Immobilization of halophilic yeast for effective removal of phenol in hypersaline conditions

Yu Jiang, Kai Yang, Tao Deng, Bin Ji, Yu Shang and Hongyu Wang

ABSTRACT

A halotolerant yeast strain of *Candida* sp. was purified for phenol biodegradation and was immobilized in alginate and nano-SiO_2. The concentration of nanoscale SiO_2 was optimized and phenol degradation performance with different initial phenol concentrations was evaluated. Three common kinetic models were used to correlate the experimental data. The effects of pH and salinity on phenol biodegradation were also investigated. It was found that 1.0% (w/v) was the optimal nano-SiO_2 concentration and the immobilized cells had a better phenol removal performance compared to free cells. More than 99% of 600 mg l^{-1} phenol was removed by the immobilized strains within 48 h. The immobilized cells also showed highest phenol degradation rates when pH and salinity were 6.5 and 0%, respectively. The high removal efficiency of phenol in reusability tests indicated the promising application of the immobilized *Candida* strain in phenol degradation under hypersaline conditions over a long period.

Key words | alginate, halophilic yeast, immobilization, kinetics, phenol biodegradation, SiO_2 nanoparticles

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INTRODUCTION

As society develops rapidly, large amounts of pollutants are discharged into areas of soil or water which does great harm to human health as well as to the ecosystem. Aromatic compounds are one of the most common kinds of contaminants in the environment due to both natural distribution and anthropogenic activities (Zhang *et al.* 2013). Among them, phenol has a simple structure as well as prominent application. It is generated from a variety of industrial processes such as petrochemical, oil refineries, paper manufacturing, plastics, pesticides, explosive production and rubber industries (Singh & Balomajumder 2016). It has been found that a small amount of phenol can be toxic to humans and aqueous creatures (Liu *et al.* 2012). Phenolic substances are water-soluble and capable of diffusing in liquids in a short period even at relatively low concentrations. They can also lead to an unpleasant odor and taste in water bodies. Therefore, considerable attention should be paid to the treatment of industrial effluents with these pollutants (Massalha *et al.* 2010).

Many treatment technologies are utilized for the removal of phenol from wastewater. Biological methods are preferred in contrast with physical and chemical methods since they are cost-effective and environmentally safe (Singh & Fulekar 2010). Based on highly efficient microbial metabolisms, these biological processes including activated sludge sequencing batch reactors (SBRs), aerobic granular sludge reactors, membrane bioreactors, and many other systems are considered to have relatively satisfactory removal performances owing to the fact that aromatic compounds are not only environmental pollutants but also growth substrates for microorganisms (Tay *et al.* 2005; Praveen & Loh 2016). However, hypersaline conditions cause the strong inhibition of microbial metabolisms and further bring about a failure to pollutant biodegradation since each kind of microbial species has optimum and limited salinity for growth. When the salinity of environments is beyond the tolerant level, dehydration and plasmolysis of cells will happen (Shen *et al.* 2015). For instance, van den Brand *et al.* (2015) reported the effect of salinity on sulfate reducing bacteria, noting that the sulfate reduction rate decreased by 41% with the increase of salt concentration. A forward osmosis and continuous airlift nitrification bioreactor were integrated to treat high strength ammonia wastewater. It could be observed that salinity higher than 17 g-TDS l^{-1} inhibited

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the activity of the nitrite-oxidizing bacteria in the process (Jeong *et al.* 2016). Wang *et al.* (2016) investigated the toxicity of inorganic salts to denitrifying granular sludge and found that the toxicity of low salinity was due to the inhibition of denitrifying activity, whilst the toxicity of high salinity was because of both the inhibition of denitrifying activity and the lethality of the denitrifying cells. Although most microorganisms are deactivated in high salt environments, there are still a few halophilic strains capable of withstanding hypersaline environments, which makes it possible for biological systems to be considered in treating some kinds of industrial wastewater such as leather and petroleum effluents which always contain both high salt and phenolic pollutants (Castillo-Carvajal *et al.* 2014).

