Journal of Hazardous Materials 183 (2010) 366–371

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Biodegradation of phenol at high concentration by a novel fungal strain *Paecilomyces variotii* JH6

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ARTICLE INFO

*Article history:*
Received 18 February 2010
Received in revised form 5 July 2010
Accepted 9 July 2010
Available online 15 July 2010

*Keywords:*
Phenol
Biodegradation
Kinetics
*Paecilomyces variotii*

ABSTRACT

A novel phenol-degrading filamentous fungus, strain JH6, was isolated from activated sludge and identified as a member of *Paecilomyces variotii* based on standard morphological and phylogenetic analysis. The degradation assays suggested that the strain was able to utilize phenol as the sole source of carbon and energy at concentrations up to 1800 mg/l. The strain exhibited optimum phenol degradation performance with the addition of 100 mg/l glucose at pH 5, 37 °C. Haldane's model could be fitted to the growth kinetics data well over a wide range of initial phenol concentrations (100–1800 mg/l), with kinetic values $\mu_{max}$ = 0.312 $h^{-1}$, $K_s$ = 130.4 mg/l, and $K_i$ = 200 mg/l. The decay coefficient was found to be 0.0073 $h^{-1}$. Complete phenol degradation by strain JH6 could be achieved in the presence of other toxicants, such as m-cresol and quinoline, which were often found in the real phenol-containing wastewater.

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## 1. Introduction

Phenol is a major pollutant which can be found in several types of industrial wastewater, for example, the wastewater from coal refineries, phenol manufacturing pharmaceuticals, and industries of resins, paints, dyes, petrochemicals, textiles, pulp and paper mills [1]. Phenol is difficult to be decomposed biologically. It is toxic to plants, microorganisms, animals and humans, causing serious environmental problems [2]. In addition, it is water soluble and highly mobile, and so it is likely to reach drinking water sources downstream from discharges, where, even at low concentrations, it can cause severe odor and taste problems and pose risks to populations.

As a result of growing awareness over pollution caused by phenol release, efforts are being made to minimize their adverse effect. Currently, many treatment techniques such as activated carbon adsorption, solvent extraction, chemical oxidation, electrochemical oxidation and biodegradation have been developed to remove phenol from contaminated environment [3]. Of these options, physicochemical methods have proven to be costly and have the inherent drawbacks of causing a secondary pollution. However, biodegradation technique, which is environmental friendly and cost effective, has turned out to be a favorable alternative [4].

Aerobic microorganisms are efficient at degrading toxic compounds and usually transform the organic compounds into inorganic compounds such as $CO_2$ and $H_2O$ [5]. However, most of the phenol-degrading strains belong to bacterial family. Fungi, such as *Aspergillus fumigatus* [6,7], *Graphium* sp. FIB4 [8], *Trichosporon cutaneum* [9], *Candida tropicalis* [10], and *Penicillium chrysogenum* have also been reported for the biodegradation of phenol [11]. However, to best of our knowledge, information about phenol degradation by *Paecilomyces* sp. is still missing.

In this study, phenol degradation by a newly isolated fungus, named after *Paecilomyces variotii* JH6, was described. The effects of the various abiotic factors, such as temperature, pH, additional glucose concentration, on the biodegradation performance of phenol by strain JH6, were evaluated. The kinetics of phenol removal was also investigated in a batch reactor. Since phenol-containing raw wastewater usually contains other toxicants, such as m-cresol and quinoline, the phenol degradation by strain JH6 in the presence of these toxicants was also tested. The overall purpose of this study was to demonstrate the degradation effectiveness and application potential of *P. variotii* JH6.

## 2. Materials and methods

### 2.1. Isolation of phenol-degrading strain

Microorganisms were isolated from activated sludge from a local coking wastewater treatment plant located in Wuhan, Hubei Province, PR China. Selection of microorganisms capable of degrading phenol was performed in mineral salts medium (MSM) containing $KH_2PO_4$ (1 g/l), $(NH_4)_2SO_4$ (1 g/l), $MgSO_4{\cdot}7H_2O$ (0.5 g/l), supplemented with 300 mg/l phenol as the sole carbon and energy source. Agar plates contained 20 g of agar per liter of medium.

