

## Phenol biodegradation by isolated *Citrobacter* strain under hypersaline conditions

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### ABSTRACT

Phenol is a toxic pollutant in many kinds of hypersaline industrial effluents that should be treated properly before discharged into water bodies. In this work, a halophilic strain which could utilize phenol as the sole source of carbon and energy was isolated. Based on 16S rRNA results, it was identified as a member of *Citrobacter*. The phenol biodegradation ability and cell growth of the strain was evaluated with the variation of initial phenol concentration and salinity. The effect of temperature and pH on phenol removal was also investigated. The results showed that the strain was capable of withstanding high phenol (up to 1,100 mg L<sup>-1</sup>) environment with varying salinity conditions (0–10% of NaCl). The optimal initial phenol concentration was 400 mg L<sup>-1</sup>, at which the average removal rates of phenol peaked at 10.8 mg L<sup>-1</sup> h<sup>-1</sup>. The higher initial concentration of phenol could inhibit the microbial metabolism. The optimal temperature, pH, and salinity were 35 °C, 6.0, and 0%, respectively. Under these conditions, 400 mg L<sup>-1</sup> of phenol could be completely degraded within 20 h. The high removal rates of phenol by the strain might provide an alternative for treating phenolic wastewaters containing high salinity.

**Key words** | biodegradation, hypersaline environment, optimization, phenol, strain isolation, 16S rRNA

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### INTRODUCTION

With the rapid development of industrialization, environmental pollution has attracted increasing concern in recent days. Aromatic hydrocarbons are widespread pollutants in soils and freshwaters, as well as marine environments (Vedler *et al.* 2013). Phenol is a common toxic aromatic compound in anuran amphibians, fish, and crustaceans and it also does great harm to human beings, inducing carcinogenicity and causing reproductive and developmental toxicity, neurotoxicity, and acute toxicity (Kumar *et al.* 2013). Without proper treatment, the wastewater exposed to phenol will cause lots of problems to ecosystems as well as human health because phenol is widely used as a raw material and by-product in many industrial sectors such as textiles, phenol-formaldehyde resin, oil refinery, coking plant, pharmaceutical, and coal conversion (Mandal *et al.* 2013). Many treatment technologies have been applied in phenol reduction. Physicochemical methods including distillation, adsorption, extraction, membrane pervaporation, etc. were mentioned in previous studies (Busca *et al.* 2008). Biological methods are also available for the degradation of phenol.

They are preferred to physicochemical methods in wastewater treatment because biological processes like microbial biodegradation have been shown to be practical, cost-effective and environmental friendly (Basak *et al.* 2013). However, microbial biodegradation could be inhibited by higher concentration of phenol, which is a bottleneck for successful bioremediation (Basak *et al.* 2014). Therefore, isolation and identification of proper microorganism for biodegradation is important and potent microbial strain can be obtained after screening (Choudhury *et al.* 2017). Despite high toxicity of phenol, several microorganisms including bacteria and fungi were reported to be capable of tolerating relatively high concentration of phenol and utilizing phenol as sole source of carbon and energy. *Pseudomonas putida*, *Candida* sp., *Cryptococcus terreus*, *Rhodotorula creatinivora*, etc. were isolated from a variety of sources and have been found to be adequate microorganisms with high metabolic activity that would be able to remove phenol with relatively high efficiency (Kumar *et al.* 2005; Krallish *et al.* 2006; Jiang *et al.* 2015).