Actual industrial effluents are always mixtures of hazardous contaminants and the conditions of these wastewaters vary distinctly, which is challenging for proper functioning of free cells. Compared to freely suspended microbial strains, immobilized cells have a lot of advantages such as resistance to adverse environments, easy separation, long-time reuse and high biomass concentration in the system. Alginate is one of the most common polymer matrices applied to immobilization technology not only because it is easily accessible, biocompatible, and economic, but also because it has the advantages of a highly porous structure, leading to good diffusion performance of solutes and dissolved gases (Bergero & Lucchesi 2015). With the addition of silica nanoparticles, a nanoporous monolithic structure can be formed. The microsized pores on the surface are able to induce and adsorb various molecules into the pores, which is beneficial for pollutant reduction (Perullini *et al.* 2010). Furthermore, during the formation of a hybrid matrix, nano-scale silica can be embedded through hydrogen bonds. As a consequence, an increase of the modulus of elasticity and tensile strength of the material can be observed (Sheykhnazari *et al.* 2016). Therefore, nano-silica was chosen to improve the phenol removal performance of alginate beads containing *Candida* strains. In this study, a halophilic strain of *Candida* sp. was entrapped in alginate and SiO_2 nanoparticles for phenol removal under hypersaline conditions. The dosage of nano-SiO_2 was optimized and the kinetics of phenol biodegradation were investigated. Phenol removal performance was evaluated at different pH values and salinities. Furthermore, the immobilized cells were used repeatedly for long time operation. The goal of this work was to provide some guidance for the development of salinity-tolerant biological processes for hypersaline phenol-rich industrial wastewater treatment.

METHOD

Strain isolation and identification

The halophilic strain was isolated from activated sludge which was collected from the biological tank of a pharmaceutical plant from Wuhan, Hubei Province, China. Since the pharmaceutical wastewater in the plant was hypersaline, the activated sludge was salt-tolerant. After the sludge was cleaned by water several times to remove sulfates, the isolates were separated by streaking inoculation and multipoint inoculation on plates with selective media. The components of the selective media were ($g\,l^{-1}$): phenol 0.5, NaCl 50.0, KH_2PO_4 2.0, K_2HPO_4 1.3, NH_4Cl 0.1 and 1 ml of trace mineral solution. The compositions of the trace mineral solution (per liter) were: $FeCl_3{\cdot}6H_2O$ 0.01 g, H_3BO_3 0.15 g, $CuSO_4{\cdot}5H_2O$ 0.03 g, KI 0.18 g, $MnCl_2{\cdot}4H_2O$ 0.12 g, $Na_2MoO_4{\cdot}2H_2O$ 0.06 g, $ZnSO_4{\cdot}7H_2O$ 0.12 g, $CoCl_2{\cdot}6H_2O$ 0.15 g, EDTA 10 g. All chemicals were of analytical grade. The morphology of the isolate was observed by using an optical microscope (CX31, Olympus Co., Ltd, Tokyo, Japan). The operations for purified strain identification were according to our previous paper (Jiang *et al.* 2016). The sequence of the strain was submitted to an online BLAST search in the GenBank databases (National Center for Biotechnical Information, NCBI, http://www.ncbi.nlm.nih.gov) under accession number KP243194.

Strain immobilization

To improve the isolated strain's tolerance to disadvantageous conditions, Na-alginate and nanoscale SiO_2 were used to entrap the microbial cells. The SiO_2 nanoparticles applied in the present work were purchased from Aladdin Industrial Corporation Ltd of Shanghai, China. In detail, the purified strain on the agar plate was first transferred into 100 ml of beef extract peptone media and incubated in a rotary shaker at 30 °C and 160 rpm. When the strain was enriched, the mixture was added to selective media with the inoculation ratio of 10% (v/v). After 72 h of incubation under the same condition, the cells and nano-SiO_2 were transferred to sodium alginate solution which was sterilized by autoclaving at 121 °C for 20 min. The final concentration of Na-alginate was 3% (w/v) and the inoculation ratio of the strain was 25% (v/v). The mixture was dropped into 3% (w/v) $CaCl_2$ solution to produce Ca-alginate beads after being fully stirred. The beads were subsequently stored in the $CaCl_2$ solution at 4 °C for 8 h aiming at

formatting the gel completely. Before used for further tests, the beads were washed several times with deionized water to remove $CaCl_2$ residues thoroughly.

