# Isolation, Kinetics, and Performance of a Novel Phenol Degrading Strain



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Efficient phenol-degrading bacteria is still the key to the biological treatment of phenol-containing wastewater. In this research, a novel phenol-degrading strain N8 was isolated. According to the 16S rDNA identification, it was concluded that the N8 strain was *Bacillus* sp. IARI-J-20. The wastewater treatment experiments showed that the phenol degrading rate of N8 reached 92.8 % at 24 h with the inoculation amount of 15 %, temperature of 30 °C, pH of 7.2, yeast extract addition of 0.08 %, and initial phenol concentration of 225 mg $L^{-1}$. Haldane's model was fit for the growth kinetics of the phenol-degrading strain N8 over a wide range of initial phenol concentrations (50–1200 mg $L^{-1}$), with kinetic values $\mu_{max}$ = 0.33 $h^{-1}$, $K_s$ = 79.16 mg $L^{-1}$, and $K_i$ = 122 mg $L^{-1}$. The yield coefficient reached maximal value when the phenol concentration was 400 mg $L^{-1}$. When the initial phenol concentration was more than 400 mg $L^{-1}$, the inhibition effect of phenol became predominant.

*Keywords:*
phenol-degrading strain, phenol wastewater, biological treatment

## Introduction

Phenol is an important raw material in paper, plastics, pesticides, and pharmaceutical industries. Phenol-containing wastewater from these industrial productions is always toxic and hardly degradable. Therefore, phenol wastewater treatment has been a hot research field in the world[1,2]. High phenol content in water is harmful to fish (death, and even extinction)[3,4]. Moreover, phenol-containing wastewater has an adverse effect on plants and farmland crops. For example, the crops are inedible when irrigated with phenol-containing water. When the phenol content is high, the seedlings die, which affects the crop yields. Phenol is one of the most serious pollutants in many countries. It is of great significance to study the treatment technology of phenol-containing wastewater.

Phenol wastewater can be treated by various methods, including physical, biological, chemical, and combinative. The common treatments include solvent extraction method[5], adsorption[6], membrane separation[7], biological treatment[8,9], and advanced oxidation processes[10,11]. Solvent extraction method uses the principle of different solubility and distribution coefficient in immiscible solvents. This method is always complicated, expensive, and toxic. The adsorption method is mainly used for the treatment of low concentration phenol-containing wastewater. Membrane separation employs the special structure of the membrane to separate the different components in water. However, the cost is too high. Advanced oxidation process oxidizes phenol into $H_2O$ and $CO_2$ through different processes, such as photocatalysis, ultrasonic catalysis, ozone oxidation, Fenton oxidation, and electrochemical oxidation. Advanced oxidation process shows rapid oxidation rate and high removal efficiency on various organic pollutants, but it is also complicated and expensive.

Phenol can also be degraded by microorganisms. Biological methods take advantage of the metabolic activity of the microorganisms to remove toxic substances. The biological wastewater treatment is always economic, secure, and with no secondary pollution. The degradation performance of microorganisms is an important factor in deciding the biological treatment effect. Many phenol-degrading strains have been separated, such as *Pseudomonas*[12–14], *Acinetobacter*[15,16], *Rhodococcus*[17], and *Clostridium*[18]. Microorganisms can easily be isolated from the wastewater, sludge, and soil polluted by phenol[19]. At the same time, the phenol-degrading ability can be improved by the combination of mixed strains and the domestication of genetically engineered bacteria.

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Efficient phenol-degrading bacteria is still the key to the phenol biodegradation technology. In this research, the phenol resistant strains were isolated from the soil polluted by phenol. In addition, the phenol-degrading bacteria were separated from the cultivation medium with phenol as the single carbon source. Efficient phenol-degrading bacteria were obtained from the cultivation medium with high phenol concentration. Moreover, the microorganism was identified by conventional methods. Finally, the degradation performance of phenol was tested and the degradation kinetics analyzed.

## Materials and methods

### Materials

All chemicals of analytical reagent grade were purchased from Beijing Chemical Factory (Beijing, China). All the biochemical reagents were produced by Beijing Biological Technology Factory (Beijing, China). The strains were stored in the tube culture at 4 °C. The wastewater and soil samples for the experiment were provided by Qinhuangdao Third Sewage Treatment Plant (Hebei, 066004).