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doi:10.1016/j.jhazmat.2010.07.033

The activated sludge was serially diluted and streaked onto phenol mineral salts agar plates. The cultures were incubated at 37 °C for 120 h, before the predominant microorganisms were picked and incubated in MSM supplemented with 300 mg/l phenol. When the phenol depleted, 5 ml of the cultures were transferred to fresh MSM to initiate the degradation. From then on, the cultures were transferred successively to fresh MSM using identical growth conditions at each transfer, except that the phenol concentration increased stepwise, varying from 300 mg/l to final 1800 mg/l at 300 mg/l interval. During this process, 100 mg/l glucose was added to stimulate the growth of the strains. Of the isolates obtained, strain JH6, which exhibited the best phenol degradation ability was selected for further degradation study. The strain JH6 can be primarily identified as a member of fungi based on standard morphological characteristics.

### 2.2. Identification of the phenol-degrading strain

Genomic DNA was extracted from fresh fungal mycelium in the mineral salts medium according to Li et al. [12]. The extracted genomic DNA was used as template for PCR. The ITS1-5.8S rRNA gene-ITS2 DNA fragment of the fungi was amplified by PCR using primers ITS1 (5′-TCCGTAGGTGAACCTGCCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR amplification was performed in a total volume of 50 μl containing 0.1 μg of template DNA described above, 5 μl of 10× PCR buffer (contained 17.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 μM of each primer, 2.5 units of *Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR amplification was performed in an automated thermal cycler (PTC-100, MJ Researcher, U.S.A.) under the following conditions: predenaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; with a penultimate step of 72 °C for 10 min, final cooling to 4 °C. For each PCR reaction, 5 μl of PCR products were also examined by electrophoresis. The ITS1-5.8S rRNA gene-ITS2 sequence was determined directly by Tianyihuiyuan Bio. Tech Company. The sequences obtained were compiled and compared with sequences in the GenBank databases using BLAST program.

A maximum likelihood phylogenetic tree was generated by neighbor-joining methods. Evolutionary distance bootstrap values were determined using DNADIST program in PHYLIP.

### 2.3. Biodegradation experiments

The biodegradation experiments were conducted in a series of 250 ml sterile flasks. Each flask contained 50 ml sterile MSM with phenol concentrations varying from 100 to 1800 mg/l. The inoculum in this study was prepared by growing JH6 in MSM supplemented with 600 mg/l phenol and 100 mg/l glucose, on a rotary shaker at 200 rpm and 37 °C. The strains were incubated for 20–30 h in order to obtain similar growth condition and to minimize variations in the starting inoculum. The inoculum biomass concentration was maintained at 25 mg/l.

To investigate the phenol biodegradation performance at different pH, temperature and additional glucose concentration, strain JH6 was transferred into flasks with an initial biomass concentration of 25 mg/l. Because the initial pH of mineral salts medium (MSM) was 4.8–5.4, so the pH of the medium was adjusted by adding 1 N HCl or NaOH.

In order to estimate the kinetic parameters of phenol degradation, the net effect of phenol concentration on fungal growth was investigated. Initial phenol concentrations varied from 100 to 1800 mg/l. Under each condition, three flasks were used to test for consistency. All of above experiments were carried out at initial pH of 5.0 and the flasks were incubated on rotary shaker at 200 rpm and 37 °C.

The same experimental procedure was used for testing the capacity of strain JH6 to degrade phenol in the presence of m-cresol or quinoline. Phenol concentrations were 600 mg/l with m-cresol concentration ranged from 100 to 300 mg/l and quinoline concentrations ranged from 100 to 200 mg/l. Samples were taken periodically for determining the concentrations of phenol, m-cresol and quinoline.

The results were reported as an average of three independent experiments. Standard deviations in the mean values (error bars) were indicated.

### 2.4. Phenol degradation kinetics

In our work, it was presumed that the aeration provided by shaking the flasks was able to keep the oxygen concentration sufficient and not limited, the influence of oxygen was not considered. Thus, the strain JH6 growth rate and phenol degradation rate were only limited by substrate concentration at fixed initial pH, temperature and shaking rate.

Cell growth kinetics in a batch reactor may be modeled by the following equation:

$$\frac{dX}{dt} = \mu_x X - k_d X \quad (1)$$

where $X$ is the biomass concentration (mg/l), $\mu_x$ is the specific growth rate of the biomass ($h^{-1}$), $k_d$ is the endogenous coefficient ($h^{-1}$).

