Comparing biofilm reactors inoculated with Shewanella for decolorization of Reactive Black 5 using different carrier materials

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Abstract

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Shewanella was the dominant functional bacteria in the MBBR and PPC carriers, while highly efficient degrading microbial communities were observed on the biochar carrier. Overall, the physicochemical properties of the carrier materials had a significant impact on the microbial community in the reactor, which affected the degradation efficiency of RB5. These findings provide valuable insights into the optimization of biofilm reactors for dye-containing wastewater treatment.

Keywords: Biofilm reactors, *Shewanella* , decolorization, carrier materials, microbial community.

1 Introduction

Azo dyes are widely used in various industries, and the discharge of azo dye-containing wastewater poses a significant threat to human health and the environment. Microbial decolorization has been recognized as one of the most effective and environmentally friendly methods for treating azo dye wastewater (Pandey et al., 2007; Yin et al., 2016). Immobilization of microorganisms on the surface of a biofilm carrier has become an increasingly popular technology in this field. Microbial immobilization technology employs physical or chemical methods to restrict cell migration, and the carrier provides optimal conditions for microbial reproduction and a protective surface for the biofilm (Mjla et al., 2021; Shao et al., 2017). Embedded or attached microorganisms can create aggregates on the carrier's surface, enhancing the efficiency and stability of microbial dye treatment (Sarti et al., 2016).

The choice of carrier material is critical to the effectiveness of wastewater biological treatment. Various types of carrier fillers, including thermoplastic plastics, minerals, fiber materials, and waste materials, can be used in the production of carrier fillers (Jagaba et al., 2021). For example, the removal rate of Congo red dye by the low-density polyethylene-polypropylene (LDPE-PP) carrier-based moving bed biofilm reactor (MBBR) using microorganisms isolated from dye-polluted soil as inoculum was as high as 99.2% (Sonwani et al., 2021). The decolorization rate of Anthraquinone Blue RS by a continuous up-flow packed bed reactor using *Bacillus flexus TS8, Proteus mirabilis PMS,* and *Pseudomonas aeruginosa NCH* isolated from textile wastewater as inoculum and corn cob biochar as carrier material was up to 90% (Mohanty and Kumar, 2021). Studies have shown that the carrier is a medium for biofilm proliferation, and its surface and material characteristics determine the properties of the biofilm, which may affect the diversity of the biofilm community (Chu and Wang, 2011; Abu Bakar et al., 2020). The type of polymer used in the biofilm carrier has a significant impact on the performance of the reactor (Krsmanovic et al., 2021).

In this study, we investigated the effect of different carriers on the decolorization efficiency and degradation pathway of Reactive Black 5 (RB5) biofilm reactors inoculated with *Shewanella* india. We employed three different types of carriers, namely almond shell biochar, Moving Bed Biofilm Reactor (MBBR) polyethylene, and Porous Polyurethane Carrier (PPC), and examined their effectiveness in achieving high and stable decolorization rates. We analyzed the microbial community structure and identified highly efficient degradation strains in the reactors to investigate the variations in microbial composition among different carriers. Through 16S rDNA community analysis, we explored the composition of microorganisms on the carrier and highly efficient RB5 degradation bacterial communities in the reactor. The findings from this study will provide guidance for expanding the selectivity of biofilm reactor fillers and improving the decolorization efficiency of azo dyes.

2 Materials and methods

2.1 Carrier material

Almond shell biochar was produced through high-temperature pyrolysis of almond shells at 500 , with a particle size of 4-8 mesh and an ash content of less than 10. The MBBR carrier was purchased from a local supplier and was a high-density polyethylene carrier with strong hydrophilicity, a large specific surface area,

an excellent microbial growth environment, a high surface roughness, and a fast biofilm formation rate. The PPC carrier was a polyurethane porous gel carrier with an average diameter of 1cm, a bulk density of 12.6 kg/m^3, a specific surface area (Sa) of 4000 m^2/m^3, good corrosion resistance, and high specific surface area, and was not easily deformed.