Saline and hypersaline environments are often contaminated with organic compounds due to industrial processes and urban water effluents (García *et al.* 2004). Many kinds of industrial effluents in the presence of phenol contain a large amount of salinity, which leads to more difficulties in microbial degradation of phenol without proper pretreatment (Lin *et al.* 1998). The biodegradation efficiencies of pollutants by non-extremophilic microorganisms in high-salt conditions are always lower because high concentration of salinity can lead to the loss of cellular water or recession of the cytoplasm. It is known that cytoplasm is induced by an osmotic difference across the cell wall. The outward flow of intracellular water happens at high salinity (>2%), which will cause cell dehydration and have an adverse effect on activities of strains (Kargi 2002). Considering the high cost of physico-chemical processes before biodegradation, halophilic microorganisms are promising for treating hypersaline wastewater. The previous studies have shown that halophilic strains such as *Halomonas elongata*, *Marinobacter*, and *Debaryomyces* were able to grow in hypersaline environments and could be used for decolorization, biodegradation of aromatic compounds, etc. These results indicated that microbial isolates can be reasonable approaches for treating hypersaline phenol-containing wastewater (Arun Prasad *et al.* 2013; Eslami *et al.* 2016; Jiang *et al.* 2016).

In this study, a halotolerant strain that was able to grow in hypersaline environments with phenol as sole source of carbon and energy was isolated and purified from saline wastes of petroleum industry. The strain was identified by 16S rRNA. The microbial growth and biodegradation of phenol were evaluated with different initial phenol concentrations. The tolerance of high salinity was also investigated with the variation of salt concentration in the media. In addition, the tests were performed to show the effect of pH and temperature on phenol removal efficiency. To our knowledge, this is the first report of phenol degradation by *Citrobacter* strain in hypersaline conditions. The study aims to optimize the culture condition for growth and phenol degradation of the isolate and contributes to utilization of purified halophilic strains for treating hypersaline phenol-rich wastewater.

## MATERIAL AND METHODS

### Source and media

The oil waste was collected from a petroleum plant in Nanyang, China. The waste was placed into a flask containing

selective media in which phenol acted as the sole source of carbon and energy. The salinity during cultivation of bacterial seed was 5% (w/v). The initial selective media contained the following components (per liter):  $\text{KH}_2\text{PO}_4$  2.0 g,  $\text{K}_2\text{HPO}_4$  1.3 g, NaCl 50 g,  $\text{NH}_4\text{Cl}$  0.1 g, phenol 0.1 g. The constituents of trace mineral solution (per liter) were as follows:  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.01 g,  $\text{H}_3\text{BO}_3$  0.15 g,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.03 g, KI 0.18 g,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.12 g,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.06 g,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.12 g,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.15 g, EDTA 10 g. All the chemicals used in the study were of analytical grade. After the initial concentration of phenol could be completely removed within one cycle, the initial phenol concentration in the media of the next cycle was increased by  $100 \text{ mg L}^{-1}$ . Until initial concentration of phenol reached  $600 \text{ mg L}^{-1}$ , the flask was used as seed for isolation of halophilic phenol-degrading strains. The enrichment of isolated strain was conducted in beef extract peptone media containing  $10.0 \text{ g L}^{-1}$  peptone,  $5.0 \text{ g L}^{-1}$  beef extract, and  $5.0 \text{ g L}^{-1}$  NaCl.

### Strain isolation and enrichment

When  $600 \text{ mg L}^{-1}$  of initial phenol was degraded thoroughly after 48 h of the flask being incubated at  $30^\circ\text{C}$  and 150 rpm in a rotary shaker, the mixture was diluted in gradients of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and spread onto solid selective media supplied with 2% (w/v) agar. The plates were incubated under mesophilic condition of  $30^\circ\text{C}$  in an incubator for 72 h. The isolation of pure strains and preparation of seed culture were carried out according to our previous report (Jiang *et al.* 2016). After cultivation in extract peptone media and further incubation in selective media for 72 h, the cultures containing each isolated strain were inoculated into another fresh selective media to make the final inoculation ratio of 5% (v/v) followed by incubation in the rotary shaker at  $30^\circ\text{C}$  and 150 rpm. The samples were taken from each flask at intervals of 4 h for testing residual phenol concentration and cell density. The strain which showed the highest phenol degradation efficiency and cell growth rate was picked for further tests.