Also, it can be assumed that endogenous coefficient $k_d$ in Eq. (1) may be neglected during exponential phase. Eq. (1) therefore reduces to the following equation:

$$\frac{dX}{dt} = \mu_x X \quad (2)$$

During initial phase, $X$ may be taken equal to $X_0$. Therefore,

$$\ln\left(\frac{X}{X_0}\right) = \mu_x t \quad (3)$$

On the basis of the literature [13,14], phenol exerts substrate inhibition on cells. Of the kinetics models describing the growth kinetics of inhibitory compound, Haldane's model is widely studied due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates. The Haldane's inhibitory growth kinetics equation is as follows:

$$\mu_x = \frac{\mu_{max} S}{K_s + S + (S^2/K_i)} \quad (4)$$

where $S$ is the substrate concentration (mg/l), $\mu_{max}$ is the maximum specific growth rate ($h^{-1}$), $K_s$ is the half saturation coefficient (mg/l), $K_i$ is the inhibition coefficient (mg/l).

The yield coefficient (dry weight of biomass/weight of substrate) $Y_x$ can be calculated by the following equation:

$$Y_x = \frac{X_{max} - X_0}{S_0 - S_a} \quad (5)$$

where $X_{max}$ is the maximum biomass concentration (mg/l), $X_0$ is the initial biomass concentration (mg/l), $S_0$ is the initial phenol concentration (mg/l), and $S_a$ is the substrate concentration when biomass concentration reached maximum (mg/l).

### 2.5. Analytical methods

To determine the concentration of phenol, m-cresol and quinoline by high performance liquid chromatography (HPLC), samples were passed through a 0.22 μm filter and submitted for analysis. HPLC was performed on a reverse phase C18 column (150 mm × 4.6 mm, Shimadzu, Japan) with a methanol/water (60:40, v/v) mobile phase at a flow rate of 0.5 ml/min. Detection

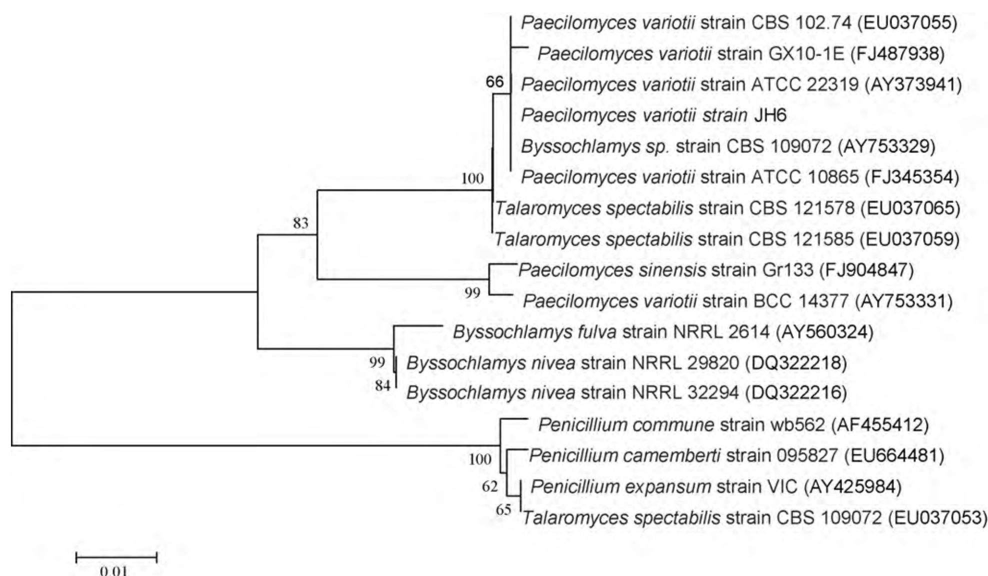


**Fig. 1.** Phylogenetic tree of strain JH6 based on ITS1-5.8S rRNA gene-ITS2 sequence comparison.

was performed with a UV detector (SPD-10A, Shimadzu, Japan). Analysis was performed at 270 nm for phenol and m-cresol, and at 313 nm for quinoline. The dry weight of biomass was measured by calculating the biomass concentration. Dry weight was determined by filtering a specific volume (50 ml) of suspended culture through per-weighted 0.22 μm pore size filters, drying the cells at 105 °C until the weight was constant.

