the top of the three biofilters with a spray intensity of 1.5 l h$^{-1}$ by a peristaltic pump for one hour out of every three hours and the nutrient solution was changed every seven days.
2.3 Toluene Concentration Analysis

The determination of toluene concentration was carried out by adsorption of activated carbon and desorption of carbon disulfide, and then the toluene gas was injected into a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a packed column (free fatty acid phase (FFAP) capillary column, 30 m × 0.25 mm × 0.25 μm) and a flame ionisation detector (FID). The gas chromatography nitrogen was used as the carrier gas with a flow rate of 1 ml min\(^{-1}\). Temperatures of the injection port, column and detection port were set to 150°C, 65°C and 150°C, respectively. Gas samples were collected from the inlet and outlet of the biofilter with a gas-tight syringe and injected into the GC daily (14). Data were obtained from the workstation by automatic comparison of the peak area of the inlet and outlet samples with the baseline of toluene. The performance of the biofilter was evaluated in terms of (%) RE and the elimination capacity (EC) as a function of toluene loading. The RE and EC were calculated as in Equations (i)–(iii):

\[
\text{Removal efficiency} = \frac{(C_{in} - C_{out})}{C_{out}} \times 100\% \quad (i)
\]

\[
\text{Inlet loading} = \frac{Q \times C_{in}}{V} \quad (ii)
\]

\[
\text{Elimination capability} = \frac{Q \times (C_{in} - C_{out})}{V} \quad (iii)
\]

where the \(C_{in}\) and \(C_{out}\) are the inlet and outlet toluene concentration (mg m\(^{-3}\)), the \(V\) is the volume of the whole biofilter (l) and \(Q\) is the gas flow rate (l min\(^{-1}\)).

2.4 Physical and Chemical Property Analysis

The specific surface area and the porosity of the filler were measured by a surface area analyser (Gemini\textsuperscript{®} VII 2390, Micromeritics\textsuperscript{®,} USA). Solid samples were filtered and the pH value of the filtrate was detected using a Bioblock 90431 electrode connected to a C-835 Bioblock multiparameter analyser (Fisher Scientific, France).

The mechanical strength of the composite filler was measured by using a compressive strength-testing instrument (YHKC-2A, Taizhou Yinhe Instrument Plant, China). The pressure drop of the packed bed was measured using a digital pressure gauge (testo 510, Testo SE & Co KGaA, Germany) connecting two ends from the inlet and outlet. The pressure gauge had a measuring range of 0–100 kPa, a resolution of 1 Pa and an accuracy of ±0.3 Pa.

The saturated moisture content: some packing fillers were chosen randomly and immersed into distilled water for 2 h to adsorb as much water as possible. Then the packing fillers were removed and placed in a vacuum oven (DZF6050, Yiheng Scientific Instrument Co Ltd, China) at 105°C for at least 12 h until its weight remained stable.

The concentration of microorganisms in the filler was determined by plate counting. Approximately 10 g fillers were taken out homogeneously from the three modules of the running biofilter, and then put into a conical flask with 90 ml distilled
water. After that, the mixture was shaken in a thermostatic shaker bath for 2 h at 25°C to obtain the liquid containing microorganisms. Next, a series of solutions were prepared by different dilution factors (1, 10, 10², 10³, 10⁴ and 10⁵ times). Each 0.1 ml solution was taken and inoculated into three types of plate cultures (beef-protein, Rose Bengal medium and Gause’s No.1 medium) for bacteria, fungi and actinomycetes, respectively. The plates were placed in a biochemical incubator (CLIN-250, Tianjin Huabei Experimental Instrument Co Ltd, China) for 2–7 days at 28°C. Finally, the number of microorganism colonies in each plate was counted. Moreover, all the glass vessels used in this experiment were sterilised by using a seating automatic electro-thermal pressure steam steriliser (Model ZDX-35B, Shanghai Medical Instrument Manufactory, China) (15, 16).

2.5 DNA Extraction and Sequencing

Approximately 10 g fillers were randomly sampled from the lowest module of BF1 system at the 25th day, 65th day, 95th day and 145th day. Then the samples were sealed with aluminium foil and frozen at −4°C in a fridge.

Microbial DNA was extracted from the above four samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek Inc, USA) according to the manufacturer’s protocols. The final DNA concentration and purification were determined by a NanoDrop™ 2000 UV-vis spectrophotometer (Thermo Scientific™, USA), and DNA quality was checked by 1% agarose gel electrophoresis. Polymerase chain reaction (PCR) was conducted according to the following: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s of annealing at 55°C, 45 s of elongation at 72°C and a final extension at 72°C for 10 min. PCR was performed in triplicate in 20 μl mixtures containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM deoxyribonucleotide triphosphates (dNTPs), 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu Polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel, further purified using the Axygen® AxyPrep DNA Gel Extraction Kit (Corning Inc, USA) and quantified using QuantiFluor®-ST fluorometer (Promega, UK) according to the manufacturer’s protocol (16).