### Strain identification

For identifying the isolated halophilic strain, genomic DNA of the isolate was extracted using the Ezup Pillar bacterial genomic DNA Extraction Kit (Sangon Biotech Co., Ltd, Shanghai, China). The part of the 16S rRNA genes were amplified using a pair of forward (5'-CAGAGTTT GATCCTGGCT-3') and reverse (5'-AGGAGGTGATCCAG CCGCA-3') primers. The composition of polymerase chain

reaction (PCR) reaction system and amplifications procedure were described in the report of Jiang *et al.* (2016b). The amplified products were purified using agarose gel electrophoresis and the sequencing was carried out at Sangon Biotech Company (Shanghai, China). The nucleotide sequence was submitted to the GenBank databases for comparison and identification using the BLAST package software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A maximum likelihood phylogenetic tree was generated by neighbor-joining methods with MEGA 7. The biochemical tests such as motility and citrate utilization were also carried out for identification of the strain.

### Phenol biodegradation and cell growth

Phenol biodegradation and cell growth of the isolated strain were evaluated with different initial concentrations of phenol. After the strain was enriched in beef extract peptone media and transferred to selective media for 72 h incubation at 30 °C and 150 rpm, the mixture was added into another fresh selective media with initial concentration of phenol ranging from 100 to 1,300 mg L<sup>-1</sup>. The inoculation ratio was 5% (v/v). The flasks were subsequently placed in the rotary shaker at 30 °C and 150 rpm. Residual concentrations of phenol and cell density were examined every 4 h. The flasks were shaken in the dark to avoid phenol photo-destruction. All tests were done in triplicate.

### Effect of temperature and pH

After the optimal initial concentration of phenol was found, the effect of temperature and pH on phenol degradation by the isolated strain was carried out. The strain was inoculated into selective media and the six flasks were put into the rotary shakers at 20, 25, 30, 35, 40, and 45 °C. For pH tests, the pH of six flasks containing selective media were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. The inoculation ratio was 5% (v/v) and the tests lasted for 48 h. The residual concentrations of phenol were determined every 12 h and all tests were done in triplicate.

### Evaluation of salt tolerance

The tolerance of high-salt conditions was investigated by transferring microbial seed to selective media in salinity ranging from 0% to 12% (w/v). The incubation was performed at optimal pH and temperature. The other operations were the same as for temperature and pH. The cell densities as well as residual phenol concentrations were examined at

4-h intervals to show whether low and high concentrations of salinity had negative effect on cell growth and phenol biodegradation. The assays were carried out in triplicate.

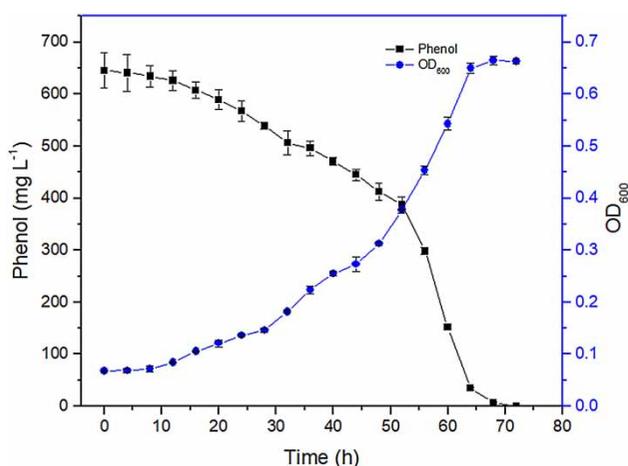
### Analytical methods

After the samples were periodically withdrawn from the flasks, they were centrifuged at 6,000 rpm for 10 min to remove biomass before measuring residual phenol concentration. The phenol concentrations were determined using 4-aminoantipyrine spectrophotometric method according to APHA (2012). Cell concentration was determined by measuring dry-cell weight. The culture was centrifuged at 6,000 rpm for 10 min and the sediment was washed, resuspended, and filtered through a pre-washed 0.45 mm filter paper and dried at 105 °C until constant weight. The growth of the microorganism was monitored as absorbance at 600 nm. The pH values of the cultures were measured by pH meter (PHS-3C).