## 3. Results and discussion

### 3.1. Characterization of phenol-degrading strain

A fungal strain, JH6, able to grow on phenol was isolated from active sludge of a coking wastewater treatment plant. Colonies of strain JH6 are powdery on phenol solid plates. Mycelia are hyaline, septate, with conidiophores and chlamydospores observed. Conidiophores are often branched and carry the phialides at their tips. The phialides are swollen at their bases and taper towards their apices. They are usually grouped in pairs or brush-like clusters. Conidia are unicellular, hyaline to darkly colored, smooth, oval to fusoid, and form long chains. Chlamydospores are occasionally present. These features are consistent with the report by Wang et al. [15].

The 5.8S rRNA gene is highly conserved gene and was used for the phylogenetic analysis of higher taxonomic levels, whereas the highly variable ITS regions was used for analysis of lower taxonomic levels. The sequence of strain JH6 obtained by sequencing was found to be 603 bp and was submitted to the GenBank database under accession no. GQ386857. A maximum likelihood tree was shown in Fig. 1. A similarity BLAST search was performed, showing the strain JH6 to be closely related to *P. variotii* ATCC 10865 (GenBank accession no. FJ345354) and *P. variotii* ATCC 22319 (GenBank accession no. AY373941), with 99.8% sequence identity. Therefore, the isolate was designated as *P. variotii* JH6.

### 3.2. Effect of additional glucose concentration on phenol biodegradation

The effect of supplementary glucose on phenol biodegradation by strain JH6 was performed in the presence of different concentrations of glucose. The concentration of phenol was set at 1200 mg/l, while the concentrations of glucose were altered to 100, 200, 300 and 400 mg/l (Fig. 2a). The lag phase of phenol degradation was about 47.5 h when the glucose concentration was reduced to 0 mg/l. However, in the presence of glucose, lag phase of phenol biodegradation was always shorter than 20 h. The experimental results showed that the biodegradation of phenol could be greatly enhanced in the presence of low concentration of glucose (100 mg/l). However, no obvious improvement on the phenol degradation performance was observed when the glucose concentration further increased. For example, in the presence of 400 mg/l glucose, the phenol degradation profile was slightly different from that in the presence of 100 mg/l glucose. This observation can be accounted for as that glucose is the preferable substrate for degradation/metabolism, which can lead to a faster growth rate and higher biomass yield [16]. Leitao et al. [11] reported that phenol degradation by *P. chrysogenum* was delayed in cultures containing 3% glucose, indicating that high glucose concentrations may reduce the phenol degradation efficiency through competitive inhibition. Wang et al. [17] also used a general additive cell growth equation to describe the cell growth of *Pseudomonas putida* on phenol and glucose. Their results suggested that phenol and glucose were involved in uncompetitive inhibition. The specific phenol degradation rate was observed to be reduced in the presence of high concentration phenol. So, it is our view that the presence of low concentration of

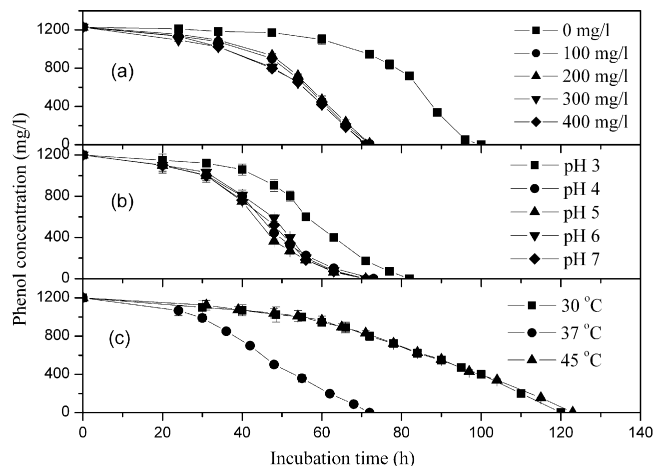


**Fig. 2.** Biodegradation of phenol at different glucose concentrations (a), initial pH values (b) and temperatures (c).