### Culture media and microorganism culture

The composition of the solid culture medium was as follows: beef extract 3 g $L^{-1}$, peptone 10 g $L^{-1}$, NaCl 5 g $L^{-1}$, and agar 20 g $L^{-1}$. The composition of liquid medium (with phenol as carbon source) was as follows: $NH_4Cl$ 1.0 g $L^{-1}$, $K_2HPO_4$ 0.6 g $L^{-1}$, $KH_2PO_4$ 0.4 g $L^{-1}$, $MgSO_4$ 0.06 g $L^{-1}$, $FeSO_4$ 3 mg $L^{-1}$, and a certain amount of phenol (from 0.4 g $L^{-1}$ to 1.6 g $L^{-1}$). The composition of solid medium was as follows: beef extract 3 g $L^{-1}$, peptone 10 g $L^{-1}$, NaCl 5 g $L^{-1}$, agar 20 g $L^{-1}$, and phenol (0 g $L^{-1}$ or 0.1 g $L^{-1}$). The composition of liquid culture medium was as follows: beef extract 3 g $L^{-1}$, peptone 10 g $L^{-1,}$ and NaCl 5 g $L^{-1}$.

### Isolation of efficient phenol-degrading bacteria

Firstly, 8 mL solid culture medium containing phenol (0.1 g $L^{-1}$) was poured into a sterile culture dish. One side of the dish was placed on a wooden strip. The culture medium in the dish was inclined and completely covered the bottom of the dish. After solidification of the medium, another 8 mL solid culture medium without phenol was poured into the medium. A gradient plate was then formed. On the concentration gradient plate, the phenol concentration was distributed from low to high. Therefore, the colony that formed in the high concentration zone was more tolerant to phenol. The soil sample was mixed with deionized water at the ratio of 1:10 (g $mL^{-1}$) and the mixture was stirred for 15 min on a magnetic stirrer, and then left to rest for half a minute. Different dilutions of the supernatant liquor were prepared and then poured into the solid culture medium. The culture dish was put in the incubator at 30 °C for 24–48 h. The colonies of bacteria were distributed from dense to dilute in the plates. The phenol-resistant strains were picked from high phenol concentration areas. After cultivation, these strains were stored in the tube culture at 4 °C.

The liquid medium was prepared by adding some amount of phenol (from 0.4 g $L^{-1}$ to 1.6 g $L^{-1}$) as a single carbon source to the basic culture medium. After cultivation of 48 h, the $OD_{600}$ values of the solution were measured with a spectrophotometer and the results recorded. The solution with low OD values meant that the strain in the medium could not use phenol as carbon source. The strains that could better utilize phenol were isolated and employed for the following experiments.

The phenol solid medium was prepared at different phenol concentrations (0.4 g $L^{-1}$, 0.6 g $L^{-1}$, 0.8 g $L^{-1}$, 1.2 g $L^{-1}$, 1.4 g $L^{-1}$, 1.6 g $L^{-1}$). The suspended cells liquid of isolated strains was poured into the culture dish. The culture dish was put in the incubator at 30 °C for 24–48 h. The strain that grew better under high phenol concentration was picked and inoculated on the solid culture medium. After cultivation, the strain was stored in the tube culture at 4 °C. The genomic DNA of strain was extracted by the method of precipitation. The 16S rDNA was amplified using the primers: 16F (AGAGTTTGATCCTGGCTCAG) and 16R (GGTTACCTTGTTACGACTT). PCR amplification was performed under the following conditions: 3 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and an additional 5 min cycle at 72 °C. The automatic sequence was carried out by Beijing Sun Biotech Co., Ltd. The 16S rDNA sequence was checked in GenBank.

### Biological treatment of phenolic wastewater

The biological treatment was limited to 24 h to study the efficient phenol-degrading strain. The preserved strain was activated in the culture medium and then inoculated to phenol wastewater with the phenol concentration of 225 mg $L^{-1}$. After 24 h, the phenol concentration was measured and the phenol removal percentage calculated. The effects of temperature, pH, inoculum amount, and nutrient concentration were considered in this research. All the mentioned experiments were repeated in triplicate and the average data reported.

### Phenol degradation kinetics model

In this research, the strain growth rate and phenol degradation rate were only limited by substrate

concentration at a fixed initial pH, temperature, and shaking rate. Cell growth kinetics of the exponential phase in a batch reactor may be modeled by the following equation:

$$\mu = \frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} \quad (1)$$

where $X$ is the cell weight concentration (mg $L^{-1}$), $\mu$ is the specific growth rate of the biomass ($h^{-1}$), and $t$ is the reaction time.

After the integral, the following equation can be obtained:

$$\ln X = \mu t + A \quad (2)$$

where $A$ is the constant.

In addition, through plotting ln$X$ *v*s. time, the slope of the line is the value of $\mu$.

