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Research article

Sustainable biodegradation of phenol by immobilized *Bacillus* sp. SAS19 with porous carbonaceous gels as carriers

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ABSTRACT

In this study, high-efficient phenol-degrading bacterium *Bacillus* sp. SAS19 which was isolated from activated sludge by resuscitation-promoting factor (Rpf) addition, were immobilized on porous carbonaceous gels (CGs) for phenol degradation. The phenol-degrading capabilities of free and immobilized *Bacillus* sp. SAS19 were evaluated under various initial phenol concentrations. The obtained results showed that phenol could be removed effectively by both free and immobilized *Bacillus* sp. SAS19. Furthermore, for degradation of phenol at high concentrations, long-term utilization and recycling were more readily achieved for immobilized bacteria as compared to free bacteria. Immobilized bacteria exhibited significant increase in phenol-degrading capabilities in the third cycle of recycling and reuse, which demonstrated 87.2% and 100% of phenol (1600 mg/L) degradation efficiency at 12 and 24 h, respectively. The present study revealed that immobilized *Bacillus* sp. SAS19 can be potentially used for enhanced treatment of synthetic phenol-laden wastewater.

1. Introduction

Phenol and its derivatives are hazardous and carcinogenic compounds, which are ubiquitous in industrial wastewater generated from steel industry, coal conversion, coking plant, and oil refineries (Bajaj et al., 2008; Basak et al., 2014; Li et al., 2015; Praveen and Loh, 2013; Senthilvelan et al., 2013). For removal of phenol in wastewater, several methods including physical, chemical, and biological technologies have been extensively studied (Basak et al., 2014; Liang and Zhu, 2016; Praveen and Loh, 2013). In comparison with physico-chemical treatment techniques, biological treatments are considered to be efficient, low-cost and environmentally benign technique (Liu et al., 2018; Martinkova et al., 2016). However, the toxicity of phenol to bacterial cells inhibits bacteria activity resulting in low phenol removal performance (Peyton et al., 2002). Moreover, the limitations in using of free bacteria for wastewater treatment include substrate inhibition, sensitivity to environmental conditions, and difficulties with recovery and reuse (Bai et al., 2018; Lin et al., 2014).

Immobilization of microorganisms with the multifunctional materials has recently been developed to overcome these problems (Jiang et al., 2013; Wang et al., 2015b). Immobilization technologies with low energy and chemical consumption are needed for sustainable remediation application, which have many advantages such as protecting bacterial cells from the stress of harsh environmental conditions, preventing cells washout during continuous degradation, reducing cost through recycling, and providing higher bacterial cell densities with elevated degradation capacity (Bai et al., 2018; Chen et al., 2016; Liu et al., 2018; Wang et al., 2015a). All these advantages of immobilized bacterial cells suggested that application of immobilization technologies to phenolic wastewater treatment systems has the potential of enhancing the performance of phenol degradation. Up to now, although extensive studies on carrier materials for immobilization have been investigated, such as bamboo (Chen et al., 2016), bagasse (Basak et al., 2014), cinders bead (Huang et al., 2016) and ceramic (Lin et al., 2015), the selection of matrices was one of the major drawbacks for immobilization technologies (Chris Felshia et al., 2017). The sponge-like porous carbonaceous gels (CGs) (Wu et al., 2013), which had large specific surface areas, unique sorption capacity and low cost, are promising carrier materials for bacteria immobilization. Notably, until now there is still little information available about CGs as the carrier for

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bacteria immobilization.

Moreover, the low phenol-degrading capability of bacterial species was another obstacle. The application of immobilized bacteria in phenolic wastewater treatment depends on the phenol-degrading capabilities of bacterial species used for immobilization. Therefore, the search for high efficient phenol-degrading bacteria is crucial for bacteria immobilization. However, a very small fraction of functional bacteria can be obtained by the conventional plate purification, because most bacteria are in a viable but non-culturable (VBNC) state in response to a wide variety of adverse environmental conditions (Oliver, 2010; Su et al., 2018). For instance, the genera of *Pseudomonas*, *Corynebacterium*, *Rhodococcus*, *Bacillus* and *Alcaligenes* which contained well-known phenol-degrading bacteria have been reported to enter the VBNC state under stress conditions (Li et al., 2018; Oliver, 2010; Su et al., 2015b, 2018). Resuscitation of VBNC bacteria is essential for isolating novel highly efficient phenol-biodegraders. Resuscitation-promoting factor (Rpf), which is a bacterial cytokine secreted by *Micrococcus luteus* (Mukamolova et al., 1998) is the most notable and extensively used for recovery of VBNC bacteria. The resuscitation and stimulation functions of Rpf on bacteria for enhanced salt-tolerant phenol-degrading capability, nitrogen removal, biphenyl/polychlorinated biphenyl (PCB) degradation have been verified (Liu et al., 2016; Su et al., 2015a, 2018). Thus, resuscitated bacteria are promising candidates for bacteria immobilization since they are vast majority of microbial sources for exploring high-efficient phenol-degrading bacteria.

In this study, the sponge-like porous CGs were employed as the carrier and a strain *Bacillus* sp. SAS19 which was isolated by Rpf addition was used for immobilization. The aims of this study were to (i) investigate the phenol-degrading capabilities of *Bacillus* sp. SAS19; (ii) compare the biodegradation capability of phenol by immobilized and free SAS19 cells at different phenol concentrations; and (iii) evaluate the reusability of immobilized *Bacillus* sp. SAS19. To the best of our knowledge, this is the first attempt to use CGs for immobilizing phenol-degrading bacteria which was isolated by Rpf addition.