To test the physicochemical properties of carriers, firstly, pH measurement was conducted by preparing a carrier-water solution with a weight ratio of 1:50, where 2 g of carrier was added to 100 mL of water. The mixture was allowed to reach sample equilibrium by standing for 45 minutes, and the pH value of the solution was measured to determine the pH value of the carrier samples. Three replicates were prepared for each group and mixed appropriately. This method was based on previous studies (Bharti et al., 2019; Cao and Harris, 2010). Secondly, scanning electron microscopy (SEM) analysis was conducted by cutting biofilm-adhered carriers into 5 mm thick slices. The cut carriers were fixed in a 2.5% glutaraldehyde solution at 4 for 3 hours, washed repeatedly with a phosphate buffer solution to avoid residual fixative, and then dehydrated using ethanol solutions of different concentrations (30%, 50%, 70%, 100%) for 10 minutes each. Three rounds of dehydration with anhydrous ethanol were then conducted. The dehydrated carriers were dried, sprayed with gold, and mounted onto the scanning electron microscope sample stage for observation. This method was employed to investigate the surface morphology and roughness of the carriers.

2.2 Reactor equipment

The reactor system (Figure 1) consisted of an inlet tank, fixed microbial reactor, outlet tank, insulation tank, and lift pump.*Shewanella* bacteria were inoculated into all three reactors using almond shell biochar, MBBR carrier, and PPC carrier as fillers. The fixed microbial reactor was made of organic glass pipes with a diameter of 6 cm, a height of 50 cm, and an effective volume of 1.4 L. The inlet water was pumped into the fixed microbial reactor by a constant flow pump. The inlet water contained 100 mg/L RB5 synthetic wastewater, with sodium formate and MSM as the carbon and nutrient sources and operated in a continuous flow mode. The outer wall of the fixed microbial reactor was wrapped with rubber tubing, and water in the insulation tank circulated through the rubber tubing to the outer wall of the fixed microbial reactor to ensure stable operation at 30-35 .

2.3 Reactor performance analysis

2.3.1 Measurement of decolorization rate

The hydraulic retention time in the reactor was maintained at 24 hours. The absorbance A_0 of RB5 wastewater before decolorization, i.e., in the inlet bucket, was measured at 595 nm. At the outlet of the reactor, 10 mL of effluent was collected, centrifuged for 15 min at 4000 g, and the filtered solution was measured for absorbance A at the maximum absorbance value of RB5 (595 nm). The decolorization percentage was calculated using the initial (A_0) and final (A) absorbances as follows:

$$\text{Decolorization rate}\,(\%) = \frac{(A_0 - A)}{A_0} \times 100\%\#\,(1)$$

2.3.2 Analysis of RB5 degradation pathways

In this study, we analyzed the differences in degradation pathways using three different techniques: Ultraviolet-Visible spectroscopic analysis (UV-Vis), Fourier transform infrared spectroscopy analysis (FTIR), and Liquid chromatography-mass spectrometry (LC-MS).

For the UV-Vis analysis, samples of influent and effluent (10 mL) from each reactor were collected and centrifuged at 4000 g for 15 minutes. After filtering through a 0.22 μm membrane filter, the samples were subjected to full wavelength scanning using a UV-Vis-NIR spectrophotometer to compare changes in the absorption peaks before and after dye decolorization.

For the FTIR analysis, samples of almond shell biochar, MBBR, and PPC reactor effluent (60 mL) were collected and centrifuged. The filtered liquid was evenly distributed in multiple plastic culture dishes and sealed with sealing film. The samples were then frozen overnight at -80 °C, removed from the freezer, and immediately dried in a drying oven until the sample moisture was completely vaporized. The dried samples were then analyzed using a Fourier transform infrared spectrometer.