The most common cell growth dynamics equation is the Monod equation. However, based on the literature[20], phenol shows substrate inhibition on cell growth. With the kinetic models describing the growth kinetics of inhibitory compounds, Haldane's model is widely studied due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates. Haldane's inhibitory growth kinetics equation is as follows:

$$\mu = \frac{\mathrm{d}X}{X\mathrm{d}t} = \frac{\mu_{\max} S}{K_s + S + S^2 / K_i} \quad (3)$$

where $S$ is the substrate concentration (mg $L^{-1}$), $\mu_{max}$ is the maximum specific growth rate ($h^{-1}$), $K_s$ is the half-saturation coefficient (mg $L^{-1}$), and $K_i$ is the inhibition coefficient (mg $L^{-1}$). Through plotting $\mu$ *vs.* $S$ and the curve fitting, the parameter values could be calculated by Origin software.

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

$$Y = \frac{X_{\max} - X_0}{S_0 - S_m} \quad (4)$$

where $X_{max}$ is the maximum biomass concentration (mg $L^{-1}$), $X_0$ is the initial biomass concentration (mg $L^{-1}$), $S_0$ is the initial phenol concentration (mg $L^{-1}$), and $S_m$ is the substrate concentration when biomass concentration reached maximum (mg $L^{-1}$).

At different initial phenol concentrations, the yield coefficient $Y$ was calculated, and the relationship between $Y$ and phenol concentration was plotted.

### Analysis method

The phenol concentration was measured by the colorimetric method. Different volumes of 50 mg $L^{-1}$ phenol standard solution (0, 0.5, 1.00, 1.50, 2.00, and 2.50 mL) were measured, respectively, and taken in 25-mL flasks; then, 1 mL water, 0.5 mL buffer solution, and 1.0 mL of 4-aminophenazone solution were added into the solution. Subsequently, a 1.0-mL potassium ferricyanide solution was added and thoroughly mixed. Finally, water was added to set the volume to 25 mL. The mixture was settled for 15 min, and the absorbance was measured in the spectrophotometer at the wavelength of 510 nm. The standard curve is $y = 7.7014x + 0.0334$, where $y$ is the concentration of phenol in the wastewater, and $x$ is the absorbance.

Concentration of ammonia nitrogen (AN) was determined according to standard methods of Nessler's reagent spectrophotometry. COD (mg $L^{-1}$) was measured on HACH equipment, (HACH Co., USA), consisting of HACH, DR/4000U spectrophotometer and COD heating reactor, using the standard HACH testing kits. BOD (mg $L^{-1}$) was measured on a HACH equipment (BOD Trak II, HACH Co., USA). The pH was measured in a pH meter (PHS-3B, Shanghai Precision & Scientific Instrument Co. Ltd., China). The optical density (OD) of cell growth at regular intervals was determined at 600 nm using a UV-VIS spectrophotometer (TU-1901, Purkinje General Instrument Co. Ltd., China) throughout the studies. The cell concentration was represented by $OD_{600}$. The dry cell weight (mg $L^{-1}$) was measured by the centrifugal drying method.

## Results and discussion

### The isolation of phenol-resistant strains

The phenol concentration was distributed from low to high on the gradient plate. Phenol had an inhibition effect on cell growth[21]. Therefore, the colony that formed in the high concentration zone was more tolerant to phenol. After culturing for 48 h, the colonies of bacteria were distributed from dense to dilute in the plates. Eleven phenol-resistant strains were isolated and inoculated in the culture medium for cultivation at 30 °C for 24–48 h. After cultivation, the strains were stored in the tube culture at 4 °C.

### The isolation of phenol-degrading strains

Eleven phenol-resistant strains were isolated in the first step, but the phenol-resistant strains and the phenol-degrading strains were not distinguished. It is well known that the growth of microorganisms is dependent on the carbon source. If phenol is the only carbon source in the culture medium, the growth of the bacteria must depend on the degradation of phenol. Therefore, phenol would be removed by the phenol-degrading strains. The screened eleven resistant strains were inoculated into the culture

medium (phenol as the sole carbon source) and cultured for 48 h. The cell concentration was represented by $OD_{600}$. As shown in Fig. 1, all the resistant strains could grow on the culture medium with phenol as the sole carbon source, indicating that these strains were phenol-degrading strains. However, the growth of strains N1, N4, N8, and N11 was much better.

Strains N1, N4, N8, and N11 were then inoculated on the solid medium containing phenol in order to isolate the efficient phenol-degrading strains. Strain N8 had relatively good performance and grew better. Strain N8 was chosen for further experiments. The 16S rDNA gene sequence of N8 (compared with those available sequences in GenBank) showed that it was 99 % identical to *Bacillus* sp. IARI-J-20 (Accession No. JN411414). The phylogenetic tree of the strain is shown in Fig. 2. The isolated phenol-degrading strain N8 was a member of *Bacillus*. There is another literature example[22] of a hyper phenol tolerant *Bacillus* sp., isolated from oil refinery and exploration sites, which showed good performance in degrading phenol.