Optimization of nano-SiO_2 concentration for phenol degradation by immobilized cells

The concentration of nanoscale SiO_2 was optimized by investigating phenol degradation rates with immobilized strains containing different dosages of SiO_2 nanoparticles. Briefly, four 250 ml flasks with 150 ml selective media were sterilized for the tests. Ca-alginate beads were prepared with the addition of nanoscale SiO_2, the concentration of which ranged from 0.5% to 3% (w/v). After four kinds of beads were fully formatted, 40 ml of each kind of the immobilized cells was transferred into selective media with 600 mg l^{-1} of initial phenol and 5% (w/v) NaCl. The flasks were subsequently placed into a rotary shaker at 30 °C and 160 rpm for 48 h. The samples were taken from the flasks at intervals of 4 h to measure residual concentrations of phenol. The flasks were kept in darkness to avoid photo-destruction of phenol.

Phenol biodegradation by free and immobilized cells

After the optimal concentration of nano-SiO_2 to be added to the sodium alginate solution was found, phenol degradation by both free and immobilized cells was evaluated. 40 ml of isolated strains entrapped in alginate and nanoscale SiO_2 were inoculated into 250 ml flasks with 150 ml selective media containing 300, 600 and 800 mg l^{-1} of initial phenol at a salinity of 5%. Regarding free cells, 10 ml of freely suspended strains were transferred into another three flasks with the same three initial concentrations of phenol. The flasks were then put into a rotary shaker at 30 °C and 160 rpm for 72 h. The supernatants of the media were taken out from the flasks at intervals of 4 h and the remaining concentrations of phenol were tested after sample collection. All flasks were placed in darkness to avoid phenol photo-destruction.

Phenol degradation kinetics

In order to represent the degradation kinetics of phenol by the immobilized cells, three common kinetics models, namely the Haldane model (Haldane 1965), the Yano model (Yano *et al.* 1966), and the Edward model (Edwards 1970) were used to fit the experimental data of the present study at a salinity of 5%. As shown in Table 1, S_0 represented the initial substrate concentration (mg l^{-1}), K_S is the substrate-affinity constant (mg l^{-1}), K_I is the substrate-inhibition constant (mg l^{-1}), and K is the Yano constant. q is the degradation rate (h^{-1}) which is obtained as follows:

$$q = -\frac{1}{X}\frac{dS}{dt}$$

where S is the residual phenol concentration in the media (mg l^{-1}), X is the dried cell concentration at the end of the logarithm phase.

With each initial concentration of phenol, a specific degradation rate q was calculated accordingly. The values of the kinetic parameters of the three models mentioned above were obtained from the values of q versus S_0 by MATLAB 2012.

Effect of pH and salinity

The effects of pH values as well as salinity in the media were investigated by adjusting pH and NaCl concentrations from 6.0 to 7.5 and 0% to 11% (w/v), respectively. 40 ml of Ca-alginate beads containing the strains were added into four flasks with selective media containing NaCl of 5% (w/v) at pH of 6.0, 6.5, 7.0, and 7.5, respectively. The pH was adjusted by using sterilized hydrochloric acid and sodium hydroxide. With respect to the variation of salinity, free and immobilized cells were inoculated into selective

Table 1 | Parameters of kinetic models for phenol biodegradation by immobilized cells

Kinetic model	q_{max} (h^{-1})	K_S (mg L^{-1})	K_I (mg L^{-1})	K (mg L^{-1})	R^2
Haldane: $q = \frac{q_{max}S_0}{K_S + S_0 + (S_0^2/K_I)}$	15.92	42,603	1.10	–	0.993
Yano: $q = \frac{q_{max}S_0}{K_S + S_0 + (S_0^2/K_I)(1 + S_0/K)}$	1.06	2,941	22.30	1,712	0.999
Edward: $q = q_{max}\left[\exp\left(\frac{-S_0}{K_I}\right) - \exp\left(\frac{-S_0}{K_s}\right)\right]$	0.088	136.45	504.40	–	0.991

media with 0%, 3%, 5%, 7%, and 11% (w/v) of NaCl. The samples were withdrawn from the flasks every 4 h to measure phenol concentrations.