**Table 1**
Comparison of phenol degradation kinetic constant values in batch experiments with pure culture.

| $\mu_{max}$ ($h^{-1}$) | $K_s$ (mg/l) | $K_i$ (mg/l) | Maximum phenol concentration (mg/l) | Temperature (°C) | Culture | Reference |
|---|---|---|---|---|---|---|
| 0.436 | 6.2 | 54.1 | 100 | 25 | *Pseudomonas putida* DSM 548 | [14] |
| 0.800 | – | 188.0 | 350 | 30 | *Acinetobacter* sp. | [21] |
| 0.051 | 18.0 | 430.0 | 1750 | 30 | *Pseudomonas putida* F1 | [22] |
| 0.305 | 36.3 | 129.8 | 1000 | 30 | *Pseudomonas putida* MTCC | [23] |
| 0.026 | 29.3 | 2434.7 | 1750 | 34 | *Bacillus brevis* | [24] |
| 0.656 | 33.1 | 1470 | 2500 | 30 | *Corynebacterium* sp. DJ1 | [25] |
| 0.312 | 130.4 | 200 | 1800 | 37 | *Paecilomyces variotii* JH6 | This work |

easily degraded substrate is beneficial for the phenol biodegradation.

### 3.3. Effect of initial pH on phenol biodegradation

In order to investigate the effect of pH on phenol biodegradation, the biodegradation of phenol was studied at different initial pH values, varying from 3 to 7. The phenol biodegradation performance of strain JH6 at different pHs is shown in Fig. 2b. Although the best degradation performance occurred at pH of 5, no notable difference was observed within the 4–7 range. At pH of 3, phenol degradation by strain JH6 was delayed. pHs below 3 were not studied as it is well known that most microorganisms were unable to tolerate such acidic levels [18]. So it can be inferred that *P. variotii* JH6 can adapt over a wide pH range, indicating that the *P. variotii* JH6 can be applied to different climates without altering the pH.

### 3.4. Effect of temperature on phenol biodegradation

The effects of temperature on the biodegradation processes of phenol were explored. The lag phases of phenol degradation were extended at 30 °C and 45 °C, however, strain JH6 was able to completely degrade phenol within 125 h (Fig. 2c). The degradation rates dropped significantly with decreasing temperature, indicating prominent inhibition of strain JH6 at low temperature. This was in agreement with the reports that *P. variotii* was a mesophilic fungus [19]. In fact, the strain JH6 was isolated from the coking wastewater treatment plant, where the water temperature was almost above 37 °C. Earlier studies reported that the most favorable temperature range for the phenol-degrading strain was 25–30 °C (Table 1). However, for most strains, the phenol degradation rate declines significantly at temperature lower than 25 °C or higher than 40 °C [20]. As a mesophilic fungus, *P. variotii* JH6 was able to survive in high temperature range.

### 3.5. Kinetic studies of phenol degradation by strain JH6

In order to obtain the kinetic model parameters of phenol biodegradation, cell and substrate concentrations were measured over time for different initial phenol concentrations (100–1800 mg/l).

The biodegradation of phenol and growth of JH6 at different initial concentrations were shown in Fig. 3. The specific growth rates of the biomass $\mu_x$ under different initial phenol concentrations were calculated by Eq. (3).

In order to estimate the kinetic parameters of phenol, Haldane's growth kinetics model was used. The model had been used on the premise that this had less number of parameters and lent itself to be used easily in model equations. However, the estimation of these three parameters values required the use of a non-linear least squares technique. Non-linear regression analysis was performed using Matlab 7.0, based on Windows XP.

Experimental and predicted specific growth rates of the culture due to Haldane's model are shown in Fig. 4. The values of kinetic constants for phenol degradation obtained in this work were $\mu_{max}$ = 0.312 $h^{-1}$, $K_s$ = 130.35 mg/l, $K_i$ = 200 mg/l ($r^2$ = 0.949, standard deviation = 0.064). The maximum specific growth rates occurred at low substrate concentration. On further increasing the initial substrate concentration, significantly lower values of the specific growth and degradation rates were achieved. The values of kinetic constants for phenol degradation reported by other laboratories are also listed in Table 1. The $K_s$ value in this study was comparatively larger than that previously reported, indicating that the inhibitory effects of phenol occurred at substantially higher concentrations. Therefore, it can be concluded that the higher the