2. Materials and methods

2.1. Phenol-degrading capability of free bacteria

A phenol-degrading strain SAS19 (GenBank accession number MG591719) was isolated from activated sludge by Rpf addition and identified as *Bacillus* sp. (Li et al., 2018). The obtained *Bacillus* sp. strain SAS19 was inoculated at 30 °C on a rotary shaker at 120 rpm for 24 h in Luria-Bertani (LB) liquid medium. The bacterial culture was centrifuged (6000 g, 20 min) and washed three times with sterilized phosphate buffer saline (PBS, pH 6.8). The washed pellets were resuspended in sterilized PBS (OD_{600} = 1.06). Then the cell suspension was inoculated (4%, v/v) into mineral salt medium (MSM) which contained (per liter): KH_2PO_4 2 g, Na_2HPO_4 1.3 g, $(NH_4)_2SO_4$ 0.1 g, $FeCl_3$ 0.01 g, and the solution pH was adjusted to 7.0. At last, various initial phenol concentrations of 800, 1000, 1200, 1400, 1600 and 1800 mg/L in the MSM were prepared to investigate phenol-degrading capability. Residual phenol concentration in each culture was measured twice a day at regular intervals of 9 and 15 h using 4-aminoantipyrine spectrophotometric method (Fiamegos et al., 2002). All experiments were performed in triplicates.

2.2. Immobilization of phenol-degrading bacteria on CGs

CGs were synthesized by a simple one-pot hydrothermal process according to Wu et al. (2013). The obtained CGs were cut into 1 cm^3 volume with a knife and freeze-dried to remove the absorbed water. Freeze-dried CGs were placed in a dry environment and used to immobilize bacteria. The cell suspension (OD_{600} = 1.06) described above was added to CGs overnight at 4 °C. CGs with bacteria were mixed with 1.5% (w/v) sterilized alginate solution, and then the mixture was dropped into 3.5% sterilized $CaCl_2$ (w/v) solution using peristaltic pump. Immobilized bacteria were collected and washed with sterile water for several times. Meanwhile, immobilized bacteria autoclaved at 121 °C for 20 min were studied as the control group. Morphological characteristics of CGs and immobilized bacteria were observed using scanning electron microscopy (SEM, Hitachi S-4800).

2.3. Comparison of free and immobilized bacteria of phenol degradation

The cell suspension (6%, v/v), immobilized bacteria (3 cm^3) and autoclaved immobilized bacteria (3 cm^3) were inoculated to sterilized 15 mL MSM containing different initial phenol concentrations of 800, 1200 and 1600 mg/L, respectively, in a shaker at 120 rpm and 30 °C. After complete removal of phenol, the immobilized bacteria were collected and washed three times with PBS for further reusability analysis. All experiments were performed in triplicates.

2.4. Reusability of immobilized bacteria

The washed immobilized bacteria described above were placed into sterile fresh MSM with the same initial phenol concentrations of 800, 1200 and 1600 mg/L, respectively, and incubated in a shaker at 120 rpm and 30 °C. The residual concentration of phenol in each culture was measured every 12 h until complete degradation. Then the immobilized bacteria were collected from the first cycle and washed three times with PBS for reusing in the second cycle. After the second cycle, the immobilized bacteria were collected, washed and transferred into fresh MSM for the third cycle. The initial phenol concentration of each cycle was constant. All the experiments were performed in triplicate, and standard deviation (SD) was calculated by SPSS software version 19.0 (SPSS, Inc., Chicago, IL, USA) for analysis of statistical significance ($p < 0.05$) of the triplicate samples.

3. Results and discussion

3.1. Phenol-degrading capability of free bacteria

The morphological of strain SAS19 was observed to be consistent with its assignment to genus *Bacillus* (Supplementary Figs. 1S–2S). Colonies of the strain SAS19 were observed to be smooth, white dots, dry and opaque with regular edges on MSM agar with phenol as the sole carbon and energy source (Supplementary Fig. 1S). In order to investigate the phenol-degrading capabilities of *Bacillus* sp. SAS19, the phenol-degradation curves at different initial phenol concentrations from 800 to 1800 mg/L are depicted in Fig. 1. *Bacillus* sp. SAS19 could achieve complete degradation of phenol within 30 h at an initial phenol concentration of 1000 mg/L, while achieved 100% phenol degradation within 78 h at a concentration of 1800 mg/L. Meanwhile, when the initial phenol concentration was higher than 1200 mg/L, the lag phase of 20 h was needed for bacterial cells with higher tolerance and faster phenol degradation rate. The obtained results indicated that efficient phenol degradation could be achieved for phenol concentration between 800 and 1800 mg/L using free cells of *Bacillus* sp. SAS19.

Up to now, a variety of species have been reported to utilize phenol including *Bacillus*, *Halomonas*, *Chlorella*, *Acinetobacter*, *Aeribacillus* and *Pseudoalteromonas* (Banerjee and Ghoshal, 2010; Das et al., 2015; Jiang et al., 2007; Sun et al., 2012; Wang et al., 2000). Banerjee and Ghoshal (2010) reported that *Bacillus cereus* strain AKG1 was able to completely degrade phenol within 40 h at concentration of 500 mg/L. Das et al. (2015) demonstrated that *Chlorella pyrenoidosa* (NCIM 2738) could completely degrade phenol within 72 h at a concentration of 200 mg/L. The strain *Acinetobacter* sp. SJ-2 required 50 h to completely degrade 400 mg/L phenol (Sun et al., 2012). Jiang et al. (2007) reported that 1600 mg/L phenol can be completely degraded within 76 h by *Alcaligenes faecalis*. Moreover, Wang et al. (2016) found that *A. faecalis* WY-