Purified amplicons were pooled in equimolar fashion and paired-end sequenced on a MiSeq platform (Illumina Inc, USA) according to the standard protocols established by Shanghai Majorbio Bio-Pharm Technology Co Ltd (Shanghai, China). The acquired sequences were compared with 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) database.

3. Results and Discussion

3.1 Physicochemical Properties of the Filler

Physicochemical properties of the experimental filler used in this study and some other materials from the references are listed in Table I (13). As shown in Table I, the experimental filler is spherical with a diameter of approximately 10 mm. The bulk density of the experimental filler is approximately 271 kg m⁻³, similar to that of pine bark, and lighter than most of the reference fillers. The mechanical strength is greater than that of pine bark but

<table>
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<tr>
<th>Table I Physicochemical Properties of the Fillers</th>
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<td>Filler</td>
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<tr>
<td>Experimental filler</td>
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<td>Pine bark (17)</td>
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<td>Lava rock (17)</td>
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<td>UP20 (7)</td>
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<td>Composite filler (8)</td>
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<td>Slow-release filler (16)</td>
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Due to the irregular shape of pine bark and lava rock, there is no corresponding size data. Other “—” data show that the author did not determine its physicochemical property data in the corresponding literature.
smaller than that of volcanic stone (>500 N) (17). The porosity rate is approximately 13%, which is significantly smaller than other fillers and helps toluene to better contact microorganisms in the filler when entering the biofilter (18, 19). The initial pH of the filler is 7.0 ± 0.2. The specific surface area is approximately 1.3 ± 0.1 m² g⁻¹, which is similar to that of lava rock and composite filler. Compared with lava rock, UP20 and slow-release filler (7, 8, 16), the saturated moisture content and organic matter rate are higher, which can provide water and nutrients for microorganisms in fillers. In addition, the decomposed plant fibre contained within the filler can provide nutrients for microbial growth during experimental operation (20). The selected microbial source added to the filler was _P. putida_, and the activated carbon was contained in fillers, which can adsorb toluene quickly, promoting toluene to enter the biofilter. The filler in the biofilter did not appear to have deformation, accumulation or other phenomena after operating approximately 150 days. The results indicated that the fillers had favourable properties as biofilter media, and maintained characteristics under long-term operation.

### 3.2 Start-up Performance

The removal efficiency of the three biofilters during the start-up period is presented in Figure 2. Three biofilters, operated at low toluene concentrations (100–120 mg m⁻³) and an EBRT of 35 s, demonstrated different removal performance for toluene at the start-up period. The removal efficiency of BF1 increased from the initial 40% to 80%, and stabilised between 82% and 85% after the eighth day (21, 22). The removal efficiency of BF2 showed a downward trend in the first few days and then rose to approximately 85% at the 14th day. The removal efficiency of BF3 gradually declined from the beginning, and it decreased to almost zero on the 16th–18th days (14, 23). The results showed that fillers embedded with activated carbon and polypropylene fibres have a certain adsorption capacity. However, the removal efficiency was gradually reduced when the filler reached adsorption saturation, as shown in the BF3 trend line in Figure 2. For the same reason, the BF2 line also showed a downward trend at the beginning. Due to the substantial growth of microorganisms, the subsequent removal efficiency gradually increased as shown in the BF2 trend line. Compared with BF2, the fillers in BF1 embedded with _P. putida_ showed unique degradation of toluene at the beginning. The filler-embedded microorganisms entered the working state faster than those cultured with the bacterial solution. These results indicated that the biofilter packed with the composite fillers prepared by micro-embedding could be quickly started up and the microorganisms in the biofilter could well utilise toluene as the carbon source (22).

