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Sustained degradation of phenol under extreme conditions by polyurethane-based *Bacillus* sp. ZWB3

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ABSTRACT

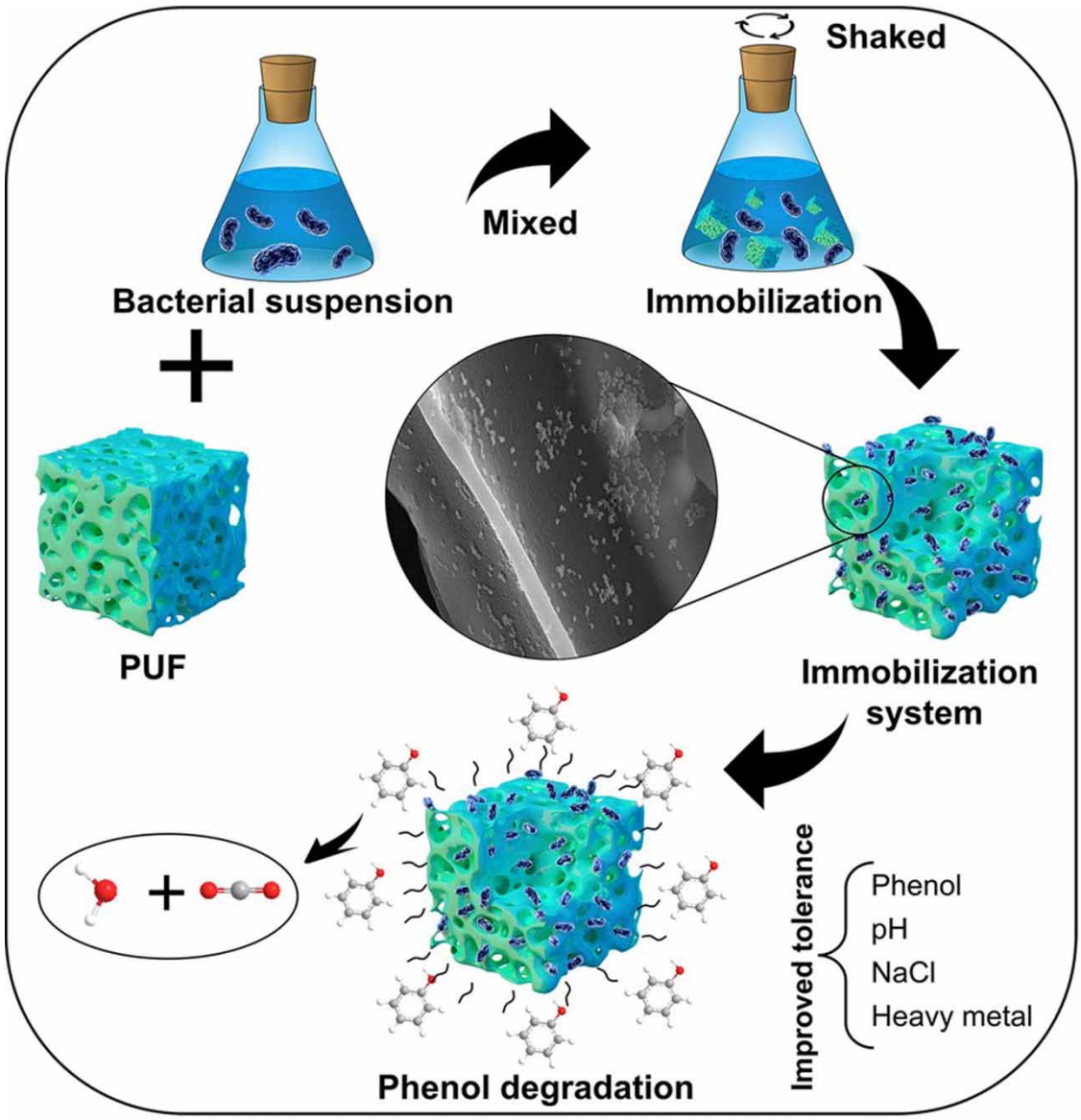
Phenol is a serious pollutant to the environment, therefore, it is urgent to find a rapid and effective method for its removal. In this study, *Bacillus cereus* ZWB3 immobilized on a polyurethane (PUF) carrier was studied. The PUF-ZWB3 required only 20 h for the degradation of 1,500 mg L^{-1} of phenol, shortened by 8 h than the free bacteria. In addition, the PUF-ZWB3 could increase the degradation concentration of phenol from 1,500 to 2,000 mg L^{-1}, and the complete degradation of 2,000 mg L^{-1} phenol only used 44 h. In addition, the PUF-ZWB3 showed much higher removal of phenol than the free bacteria at different pH values, salt concentrations, and heavy metal ions. Particularly, the PUF-ZWB3 could still completely remove phenol in a strongly alkaline environment, such as pH 10 and 11. In addition, the removal efficiency of phenol by PUF-ZWB3 was still 100% after 10 cycles. This study showed that the PUF immobilization system had great potential in the field of remediation of organic pollution.

Key words: biodegradation, immobilization, phenol, polyurethane

HIGHLIGHTS

- PUF can increase the degradation rate and concentration of phenol by ZWB3.
- The PUF-ZWB3 system can effectively degrade high concentrations of phenol at a higher pH.
- The PUF-ZWB3 system has an excellent cyclic degradation performance.

GRAPHICAL ABSTRACT



INTRODUCTION

Phenol is one of the universal aromatic pollutants in the environment and a by-product of many industrial processes (Long *et al.* 2019; Mei *et al.* 2019; Nogina *et al.* 2020). Due to its toxicity and persistence, phenol is not only harmful to plants and animals but also can cause cancer (Nouri *et al.* 2020; Li *et al.* 2021). Thus, the complete removal of phenol from industrial wastewater, before being discharged into the environment, is necessary for maintaining a healthy ecosystem.