## Degradation of phenol by strain N8

### *Effect of temperature on phenol degradation*

Strain N8 was inoculated in the liquid medium containing phenol as the only carbon source with the inoculation amount of 15 %, pH of 7.0, and the initial phenol concentration of 225 mg $L^{-1}$. The effect of temperature on the degradation of phenol by

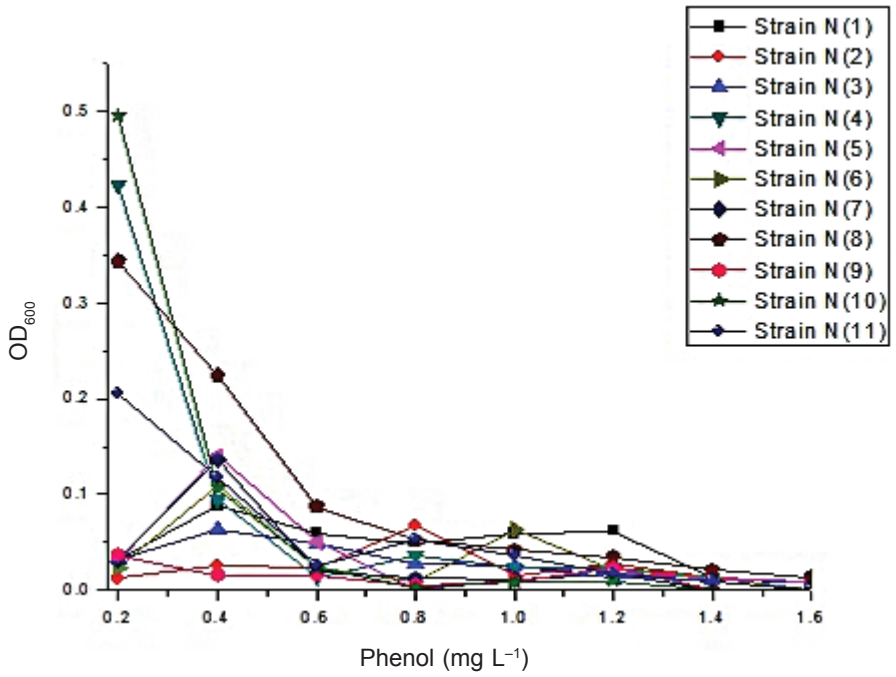


Fig. 1 – *OD value of different microorganisms in the phenol medium*

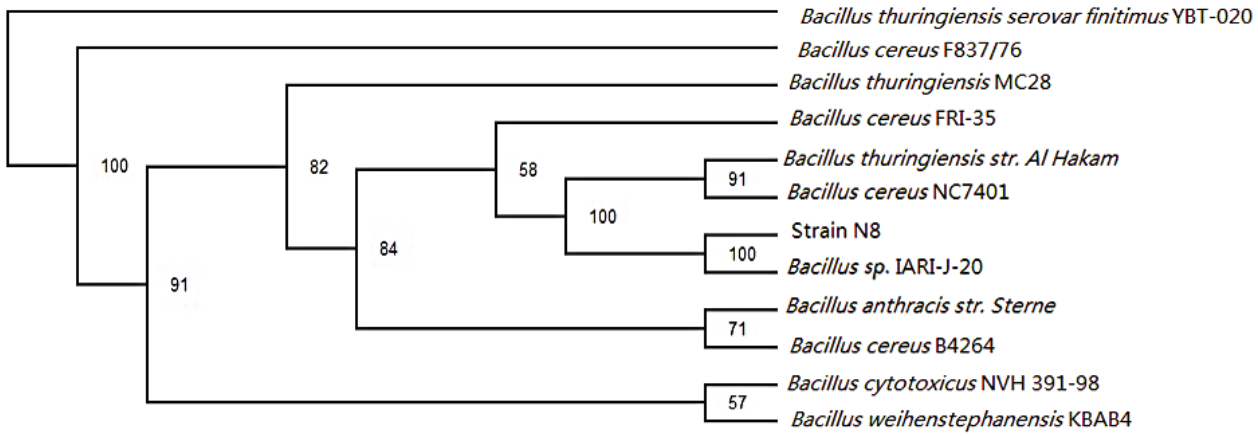


Fig. 2 – *Phylogenetic tree of strain N8*

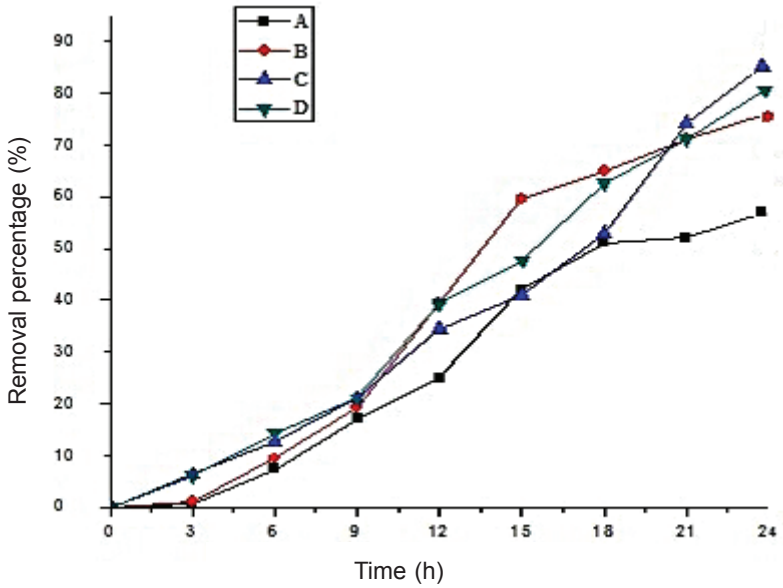


Fig. 3 – *Effect of temperature on the phenol degradation of strain N8 (A 20 °C; B 25 °C; C 30 °C; D 35 °C)*

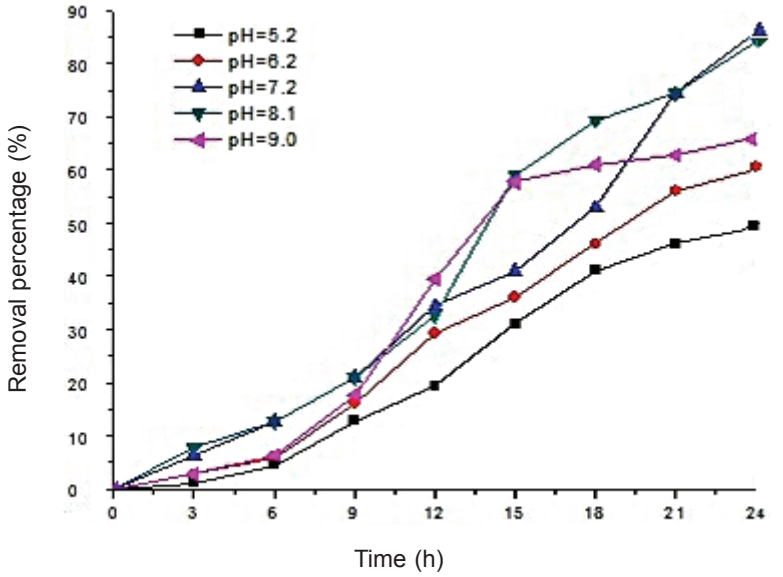


Fig. 4 – *Effect of pH on the phenol degradation of strain N8*

strain N8 was analyzed. The temperature ranged from 20 °C to 35 °C. The residual phenol concentration was measured every three hours, and the removal percentage calculated. The result is shown in Fig. 3. The phenol removal percentage at 24 h was the highest, amounting up to 86.21 % at a temperature of 30 °C. Furthermore, the data statistics of three parallel experiments showed that the standard deviation of the experimental data was less than 5 %, and the experimental data were stable and reliable. The microorganism was more active at 30 °C, resulting in a higher degradation effect[23]. In this research, the effect of temperature in the range of 25–35 °C was not obvious. The phenol-degrading bacteria had good adaptability to temperature.

*Effect of pH on phenol degradation*

Strain N8 was inoculated in the phenol wastewater with the inoculation amount of 15 %, temperature of 30 °C, and initial phenol concentration of 225 mg $L^{-1}$. The effect of pH on the degradation of phenol by strain N8 was analyzed. The pH ranged from 5 to 9. The residual phenol concentration in the liquid was measured every three hours and the removal percentage calculated. The result is shown in Fig. 4. The pH of solution had a significant influence on the growth of microorganisms. Vital activities and material metabolism of microbe are closely related to pH value[24]. When the pH was 7 to 8, the phenol removal percentage was the highest. Be-

sides, other references[25] have also reported that phenol degradation could achieve the best effect under neutral pH This indicated that phenol-degrading bacteria were more adaptable to neutral pH. Furthermore, the data statistics of three parallel experiments showed that the standard deviation of the experimental data was less than 3 %, and that the experimental data were stable and reliable.

