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Author

Li, J, Jia, Y, Zhong, J, Liu, Q, Li, H, Agranovski, I

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# Use of calcium alginate/biochar microsphere immobilized bacteria *Bacillus* sp. for removal of phenol in water



Jian Li<sup>a,b</sup>, Yinjuan Jia<sup>a</sup>, Jiaochan Zhong<sup>a</sup>, Qinghui Liu<sup>a</sup>, Han Li<sup>a</sup>, Igor Agranovski<sup>b,\*</sup>

<sup>a</sup> School of Environmental and Chemical Engineering, Nanchang Hangkong University, Nanchang 330063, China

<sup>b</sup> School of Engineering and Built Environment, Griffith University, Brisbane, QLD 4111, Australia

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## ABSTRACT

A highly efficient phenol-degrading bacteria strain, identified as *Bacillus cereus* (GenBank No:MN784421) by 16S rDNA analysis, was used for phenol treatment in the water. Under the optimal degradation conditions (10% inoculation of the strain suspension into beef extract peptone medium; 35 °C; pH 7.0; 1% NaCl), the bacterial strain provided 97.71% degradation rate within 46 h of treatment. For improving the tolerance of the strain to phenol toxicity, water hyacinth (*Eichhornia crassipes*) was used as a raw material to produce a biochar under different pyrolysis temperatures. It has been shown that treatment of 1400 mg/L phenol by a combination of a bacterial strain and 0.6% (w/v) biochar prepared at 550 °C can achieve a degradation rate of 99.5% within 60 h of treatment. It has also been shown that inoculated biochar, embedded and immobilized by calcium alginate to form calcium alginate/biochar microbial microsphere (CABMM with 3-4 mm diameter), could greatly improve the tolerance of bacteria to phenol toxicity. At the initial phenol concentration of 1400 mg/L, using of CABMM enables to achieve 99.1% of the phenol degradation over 40 h of treatment.

## 1. Introduction

Phenol is a highly toxic and carcinogenic organic pollutant, that is commonly found in wastewater from the pharmaceutical, textile, oil refining, coal gasification, and resin synthesis industry (Barik et al., 2021; Priyadharshini et al., 2019; Singh et al., 2020). The concentration of phenol in various industrial waste water is petrochemicals: 2.8–1220 mg/L, coke oven plants: 60–1200 mg/L, coal mining: 9–6800 mg/L and petroleum oil refineries is 6–500 mg/L (Panigrahy et al., 2022). Phenol pollution of water bodies has devastating effects on aquatic organisms, including algae and aquatic spermatophytes (Park et al., 2012). The biodegradation process of phenol will cause a large amount of dissolved oxygen in the water body is consumed, which may lead to the death of other aquatic organisms due to hypoxia. During the degradation process will produce odorous gases such as hydrogen sulfide, mercaptan, ammonia, etc., resulting in unpleasant odor in water bodies, destruction of biological growth environment, and impact on growth and reproduction. Therefore, phenol has long-term effects and bioaccumulation on animals and plants. However, when the concentration of phenol in the water is greater than 10mg/L, aquatic organisms cannot survive (Brink et al., 2017; Sam et al., 2021).

At present, the removal of phenol from water mainly involves physical, chemical and biological methods. Physical methods such as adsorption, extraction and ion exchange are cheap and easy to operate, how-

ever the adsorbent regeneration rate was found to be low. It is known that phenol is removed from the water without any real degradation (Lin et al., 2009). Although chemical methods, such as chemical oxidation, have the advantage of high degradation rates, they readily form toxic intermediates and secondary pollutants (Huang et al., 2020). In addition, the chemical methods are easily disturbed by unknown components when dealing with phenol in real wastewater (Norouzi et al., 2020; Rachna et al., 2020; Zambrano et al., 2019).

Compared to the physical and chemical methods, biodegradation is the most promising method because of low cost, high efficiency, and environmentally harmless products production after degradation (Barik et al., 2021; Bhattacharya et al., 2015; Zou et al., 2018). Microorganisms with phenol removal capacity are very diverse in nature. An increasing number of phenol degrading microbial strains have been reported, including bacterial strains such as *Rhodococcus ruber* C1 (Zhao et al., 2021), *Pseudomonas citronellolis* NS1 (Panigrahy et al., 2020), *Rhodotorula* sp. ZM1 (Su et al., 2019), *Bacillus badius* D1 (Sarwade et al., 2014), *Acinetobacter* sp. PD12 (Wang et al., 2007), fungi such as *Candida tropicalis* ATCC 750 (Silva et al., 2019), algae such as *Chlorella pyrenoidosa* (Priyadharshini et al., 2019), *Chlorella* sp. (Wang et al., 2016) and yeast such as *Candida oregonensis* B021 (Filipowicz et al., 2020) and *Rhodospiridium kratochvilovae* HIMP1 (Patel et al., 2017). Among various microorganisms, the bacterial strain of *Rhodococcus* sp. is considered as the most promising candidate for po-

\* Corresponding author.