Recently, lots of research works have been investigated to remove phenol from wastewater (Wu *et al.* 2018; Gracioso *et al.* 2019; Liang *et al.* 2020; Mareai *et al.* 2020; Nogina *et al.* 2020), such as physical, chemical, and biological methods. Compared with other methods, biological technologies which have the ability to completely mineralize contaminants without causing secondary pollution to the environment, are more cost-effective. Among them, microbial degradation has been proven to be a practical, economical, and environmentally friendly method, for converting phenols by adding exogenous dominant degrading bacteria or indigenous microorganisms to pollution (Su *et al.* 2019; Barik *et al.* 2021; Gong *et al.* 2021). However, microbial biodegradation is inhibited by high concentrations of phenol. Exposure to high concentrations

of phenol exceeding what bacteria can tolerate, would result in that bacteria needing a long adaptation period to grow and proliferate, further decreasing the degrading ability of phenol (Shahryari *et al.* 2018). In addition to phenol, industrial effluents possess uncertain pH and salinity, and heavy metals as co-pollutants, which are not only highly toxic, persistent and non-degradable, but also severely inhibit the degradation process. For example, Liu *et al.* (2020) investigated the effect of pH on the degradation of phenol by *Acinetobacter* APH1 and found a long delay in the degradation at pH 9, which was unable to degrade phenol in acidic or alkaline environments such as pH 5 and pH 10. Deng *et al.* (2018) evaluated the salt tolerance of *Citrobacter* and showed that an increase in salt concentration resulted in a significant decrease rate of phenol, which was completely inhibited with a salt concentration of 12%. Heavy metal ions also have a serious effect on the degradation of phenol by microorganisms, for example, heavy metals Co^{2+} and Ni^{2+} inhibited the degradation of phenol by *Debaryomyces* JS4, which could only degrade 19.1 and 15.1% (Jiang *et al.* 2016). In addition, the genotoxicity of phenol makes the sequential batch degradation of free bacteria become less stable (Ghorbannezhad *et al.* 2018). Thus, these unsuitable factors become a bottleneck for phenol bioremediation.

Microbial immobilization technology (MIT) has attracted attention which adheres to microorganisms on specific materials by physical or chemical methods (Bouabidi *et al.* 2019). MIT has been widely used for the degradation of phenol, showing many advantages, such as low cost, wide applicability (Li *et al.* 2015), and a high amount of biomass on the carrier for efficient degradation (Ghorbannezhad *et al.* 2018). Moreover, MIT makes the microorganisms significantly more tolerant to phenol, which results in the treatment of phenol being more complete and without secondary pollution. In addition, the immobilization of bacteria increases their stability, which is easier to recover and recycle (Emelyanova & Solyanikova 2020). Usually, most studies focused on the concentration of phenol at 250–1,500 mg L^{-1} (Jiang *et al.* 2017a; Ghorbannezhad *et al.* 2018; Gomes e Silva *et al.* 2019). While, the degradation of phenol at high concentrations by immobilized microorganisms has rarely been reported. In addition, the complexity of the actual wastewater, such as undesirable pH, heavy metals, and salts, destroy the growth stability of microorganisms and the structure of carrier materials, which can lead to problems such as short life and poor reusability, hindering the practical application of immobilized microorganisms. Gomes e Silva *et al.* (2019) reported that fruit pomace immobilizing microorganisms could degrade phenol for eight cycles, and the degradation rate decreased gradually from the third cycle. Meanwhile, many studies pay attention on the mild conditions for phenol degradation, lacking consideration of realistic factors such as the interference of adverse external environment. Thus, it is necessary to construct an efficient immobilized cell system to improve the tolerance of microorganisms under high concentration phenol and adaptability under extreme environments, for better application in the remediation of phenol wastewater.

The choice of carriers and strains determine the effectiveness of phenol degradation. Therefore, many carriers have been developed, such as polyurethane (PUF). PUF is a porous medium, whose preparation technology is mature and in low cost, becoming an excellent carrier for microbial attachment and growth (Moghaddam & Naimi-Jamal 2017; Joy *et al.* 2020; Zhao *et al.* 2021). It has been reported that the use of PUF as a carrier to treat organic pollutants can improve the degradation and recyclability of microorganisms, for high concentrations of toxic substrates. For example, PUF-immobilized *micrococcus* SMN-1 could degrade high concentrations of 2-nitrotoluene more efficiently, and has a better stability with 24 cycles of reuse (Mulla *et al.* 2013). Another study claimed that PUF-immobilized microorganisms have good resistance to acid and alkali, heat and salt, during the degradation of toxic pollutants (Zheng *et al.* 2009b). Therefore, PUF is an ideal carrier for the biological treatment of phenol wastewater, which is expected to achieve efficient microbial degradation and rapid adaptation of microorganisms to the extreme environment and genetic stability.

The main challenge in making the bioremediation process applicable is to consider many issues related to practical situations. To address the drawbacks of microorganisms, we established a PUF-immobilized microbial system for phenol wastewater remediation, to improve the efficiency of phenol degradation and stability during reuse. In addition, the tolerance of immobilized bacteria to various pH, salt concentration and heavy metal ions was investigated. The aim is to explore a method of immobilized cells in phenol wastewater treatment, which provides an important basis and pathway for the degradation of phenol in wastewater.