## RESULTS AND DISCUSSION

### Isolation of the strain and cell growth

After several times of purification, five strains with different properties of morphology were isolated from plates containing selective hypersaline media in which phenol acted as the sole source of carbon and energy. The five isolates were enriched and transferred to fresh selective media to compare their growth rates as well as phenol degradation efficiencies in hypersaline phenol-rich environment. Strain HP-1, which showed the highest degradation rates of phenol among these isolates, was selected for further tests. As shown in Figure 1, the phenol removal rate was relatively slow in the first 12 h. Only 19.3 mg L<sup>-1</sup> of phenol was reduced during this period. The same trend can also be found in the variation of cell density, indicating that the inoculated strains were in the lag phase. In the next 52 hours, residual concentration of phenol in the media decreased significantly. 94.7% of over 600 mg L<sup>-1</sup> of initial phenol was degraded within 64 h of shaking and phenol could be completely removed in the next 8 h. Regarding cell density, the strain showed a rapid growth during this period. The OD<sub>600</sub> at 64 h was 7.7 times more than that at 12 h. After 64 h of shaking, the cell growth became slower, which was due to the lack of phenol in the media. The substrate inhibition can also be observed in many other previous studies along with the consumption of carbon source (Kandil *et al.* 2015; Okai *et al.* 2015). It can also be



**Figure 1** | Phenol biodegradation and cell growth when utilizing phenol as the sole source of carbon and energy at salinity of 5% (w/v).

seen that the cell concentration reached its highest value at 68 h and the  $OD_{600}$  was over 0.66. After that the cell density started to decrease slightly. The variation of  $OD_{600}$  was consistent with the residual phenol concentration during the whole process, suggesting that the strain HP-1 utilized phenol to achieve metabolism and to reproduce (Perruchon et al. 2016).

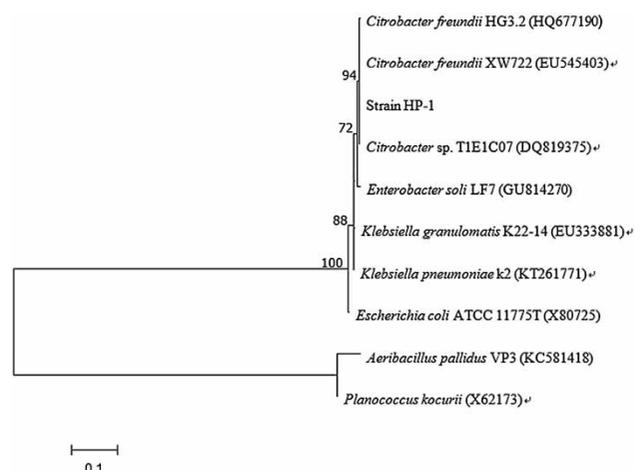
### Identification of the strain

The Gram and biochemical tests were performed for strain identification and the results are summarized in Table 1. Part of the 16S rRNA genes was amplified and 1,433 bp of sequences were obtained. The strain HP-1 sequence was compared with that in the NCBI database using homology-searching. The results showed that the isolate had the closest relation with the genus *Citrobacter*. The amplified rRNA of strain HP-1 had 98.9% similarity with *Citrobacter freundii* and 98.3% similarity with *Citrobacter* sp., which indicated

**Table 1** | Results of biochemical and gram reaction tests of the isolated strain

Name of test	Results
Gram reaction	-
Motility	+
Catalase	+
Oxidase	-
Citrate	+
Voges-Proskauer	-
Indole production	-

(+): positive, (-): negative.