For the LC-MS analysis, influent and effluent samples (10 mL) from the almond shell biochar, MBBR, and PPC reactors were taken and centrifuged at 4000 g for 15 minutes. The supernatant was concentrated by rotary evaporation and analyzed by LC-MS. Three replicates were performed for each sample. The LC-MS flow and parameter settings used were as follows: a liquid chromatography-mass spectrometry instrument was used for sample analysis, with the following conditions: chromatographic column: Acclaim 120–3 C18, 150 mm x 2.1 mm; mobile phase: pure water (A), methanol and 2.5 mM triethylamine acetate (TEAA, a mixture of acetic acid and triethylamine in equimolar amounts, B); flow rate: 0.25 mL/min; column temperature: 40 degC; mode: negative ion mode; scanning range: 50-1000 m/z. The mobile phase composition during elution was as follows: 0-30 min, 10-70% B; 30-40 min, 70-10% B; 40-45 min, 10% B. The column temperature was maintained at 40 degC. The injection volume was 50 μL.

2.4 Analysis of microbial community structure in reactors

For analysis of the microbial community structure, carrier materials from three stable biofilm reactors were sampled, with three replicates from each reactor, for a total of nine samples. High-throughput sequencing was conducted at BMK in Beijing using the Illumina HiSeq sequencing platform. The RB5-degrading bacterial communities were also isolated using the dilution spread method, with two replicates taken from each reactor for a total of six samples. These samples were also sequenced and compared with the sequencing data.

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the effect of carriers on reactor performance. Statistical significance was determined at $p < 0.05$.

3 Results and discussion

3.1 Reactor performance analysis

3.1.1 Decolorization performance

The decolorization performance of the three reactors was evaluated over a period of nearly 100 days, as shown in Figure 2. All three reactors showed high decolorization efficiency for RB5, but the MBBR reactor exhibited better decolorization stability. One-way ANOVA results indicated significant differences ($p < 0.05$) in RB5 decolorization rates among the three reactors, which was probably due to the fluctuation observed in the biochar and PPC reactors. At around day 21, samples of the carrier material were taken and preserved, and almond shell biochar was added to the biochar reactor to supplement the insufficient amount of almond shell biochar. Despite a slight decline due to the lack of biochar, the biochar reactor's discoloration rate for RB5 remained stable above 80%. Around day 70, partial detachment of the biofilm in the PPC reactor caused fluctuations in its treatment performance for RB5. Overall, all the reactors' treatment performance was relatively stable, but the partial detachment of the biofilm caused fluctuations in the biochar and PPC reactors. Obvious fluctuation in the decolorization rate was observed in the biochar and PPC reactors.

3.1.2 Analysis of RB5 degradation pathways

(1) UV-Vis analysis

The high concentration of dye in the influent exceeded the upper limit of the UV spectrophotometer; therefore, a four-fold dilution was necessary for both the influent and effluent samples. Figure 3 presents the full spectrum scan results of the diluted samples. The characteristic peaks of RB5 at 310 nm and 595 nm, which were caused by the auxochrome and chromophore of RB5, were significantly reduced (Droguett et al., 2020). The biological decolorization of the dye could have resulted from adsorption onto biochar or biodegradation. Biochar adsorption caused all peaks in the full spectrum to decrease proportionally, while biodegradation was accompanied by the disappearance of absorption peaks and the formation of new peaks (Chen et al., 2003). The UV-Vis absorbance of the biochar reactor effluent was slightly lower than that of the MBBR and PPC reactors in the 260-700 nm range, which could be attributed to the adsorption effect of biochar. The characteristic peaks of RB5 in the effluent at 310 nm and 595 nm gradually decreased and tended to be flat after passing through the biochar, MBBR, and PPC reactors, indicating that the auxochrome and chromophore of RB5 were destroyed, and the reactors had a biodegradation effect on RB5. A new characteristic peak was formed at around 250 nm and 380 nm in the effluent of the biochar, MBBR, and PPC reactors, which might have been due to the absorption peak of bacterial metabolic products or colorless intermediate products (Daneshvar et al., 2007). According to the location of the new absorption peak (200-400 nm), it could be preliminarily determined that they were likely to be monocyclic compounds, and it was speculated that substances such as amine might have been produced (Tan et al., 2016). It is speculated that there might be some similarity in the effluent products between the biochar reactor and the MBBR/PPC reactors.