E-mail address: [i.agranovski@griffith.edu.au](mailto:i.agranovski@griffith.edu.au) (I. Agranovski).

tential use as phenolic degrading agent. Existing literature showed that the degradation efficiency of bacteria was higher than that of other kinds of microorganisms and the descending order of degradation efficiency was bacteria > fungi > yeast > algae (Namita et al., 2022; Zhou et al., 2018).

Biofortification is known to be an effective way to enhance degradation rate of target pollutants but protecting microorganisms from pollutant toxicity remains a challenge. Biochar can provide space for microorganism to live and multiply in water and thus can improve the ability of microorganisms to resist pollutants. Various feedstock representatives can be used to produce biochar. Due to its unique characteristics of large surface area, high porosity, multiple functional groups, large cation exchange capacity and good stability, biochar has been widely used to adsorb and decompose pollutants, such as using palm shell biochar to decompose nitrotoluene (59.92 mg/g), as was reported by Saleh et al. (2016). Also some other literature outlines successful use of the bamboo biochar to treat Pb(II) (14.3 mg/g) (Zhou et al., 2013), alfalfa biochar to degrade bisphenol A and sulfamethoxazole (39–49 mg/g and 99 mg/g) (Choi et al., 2019), peanut shell biochar for managing doxycycline (52.37 mg/g) (Liu et al., 2017), and so on.

The immobilization technology includes immobilized enzyme and microorganism technology (Zhang et al., 2021). Most studies have shown that immobilized microorganism had the potential to enhance sealing and control biological processes in the bioreactor systems due to its advantages of solid-liquid separation, biomass reuse and operational stability (Bouabidi et al., 2018; Gennaro et al., 2008). Embedding immobilization is a common immobilization technique. Using alginate silica gel and polyvinyl alcohol (PVA) as embedding materials, the degradation efficiency of immobilized microorganism usually varied with the types of embedding materials (Shi et al., 2014). In general, embedding materials produced from both natural and non-synthetic polymers (such as algae polysaccharides), have high mass transfer properties. Alginate is a natural polymer that can be cross-linked to hydrogel by  $\text{Ca}^{2+}$  which can form stable microspheres structure (Alsamman et al., 2021).

With the development of immobilization technology, many embedding materials have been studied and applied. Natural polymer materials received increasingly growing attention due to an absence of biological toxicity and good mass transfer capabilities. However, it was found that the natural polymer embedding materials are less reusable (Chang et al., 2021; Li et al., 2015). Furthermore, the embedding materials could be eventually degraded by presented microorganisms, which worsens the mechanical properties of the natural polymer and shortens their service life (Ma et al., 2020; Shin et al., 2020). Adding biochar as the carrier for microbial growth will greatly alleviate the decomposition problem of embedded materials, thus prolonging their lifetime.

The water hyacinth EC (*Eichhornia crassipes*) is an invasive floating aquatic plant capable of rapid growth in some natural water reservoirs due to efficient adaptation and strong nutrient absorption capacities (Liu et al., 2020). EC is a good biochar raw material because of its high carbon content (33.1–46.5%), hydrogen content (4.4–6.6%) and calorific value (13.1–18.4 MJ/kg) (Hu et al., 2015). Liu et al. (2021) used EC waste straw to make a new type of biochar-iron composition (FBC) to remove Cr(VI) from aqueous solution which had a wide pH working range of 2.03–9.00. Qu et al. (2021) also prepared a new magnetic porous water hyacinth-derived biochar (MPBCMw3) under microwave-assisted condition combined with different Fe/C mass ratios for Cr(VI) and tetracycline removing. The maximum adsorption capacity of Cr(VI) and tetracycline by MPBCMw3 was 202.61 mg/g and 202.62 mg/g within 150 and 200 min. Liu et al. (2020) reported the potential of water hyacinth biochar-alginate capsules to remove  $\text{Cd}^{2+}$  from an aqueous solution with a maximum adsorption capacity of 46 mg/g. Although water hyacinth biochar has been proved to play an important role in the removal of heavy metals from water, its application in the degradation of organic compounds, especially in combination with microbial degradation of organic compounds, is rarely reported.

Water hyacinth biochar has the characteristics of large surface area and high porosity, providing very promising environment for microorganisms' growth and is a good carrier of microbial materials. In addition, it ought to be noted that the water hyacinth is a heavily invasive pest of creeks, rivers and dams, commonly present worldwide. Using it as a raw material for the production of porous media adding an important disposal component to the proposed technological approach. This study will explore a biological enhancement method for phenol removal from waste water.