*Effect of inoculation amount on phenol degradation*

In this section, the effect of inoculation amount on the degradation of phenol by strain N8 was analyzed. The inoculation amount ranged from 2 % to 20 %. The residual phenol concentration in the liquid was measured every three hours, and the degradation rate calculated. The result is shown in Fig. 5. The phenol degradation rate was the highest when the inoculation amount was 10 %–15 %. The inoculation amount had an influence on the phenol degradation. In addition, the data statistics of three parallel experiments suggest that the standard deviation of the experimental data was less than 5 %. The value of parallel experiments was close to the average value, and the experimental data were stable and reliable.

*Effect of applied nutrients on phenol degradation*

Some nutrients, such as yeast extract and glucose, are regularly added to the wastewater to improve the phenol degradation rate. As shown in Fig. 6, the degradation of phenol had increased when yeast extract had been added. With the increase in

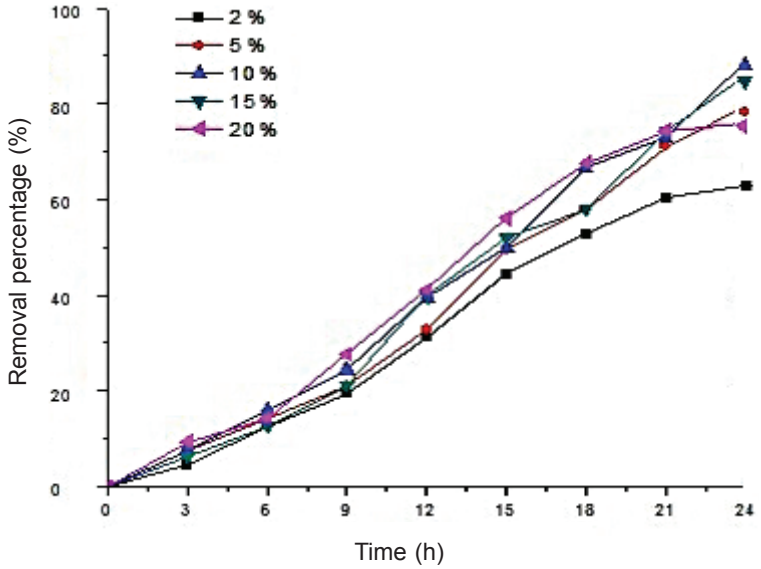


Fig. 5 – *Effect of inoculation amount on the phenol degradation of strain N8*

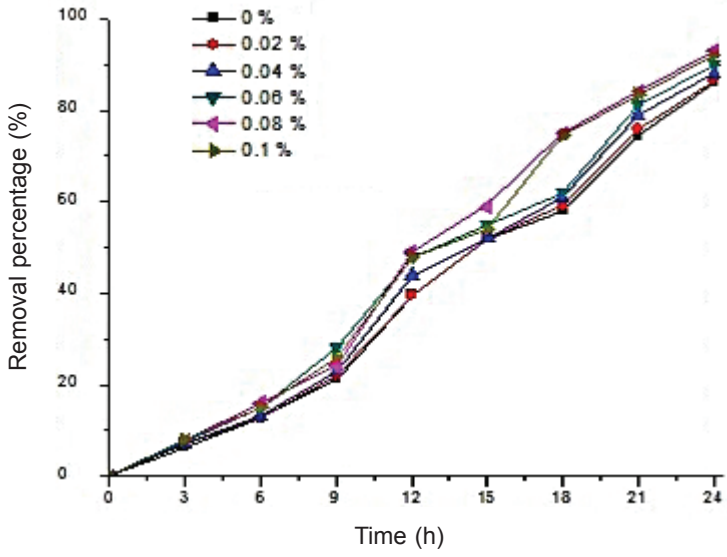


Fig. 6 – *Effect of yeast extract on the phenol degradation of strain N8*

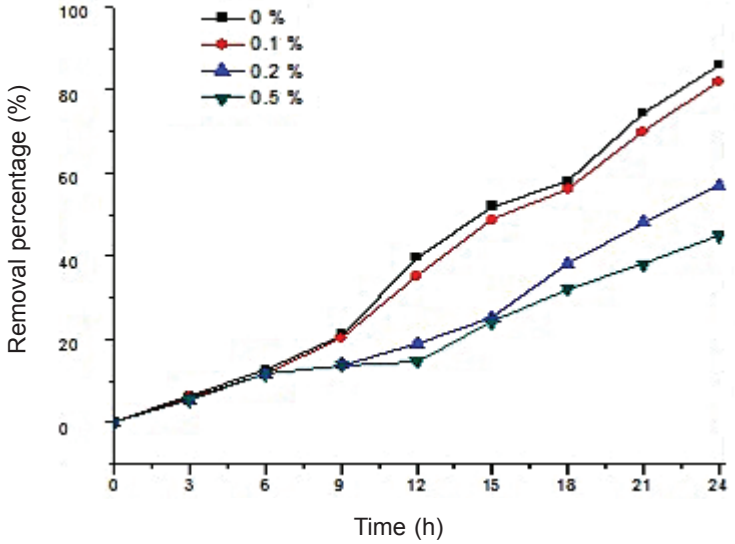


Fig. 7 – *Effect of glucose on the phenol degradation of strain N8*

yeast extract concentration, the growth of the strain had accelerated. With 0.08 % yeast extract addition, the phenol removal percentage reached 92.8 % at 24 h. The standard deviation of the experimental data was less than 3 %, and the experimental data were stable and reliable. Yeast extract is a kind of high-quality organic nitrogen source rich in protein, amino acids, peptides, nucleotides, and B vitamins. Their addition could accelerate microbial degradation of organic matter.

Next, glucose was added to the wastewater. The result is shown in Fig. 7. The degradation of phenol was affected by the addition of glucose. The degradation of phenol decreased when glucose was added. With 0.5 % glucose addition, the phenol removal percentage reduced to 45 %. If phenol and glucose were added into the medium at the same time, glucose was a preferred carbon source of microorganisms. When glucose was depleted, the synthesis of phenol degradation enzyme proceeded. The consumption of glucose could usually inhibit the synthesis of enzyme for the decomposition of other carbon source.