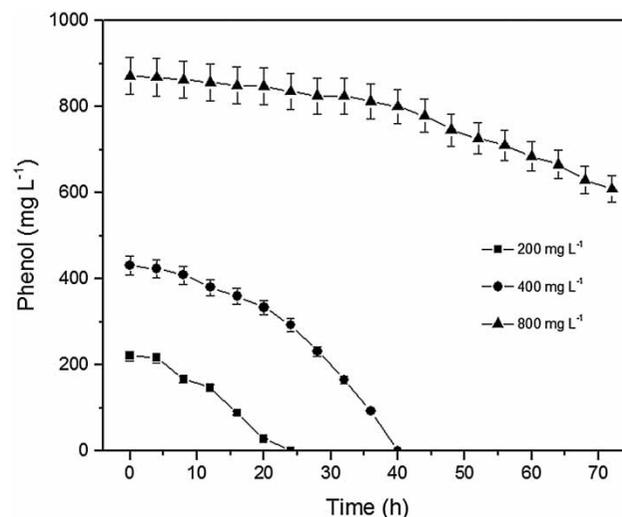


**Figure 2** | Phylogenetic tree based on 16S rRNA sequences by using the Neighbor-joining method, showing the position of strain HP-1 and representatives of some related taxa.

that the strain HP-1 was a member of *Citrobacter*. The phylogenetic relationship of strain HP-1 with several other bacteria can be seen in Figure 2.

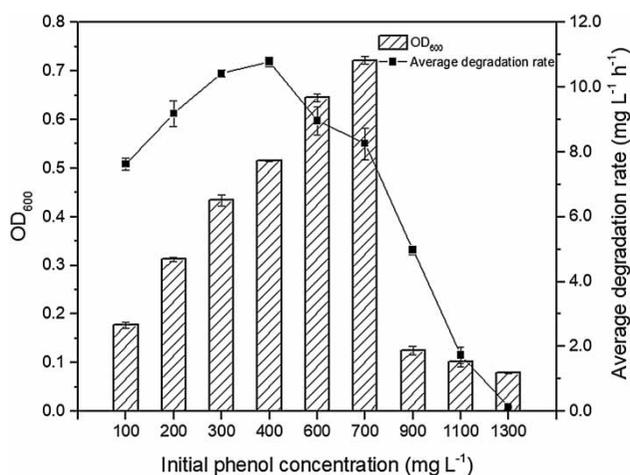
### Phenol biodegradation

The biodegradation of phenol by strain HP-1 at salinity of 5% is shown in Figure 3. It took only 24 h for the strain to remove over 200 mg L<sup>-1</sup> of initial phenol. As with 400 mg L<sup>-1</sup> of initial phenol, it could be completely degraded within 40 h. However, when initial concentration of phenol increased beyond 800 mg L<sup>-1</sup>, the removal rate of phenol became significantly lower. Phenol degradation efficiencies were



**Figure 3** | Phenol removal performance with different initial phenol concentrations at salinity of 5% (w/v).

14.4% and 30.2%, respectively, after 48 and 72 h of operation, which indicated the inhibition of high concentration of phenol in the environment to metabolism of the strain. This can also be demonstrated by the time of the lag phase. With relatively low initial concentration of phenol, the lag phase of the strain was shorter than that with high concentration of phenol, which meant that the isolate need more time to adapt to the toxic environment provided by relatively high initial concentration of phenol. The average removal rates of phenol with different phenol initial concentrations are shown in Figure 4. The average removal rates of phenol increased from 7.6 to 10.4 mg L<sup>-1</sup> h<sup>-1</sup> when the initial concentration of phenol increased from 100 to 300 mg L<sup>-1</sup> and peaked at 10.8 mg L<sup>-1</sup> h<sup>-1</sup> with approximately 400 mg L<sup>-1</sup> of initial phenol. Further increase of the initial concentration of phenol led to the decrease of phenol degradation efficiency, which suggested that 400 mg L<sup>-1</sup> was the optimal initial concentration of phenol for strain HP-1. When the initial concentration of phenol increased to 1,300 mg L<sup>-1</sup>, the average removal rate of phenol was nearly 0, indicating that microbial metabolism was almost completely inhibited. The variation of OD<sub>600</sub> with different initial concentrations of phenol in Figure 4 also showed a similar trend. When the initial concentrations of phenol were less than 700 mg L<sup>-1</sup>, the maximum OD<sub>600</sub> increased with the rise of initial phenol concentration because more substrate in the media promoted cell growth. However, when the initial concentration was over 900 mg L<sup>-1</sup>, the OD<sub>600</sub> notably decreased, which was due to the inhibition of microbial growth. The results indicated that although several bacteria could tolerate toxic pollutants, the high concentration of these