### 3.3 Continuous Biodegradation Performance

Toluene continuous removal experiments were performed in three phases based on controlling the

![Fig. 2. Removal performances of BF1 (packed with the fillers micro-embedded with _P. putida_), BF2 and BF3 during the start-up period](image-url)
EBRT of BF1 to 35 s (Phase 1, day 10 to day 49), 18 s (Phase 2, day 50 to day 80) and 12 s (Phase 3, day 81 to day 110). The results of these experimental stages (Figure 3) are described below. Initially, the biofilter was operated at a low loading rate of toluene (10.5 g m\(^{-3}\) h\(^{-1}\)) corresponding to a low inlet concentration (100–120 mg m\(^{-3}\)) and high EBRT (35 s) to facilitate proper microbial growth and establish steady-state conditions (8, 23). Steady state was achieved on the 10th day of operation, which was evident from the constant value of the removal efficiency (83%). On the 18th day, the inlet concentration increased to 200 mg m\(^{-3}\), the removal efficiency was almost stable at 88% after a slight decrease. On the 28th day, the inlet concentration increased to 400 mg m\(^{-3}\), and the removal efficiency dropped rapidly to 72% and finally stabilised at 90% after five days of continuous operation. However, when the inlet concentration was controlled at 800 mg m\(^{-3}\), the removal efficiency did not reach a correspondingly high state (less than 80%). In Phase 1, the initial rapid increase within 90% of RE may be due to some extent to competition among microorganisms in the filter unit (14, 21, 23). Again, in Phase 2, the inlet loading rate was increased and maintained at 81.2 g m\(^{-3}\) h\(^{-1}\) with a corresponding EBRT of 18 s, and the toluene inlet concentration varied between 100 mg m\(^{-3}\) and 400 mg m\(^{-3}\). The removal efficiency reached a maximum when the inlet loading rate was less than 41.4 g m\(^{-3}\) h\(^{-1}\) and was stable above 90%. However, the removal efficiency was only slightly decreased and then stabilised close to 86% at the end of this phase. This result might be attributed to the decrease in residence time of toluene in the biofilter. At a higher flow rate, the contact time between the toluene and the microorganisms in the fillers was shortened and that resulted in deterioration of the biodegradation ability of the filter bed, leading to lower removal efficiency (24). Similarly, in Phase 3, the toluene inlet concentration increased from 100 mg m\(^{-3}\) to 400 mg m\(^{-3}\), and the intake load increased to 123.3 g m\(^{-3}\) h\(^{-1}\) with a corresponding EBRT of 12 s. During this phase, the removal efficiency of toluene gradually decreased to 80%, and no significant improvement in removal efficiency was observed (17, 22).

Elimination capacity, another important indicator of the biofilter, was also used to assess the ability of the biofilter in terms of toluene removal. Figure 4 demonstrates the relationship of elimination capacity upon the inlet loading. It could be seen from Figure 4 that the elimination capacity presented a slow increase with the increase of inlet loading rates. The maximum elimination capacity of the biofilter was 101 g m\(^{-3}\) h\(^{-1}\), which is better than other typical biofilters. For example, Zhu et al. (10) used composite packing materials to remove H\(_2\)S and observed a maximum elimination capacity of 65 g m\(^{-3}\) h\(^{-1}\). Liu et al. (18) reported compost-based biofilter with a maximum elimination capacity of 50 g m\(^{-3}\) h\(^{-1}\) for toluene.

The concentration of toluene in the nutrient solution was 0.3 ± 0.1 g l\(^{-1}\) (the saturated solubility of toluene in water was 0.5 ± 0.1 g l\(^{-1}\)). This may be due to the short contact time between toluene and the nutrient solution. In addition, part of the toluene dissolved in the nutrient solution was utilised by the filler with circulation of the nutrient solution.

Fig. 3. Time course of the inlet and outlet concentration and the removal efficiency of BF1
The above results showed that a sudden increase in the inlet loading will cause the removal rate to decrease within a certain period of time. As the experiment proceeds, the system will gradually return to a higher removal rate. When the microorganisms grew under suitable conditions, the recovery ability of the system also increased. However, when the inlet loading rate was too high, the degradation ability of the microorganisms was exceeded, resulting in a relatively low removal rate. After entering the biofilter, toluene is first adsorbed by activated carbon and biofilms in the filler, and then biodegraded by microorganisms in the filler. A certain amount of toluene will be dissolved in the nutrient solution, but with the circulation of the nutrient solution, part of the toluene will be degraded by the microorganisms in the filler again.

3.4 Tolerance for Transient Shock Loading

To test the ability of the biofilter to resist sharp load change, two interference-shutdown experiments were operated after running for 114 days.