MATERIALS AND METHODS

Materials

Phenol (99%, w/v), KH_2PO_4, K_2HPO_4, NaCl, NaOH, and HCl were obtained from Sinopharm Group (China). Tryptone and yeast extract were obtained from Hangzhou Best Biotechnology Co. Ltd. $FeCl_3$, NH_4NO_3, agar, 4-aminoantipyrine

(98.5%, w/w), potassium ferricyanide, $NH_3{\cdot}H_2O$, $MgSO_4$ and $CaCl_2$, were the products of Yongsheng Fine Chemicals Co. Ltd., Beijing Chemical Reagent Co. Ltd., Beijing Aobox Biotechnology Co. Ltd., Shanghai Yuanye Biotechnology Co. Ltd., Yongda Chemical Reagent Co. Ltd., Xilong Chemical Co. Ltd., Beijing Chemical Works and Kermel Chemical Reagents Co. Ltd., respectively. The PUF was purchased from Jiangsu Yingzi Environmental Protection Factory.

Source of strain and cultivation conditions

The strain *Bacillus cereus* ZWB3 (ON738664) preserved in our laboratory was isolated and purified from the contaminated soil near the sewage discharge outlet of Hongqi Plastic Factory in Anqing City, Anhui Province, China. The strain ZWB3 was cultured in inorganic salt medium (MSM: 0.2 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2$, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} K_2HPO_4, 0.05 g L^{-1} $FeCl_3$, 1 g L^{-1} NH_4NO_3, 1 g L^{-1} phenol, pH 9), in which phenol (0.5 g L^{-1}) acted as the sole carbon source, under the inoculation amount 5%, 37 °C, and 180 rpm.

Methods of immobilization of bacteria

The PUF was cut into small squares of 1 cm × 1 cm × 1 cm, soaked in 95% ethanol solution for 30 min, repeated twice to remove the organic matter on the PUF, then washed with sterile water three times, and finally dried in an oven at 80 °C. The treated PUF and the MSM (1 g L^{-1} phenol, pH 9) were sterilized in an autoclave at 121 °C for 20 min, respectively. *B. cereus* ZWB3 were cultured using MSM with phenol as the sole carbon source (inoculation amount 5%, initial pH 9, temperature 37 °C, 180 rpm). The fermentation broth containing the strain was then centrifuged at 5,000 rpm for 20 min to remove the supernatant, then the strain was resuspended in phenol-free MSM. Sterilized PUF with 100 mL of strain suspension were mixed in a constant temperature oscillation incubator at 30 °C and 120 rpm for 6 h. The PUF with adsorbed bacteria was filtered out, and stored at 4 °C. The linear relationship between the number of bacteria and optical density can be obtained by dilution coating plate counting method, and the number of immobilized bacteria was determined according to the linear curve (Yue *et al.* 2013). The number of immobilized bacteria on PUF was 1.2×10^9 CFU/g.

Degradation of phenol by PUF-immobilized ZWB3

0.5 g of immobilized material was added to 100 mL of MSM medium with phenol as the sole carbon source. The phenol concentrations were set at 1,200, 1,500, 1,800, 2,000, and 2,200 mg L^{-1}. Finally, the immobilized bacteria and free bacteria were placed in a constant temperature oscillating incubator at 37 °C, pH 9, and 180 rpm for phenol degradation, and samples were taken at regular intervals to determine the concentration of remaining phenol. The other control group was a PUF adsorption phenol experiment. Each group of experiments was set up in three parallel.

Effect of pH on the degradation of phenol by immobilized ZWB3

The pH was adjusted to 6, 7, 8, 9, and 10, with 1 mol L^{-1} HCl or NaOH. After that, the immobilized microbial materials and free bacteria were inoculated in different pH medium with phenol concentration of 1,200 mg L^{-1} under 37 °C and 180 rpm. Samples were taken at regular intervals to detect the remaining phenol concentration. Each group of experiments was set up in three parallel.

Effect of salt concentration on the degradation of phenol by immobilized ZWB3

NaCl was added to the MSM at 1, 3, 5 and 7% (w/v), respectively, and the phenol concentration was 1,200 mg L^{-1}. The immobilized bacteria and free bacteria were cultured at the above condition.

Effect of heavy metals on the degradation of phenol by immobilized ZWB3

The MSM was added with 5 mmol of metal ions Cd^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+}, which were added as salts of $CdCl_2$, $MnCl_2{\cdot}4H_2O$, $CuCl_2{\cdot}2H_2O$, $ZnCl_2$ and $HgCl_2$, respectively. The immobilized bacteria and free bacteria were cultured at the above condition.

Recycling experiments

After immobilization, 0.5 g of the above-mentioned PUF material was added to MSM medium with phenol as the only carbon source and incubated under the following conditions: phenol concentration of 1,500 mg L^{-1}, pH 9, and temperature of 37 °C. After the first batch of phenol degradation, the waste solution was discarded and the immobilized material was removed, gently rinsed with sterilized water and placed in fresh medium for the second batch of degradation until the 10th batch.

Measurements

Scanning electron microscopic (SEM) images were obtained applying a Hitachi Regulus 8220 field emission scanning electron microscope. The L5S UV-Vis spectrophotometer from Shanghai Yidian Analytical Instrument Co. Ltd. was used to measure the phenol content and the biomass of the strain, and the pH change was detected by the pH meter of Mettler Toledo Instrument (Shanghai) Co. Ltd. Constant temperature oscillation incubator was from Harbin Donglian Electronic Technology Development Co., Ltd. Autoclave was from Shanghai Boxun Industrial Co., Ltd. High-speed refrigerated centrifuges were from Thermo Fisher Scientific Inc. SPSS software was used for data analysis.