Simultaneously, this kind of phenol-degrading strain was used for the real phenol wastewater treatment. The data of raw wastewater was COD of 1017 mg $L^{-1}$, BOD of 321 mg $L^{-1}$, phenol of 214 mg $L^{-1}$, and ammonia nitrogen of 52 mg $L^{-1}$. After the treatment, the wastewater concentration was different; it was COD of 289 mg $L^{-1}$, BOD of 58 mg $L^{-1}$, phenol of 45 mg $L^{-1}$, and ammonia nitrogen of 21 mg $L^{-1}$.

## Phenol degradation kinetics

In this section, the growth curve of strain N8 was measured. The period of logarithmic growth ranged from 12 h to 22 h; after 22 h, the strain was in the stable phase. Cell and substrate concentrations were measured over time for different initial phenol concentrations (50–1200 mg $L^{-1}$) in order to obtain the kinetic model parameters of phenol biodegradation. Moreover, Haldane's growth kinetics model was used to estimate the kinetic parameters of phenol. Experimental and predicted specific growth rates of the culture due to Haldane's model are shown in Fig. 8.

In Fig. 8, with the increase in initial phenol concentration, the specific growth rate $\mu$ was firstly increased and then decreased, which was typical of the inhibition growth mode. The experiment value was fitted with Haldane's model. By plotting with the quantity of $\mu$ and $S$ and the curve fitting, the parameter values could be calculated by Origin software. The Haldane's inhibitory growth kinetics equation was as follows: $\mu_{max}$ was 0.33 $h^{-1}$, $K_s$ was 79.16 mg $L^{-1}$, and $K_i$ was 122 mg $L^{-1}$. The actual values and the model values were matched.

$$\mu = \frac{0.33S}{79.16 + S + S^2/122} \tag{5}$$

The growth and biochemical reactions of the strain would be affected if the concentration of phenol were too high. Simultaneously, the toxicity depends not only on the concentration of the different inhibitors, but also on the tolerance of the strains. In this research, the microbial kinetic parameters were compared to values reported in the literature[26–28], and the $\mu_{max}$ value of the strain isolated in our laboratory was large, indicating that this strain had strong phenol degradation ability.