Reusability study

The strain immobilized in alginate and nanoscale SiO_2 was used in repeated batch-experiment degradation of phenol for investigating the reusability of the immobilized cells. Ca-alginate beads were inoculated into selective media containing NaCl of 5% (w/v) and incubated in a rotary shaker at 30 °C and 160 rpm. After phenol in the media ran out, the beads were taken from the flasks and washed several times with sterilized deionized water. Subsequently, the beads were transferred into another fresh media with the required initial concentration of phenol and placed in the rotary shaker under the same experimental condition for the next cycle of phenol biodegradation. The samples were collected from the flasks periodically and phenol concentrations were determined.

Analytical methods

Samples obtained at different times were centrifuged at 5,000 rpm for 10 min before measuring phenol concentrations. The concentrations of phenol were determined according to a 4-aminoantipyrine spectrophotometric method based on the rapid reaction between 4-aminoantypirine and phenol which produces red dye under alkaline conditions (APHA 2005). The tests were done in triplicate. The pH values of the media were monitored by a pH meter (PHS-3C, Shanghai INESA Scientific Instrument Co., Ltd, Shanghai, China).

RESULTS AND DISCUSSION

Effect of nanoscale SiO_2 concentrations

The SiO_2 nanoparticles, as part of the immobilization support, have a large surface area. Therefore, the concentration of nano-silica was expected to influence biodegradation of pollutants. The optimal concentration of nano-silica should be found to achieve the highest removal rates of phenol before the practical application of strain-containing beads. The isolated strain identified as *Candida* sp. was entrapped in Ca-alginate beads with different concentrations of SiO_2 nanoparticles in this study. As shown in Figure 1, the phenol removal rates were relatively high when adding

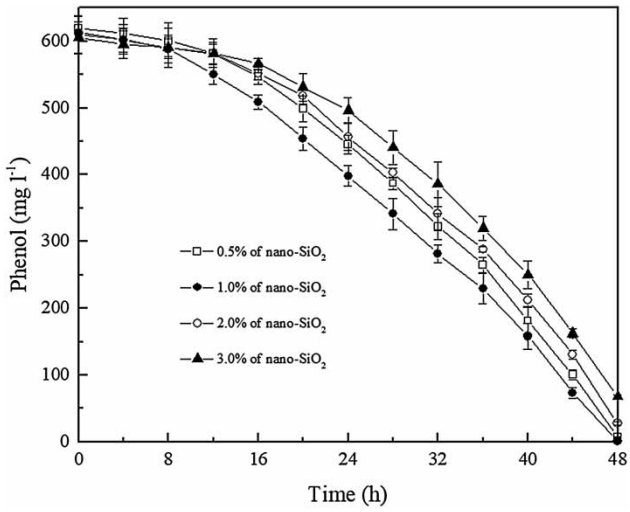

Figure 1 | Phenol removal performance of *Candida* sp. immobilized with alginate and different concentrations of nano-SiO_2.

nano-SiO_2 to the beads. The optimal dosage of nanoscale SiO_2 was 1 g per 100 ml since the phenol degradation rate was the highest. The phenol removal efficiencies within 48 h was 99.8% when initial concentration of phenol was over 600 mg l^{-1}. The phenol degradation rates of immobilized cells containing 0.5% SiO_2 nanoparticles ranked second and 98.9% of initial phenol was able to be removed after 48 h shaking. When the concentration of nanoscale SiO_2 increased to 2% and 3%, the phenol removal rates showed a slight drop. The results indicated that an exceeded amount of nanoparticles could reduce the space utilization for the microorganisms and led to a decrease of pollutant removal performance. Considering phenol removal efficiency, during the tests of the next stage the immobilized cells were prepared by adding 1% of SiO_2 nanoparticles.