RESULTS AND DISCUSSION

Preparation and characterization of PUF-ZWB3 materials

B. cereus ZWB3 was previously reported as an excellent strain with high phenol degradation performance (Zhang *et al.* 2022). Therefore, in this study, PUF was used as carrier for immobilization of ZWB3 phenol-degrading strain, to prepare bio-material PUF-ZWB3, improving the tolerance of ZWB3 on adverse environment, through the sheltering effect of PUF. Here, bacteria are first immobilized on PUF by adsorption, and the number of immobilized microorganisms was obtained using the correlation curve ($R^2 = 0.97553$) between colony forming units (CFU) and biomass (OD_{600}) of the bacterial solution, with a loading amount of 1.2×10^9 CFU·g^{-1}. Afterwards, the attachment of ZWB3 on PUF carrier was observed by SEM. As can be seen from Figure 1(a) and 1(b), the high porosity and large pore size of PUF provide a larger specific surface area for bacterial attachment and immobilization, which can also increase the mass transfer efficiency of the nutrient. In Figure 1(c) and 1(d), the free ZWB3 was short rods of 1–3 μm. Their growth on the PUF material is shown in Figure 1(e) and 1(f). The dense distribution of degrading bacterial cells on the surface of PUF indicated that PUF had a good adsorption effect on microorganisms and provided a relatively suitable environment for bacterial metabolism and proliferation, which offered a prerequisite for later experiments on phenol degradation by immobilized bacteria.

Degradation of phenol by PUF-ZWB3 materials

A large number of strains have been reported that could degrade phenol in the concentration range of 1,000–1,500 mg L^{-1} (Shahryari *et al.* 2018; Long *et al.* 2019; Su *et al.* 2019; Duraisamy *et al.* 2020; Nouri *et al.* 2020; Gong *et al.* 2021). The degradation ability of microorganisms is poor under high levels of pollution. Thus, it is in great demand to improve the tolerance of microorganisms to phenol. Immobilized microbial technology is an excellent method that can facilitate microbial

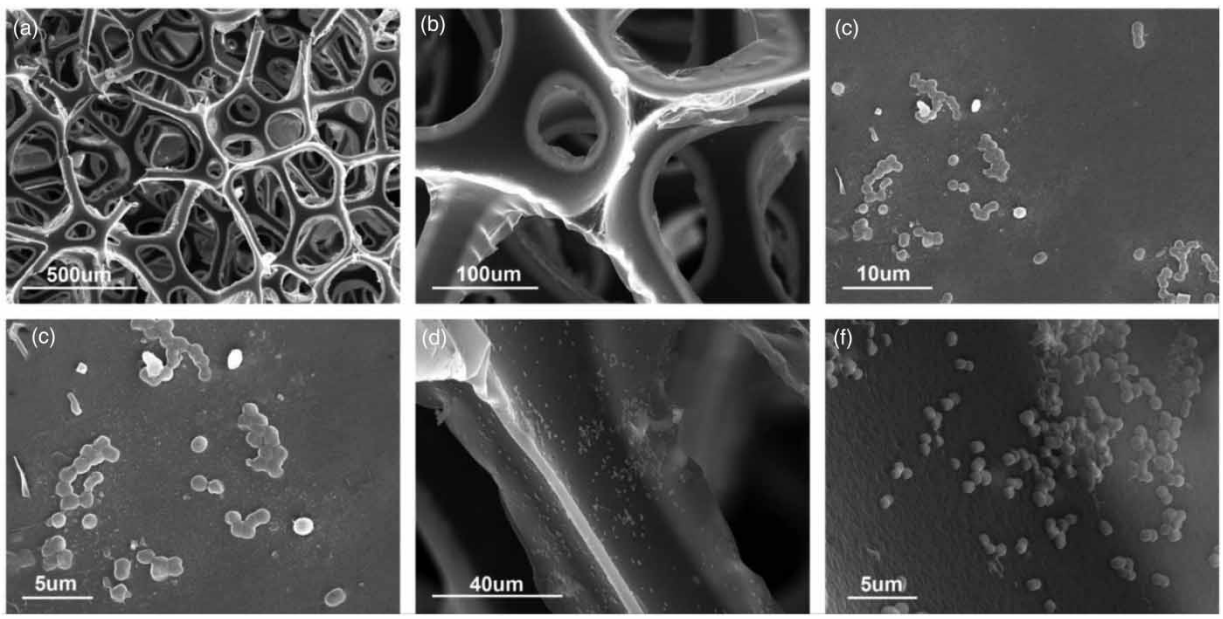


Figure 1 | SEM images of (a and b) PUF, (c and d) free cells, and (e and f) PUF-immobilized cells.

remediation. To investigate whether PUF can help microorganisms to resist highly toxic contaminants, the degradation of phenol by immobilized bacteria at different initial concentrations was investigated. It was found that the degradation of phenol was significantly improved at all phenol concentrations compared with free bacteria (Figure 2).