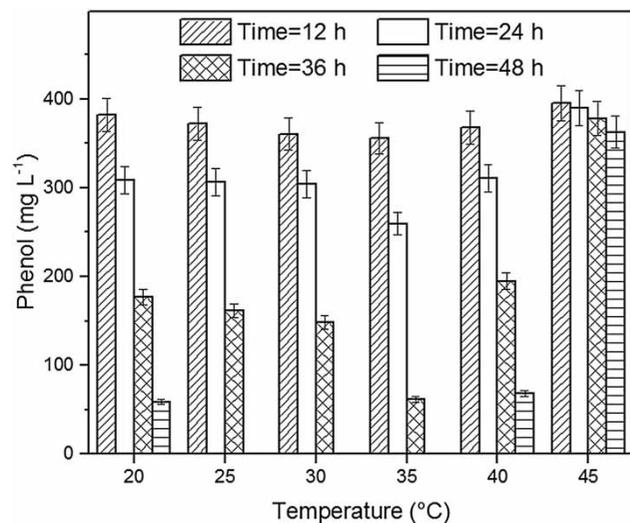


**Figure 4** | Cell growth (presented as OD<sub>600</sub>) and phenol removal rates with different initial phenol concentrations at salinity of 5% (w/v).

contaminants could have negative effect on cell growth as well as biodegradation rates (Ahmed & Ahmed 2016).

### Phenol removal at different temperature and pH

Phenol degradation performance of the strain HP-1 with the variation of temperature and pH values was investigated because enzymes play an important role in biodegradation and can be remarkably affected by these two parameters (Bhunia *et al.* 2013). As shown in Figure 5, the optimal temperature was 35 °C. The removal efficiencies of phenol within 12, 24, and 36 h were 11.7%, 35.5%, and 84.6%, respectively. More than 400 mg L<sup>-1</sup> of initial phenol could be completely removed within 48 h. Figure 5 also shows that there was no phenol left in the media when temperature was 30 °C and 25 °C, which indicated high degradation efficiency of phenol in 48 h. When the temperature dropped to 20 °C or rose to 40 °C, phenol removal rates decreased slightly. The removal efficiencies of phenol within 36 h were 56.1% and 51.6%, respectively, at 20 °C and 40 °C and more than 83% of initial phenol was degraded within 48 h. However, when the temperature increased to 45 °C, there was only a slight decrease of the residual concentration of phenol. The phenomenon along with the variation of temperature was similar to the results of many previous studies of biodegradation by isolated microorganisms where removal performance of pollutants became worse at temperatures that were too low or too high (Meena *et al.* 2016; Satapute & Kaliwal 2016). Regarding pH, phenol biodegradation shows satisfactory performance when pH of the media was 5.0, 6.0, and 7.0, at which



**Figure 5** | The effect of different temperatures on phenol biodegradation by strain HP-1 at salinity of 5% (w/v).

phenol could be completely degraded within 48 h (Figure 6). The highest phenol removal rates appeared at pH 6.0. The removal efficiencies of phenol within 12, 24, and 36 h were 15.6%, 38.0%, and 92.4%, respectively. At pH of 9.0, only 5.5% of initial phenol could be degraded in 48 h. The results at different pH indicated that the strain preferred slightly acidic and neutral environment.