## 2. Materials and methods

### 2.1. Reagents and material

#### 2.1.1. Preparation of reagents and mediums

Reagents included 4-amino antipyrine (98%), sodium alginate (90%),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (96%),  $\text{C}_6\text{H}_5\text{OH}$ ,  $\text{C}_6\text{H}_7\text{NH}_3$  (99.5%),  $\text{NH}_3 \cdot \text{H}_2\text{O}$  (25–28%),  $\text{K}_3[\text{Fe}(\text{CN})_6]$  (99.5%),  $\text{H}_3\text{PO}_4$  (99.5%),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (99.5%),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (99.0%),  $\text{NH}_4\text{NO}_3$  (99.0%),  $\text{KH}_2\text{PO}_4$  (99.5%),  $\text{K}_2\text{HPO}_4$  (99.0%),  $\text{MgSO}_4$  (98.0%),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (99.0%),  $\text{NaCl}$  (99.5%),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (99.0%),  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (99.0%), beef paste (99.5%), peptone (99.5%), agar (ash  $\leq 5.0\%$ ),  $\text{NH}_4\text{Cl}$  (99.5%),  $\text{C}_2\text{H}_5\text{OH}$  (99.7%) were purchased from the National Medicines Corporation Ltd. Of China (Shanghai, China). All reagents and calibration standards were based on analytical grade chemicals.

Beef peptone incubation medium contains (per liter): beef paste 3.0 g, peptone 10.0 g,  $\text{NaCl}$  5.0 g, deionized water 1000 mL. The nutrient solid agar medium (g/L) is made by adding 2% agar powder in the beef peptone medium. The inorganic salt incubation medium (per liter):  $\text{H}_3\text{BO}_3$  0.002 g,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.002 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.003 g,  $\text{NH}_4\text{NO}_3$  1.0 g,  $\text{KH}_2\text{PO}_4$  1.0 g,  $\text{K}_2\text{HPO}_4$  1.0 g,  $\text{NaCl}$  1.0 g,  $\text{MgSO}_4$  0.005 g,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.002 g, deionized water 1000 mL (Liang et al., 2021). All the above mediums were sterilized by autoclave (Hirayama HVE-50, Shanghai Huyue Ming Scientific Instruments Co., Ltd., China) at  $1 \times 10^5$  Pa, 121 °C for 30 min.

#### 2.1.2. Preparation of biochar

The water hyacinth (*Eichhornia crassipes*) was collected from Nanji Mountain Wetland National Nature Reserve in Poyang Lake, Jiangxi Province (116° 10'–116° 25' E, 28° 52'–29° 06' N). The leaves and roots were removed from the water hyacinth, and the petioles and stems were washed with deionized water and dried at 80 °C for 24 h. The dried water hyacinth was then heat treated in a tubular vacuum furnace at three pyrolysis temperatures of 350 °C, 550 °C, and 750 °C for 2 h. To ensure the oxygen reduced environment during the pyrolysis process, the argon gas at the flowrate of 120 mL/min was introduced to the furnace for 10 min before commencement of the treatment. Finally, the three types of produced biochar (BC<sub>350</sub>, BC<sub>550</sub>, and BC<sub>750</sub>, standing for 350 °C, 550 °C and 750 °C, treatment temperatures respectively) were crushed and screened with a 200-mesh sieve, then sealed for storage at room temperature of 25 °C.

The biochar surface area was measured by a specific surface area analyzer (77 K) (TriStar II 3020, Micrometrics Instruments Co., GA USA) for  $\text{N}_2$  adsorption-desorption at relative pressures between 0.05–0.18 MPa, and data were fitted according to the Brunauer Emmett-Teller equation (BET).

#### 2.1.3. Preparation of CABMM

The CABMM (calcium alginate/biochar microbial microsphere) used in the present study was produced by two steps procedure. The first step was focused on loading the microorganisms into the biochar, and the second step was used to embed the microorganisms loaded biochar into calcium alginate to form microsphere. In particular, 0.6% (w/v) of BC<sub>550</sub> were added into the 100 mL of inorganic salt medium with initial phenol concentration of 800 mg/L and 10% inoculum. Then, the inorganic salt medium pH value was adjusted to 7.0 and the product was placed into

the thermostatic shaker (ZD-85, Changzhou Instrument Co., Ltd., China) operated at 35 °C at 150 r/min for two days. Thirdly, the biochar loaded with microorganisms was filtered through the polyvinylidene fluoride microporous filter membrane (0.22 µm, Shanghai Xingya Purification Material Factory, China), washed with sterile saline, and centrifugated at 8000 r/min for 10 min (TGL-16G, Shanghai Anting Scientific Instrument Factory, China). All details of the process of the CABMM preparation are provided in the supplementary materials (S1).

The sodium alginate solution and CaCl<sub>2</sub> supersaturated solution were used as embedding reagents. The microorganisms' loaded biochar was firstly placed into a 2% (w/v) sodium alginate solution, and then the solution was extruded dropwise at 2-3 second intervals from 50 cm of operating height into a 2.0% (w/v) CaCl<sub>2</sub> supersaturated solution with a 5 mL syringe to produce soft and elastic CABMM material with an average diameter of 3-4 mm (supplementary material S1). The prepared CABMM were then stored at 4 °C for 24 h for following experimental runs. All of the above operations were performed under sterile conditions.