As shown in Figure 2(a), it can be seen that the growth phase of free bacteria can be divided into two stages, due to the toxicity of phenol. There is an adaptation period in the initial stage, resulting in slow degradation, and the adaptation period also becomes longer with the increase of phenol concentration. In the second stage, bacteria start to degrade phenol. With the increase of phenol concentration, the time for complete degradation of phenol was prolonged. When the phenol concentration was increased to 1,800 mg L^{-1}, the free bacteria were completely inhibited. The free bacteria could degrade phenol at 1,500 mg L^{-1}, while the immobilized system was increased to 2,000 mg L^{-1} (Figure 2(b)). The upper limit of phenol degradation in the immobilized system was increased by 500 mg L^{-1} compared to that of the free bacteria. The average degradation rates of the immobilized system for complete degradation of phenol at 1,800 and 2,000 mg L^{-1} concentrations were 75 and 45.45 mg L^{-1} h^{-1}, respectively. Due to the porous structure of PUF, it has a certain ability to adsorb phenol. At an initial concentration of 1,200 mg L^{-1} of phenol, PUF can adsorb about 212 mg L^{-1} of phenol (Figure 2(c)). Therefore, the degradation of phenol by immobilized bacteria can be divided into three stages, the first stage is the rapid adsorption of phenol by PUF. This also leads to a significantly shorter adaptation period of immobilized bacteria to enter the second stage compared to free bacteria. Thus, the bacteria could rapidly degrade phenol in the third stage. The adsorption of organic compounds by PUF effectively reduces the concentration of organic compounds in the liquid phase, avoids the toxicity of the impact effect of high concentrations of organic compounds on cells, makes the initiation of biodegradation earlier, thus shortening the time required for complete degradation, and also increases the maximum degradable concentration (Perini *et al.* 2021). For example, Zhao *et al.* (2020) prepared peanut shell-based biochar as an adsorbent for phenol and a carrier for microorganisms, and the results showed that the adsorption of the carrier led to a rapid reduction of 800 mg L^{-1} phenol in the initial stage, and then the complete utilization of the remaining phenol by microorganisms began in the second stage (20 h), while the free strain could only degrade about 20% of phenol, which was similar to the results of this study. The adsorption of phenol by the carrier material increased the survival rate of bacterial cells and significantly promoted the degradation of phenol by microorganisms. Similarly, Li *et al.* (2019b) immobilized *Alcaligenes sp*. DN25 on PUF to degrade cyanide and found that the PUF adsorbed cyanide and the degradation efficiency of the immobilized system was significantly higher than that of the free bacteria. All these studies have shown that the adsorption of immobilized materials to toxic substrates is more beneficial for microbial resistance to toxicity and degradation of high concentrations of contaminants.

In addition, in Figure 2(b), the degradation time required for the immobilized system to degrade phenol at 1,500 mg L^{-1} concentration was shortened by 8 h compared with that of the free bacteria, with an average degradation rate of 75 mg L^{-1} h^{-1}, which was 1.4 times higher than that of the free bacteria (Figure 2(a)). Therefore, compared with free bacteria, immobilized bacteria on PUF can not only degrade higher concentrations of phenol, but also accelerate the degradation rate. This is because PUF has good biocompatibility, whose porous nature and large specific surface area can make the bacteria and PUF better bonding. A large number of cells gathered on the surface of PUF makes the local cell concentration greatly increased. The protective effect of the carrier on the bacteria is also strengthened, becoming more conducive to the degradation of phenol (Zheng *et al.* 2009a; Li *et al.* 2021). Similar results were showed by Youssef *et al.* (2019) who used both PUF and polyvinyl

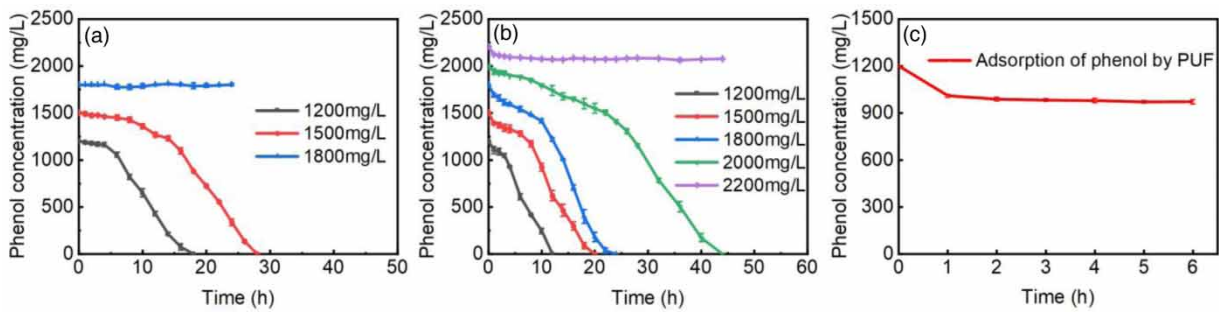


Figure 2 | Degradation of phenol by (a) free bacteria and (b) immobilized bacteria at different concentrations, and (c) adsorption of phenol by PUF. The free bacterial concentration is 1.2×10^9 CFU mL^{-1}.

alcohol–alginate carriers to immobilize the bacterial consortium. The systems were able to completely degrade 1,500 mg L^{-1} of phenol at 48 and 58 h, while the free bacteria achieved complete degradation at 72 h. In this study, the ZWB3-PUF immobilization system has a simple composition, easy operation, low cost, which can degrade phenol more rapidly and completely in a higher concentration, possessing greater potential in the application of degrading high concentration phenol wastewater.

Effect of pH

pH is also one of the important environmental factors affecting the growth of bacterial strains. The pH in wastewater is usually acidic or alkaline (Table 2), which is toxic to microorganisms, and unsuitable pH changes in wastewater can lead to a reduction or even loss of biodegradation efficiency of phenol. Therefore, it is significant to explore the effect of pH on the degradation of phenol by microorganisms. Figure 3 shows the effect of different initial pH on the removal of 1,200 mg L^{-1} phenol, which was found that PUF-ZWB3 could improve the tolerance of microorganisms to extreme pH and effectively promote the degradation of phenol at various pH values. Figure 3(a) shows that the removal rate of immobilized bacteria at pH 9 was 100% at 12 h, while the degradation rate of free bacteria was only 65%, which was 1.54 times higher than that of free bacteria. The results of this study are consistent with many reports that a neutral or alkaline environment favors the biodegradation of pollutants by free and immobilized bacteria (Jiang *et al.* 2017b). Under acidic conditions (pH 5 and 6), the degradation efficiency of free bacteria was significantly inhibited, while the immobilized system still retained some degradation capacity.