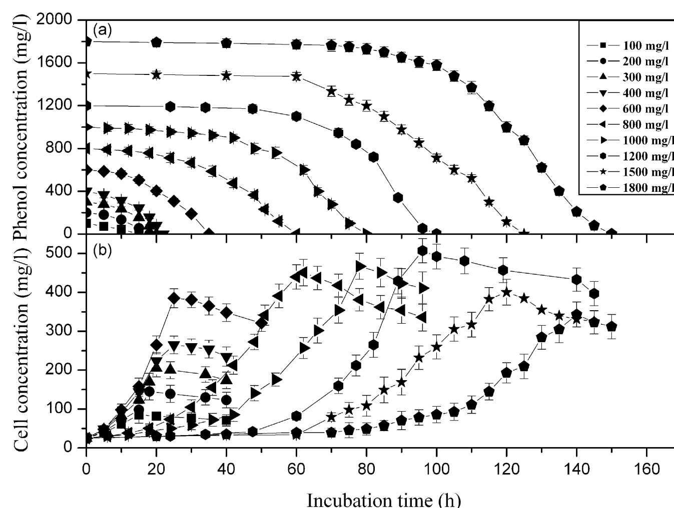


**Fig. 3.** Biodegradation of phenol (a) and growth of the strain JH6 (b) at different phenol concentrations.

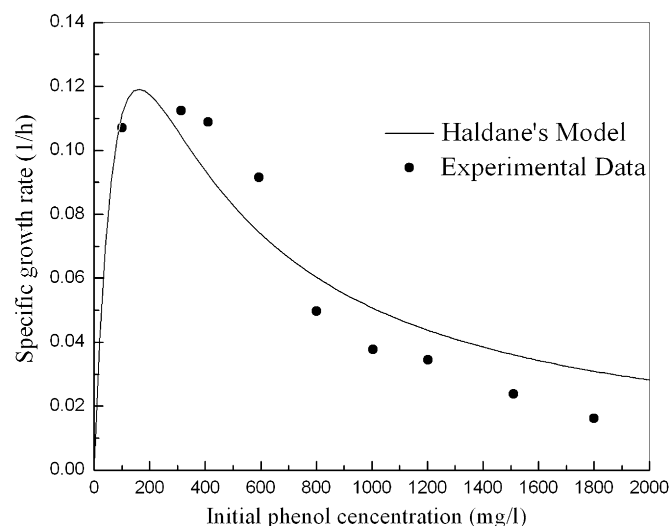


**Fig. 4.** Experimental and predicted specific growth rates of the culture due to Haldane's model.

**Table 2**
Concentration of m-cresol and quinoline during the degradation period by strain JH6.

| Multi-component system | m-Cresol concentration (mg/l) | | | | | Quinoline concentration (mg/l) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h | 0 h | 24 h | 48 h | 72 h | 96 h |
| 600 mg/l phenol + m-cresol or quinoline | 100 | 56.2 | 0 | 0 | 0 | 100 | 83.4 | 55.2 | 30.4 | 25.9 |
| | 200 | 123.7 | 52.4 | 0 | 0 | 150 | 131.6 | 81.2 | 37.8 | 29.9 |
| | 300 | 166.5 | 109.4 | 76.9 | 10.8 | 200 | 165.4 | 109 | 50.2 | 39.2 |

substrate concentration in the medium, the stronger the substrate inhibition displayed.

The slopes of all the curves during the in endogenous phase were in parallel, showing that the decay coefficient remained constant regardless of phenol concentration (Fig. 3b). The decay coefficient $k_d$ was calculated according to Shen et al. [26]. The experiment was conducted using an initial phenol concentration of 1200 mg/l. The selection of the particular growth run was arbitrary, assuming that the $k_d$ value is not dependent on initial phenol concentration. The data of the endogenous region was plotted as a natural logarithm against time. The negative slope equated to the decay coefficient, which was 0.0073 $h^{-1}$.

The yield coefficient was estimated by linearizing cell mass density increase with phenol consumption, as previously estimated by Eq. (5). The initial phenol concentration was varied from 100 to 1800 mg/l, to give a yield coefficient varying between 0.208 g/g and 0.6 g/g. the yield coefficient changed slightly at low phenol concentrations between 100 and 600 mg/l, and the yield coefficient reached maximal value at phenol concentration of 600 mg/l. At levels beyond 600 mg/l, a considerable decrease in the values of yield coefficient was observed by increasing the phenol concentration up to 1800 mg/l. Similar phenomenon of decreasing yield coefficient with increasing substrate concentration in the inhibitory region have been reported in the literature [18,27]. This phenomenon is based on the fact that the percentage of the total substrate carbon converted to energy for cell growth and maintenance is increased as the specific growth rate decreases, when the inhibition effect of phenol becomes predominant above initial phenol concentration of 600 mg/l. More energy is required to overcome the effect of substrate inhibition during the degradation of phenol. While the percentage of the total substrate carbon assimilated into biomass decreases as specific growth rates decreased. Thus substrate inhibition is known not only to reduce the specific growth rate, but also to reduce the yield coefficient.