## 2.2. Experimental procedure

### 2.2.1. Isolation and identification of bacterial strains

The activated sludge samples used for strains' isolation and screening were collected from the wastewater treatment plant located at Nanchang, Jiangxi, China. First, 10 g of the activated sludge sample was added to 100mL of sterile saline and incubated in a thermostatic shaker at 30 °C at 150 r/min for 1 h. Then, 10 mL of the bacterial saline was added to the beef peptone medium for the culture enrichment for 72 h. As the next step, 10 mL of the enriched solution was transported into 90 mL of inorganic medium containing 100 mg/L of phenol for domestication (Fan et al., 2022), with following increase of the phenol concentration up to 1000 mg/L by 100 mg/L increments at 24 h intervals. Assuming that not all groups could survive in the environment with increasing phenol concentration, those microorganisms that cannot withstand the increasing toxicity would be naturally eliminated. Phenol concentration was measured every 2 h. Finally, using the streak plate method to make several point-to-line dilutions on the surface of the plate with the inoculation ring, the strain with the highest degradation efficiency and the highest phenol resistance will be selected for subsequent phenol degradation experiments.

The selected strains with highest phenol degradation efficiency were inoculated in a solid medium to observe their colony morphology, and the surface morphology of the strains was observed by scanning electron microscopy (SEM, Nova Nano SEM450, FEI, USA) at 20000x magnification. The physiological and biochemical parameters such as Gram staining and aerobic properties of the strains were also identified.

The genomic DNA of the isolated strains was extracted using specialised kit following the manufacturer's guidelines (B518263-0050, 50 times/box, Shanghai Biotech, China). The 16S rDNA sequence of the strain was determined by amplifying universal bacterial genomic DNA primers, 1492R (5' -GGTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGA TCCTGGCTCAG-3') using PCR technique (Wu et al., 2019). The PCR cycling was as follows: denaturation at 94 °C for 30 s, annealing at 55~60 °C for 25~30 s, and extension at 72 °C for 30~50 s. The sequencing results were used for phylogenetic tree construction by using BLAST (Basic Local Alignment Search Tool) and MEGA4.1 software through gene bank comparison (<https://www.ncbi.nlm.nih.gov/genbank/>).

### 2.2.2. Degradation efficiency of phenol experiments

Under the optimum culture conditions, the maximum tolerance of the strain to phenol toxicity was further studied by gradually increasing phenol concentration to observe the amount of biomass and degradation efficiency of strains. The concentration range of phenol was set as 200–1400 mg/L. Samples of phenol concentration and biomass of microbial growth were tested simultaneously at 2 h intervals. All experimental

runs were undertaken in triplicates to avoid any random errors and to ensure their reproducibility.

The effect of the biochar dosage and pyrolysis temperature on phenol degradation was studied. Four different biochar dosages (0.2%, 0.4%, 0.6%, 0.8% (w/v)) and three biochar types (BC<sub>350</sub>, BC<sub>550</sub>, BC<sub>750</sub>) were challenged for phenol removal efficiency by adding into the inorganic salt medium containing 800 and 1000 mg/L phenol.

In addition two types of microsphere, CABMM and CMM (calcium alginate microbial microsphere, no biochar was added) were used for comparison under the same experimental conditions (pH values 7.0, 35 °C and 150 r/min).

### 2.2.3. Analytical procedures

The phenol concentration was measured by the 4-aminoantipyrene spectrophotometric method (APHA, 2005; Natan et al., 2019; Zhao et al., 2020). This measurement is based on the reaction of phenol solution with 4-amino antipyrine, in presence of the potassium ferricyanide at pH 10.0 ± 0.2 to produce an orange-red dye. After developing the color, the residual concentrations of phenol was measured with an UV-visible spectrophotometer (UV-1800, SHIMADZU, Kyoto Japan) at 510 nm within 30 min.

Since the biomass of the species is directly proportional to the turbidity of the medium, the absorbance value of the medium at a certain wavelength was used to indirectly represent the growth of the strain (Jiang et al., 2004; Panigrahy et al., 2020). The biomass amount was determined by a UV-visible spectrophotometer, taking the absorbance at 600 nm (OD<sub>600</sub>). Note, that the bacteria-free solution was used as a blank for the experiments.

### 2.2.4. Data analysis

The degradation rate (DR, %) was calculated by following equation:

$$DR(\%) = (X_i - X) / X_i, \quad (1)$$

where,  $X_i$  is initial phenol concentration, and  $X$  is phenol concentration at certain sampling time during the experiment (mg/L).

## 3. Results and discussion

### 3.1. Isolation and identification of strains

After enrichment, screening and domestication, highly efficient phenol degrading bacterial strain was selected from 9 candidates acquired from wastewater treatment plant activated sludge. It was found that the microorganism is a Gram-positive aerobic bacterium with white, round, opaque, and rough surface colony morphology, sizing in a range of 1.5–2.0 µm (Fig. 1).

Using 16S rDNA sequencing technology and comparing the results with the existing gene sequences in GenBank, the phylogenetic relationship was constructed (Fig. 2) and showed that the strain is 99.6% similar to *Bacillus cereus* MH19 and 99.7% similar to *Bacillus cereus* CCM2010. According to the homology analysis results, the strain was classified as *Bacillus* sp. strain L5-1. The gene sequence of the strain has been submitted to NCBI gene bank with the unique number of MN784421.