It is noteworthy that the degradation ability of the immobilized microorganisms under strongly alkaline conditions showed a great advantage compared to acidic conditions. In Figure 3(b), the degradation rate of free bacteria was about 37.8% at pH 10, while immobilized bacteria had reached 100%. In addition, the immobilized bacteria achieved 78.97 and 100% degradation of 1,200 mg L^{-1} phenol at pH 11 for 12 h (Figure 3(a)) and 18 h (Figure 3(b)). However, the free bacteria almost lost the ability to degrade phenol at pH 11. The pH tolerance by the PUF-ZWB3 immobilized system exhibited higher values and degradation rates (Wang *et al.* 2016; Chris Felshia *et al.* 2017; Tang *et al.* 2018; Bera & Mohanty 2020), comparing with other reported works. This discovery makes the PUF-ZWB3 immobilization system more promising for the treatment of high alkaline phenol wastewater. When the pH was 12, the removal of phenol by immobilized bacteria was almost the same as the adsorption by PUF, because the bacteria could not survive (Figure 3(b) and 3(c)). PUF maintain the stability of the bacterial cells loaded on them, improving the permeability of their cell membranes and making them more tolerant to acidic or alkaline environments (Zheng *et al.* 2009a). In addition, PUF can immobilize key enzymes for degradation, allowing them to maintain high enzymatic activity and stability (Sharari *et al.* 2013). The adsorption of certain acidic metabolites may provide a favorable microenvironment for bacterial survival, which may reduce toxicity to bacteria and promote metabolism (Gong *et al.* 2016; Shi *et al.* 2019). In this study, the degradation rate of free bacteria at pH 7–9 was not much different from that of immobilized bacteria, but the degradation rate of immobilized bacteria at pH 6, 10, and 11 was significantly greater than that of free bacteria. The findings of More *et al.* (2015) are very similar to those of this paper. They showed that the degradation of pendimethalin by *Bacillus lehensis* XJU was not significantly different from that of immobilized bacteria at pH 7, but at the rest of the pH, the degradation rate of immobilized bacteria was greater than that of free bacteria, especially at pH 4 and 10 (More *et al.* 2015). Overall, the PUF-immobilized system is able to effectively remove phenol in a wider pH range

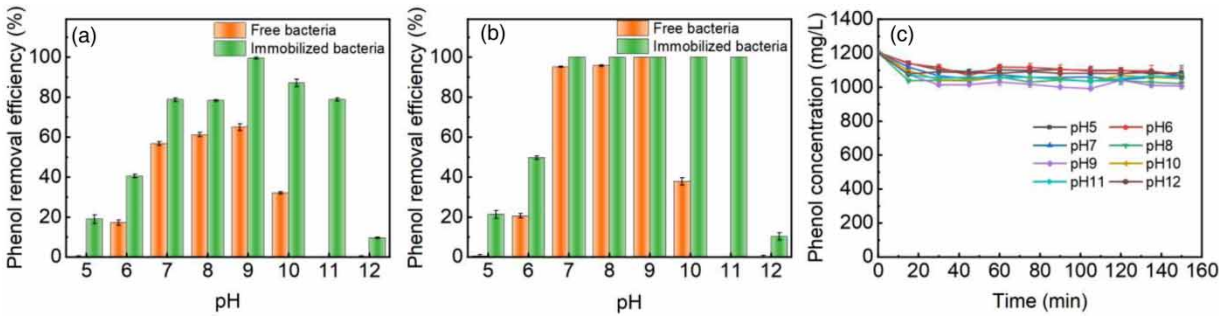


Figure 3 | Degradation of phenol at (a) 12 h and (b) 18 h for immobilized bacteria and free bacteria, and adsorption of phenol by PUF, at different pH conditions. The free bacterial concentration is 1.2×10^9 CFU mL^{-1}.

compared to free bacteria, and is more promising to cope with the unstable environmental factors in actual wastewater treatment, increasing the value of its practical application.

Effect of salt concentration and heavy metal

Industrial phenolic wastewater is often accompanied by the presence of high concentrations of salt and different heavy metals (Table 1), affecting microbial stability and reducing the biological treatment performance of industrial phenolic wastewater (Ontanon *et al.* 2017; Ren *et al.* 2018). Therefore, exploring the effect of heavy metal ions on the biodegradation of phenol is of great significance in the treatment of actual phenolic wastewater. The effect of NaCl concentrations on phenol removal by immobilized bacteria is shown in Figure 4(a). Immobilized bacteria could remove phenol rapidly in the concentration range of 0–1% NaCl, while free bacteria could not degrade phenol due to the presence of NaCl. With the increase of NaCl concentration, the degradation of phenol was also gradually inhibited, and the removal of phenol by immobilized bacteria was 26.75, 24.08, and 20.12 at 3–7% NaCl concentration (Figure 4(b) and 4(c)). The effect of NaCl concentration on phenol adsorption by PUF was negligible, and the assumed adsorption efficiency of phenol by PUF was 17.6% (Figure 3(c)). The phenol removal efficiency of immobilized bacteria was higher than the adsorption of PUF alone, indicating that the biodegradation and adsorption by bacteria still acted together at this time. When microorganisms are exposed to higher salt concentrations, the increase in osmotic pressure usually leads to dehydration and plasma membrane breakdown of microbial cells, rendering them metabolically inactive and unable to survive (Su *et al.* 2018). However, due to the presence of immobilized carriers, the removal of phenol by immobilized bacteria was significantly better than that of free bacteria, which adsorbed a portion of phenol to alleviate the dual stress on the bacteria. The carriers provided a favorable microenvironment with a certain buffering capacity for the bacteria, reducing the damage to the bacteria from salt concentration and allowing the bacteria to withstand harsher conditions (Jiang *et al.* 2017b; Li *et al.* 2019a).