(2) FTIR analysis

The FTIR analysis (Figure 4) revealed changes in the positions and intensities of certain functional groups in the effluent of biochar, MBBR, and PPC reactors. In the high wavenumber region (4000-2500 cm^{-1}), stretching vibrations of O-H, N-H, C-H, and S-H bonds were typically observed. The peak at 3386.7 cm^{-1}, which might have corresponded to the stretching vibration of -NH_2 bonds, appeared as a broad peak. The variation in peak area indicated differences in the content of functional groups. The range of stretching vibration of C-H bonds was 2850-2710 cm^{-1}, with the peak at 2829.67 cm^{-1} corresponding to the stretching vibration of -CH_2 bonds. The peak at 2829.67 cm^{-1}in the effluent of biochar, MBBR, and PPC reactors was smaller than that in the influent, indicating replacement of some -CH_2bonds.

In the wavenumber region below 1900 cm^{-1}, stretching vibrations of -C=C-, -C=N-, -C=O bonds, and skeletal vibrations of aromatic rings were observed, indicating the presence of benzene rings. The peak at 1589.77 cm^{-1} might have corresponded to the stretching vibration of -N=N- bonds, which became weaker after decolorization, indicating that -N=N- bonds might have been destroyed during the degradation process. The stretching vibration of the R-SO_2-R group was observed in the range of 1370-1290 cm^{-1} (Martorell et al., 2017). Additionally, a weaker peak at 1117.43 cm^{-1} might have corresponded to the bending vibration of -OH bonds. The characteristic peak at 835.20 cm^{-1} in the effluent of biochar, MBBR, and PPC reactors suggested the presence of polycyclic aromatic hydrocarbons, and the appearance of a small peak at 696.62 cm^{-1}indicated the occurrence of out-of-plane bending vibrations of the N-H bonds of amine compounds. The effluent of biochar, MBBR, and PPC reactors might have generated similar compounds.

(3) LC-MS analysis

Figure 5 displays the liquid chromatography profiles of the influent, biochar, MBBR, and PPC reactors. The chromatograms illustrate significant variations between the effluents of the biochar reactor and the MBBR and PPC reactors. In the MBBR and PPC reactor effluents, a prominent peak was observed around 9.86 min, which was absent in the influent chromatogram. This indicates that the production of organic compounds with higher concentrations occurred in the MBBR and PPC effluents that were not present in the biochar reactor effluent. It should be noted that the soluble nature of RB5 in water prevented its extraction in the organic phase, and therefore it was not detected in the influent liquid chromatography.

Based on the findings in Figure 6, it can be concluded that there was a significant increase in the ion peak at m/z 399 in both the MBBR and PPC reactor effluent at 9.86 min. The molecular ion peak $[M\text{-}H^+]^+$ at m/z 399 suggested a relative molecular mass of 400 for this substance, indicating that it was only present

in the effluent of the MBBR and PPC reactors. It is tentatively suggested that this substance might be a benzene-containing structure formed by further ring-opening of the naphthalene ring structure in the active black 5 molecule after the double azo bond was opened. The maximum absorption peak at around 250 nm in the characteristic absorption of the benzene ring supports this conclusion. However, the results also indicate that the MBBR and PPC reactors were unable to completely mineralize the dye, and additional reaction systems may be required to achieve thorough degradation.