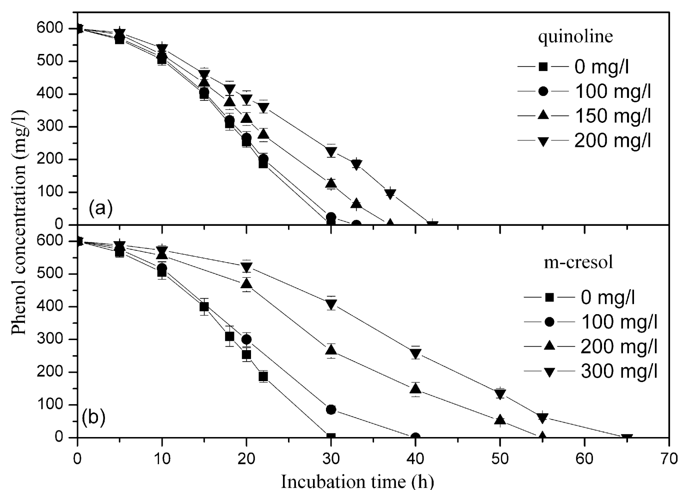


**Fig. 5.** Degradation of 600 mg/l phenol by strain JH6 in the presence of different concentrations of quinoline (a) and m-cresol (b).

### 3.6. Effect of m-cresol and quinoline on phenol degradation

Phenol industrial wastewater usually contains multi-organic compounds. The coexistence of these compounds can influence the performance of the overall biological process. The phenol degradation by strain JH6 was also tested in the presence of m-cresol or quinoline as these two toxicants are usually present along with phenol in industrial wastewaters. Fig. 5a shows that strain JH6 degradation of phenol (600 mg/l) was not inhibited by quinoline up to 200 mg/l, but the degradation rate was reduced with further increases in quinoline concentration. Similar results were shown by replacing quinoline with m-cresol (Fig. 5b). During the degradation period, when m-cresol concentration was lower than 200 mg/l, m-cresol was removed completely within 72 h. In the presence of 600 mg/l phenol, quinoline also had a very high removal rate. With initial quinoline concentrations of 100, 150 and 200 mg/l, the degradation ratio of quinoline was as high as 74.08%, 80.07% and 80.41%, respectively (Table 2). On comparing these two compounds, quinoline was shown to have a lower effect on phenol degradation than m-cresol. m-Cresol displayed higher competitive inhibition than quinoline as m-cresol is a better carbon source than quinoline for strain JH6. The multi-component system of phenol and m-cresol or phenol and quinoline can be co-utilized by strain JH6, and co-utilization influences the biodegradation rates of the involved substrates [28]. These results showed that strain JH6 degraded phenol effectively even in the presence of these toxicants. Moreover, m-cresol or quinoline can be co-metabolized with phenol by strain JH6. Strain JH6 can attain growth by using multiple carbon sources simultaneously, even when one of the components is toxic.

## 4. Conclusions

The paper describes the phenol degradation by a novel strain JH6. The following conclusions were extracted from the study.

1. A phenol-degrading strain (*P. variotii* JH6) was isolated and identified, which could degrade phenol in concentrations as high as 1800 mg/l.
2. Strain JH6 was able to actively metabolize phenol at temperatures from 30 to 45 °C and at initial pHs between 3 and 7. Supplement of low concentrations of glucose was helpful for accelerating the degradation of phenol.
3. For batch experiments carried out at 37 °C, initial pH of 5, Haldane's model was discussed in order to simulate the TNP removal profile with time. The kinetic parameters for *P. variotii* JH6 according to the Haldane model were $\mu_{max}$ = 0.312 $h^{-1}$, $K_s$ = 130.4 mg/l, and $K_i$ = 200 mg/l. The decay coefficient was found to be 0.0073 $h^{-1}$.
4. In the presence of m-cresol or quinoline, strain JH6 was able to degrade 600 mg/l phenol to completion. m-Cresol displayed higher competitive inhibition than quinoline.

## Acknowledgements

The authors wish to thank the Plan of the National Sci-Tech Major Special Item for Water Pollution Control and Management

(2009ZX07528-003-05) for financing the work. We would also like to thank Dr. Ziqian Zhang (Wuhan University) for technical assistance on PCR and helpful discussion, and Dr. Barry Wong (Wuhan University) for comments on the manuscript.

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