The presence of Mn^{2+} and Zn^{2+} retarded the removal of phenol by the immobilized bacteria. However, it was still possible to remove phenol completely within 18 h, while the degradation rate of free bacteria only reached 84.26 and 13.11% (Figure 5(a) and 5(c)). The presence of Cd^{2+}, Cu^{2+}, and Hg^{2+} had a greater effect on the removal of phenol by immobilized bacteria. At the 12 h degradation of 1,200 mg L^{-1} phenol (Figure 5(b)), the removal of phenol by immobilization was 17.13, 16.95, and 21.54%, respectively. But the free bacteria could not degrade phenol at this time. Here, the immobilization of PUF improved the phenol removal rate of the strains under heavy metal stress compared to free bacteria. Because the bacteria adsorbed inside the PUF were protected, and only a small fraction of the strains were exposed to the heavy metal environment, reducing the interruption of the heavy metal degradation process of phenol. In addition, the outer layers of bacteria exposed to heavy metals secrete some extracellular polymers with strong adsorption capacity to adsorb some of the heavy metals, reducing the concentration of heavy metal ions. Further, the formation of protective layers by extracellular polymers on the surface of PUF to mitigate the damage to the bacteria (Ibrahim *et al.* 2016). In addition, PUF has certain adsorption performance for heavy metals, which mainly uses groups on PUF molecules such as ether oxygen or carbamate nitrogen to

Table 1 | Contaminant composition of common industrial wastewater

Types of wastewaters	Composition of wastewater	pH	Inorganic salt	Heavy metal
Printing and dyeing wastewater	Benzene, naphthalene, anthraquinones, aniline, nitrobenzene, phenols, pyridine, cyanide, and other substances	Alkaline, pH > 10	15–25%, including chloride, ammonium, and sulfate salts	Fe, Cu, Cr, Cd, As, Pb, Hg, etc.
Coking wastewater	Phenols, organic nitriles, PAHs, nitrogen-containing heterocycles, oxygen-containing heterocycles and a small number of esters, alkanes, and halogen-containing organic substances	Alkaline, 8–9.	20%, including chloride, ammonium, and sulfate salts	Zn, Cu, Cd, Hg, Pb, As, etc.
Petrochemical industry wastewater	Oil, sulfur, phenol, cyanide, COD, polycyclic aromatic hydrocarbons, aromatic amines, and heterocyclic compounds	Acidic or alkaline	3.5%, Ca^{2+}, Mg^{2+}, Cl^{-}, SO_4^{2-}, and other inorganic salt ion	Ni, Cr, Pb, Hg, etc.
Pesticide wastewater	Benzene, organic phosphorus, organic nitrogen, benzene, phenols, etc.	Acidic.	10%–25%, NaCl, K_2CO_3, KCl, etc.	Cr, Pb, Cd, As, Hg, etc.

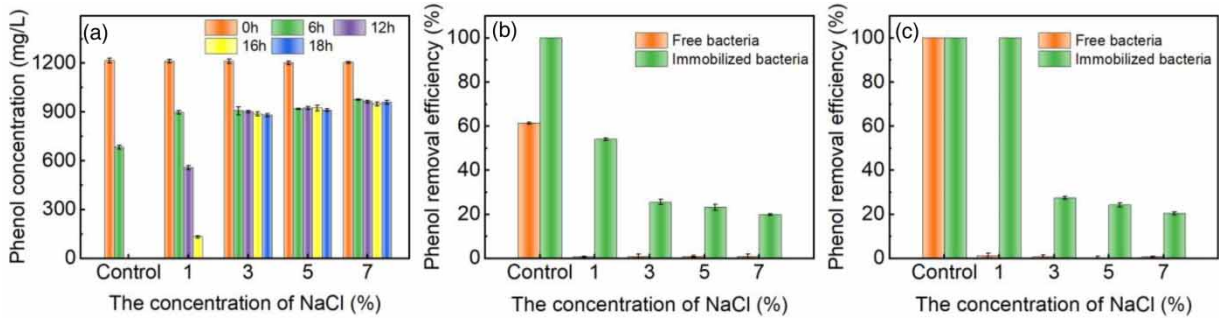


Figure 4 | (a) Phenol degradation at different salt concentrations at (b) 12 h and (c) 18 h. The free bacterial concentration is 1.2×10^9 CFU mL^{-1}.

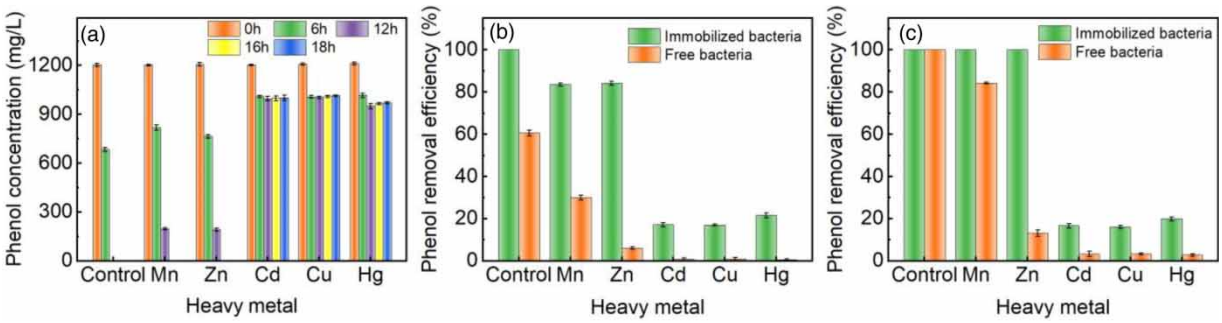


Figure 5 | (a) Phenol degradation under different heavy metal ions (5 mmol L^{-1}) at (b) 12 h and (c) 18 h. The free bacterial concentration is 1.2×10^9 CFU mL^{-1}.

form complexation with heavy metal anions (Bashammakh *et al.* 2009; Teodosiu *et al.* 2014). Thus, heavy metals can be rapidly separated from the phenol wastewater, reducing the toxicity to bacteria, which may be the reason for the high phenol removal rate of PUF-immobilized strains under heavy metal stress.