The UV-Vis and FTIR analysis results indicated that the effluent from the biochar reactor shared similarities with that of the MBBR and PPC reactors. This suggests that these reactors may have produced similar substances during the RB5 degradation process. However, the LC-MS analysis showed significant differences between the biochar reactor and the MBBR and PPC reactors. While parallel sample repeatability was good, the degradation pathways of RB5 in the biochar and polymer carrier reactors differed to some extent. High-efficiency degradation bacterial isolation results showed that the microbial species responsible for the RB5 degradation in the polymer carrier MBBR and PPC reactors were similar. Therefore, it is possible that the differences and overlaps in the degradation mechanism can be attributed to the presence of high-efficiency degradation bacteria. The carrier material could have an impact on the high-efficiency degradation bacterial community in the reactor, which may affect the RB5 degradation mechanism.

3.2 Analysis of microbial community structure in reactors

3.2.1 Analysis of microbial community structure on the carrier

(1) Statistical analysis of diversity indices

Based on Table 1, the ACE and Chao1 indices indicated that P1 had the highest richness, while Simpson and Shannon indices showed that P1 had good evenness. M2 and P1 had similar richness, as shown by the ACE and Chao1 results. A1 had good evenness according to the Simpson index, while P1 had good evenness according to the Shannon index. Overall, the richness of microorganisms on the biochar carrier was lower than that on MBBR and PPC, possibly due to the smaller pores of the almond shell biochar carrier, which could be easily covered by a dense biofilm, limiting dissolved oxygen diffusion into the inner layer of the carrier, and leading to decreased overall richness. Simpson and Shannon observations showed that the evenness of microorganisms on MBBR carriers was slightly lower than that on biochar and PPC carriers. The PD_whole_tree results indicated that the abundance of microorganisms on MBBR and PPC was higher than that on biochar, suggesting differences in microbial diversity among different carriers.

(2) Species distribution histogram

Based on the results presented in Figure 7a, the dominant phyla on the three different carriers were *Proteobacteria* , *Firmicutes* , and *Bacteroidetes* . Specifically, the biochar carrier had a higher proportion of *Firmicutes* (53.3±2.0%),while the MBBR and PPC carriers had relatively abundant *Proteobacteria* and*Bacteroidetes* . Previous studies had shown that*Proteobacteria* (Wang et al., 2008a) and *Firmicutes*(Ramalho et al., 2007a; You and Teng, 2009) were effective in degrading RB5, and a mixed community of *Bacteroidetes* could also effectively degrade RB5 (Forss et al., 2013). *Synergistetes* were more abundant on the biochar carrier, while *Chloroflexi* bacteria were more commonly found on the MBBR and PPC carriers.

At the family level (Figure 7b), the dominant families on the biochar carrier were *f-Family_XIV* , *f-Peptococcaceae* , and*f-s74e-6049* , while on the MBBR carrier, the dominant genera were*Paracoccus* , *Flavobacterium* , and *Taibaiella* , and on the PPC carrier, the dominant genera were *Paracoccus* ,*f-Peptococcaceae* , *Desulfuribacillus* , and*Desulfitibacter* . *Paracoccus* had been shown to effectively degrade dyes (Bheemaraddi et al., 2014). The results suggested that the carrier played an important role in the growth and enrichment of microorganisms. Different carriers had different microbial functional structures, depending on the characteristics of the carrier material, porosity, surface area, and other factors (Wang et al., 2014). In addition, there were very low levels of *Shewanella* on all three reactor carriers, with only

0.01%+-0.004%, 0.01%+-0.01%, and 0.004%+-0.006%, respectively, which might have been due to competition between different genera (Hibbing et al., 2010; Zhu et al., 2019).