Fig. 4. Toluene elimination capacity of BF1 versus the inlet loading

![Fig. 4. Toluene elimination capacity of BF1 versus the inlet loading](image)

\[ y = 0.7989x + 2.9602 \]

\[ R^2 = 0.995 \]

Fig. 5. Performance evaluation during shutdown and restart periods of BF1 under transient shock loading

![Fig. 5. Performance evaluation during shutdown and restart periods of BF1 under transient shock loading](image)
Figure 5 shows the performance evaluation during shutdown and restart periods of BF1 under transient shock loading. When the inlet toluene concentration decreased from 400 mg m\(^{-3}\) to 200 mg m\(^{-3}\), the removal efficiency increased to 90%. Then, the biofilter was subjected to a three-day shutdown experiment and the removal efficiency was restored to 81.2% after running three days. Compared with the shutdown experiments of Singh and Wang (22, 23), the interrupt experiment in this study better reflects the change of flow in actual operation. In the second experiment, when the inlet toluene concentration increased from 400 mg m\(^{-3}\) to 800 mg m\(^{-3}\), the removal efficiency decreased drastically to 62%, and time for the RE to reach at 80.9% was only six days after seven days of shutdown operation. This result clearly indicates that a certain amount of toluene absorbed in activated carbon was supplied to the microorganisms during the shutdown operation of the system, and the microbial activity was maintained; in addition, the decomposed plant fibres also provided a carbon source for the microorganisms, as found by Jorge and Livingston (25).

3.5 Biomass Concentration and Pressure Drop in the Biofilter

The attached growth biomass concentration and pressure inside the device were measured during 1–60 days in the biofilter, as shown in Figure 6. The pressure drop increased more obviously from 56 Pa to 373 Pa. The biomass concentration in the biofilter gradually increased from 5 \times 10^4 colony forming units (CFU) g\(^{-1}\) (the filler was placed in the refrigerator for 1 month, and the biomass concentration was reduced to 5 \times 10^8 CFU g\(^{-1}\)) to 4 \times 10^8 CFU g\(^{-1}\) on the 60th day, which was consistent with the trend in the pressure drop (24, 26). The above result indicates that the increase in system pressure drop was mainly due to the rapid growth in microbial biofilm formation and inlet loading rates. The efficient growth and reproduction of microbial biomass played an important role in the efficient operation of the system and the growth of the microorganisms affected the pressure drop across the packed bed and the ease with which the packed bed was clogged. Low biomass reduces the removal efficiency. In contrast, excess biomass reduces the space required for gas and liquid to pass through the biofilter, which leads to an increase in the system pressure drop (27). Although the biomass concentrations in the biofilter increased and the porosity of the system was reduced, this process did not cause blockage of the system and had no significant effect on the removal performance.

4. Bacterial Community Analysis

To explore the bacterial communities in the biomass attached to BF1, genetic sequencing analyses were carried out. Sequencing of 16S rRNA genes amplified from the active bacterial communities during the operational stages revealed 21 phyla, 41 classes, 96 orders, 184 families and 347 genera (28, 29). The community analysis at phylum level of the fillers is shown in Figure 7. The four operational stages were sampled at the...
25th day, the 65th day, the 95th day and the 145th day, where the 25th day, the 65th day and the 95th day had a different EBRT and the same inlet toluene concentration, and the 145th day was after two interference-shutdown experiments. The dominant phyla were Firmicutes (63.4 ± 8.7%), followed by Actinobacteria (14.6 ± 3.9%) and Proteobacteria (10.1 ± 4.2%). With decreased EBRT, the abundance of Firmicutes remained high, but the abundance of Actinobacteria decreased, and the abundance of Proteobacteria increased. This is mainly due to a reduction in residence time leading to the inability of microorganisms to fully utilise toluene, and a reduction in the carbon source leading to a change in the proportion of microorganisms (30). After two interference-shutdown experiments, the abundance of Bacteroidetes increased and the abundance of Proteobacteria decreased. This is due to a reduction in residence time leading to the inability of microorganisms to fully utilise toluene, and a reduction in the carbon source leading to a change in the proportion of microorganisms (30). However, in the start-up phase, the biofilter embedded with P. putida started quickly, and the removal efficiency of toluene remained high, which indicated that the added P. putida contributed to the efficient operation of the biofilter (33). These results indicated that the biomass could maintain itself by microbial community changes, and the rapid re-adaptation of the biofilter could contribute to the activity retention of its biomass during the starvation period.

5. Conclusions

A composite filler micro-embedded with P. putida was prepared and evaluated for the biodegradation of toluene. The biofilter packed with the fillers could start up quickly with 85% RE on the eighth day, and tolerate substantial transient shock loadings. The RE of the biofilter remained above 90% when
the EBRT was 18 s and the intake load was not higher than 41.4 g m$^{-3}$ h$^{-1}$. In the experimental period of 145 days, no filter plugging phenomenon was observed. Moreover, the high removal efficiency and elimination capacity contributed to rich bacterial communities for the efficient biodegradation of toluene. The communities mainly included *Firmicutes*, *Actinobacteria* and *Proteobacteria*, and the abundance of *Bacteroidetes* increased significantly during the recovery period. The composite filler exhibited favourable physicochemical properties in this experiment and its practicability in industrial engineering should be further investigated.

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**References**


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