### 3.2. Degradation efficiency of phenol by the bacteria strain L5-1

Time related degradation rates achieved by L5-1 strain for various phenol concentrations in the water are showed in Fig. 3a. As is seen, for the lowest initial phenol concentration of 200 mg/L, its degradation reached 89.0% within 6 h of the experiment. However, for the concentration increase to 1000 mg/L the degradation rate was only 7.32% over the same 6 h period, requiring 40 h to achieve the identical rate of 89.0%, and 46 h to get to the degradation level of 97.7%. Such significant time increase could be explained by increase of the lag

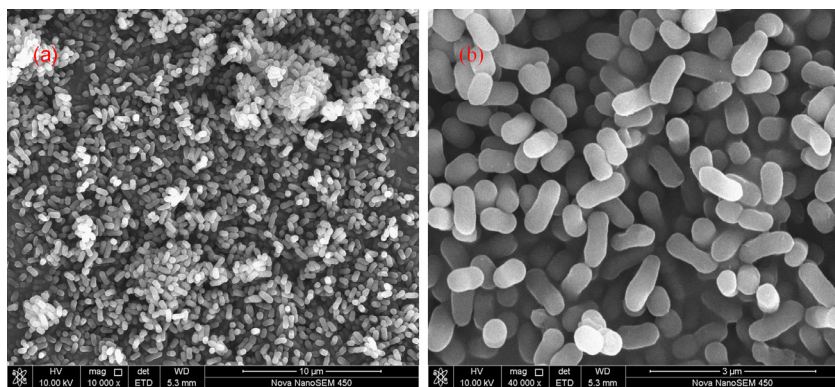


Fig. 1. SEM photos of strain L5-1.

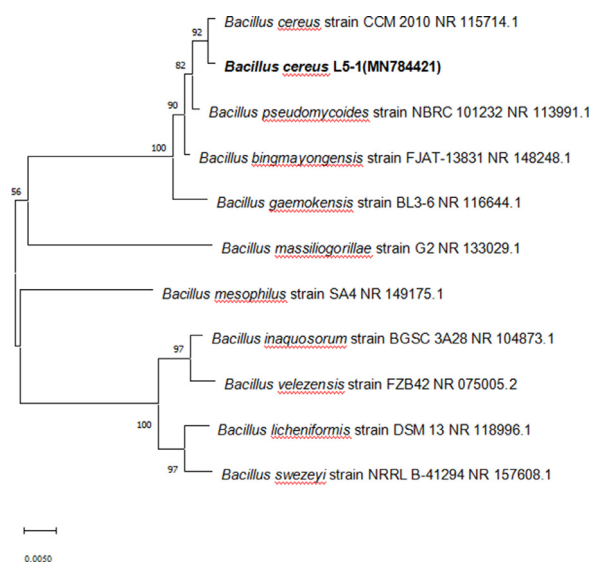


Fig. 2. Phylogenetic tree of strain L5-1.

phase of the strain prolonging the time required to degrade the chemical. It can be seen from Fig. 3b that the lag phase of the strain L5-1 at 1200 mg/L phenol concentration should experience about 16–18 h, while at 1400 mg/L, the strain was significantly inhibited and grew very slowly. There was no significant logarithmic growth period appearing within 70 h. When the concentration of phenol decreased to 1000 mg/L, the lag phase was a significant shortening. As the external stress of pollutants is aggravated, the microbial need to adapt to the new environ-

ment, their growth enters a lag phase, the cells stop proliferating and the cell number no apparent increases for this period (Kumari et al., 2013; Zhao et al., 2020). Considering all results in Fig. 3, one could conclude that high concentrations of phenol might have strong inhibitory or toxic effects on the strain, decreasing its capability to handle elevated phenol concentrations fast.

### 3.3. Microscopic observation and biodegradation of biochar at different pyrolysis temperatures

The SEM images (Fig. 4a) clearly illustrate that the biochar, prepared by using water hyacinth as raw material, has a rough, non-uniform surface and asymmetric pores. The porous structure of the biochar had a relatively large specific surface area, which was conducive to the adsorption and degradation of pollutants and the attachment growth of microorganisms (Zanella et al., 2021). The characteristics of biochar prepared at three temperatures are presented in Table 1, which shows that the higher the pyrolysis temperature, the lower the yield of biochar, but the greater the specific surface area. Although BC<sub>750</sub> has the largest specific surface area, it has higher alkalinity (pH~8.3) as compared to BC<sub>550</sub> (pH~7.1), which is not beneficial for bacterial strain growth (the optimum culture pH value must be ~7.0).