The microbial community structure on the carriers in this study was complex, and the efficient degrading bacteria for RB5 was likely to be a microbial community. Previous studies had shown that microbial communities could effectively degrade RB5. For example, the natural microbial community DDMZ1, composed of *Burkholderia* ,*Achromobacter* , *Stenotrophomonas* , *Pseudomonas* , and*Enterobacter* , showed stable decolorization efficiency for RB5 (Zhang et al., 2019). Another study isolated a microbial community consisting of *A. hydrophila, R. mucilaginosa* , *G. pseudocandidum* , and *E. coli* from dye wastewater and found that this community achieved a decolorization rate of over 95% for RB5 within 24 hours, with a more stable decolorization effect than a single strain (Florez Restrepo et al., 2018). Compared to single strains, mixed microbial communities had stronger advantages in completely mineralizing azo dyes due to the synergistic effect between different degrading microbial communities, which could better degrade azo dyes (Xie et al., 2020; Saratale et al., 2010).

3.2.2 Isolation of high-efficiency degradation bacterial community

The effluent from the reactors was acclimatized with increasing concentrations of RB5 (Figure 8), starting from 100mg/L. After 8 hours, no significant discoloration effect was observed, and the OD_{600} did not show a significant increase. However, gradual discoloration began to appear in the effluent of all three reactors after 8 hours. At a concentration of 200 mg/L, discoloration was observed in the effluent of all three reactors at 4 and 8 hours. At a concentration of 300 mg/L, discoloration first appeared in the MBBR reactor group at 4 hours, with MBBR reactor group having a slightly higher OD600 than biochar reactor group and PPC reactor group. This early discoloration may be attributed to the effect of acclimatization and the accumulation of a large number of degrading bacteria. At 24 hours, all three reactors had completed discoloration, with MBBR reactor group having the highest OD600. At 48 hours, the discoloration rate of all three reactors was nearly 97%, with MBBR reactor group showing the highest OD_{600}, indicating the completion of acclimatization. Subsequently, the enriched liquid after acclimatization was diluted and spread plated five times, resulting in the isolation of highly efficient RB5-degrading bacterial communities from the effluent of the three reactors.

3.2.3 Analysis of the RB5 efficient degradation bacterial community structure

(1) Statistical analysis of diversity indices

Table 2 shows that the ACE and Chao1 indices were highest in M1 and M2 in the MBBR reactor, indicating a higher abundance of the microbial community with high RB5 degradation efficiency in the MBBR reactor. Additionally, the Simpson and Shannon indices were also highest in M1 and M2, suggesting greater diversity in the MBBR reactor. In contrast, A1 and A2 had the lowest abundance based on the ACE and Chao1 indices. Overall, the abundance of the microbial community with high RB5 degradation efficiency was smaller in the biochar reactor than in the MBBR and PPC reactors based on the ACE and Chao1 indices. However, based on the Simpson and Shannon indices, the functional bacterial composition was more complex in the MBBR reactor than in the biochar and PPC reactors, indicating greater diversity in the MBBR reactor. The PD_whole_tree analysis showed that the abundance of the three reactors was similar.

(2) Species distribution histogram

At the phylum level, Figure 9a shows that in the efficient RB5 degrading bacterial communities of the biochar reactor, *Clostridiales* and*Pseudomonadales* had the highest proportions, accounting for nearly 100% of the community. In the MBBR efficient RB5 degrading bacterial community, *Pseudomonadales* accounted for approximately 27-30%, *Enterobacterales* accounted for approximately 23%,*Burkholderiales* accounted for approximately 25-28%, and*Bacillales* accounted for approximately 20%. In the PPC reactor's efficient RB5 degrading bacterial community, *Pseudomonadales*accounted for approximately 65-67%, and *Enterobacterales*accounted for approximately 31-33%. The biochar reactor had a higher abundance of *Clostridiales* than the other reactors, but*Enterobacterales* were not detected in the biochar reactor.*Burkholderiales*

and *Bacillales* were present in higher numbers in the MBBR reactor than in the biochar and PPC reactors.*Burkholderia* , a member of the *Burkholderiales* order, can synergistically degrade RB5 with other bacterial species, which may be why the MBBR reactor showed the earliest decolorization effect among the efficient RB5 degrading bacterial communities.