Phenol biodegradation performance

Phenol degradation performances by free and immobilized cells were evaluated with 300, 600 and 800 mg l^{-1} of initial phenol. The results are exhibited in Figure 2. It could be found from the time courses that the removal rates of phenol by the two kinds of cells were almost the same with 300 mg l^{-1} of initial phenol. The removal efficiencies of phenol by immobilized cells and freely suspended strains within 36 h were 99.5% and 96.8%, respectively. With regard to phenol removal performance with 600 mg l^{-1} of initial phenol, the phenol degradation rates were a little higher when immobilized cells were used than when free cells were used. More than 99% of initial phenol was able to be removed by alginate and nano-SiO_2 immobilized

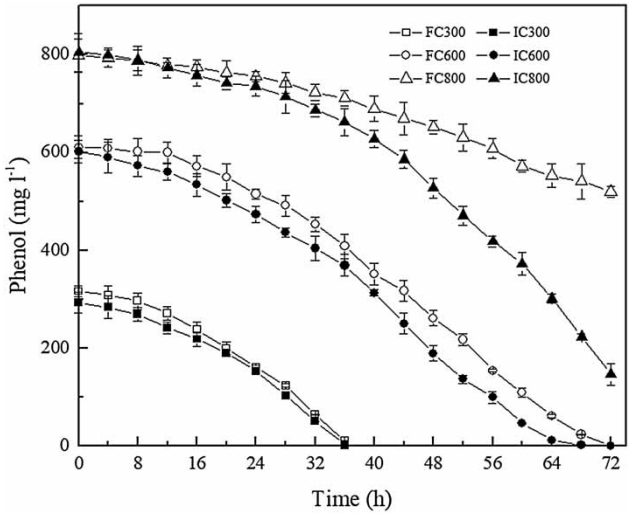

Figure 2 | Time courses of phenol biodegradation by free cells (FC) and immobilized cells (IC) with 300, 600, and 800 mg l^{-1} of initial phenol.

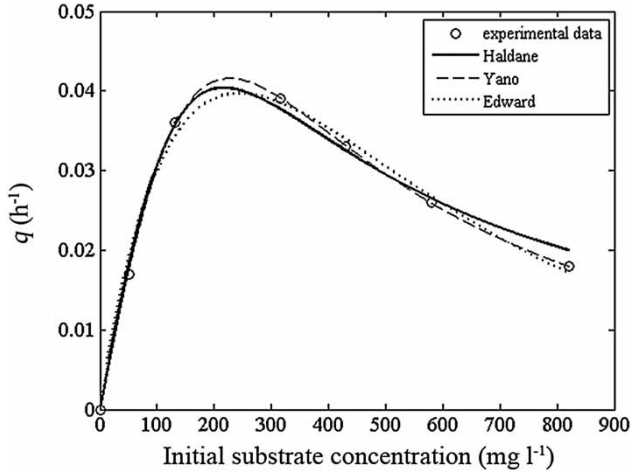

Figure 3 | Correlation of specific biodegradation rates of phenol and initial substrate concentrations by using the Haldane, Yano, and Edward models.

strains within 68 h while the removal efficiency of phenol by freely suspended cells was 96.2% during the same period of time. When the initial concentration of phenol increased to 800 mg l^{-1}, the immobilized cells showed noticeably better degradation capacity, indicating that the strains inside the beads were protected from harsh environments (Zhang *et al.* 2015). The better removal performance of the immobilized cells than the free strains when fed with high concentration of phenol indicated that alginate and nano-SiO_2 entrapped strains could be a promising alternative applied in practical industrial wastewater treatment, since some kinds of effluents have relatively high concentration of phenol. It can also be observed from Figure 2 that it took a much longer time for the strain to adapt to the media when initial concentration of phenol was beyond 600 mg l^{-1}, no matter what forms of the isolate were used. This might be attributed to the toxicity of phenolic compounds which inhibited the microbial activities (Xu *et al.* 2016). Even for phenol-degrading microorganisms, high concentration of phenol could have an adverse effect on cell growth and further reduce phenol removal rates.

Modeling of the phenol biodegradation process

The phenol degradation rates of the *Candida* strain immobilized in alginate beads containing SiO_2 nanoparticles with different initial phenol concentrations were calculated. The results are presented in Figure 3 in which three common theoretical kinetic models are used to describe the relation between the degradation rate and the initial substrate concentration. The obtained parameters in the kinetic model could indicate the inhibition of phenol due to its toxicity and find the maximum degradation rate to evaluate the phenol biodegradation capacity of the immobilized cells with the variation of initial phenol concentrations. After correlation to the kinetic models, the degradation rate of phenol by the immobilized cells could be predicted when a specific initial concentration of phenol was given in practical application. It can be found from Figure 3 that the phenol degradation rate first increased sharply and reached maximum when the initial concentration of phenol was approximately 300 mg l^{-1}. Further increasing initial phenol concentration led to a slight drop of phenol degradation rates, indicating the inhibitory effects of phenol. The values of the parameters of the three kinetic models, namely Haldane, Yano, and Edward, were obtained by the fitness of experimental observations of the degradation rate and the corresponding initial phenol concentration. The predicted curves in Figure 3 were in agreement with the experimental data. As shown in Table 1, the experimental results were well fitted to the three models since relatively high correlation coefficients (R^2) were obtained. The large value of K_I in the Edward model suggests that the immobilized cells were not very sensitive to the toxicity of the substrate (Edwards 1970). This could be ascribed to the protection of the strains by the beads against the changes in phenol concentration.