### Salt tolerance

Halophilic microorganisms could grow in saline and hypersaline conditions. The tolerance of salinity of the strain was therefore evaluated and the results were shown in Figure 7. The isolate could grow and degrade phenol in media with salinity less than 10%. When there was no salt, the strain had the highest phenol degradation rate. Over 400 mg L<sup>-1</sup> of initial phenol could be completely degraded within 20 h. With the increase of salinity, phenol degradation rates decreased. It took 28, 40, and 64 h, respectively, for complete removal of the same concentration of initial phenol when the salinity was 2%, 4%, and 6%. More than 98.5% of initial phenol was degraded in 72 h in salinity of 8%. The degradation efficiency of phenol in 72 h was only 23.3% when salinity was 10%. In addition, the residual concentration of phenol was almost the same during 72 h shaking in salinity of 12%, suggesting the strong inhibition of cell growth and phenol biodegradation in high salinity, which was also found in the variation of OD<sub>600</sub> that showed no obvious increase in media containing 12% of salinity (data not shown).

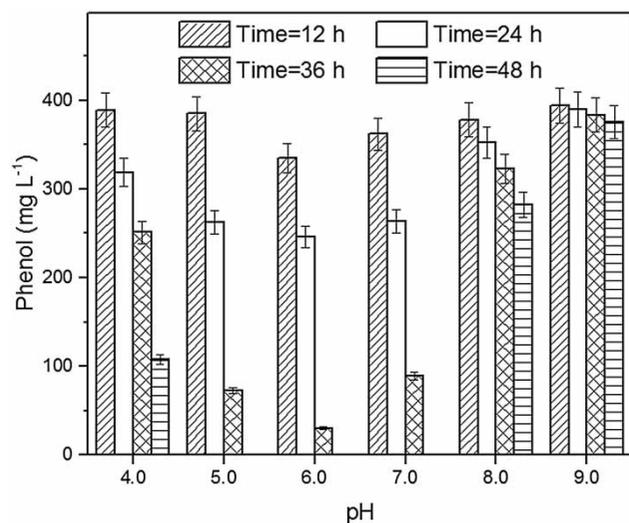


Figure 6 | The effect of pH on phenol biodegradation by strain HP-1 at salinity of 5% (w/v).

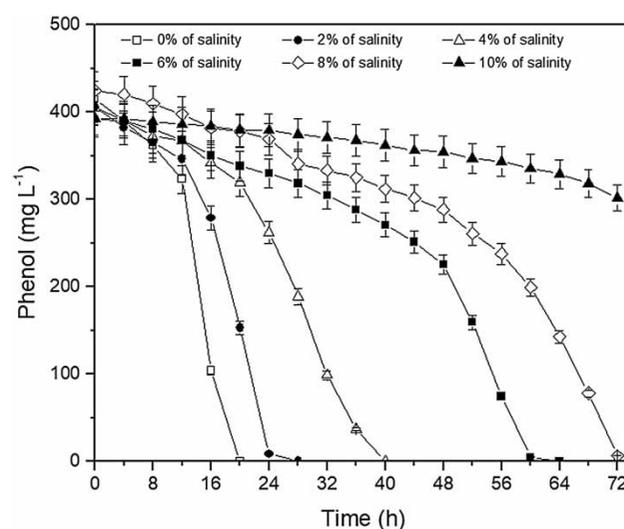


Figure 7 | Salt tolerance of the strain HP-1 at 35 °C and pH of 6.0.

### CONCLUSIONS

In this study, a halophilic strain which could utilize phenol as sole source of carbon and energy was isolated. The bacterial strain was identified as a member of genus *Citrobacter*. The strain could grow in media with phenol less than 1,100 mg L<sup>-1</sup> and could tolerate salinity less than 10%. The optimal temperature, pH, and salinity were 35 °C, 6.0, and 0%, respectively. Under optimal conditions, over 400 mg L<sup>-1</sup> of phenol could be completely degraded within 20 h. The results of the present work indicated that the strain might be promising for phenol removal in hypersaline industrial effluents.

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