It is very important to note that the procedure enabled efficient microorganism settlement both on the surface and in the pores indicating successful loading of the medium (Fig. 4b). There are two potential reasons for facilitating strain growth on the biochar. First, the microorganism adheres to micropores, and the biochar functional groups such as -COOH, -OH and C-O-C could serve as active sites on the surface, which showed the electrostatic adsorption of BC<sub>550</sub> (Jiang et al., 2022). Alternatively, suitable growth environment provided by BC<sub>550</sub>, such as suitable pH, large specific surface area and loose porous struc-

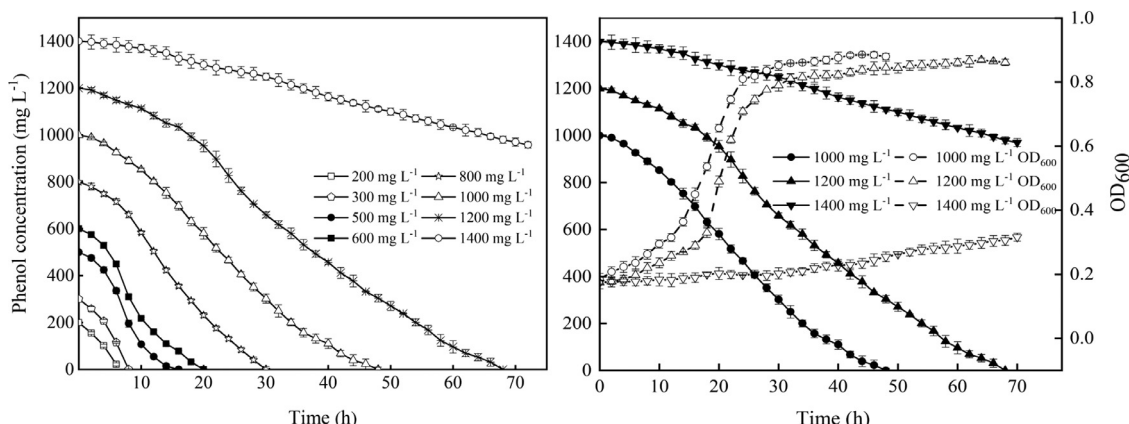
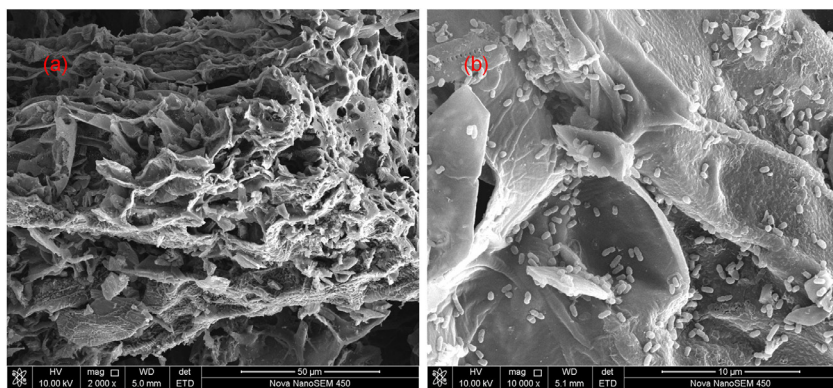


Fig. 3. Degradation of phenol by strain L5-1; (a) degradation efficiency, (b) biomass.

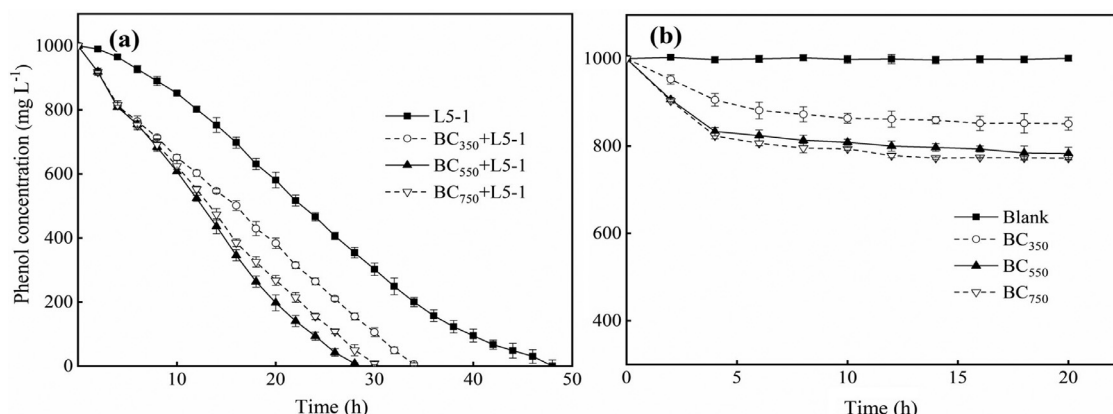
**Table 1**  
Characteristics of the biochar prepared at different pyrolysis temperatures.