According to Figure 9b, at the genus level, the highly efficient degrading bacterial communities in the biochar reactor were dominated by the *Pseudomonas* genus (approximately 62-63%), followed by the*Clostridium_sensu_stricto_1 genus* (approximately 35-37%). In the MBBR reactor, the dominant genera were *Pseudomonas*(approximately 27-30%), *Shewanella* (approximately 23-24%),*Delftia* (approximately 25-28%), and *Bacillus*(approximately 20%). In the PPC reactor, the dominant genera were*Pseudomonas* (approximately 65-67%) and *Shewanella*(approximately 31-33%). The *Shewanella* genus was not present in the biochar reactor but accounted for a large proportion in the MBBR and PPC reactors, possibly due to the electron transfer efficiency and affinity between *Shewanella* and the carrier (Shi et al., 2007).*Clostridium sensu stricto 1* was found only in the biochar reactor and was the dominant species. In the MBBR reactor, a large number of*Delftia* and *Bacillus* species were detected, but they were almost absent in the biochar and PPC reactors. *Pseudomonas* , which was present in all three reactors, had been extensively documented to have good decolorization effects on RB5 (Wang et al., 2008b; Hussain et al., 2013). *Bacillus* and *Delftia* were also capable of effectively degrading RB5 and solubilizing phosphate in the substrate, respectively, indicating their potential synergistic action (Dafale et al., 2008; Liao et al., 2013). In bottle experiments, the efficient degradation microbial community in the MBBR reactor showed earlier decolorization of RB5 than those in the biochar and PPC reactors, possibly due to the diverse functional bacteria present. The efficient degradation microbial communities in the MBBR and PPC reactors had high similarities and had consistent degradation mechanisms.*Shewanella* , which was artificially inoculated as an RB5-degrading bacterium during reactor setup, did not become the dominant species on the reactor carrier, but screening showed that it was a dominant functional bacterium for RB5 degradation in both MBBR and PPC reactors, working in conjunction with other functional bacteria to effectively degrade RB5.

3.3 The impact of carrier physicochemical properties on reactor performance

Microbial attachment and fixation on carrier surfaces is an important aspect of reactor performance, influenced by both the physicochemical properties of the carriers and the surface characteristics of the microorganisms. Previous studies have shown that carrier roughness, pH, and pore size affect the growth and stability of biofilms in packed bed reactors (Al-Amshawee et al., 2021; Jaroszynski et al., 2011).

Almond shell biochar showed slightly alkaline pH, with a pH value of 8.28+-0.20. It is worth noting that most biochar samples reported in the literature had an alkaline pH value (Greenough et al., 2021). The pH of the MBBR carrier was 7.41+-0.22, which was also slightly alkaline. Alkaline conditions were conducive to microbial growth and the development of microbial communities on the surface of the MBBR carrier, as well as the formation of biofilms and diverse functional bacteria (Dai et al., 2021). The pH of the PPC carrier was 6.80+-0.04.

Surface roughness was an important factor in carrier selection and reactor design, as it influenced mass transfer efficiency, effective contact area, and protection of microorganisms (Messing and Oppermann, 1979). As shown in Figure 10, biochar carrier had a highly porous surface with small pore sizes (0.1 - 0.5 μm), which might limit the initial growth of microorganisms (Messing and Oppermann, 1979). In the reactor that effectively degraded bacteria with a length of about 0.6 to 1.5 μm, these pores could not serve as their living place, and microorganisms attached to the top or nearby areas of the pores. After the reactor had been in operation, these pores were filled with microbial metabolites, and the pore structure was no longer obvious, resulting in the appearance of a large number of spherical substances. Most of the surface of the biochar carrier was covered with a layer of attached biofilm growth, and there were rod-shaped bacteria present on the surface and grooves of the biochar carrier.