The yield coefficient to the substrate was estimated by Eq. (4). The initial phenol concentration was varied from 50 to 1200 mg $L^{-1}$. The yield coefficient variation is shown in Fig. 9. The yield coef-

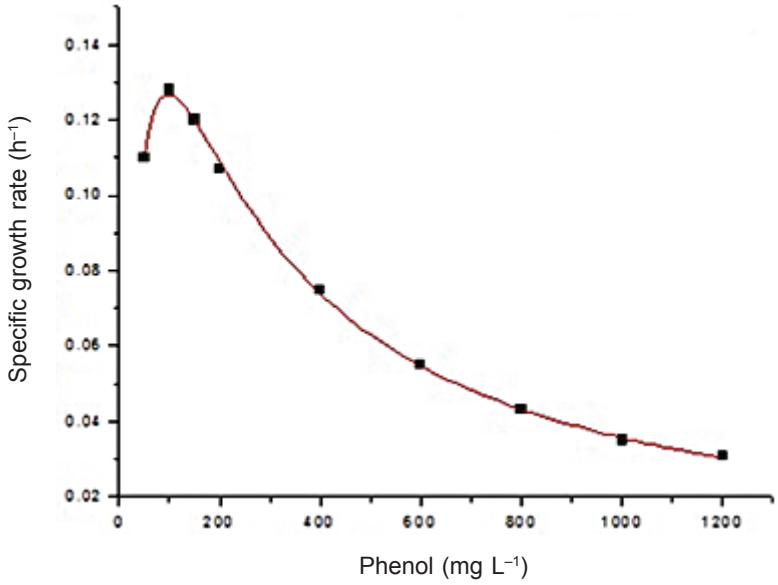


Fig. 8 – *Experimental and predicted specific growth rates of the culture according to Haldane's model*

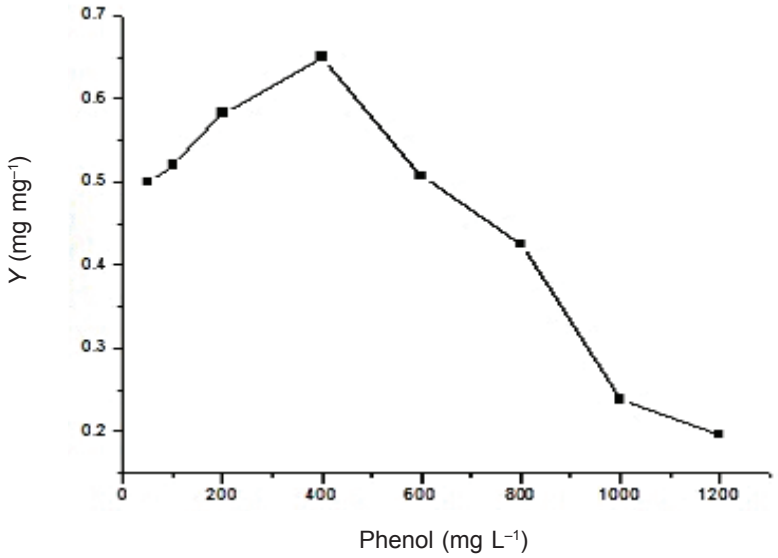


Fig. 9 – *Yield coefficient variation under different phenol concentration*

ficient changed slightly at low phenol concentrations between 50 and 400 mg $L^{-1}$ and reached maximal value at phenol concentration of 400 mg $L^{-1}$. It was different from the variation of the specific growth rate. When the phenol concentration was above 400 mg $L^{-1}$, a high reduction in the value of yield coefficient was observed. A similar phenomenon of decreasing yield coefficient with increasing substrate concentration had been reported in the literature[26,29]. Substrate consumption during the phenol degradation included the following aspects: the synthesis of new cellular material, the supply of cell energy needed for growth, and the synthesis of new metabolites. Only part of the carbon consumption is used to synthesize cells. Therefore, the variation of yield coefficient to the substrate was different from the variation of the specific growth rate.

## Conclusions

Isolating efficient phenol-degrading bacteria is important for the phenol biodegradation technology. In this research, the phenol-resistant bacteria were isolated from the sludge and sewage polluted by phenolic wastewater. The phenol-degrading bacteria were separated from the cultivation medium with phenol as the single carbon source. An efficient phenol-degrading strain N8 was obtained from the cultivation medium with high phenol concentration. The degradation treatment showed that the strain N8 was able to grow when phenol concentration amounted to 1.6 g $L^{-1}$. Moreover, the wastewater treatment experiment showed that the phenol removal percentage reached 92.8 % at 24 h with the

inoculation amount of 15 %, temperature of 30 °C, pH of 7, yeast extract addition of 0.08 %, and initial phenol concentration of 225 mg $L^{-1}$. The degradation kinetics of strain N8 was studied simultaneously. Haldane's model fitted the growth kinetics of the phenol-degrading strain N8 over a wide range of initial phenol concentrations (50–1200 mg $L^{-1}$), with kinetic values $\mu_{max}$ = 0.33 $h^{-1}$, $K_s$ = 79.16 mg $L^{-1}$, and $K_i$ = 122 mg $L^{-1}$. When the initial phenol concentration was above 400 mg $L^{-1}$, the inhibition effect of phenol became predominant.

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