Biochar	W <sub>f</sub> (g)*	W <sub>d</sub> (g)	W <sub>b</sub> (g)	W <sub>BC</sub> (g)	pH	S <sub>BET</sub> (m <sup>2</sup> /g)
BC <sub>350</sub>	1000.1 ± 0.3	366.4 ± 8.3	354.9 ± 9.8	164.7 ± 5.4	6.5 ± 0.1	5.5 ± 0.2
BC <sub>550</sub>	1000.2 ± 0.5	367.8 ± 7.6	356.1 ± 8.6	121.2 ± 4.0	7.1 ± 0.2	33.0 ± 0.3
BC <sub>750</sub>	1000.0 ± 0.7	368.7 ± 7.5	355.9 ± 8.1	95.2 ± 3.6	8.3 ± 0.3	52.0 ± 0.1

\* Mean ± STDEV (n ≥ 3); W<sub>f</sub> – fresh weight; W<sub>d</sub> – dry weight. W<sub>b</sub> – dry weight before pyrolysis (powder); W<sub>BC</sub> – weight of biochar after pyrolysis; S<sub>BET</sub> – specific surface area of biochar.



**Fig. 4.** Photos of biochar; (a) biochar, (b) biochar loaded with bacteria.



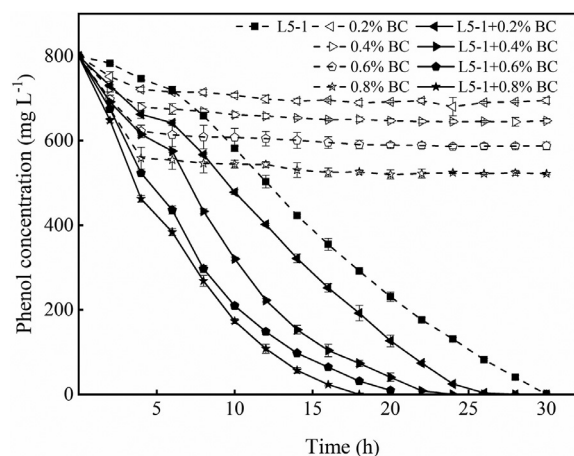
**Fig. 5.** Degradation of phenol at different pyrolysis temperatures; (a) BC with microbes, (b) pure BC.

ture, made it easier for microorganism to grow and reproduce, which could significantly improve the degradation efficiency of organic pollutants (Shi et al., 2019; Tu et al., 2020; Zhao et al., 2020).

Interestingly, as is seen in Fig. 5, the BC<sub>750</sub> on its own exhibited the highest rate of phenol degradation (Fig. 5b), however in combination with the L5-1 strain this leadership was lost, moving BC<sub>550</sub> to the top position (Fig. 5a). Such outcome could be explained by the fact that when biochar is used along with microorganisms, various parameters could influence the process of microbial growth, including pH of the environment and voidage, justifying selection of the BC<sub>550</sub> for all experimental runs within the scope of this project.

### 3.4. Degradation of phenol by different concentrations of biochar

Fig. 6 showed that it took nearly 30 h for solely used strain L5-1 to completely degrade 800 mg/L of phenol, at the degradation rate of about 3.0% during first 2 h increment. However, the removal rate of phenol was increased significantly by adding the biochar to the bacterial solution. During first 2 h of the experiment, the phenol removal rate was increased to 13.1%, 19.2%, 26.5%, 34.8% by adding 0.2%, 0.4%, 0.6%, and 0.8% (w/v) of the biochar, respectively. The results clearly show



**Fig. 6.** Effect of biochar concentration on phenol degradation in presence of the strain L5-1.

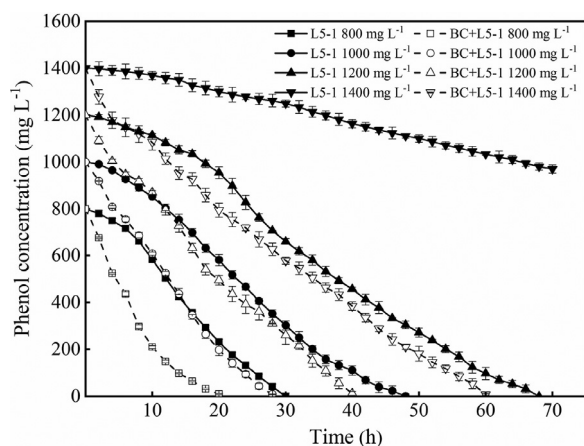


Fig. 7. Influence of BC on degradation of high-concentrated phenol solution.

that increase of the biochar concentration enhances the removal rate of phenol, reaching the highest values at 0.8% (w/v).

### 3.5. Degradation of phenol by adding the biochar

As is seen in Fig. 7, when the initial phenol concentration was 1200 mg/L, microorganisms in the system, without adding biochar, entered the logarithmic growth phase after 18 h. In particular, at 18–20 h interval, the strain biomass rapidly increased by 54.5% with the OD<sub>600</sub> values increased from 0.33 to 0.51, and the absorption rate of phenol increased from 11.52 to 12.30 mg/h (Fig. 3b). On the other hand, during earlier stages of the experiment (16–18 h interval), the strain biomass was only increased by 17.8%, and phenol absorption rate increased from 10.33 to 11.52 mg/h (Fig. 3b). Finally, for the microorganism acting on its own, the absorption rate of 17.71 mg/h was achieved after 66 h of operation. In contrast, adding of 0.6% (w/v) of the biochar significantly shortened time required to commence the logarithmic growth phase (10 h), and the absorption rate of 29.7 mg/h was achieved during much shorter time of 40 h.