In contrast, MBBR carriers had many protrusions, grooves, and micro-cracks on their surface, and their

overall structure had a toothed shape. The high surface roughness of the carrier resulted in slow growth of biofilm on it, which could form a biofilm structure that was more resistant to flushing (Al-Amshawee et al., 2020). There were mainly two reasons for this. Firstly, compared with smooth surfaces, the rough surface of the carrier increased the effective contact area between bacteria and the carrier. Secondly, the rough parts of the carrier's surface, such as holes and cracks, protected the attached bacteria (Al-Amshawee et al., 2021). After the reactor operated, microorganisms accumulated in large numbers on the carrier surface, and the biofilm structure became compact, with spherical and rod-shaped bacteria interweaving and growing together. As the biofilm matured, it became more stable and less likely to detach, which was beneficial for the sustained and stable operation of the reactor.

The PPC carriers had an average diameter of 1cm and a relatively smooth surface, but with many large pores ranging from 0.5 to 1.5 μm in diameter. These pores could serve as attachment and survival sites for microorganisms, providing protection against hydraulic shear stress. However, some studies had shown that the porous structure of biofilm carriers might have led to low mass transfer efficiency (Cao et al., 2017). After the reactor operates, these large pores were filled with a large number of rod-shaped bacteria, which grew abundantly and covered the pores and their surroundings. The microbial abundance on the surface of PPC carriers was relatively high, and this carrier could effectively enrich microorganisms. However, there was a risk of the biofilm being washed away from the polyurethane carriers (Al-Amshawee et al., 2020).

Initially, all carriers had clean surfaces without any attached biofilms. After reactor operation, dense biofilms formed on all carriers. The pore size of the almond shell biochar was too small to serve as a habitat for microorganisms. As a result, the abundance of microorganisms on the almond shell biochar carrier was lower than that on the MBBR and PPC carriers. The surface of the MBBR carrier was rougher than that of the PPC carrier, and the rough surface reduced the possibility of biofilm detachment (Huang et al., 2018). The MBBR carrier could form a robust and impact-resistant biofilm structure. At the same time, the surface heterogeneity of the MBBR carrier provided a better living space for microorganisms and increased the utilization rate of adsorbed organic matter by attached microorganisms (Dutta et al., 2014). Therefore, microorganisms could more easily utilize adsorbed compounds and convert them into final products.

4 Conclusions

This study aimed to investigate the effectiveness of biofilm reactors inoculated with azo dye degrading *Shewanella* and using different carrier materials (biochar, moving bed biofilm reactor (MBBR), and polypropylene carrier (PPC)) for the decolorization of Reactive Black 5 (RB5). The results showed that all three carriers exhibited good RB5 decolorization performance, with varying degrees of efficiency. Although the effluent from the biochar reactor and the two polymer carrier reactors showed some similarity in UV-Vis and FTIR results, LC-MS analysis revealed significant differences in the degradation pathways of RB5, indicating the role of carrier materials and microbial communities in the process.

The MBBR carrier had a rough surface with a large number of microbial aggregates clustered at the protruding tooth-like positions, which intertwined with each other and were not easily detached, resulting in good stability of MBBR operation. Furthermore, the sequencing results showed that the carrier material had a significant impact on the microbial structure in the reactor. The differences in the microbial community structure were relatively small between the MBBR and PPC carriers compared to the almond shell biochar carrier. The highly efficient degrading microbial communities on the biochar carrier were markedly different from those on the MBBR and PPC polymer carrier reactors, with *Shewanella* being the dominant functional bacteria in the MBBR and PPC polymer carrier reactors.

Overall, this study demonstrated that the physicochemical properties of the carrier materials had a significant impact on the microbial community in the reactor, which in turn influenced the degradation efficiency of RB5. The findings provide valuable insights into the optimization of biofilm reactors for the treatment of dye-containing wastewater.

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Conflicts of Interest

The authors declare no conflict of interest.

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