Phenol removal at different pH and salinity

Phenol biodegradation by immobilized *Candida* sp. with variation of pH values is presented in Figure 4. It was

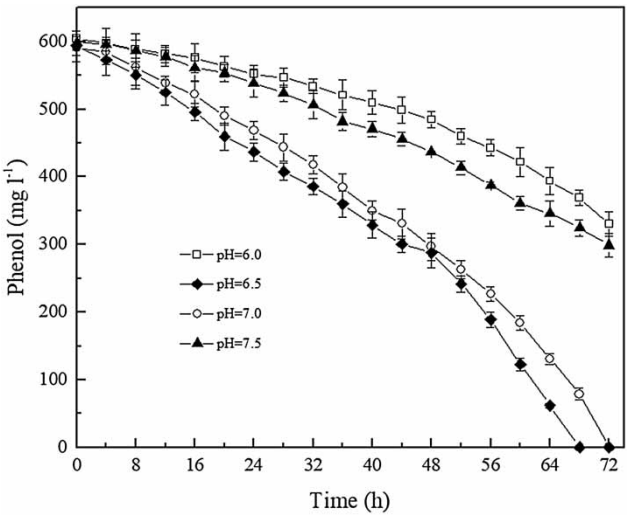

Figure 4 | Effects of pH on the phenol removal by immobilized cells.

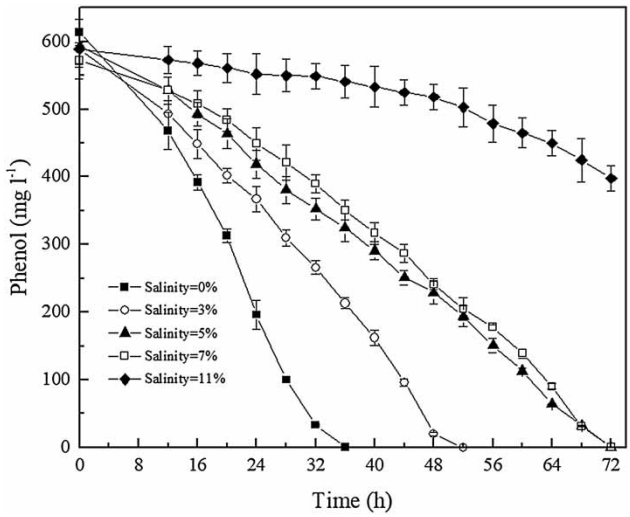

Figure 5 | Effects of salinity on the phenol biodegradation capacity of the immobilized strain.

found that the phenol degradation rate was the highest at a pH of 6.5, followed by a pH of 7.0. The removal efficiencies of phenol at pH 6.5 were 26.4%, 51.5%, and 99.96%, respectively within 24, 48 and 72 h when the initial concentration of phenol was approximately 600 mg l^{-1}. Compared with phenol removal at pH 6.0, immobilized cells at pH 7.5 showed better performances. The result of the present study was in accordance with many previous reports that the suitable pH of the environment was neutral or nearly neutral for the biodegradation of toxic pollutants by free cells and immobilized strains (Nawawi *et al.* 2015; Liu *et al.* 2016).