In general, adding of the biochar to the system significantly enhanced the process, causing rapid decrease of phenol concentration over the first 4 h (Fig. 7). Such decrease established more favorable and safer environment for the strain to enter the logarithmic growth phase during shorter time periods. This conclusion is also well supported by the results obtained for the highest phenol concentration used in this project (1400 mg/L), which is also highest concentration for the strain to tolerate without dramatic inactivation. As is seen in Fig. 7, the degradation rate was very low achieving only 29% after 66 h of the experiment involving solely bacterial strain, which could be explained by strong toxicity of phenol present in the system in high quantity. However, adding 0.6% of the biochar to the system increased the degradation to 99.5% observed after 60 h of the experimental run verifying that adding biochar improves the degradation efficiency and proving its protective effect on bacterial strains involved in chemicals' degradation processes.

### 3.6. Degradation of high concentration phenol by CABMM

Previous experiments have confirmed that adding biochar to the bacterial solution can effectively remove phenol from wastewater. However, biochar recovery is a fairly complex procedure, leaving room for improvement. Therefore, calcium alginate immobilization microsphere method was selected to immobilized strain L5-1 on biochar to further improve its degradation efficiency and tolerance to phenol.

As is seen in Fig. 8, calcium alginate/biochar immobilized microorganisms microsphere (CABMM) demonstrated even better phenol removal performance, along with further improvement of microbial tolerance to phenol. As with the addition of biochar, regardless of whether

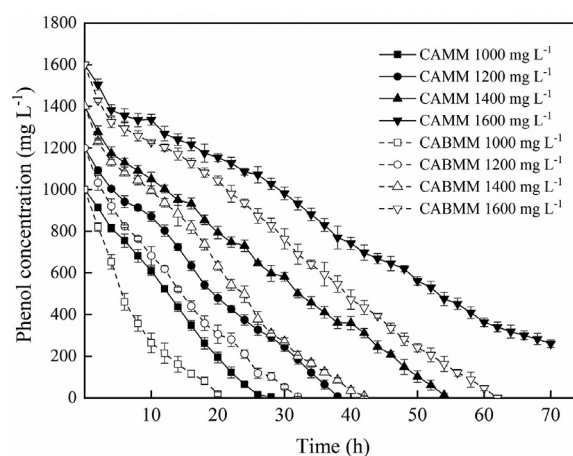


Fig. 8. Degradation of phenol in presence of CABMM and CMM.

CABMM or CMM was added, the initial concentration of phenol was rapidly reduced within the first 4 h, which has a certain protective effect on microorganisms. Even for the initial concentration of 1400 mg/L, more than 99% of phenol was degraded in presence of CABMM during 40 h of operation, which is significantly higher compared to the numbers obtained for BC. Relatively high efficiency of phenol degradation was also demonstrated in presence of CMM, however it is still lower compared to CABMM, as is clearly seen in Fig. 8.

Interestingly, color of CMM at different phenol concentrations was observed in absence of the biochar. At low concentrations, the black color of CMM was changed to gray and white, indicating that the microorganisms efficiently grow. In contrast, when the concentration of phenol was high, the color change was not distinctive, confirming slow growth or even no growth of microorganisms.

## 4. Conclusions

The bacterial strain with high degradation efficiency of phenol was successfully isolated from activated sludge samples of sewage plant and identified as *Bacillus cereus* L5-1. It demonstrated strong ability to enhance phenol degradation, better resistance to aggressive chemicals, and a wider range of environmental adaptation.

The water hyacinth (*Eichhornia crassipes*) was selected and used for production of biochar as a microbial carrier. It was confirmed that the biochar could greatly enhance the microbial capabilities on phenol degradation in liquid environment. On the one hand, the addition of biochar has a certain adsorption effect, which can reduce the initial phenol concentration. It was also shown that the biochar could significantly improve the tolerance of microorganisms to phenol toxicity, enabling bacteria to rapidly enter logarithmic growth phase and shorten its stagnation phase.

Finally, immobilization bacteria on the biochar with calcium alginate enabled to produce embedded microspheres (CABMM), further improving the ability of the strain L5-1 to tolerate high phenol concentrations. It could be concluded that CABMM has a great potential in the field of water treatment if high phenol concentration handling is required. Some interesting results might be expected if the CABMM would be used in the dynamic reactors to remove phenol from actual wastewater streams in some industrial processes.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRedit authorship contribution statement

**Jian Li:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Visualization, Investigation, Funding acquisition. **Yinjuan Jia:** Software, Writing – review & editing, Data curation, Resources. **Jiaochan Zhong:** Methodology, Writing – original draft, Data curation, Resources. **Qinghui Liu:** Writing – original draft, Resources, Data curation, Visualization. **Han Li:** Conceptualization, Data curation, Writing – review & editing. **Igor Agranovski:** Writing – review & editing, Supervision, Formal analysis, Validation.

## Data Availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envc.2022.100599.

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