Reusability

The reusability of the immobilized system is one of the key factors to evaluate the stability. Reusability was performed on immobilized bacteria at a concentration of 1,500 mg L^{-1} phenol, and the results are shown in Figure 6. The time required for complete phenol removal was almost the same in the first four cycles, and the time required for complete phenol degradation increased as the number of cycles increased. Previously, it was reported that the decrease in removal efficiency can be attributed to the toxicity of high phenol concentration and cell shedding from the carrier (Gong *et al.* 2021). However, in this study, the removal efficiency of phenol by the immobilized bacteria was still 100% when after 10 cycles (total 451 h). The phenol degradation by different microbial carriers is shown in Table 2.

Porous carbonaceous gel immobilization of *Bacillus* sp. SAS19 could degrade phenol at 1,600 mg L^{-1} three times (Ke *et al.* 2018). Alginate and SiO_2 nanoparticles immobilization of *Candida sp*. could repeat the degradation of phenol at 600 mg L^{-1} five times (Jiang *et al.* 2017b), etc. Compared with most of the immobilization systems reported in the literatures, PUF-ZWB3 can degrade higher concentrations of phenol and reuse more times. This may be due to the fact that after immobilization of bacteria, the microenvironment of immobilized bacteria is different from the surface of free bacteria, forming a physical or chemical connection between the carrier and the cell. The changes in these microenvironments lead to changes in the shape and structure, biological characteristics and metabolic activity of the cells, increasing the stability of the bacteria and making them continue to degrade phenol efficiently and continuously (Zhang 2022). This performance indicates that PUF-immobilized *B. cereus* ZWB3 has good degradation ability and stability, while also reducing economic and time

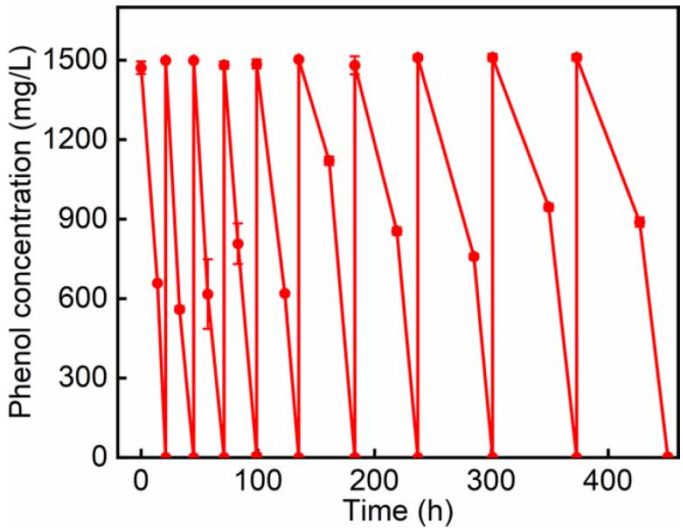


Figure 6 | Reusability of PUF-immobilized strains.

Table 2 | Phenol degradation for different microbial carriers

Immobilized materials	Microbial	Phenol concentration	Removal efficiency (%)	Number of cycles	References
Cashew apple bagasse	*Candida tropicalis* ATCC 750	1,100 mg L^{-1}	100	8	Gomes e Silva *et al.* (2019)
Ca-alginate beads with nanoscale Fe_3O_4	*Debaryomyces* sp.	500 mg L^{-1}	100	10	Jiang *et al.* (2017a)
Porous carbonaceous gels	*Bacillus* sp. SAS19	1,600 mg L^{-1}	100	3	Ke *et al.* (2018)
Alginate and nano-SiO_2	*Candida* sp.	600 mg L^{-1}	99	5	Jiang *et al.* (2017b)
Polyvinyl alcohol–alginate–kaolin beads	*Sphingomonas* sp. GY2B	300 mg L^{-1}	100	16	Ruan *et al.* (2018)
Sepiolite/ZIF-8 nanocomposites	*Pseudomonas putida*	20 mg L^{-1}	100	3	Dong *et al.* (2020)
Sugarcane bagasse	*Candida tropicalis* PHB5	400–2,400 mg L^{-1}	100	6	Basak *et al.* (2019)
Polyurethane foam	*Bacillus cereus* ZWB3	1,500 mg L^{-1}	100	10	This work

costs, providing favorable conditions for biological treatment of industrial wastewater and promising better application in the treatment of actual industrial phenol-containing wastewater.

CONCLUSIONS

In conclusion, the protective effect of PUF on *B. cereus* ZWB3 to enhance degradation rate and maximum degradable concentration for phenol, was explored. The immobilized bacteria could degrade 2,000 mg L^{-1} phenol in only 44 h. In addition, the PUF-immobilized bacteria maintained high phenol degradation performance over a wide pH range, especially under strong alkaline conditions, such as pH 10 and 11, compared to the free bacteria. Immobilization also enhanced the ability of the bacteria to tolerate salt stress as well as heavy metal ion stress, indicating their potential to treat real wastewater. In addition, the system showed no loss in degradation rate of 1,500 mg L^{-1} phenol after 10 consecutive cycles of use. The above results demonstrate that PUF-immobilized *B. cereus* ZWB3 has high efficiency, high applicability and good reusability for phenol removal, which can be further applied to the continuous degradation of phenol wastewater in bioreactors with potential practical application.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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