Considering that the salinity of phenol-containing industrial effluents varies significantly and the biological processes are always influenced by the concentration of salt, phenol removal capacity of the immobilized *Candida* strain was also evaluated when salinity ranged from 0% to 11% (w/v). As shown in Figure 5, relatively high removal rates of phenol were achieved. More than 99% of approximately 600 mg l^{-1} of initial phenol was degraded within 72 h at a salinity varying from 0% to 7%. The optimal salinity was 0%, at which over 600 mg l^{-1} of initial phenol was completely removed within 36 h. It could also be found from Figure 5 that although a high removal efficiency of phenol was performed at salinity of 7%, the phenol degradation rate decreased compared to that at lower salinity. When the salt concentration was further increased to 11%, phenol removal rates dropped significantly. This result might be due to the obvious cell growth inhibition at high salinity. The high removal efficiency of phenol at salinity ranging from 0% to 7% suggested the application potential of the immobilized *Candida* strain for treating hypersaline phenol-rich industrial effluents.

Evaluation of bead reusability

To establish the long-term stability of phenol degradation in a saline environment by immobilized cells, reusability tests of the fungal strain entrapped in alginate and nanoscale SiO_2 were carried out by five cycles of operation. The stability of the beads in a saline environment is important since the breakage of some beads will result in the loss of immobilized microorganisms, which further leads to worse phenol biodegradation performance. The beads were washed out and put into fresh media after the initial phenol was used up. The time course of residual concentrations of phenol is presented in Figure 6. It can be observed that the phenol removal performance was relatively high during the tests. Over 560 mg l^{-1} of initial phenol was able to be removed within 48 h in Cycle 1. Approximately 600 mg l^{-1} of initial phenol could also be degraded in the same period of time from Cycle 2 to Cycle 5. In Yu *et al.* (2015), which describes the decolorization by a newly isolated salt-tolerant *Bacillus* sp. strain, the removal efficiency of dyes by the Ca-alginate beads containing the bacterial cells dropped when shifted from cycle I to cycle II. In the present study, the removal rates of the pollutant remained relatively high after five cycles of phenol biodegradation, suggesting that the immobilization process had high biomass concentration and was beneficial for cell separation from the solution.

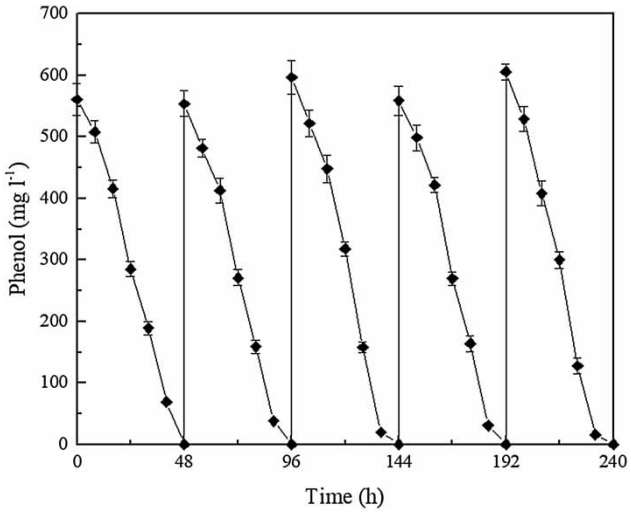

Figure 6 | Phenol biodegradation by *Candida* strain entrapped in alginate with nanoscale SiO_2 in repeated batch tests (at a pH of 6.5).

CONCLUSIONS

The immobilization of a halophilic *Candida* strain in alginate and SiO_2 nanoparticles led to better tolerance to high concentration of phenol (up to 800 mg l^{-1}) as well as variable salinity conditions (0–11% of NaCl). The alginate nano-SiO_2 containing beads with a highly porous structure could not only protect cells from a harsh environment but also maintain good diffusion performance of the substrates and dissolved gases. The optimal concentration of nanoscale SiO_2 and the pH were 1% (w/v), and 6.5, respectively. The phenol degradation rate peaked at 300 mg l^{-1} of initial phenol and exhibited inhibitory behavior which could be correlated well by the Yano model. The phenol removal performance in the reusability tests also suggested that the beads could keep stable during long periods of operation. The above parameters and results are invariably required for the design of continuous bioreactors treating hypersaline phenol-containing industrial wastewater in further research. The high removal efficiency of phenol with the variation of initial phenol concentration, pH and salinity by the entrapped cells and the reusability of the beads suggests that the immobilized *Candida* strain could be a promising process for treating real hypersaline industrial effluents rich in phenol.

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