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Dual substrates biodegradation kinetics of m-cresol and pyridine by *Lysinibacillus cresolivorans*

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ABSTRACT

Phenols and N-heterocyclic compounds are found to co-exist in actual wastewater, especially in petrochemical and coking wastewater. *Lysinibacillus cresolivorans*, a bacterium capable of phenol-biodegradation was used to study the substrate interactions of m-cresol and pyridine as single and dual substrates. The cell growth and substrate biodegradation kinetics were also investigated with initial m-cresol concentrations varying from 0 to 1200 mg/L and pyridine concentrations varying from 0 to 150 mg/L. The single substrate kinetics was well described by the Haldane kinetic models. The single-substrate parameter values of m-cresol on cell growth were $\mu_{max} = 0.89\,h^{-1}$, $K_s = 426.25$ mg/L, $K_i = 51.26$ mg/L and $\mu_{max} = 0.0925\,h^{-1}$, $K_s = 60.28$ mg/L, $K_i = 16.17$ mg/L for cell growth on pyridine. Inhibitory effects of substrates were observed when cells were grown on the mixed substrates. The interaction parameter $I_{m,p}$ (0.76) was greater than $I_{m,p}$ (0.11), which indicated that m-cresol inhibited the utilization of pyridine much more than pyridine inhibited the biodegradation of m-cresol. The study showed a good potential of *L. cresolivorans* in degrading mixed substrates of m-cresol and pyridine.

1. Introduction

Phenol and its methylated derivative o-, m- and p-cresols are characteristic pollutants in wastewater and effluents from various industries, including petrochemical, textiles, dying, varnish industries, phenolic resin manufacturing, and steel plants [1,2]. These compounds have resulted in cumulative hazardous effects on the environment [1]. Due to the toxic properties of both phenol and cresol, the complete removal of these compounds by microorganisms is of great importance [3]. Even a trace of pyridine, an important class of aromatic N-heterocycle, is a common co-contaminant in coking wastewater, petroleum and ceramics wastewater and it constitutes a danger for human beings as well as other living organisms. Its ring-structure can affect microbial biodegradation of m-cresol in some areas. Because of the presence of dual substrates or even more substrates in wastewater, the isolation and biodegradation of the pure strain will meet difficulties in practical application. Therefore it is important to figure out the interaction between the mixture substrates.

Authors have reported the biodegradation of phenol and its derivatives by pure and mixed cultures [4–8]. Jiang et al. [9] found that 1600 mg/L of phenol was completely degraded by the strain of *Alcaligenes faecalis* within 76 h. Jiang et al. [2] isolated a pure culture of *Candida tropicalis*, which can degrade phenol and m-cresol. They found that the presence of m-cresol intensely inhibited phenol biodegradation while m-cresol rate of biodegradation was greater than that without phenol. Gong et al. [10] reported that Mn^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} promoted phenol degradation in certain concentrations, but 200 mg/L of α-naphthol and aniline strongly inhibited phenol degradation.

However, there are few reports on different co-substrates' impact on the growth of pure pyridine-degrading culture and the biodegradation of pyridine [11]. Kim et al. [12] observed that the biodegradation of pyridine by freely suspended and immobilized *Pseudomonas putida* MK1 was inhibited in the presence of phenol. Rhee et al. [13] isolated a pyridine degrading strain and found that glucose and acetic acid promoted pyridine biodegradation.

In this study, we focus on the biodegradation, applicability, and kinetics of single and dual substrates with a pure strain. Recently, the biodegradation of m-cresol by *Lysinibacillus cresolivorans* in the presence of pyridine was investigated. The work discusses the possible inhibitory effects of pyridine during m-cresol biodegradation. The results will provide more knowledge on the application and the possibility of a pure culture microorganism for biodegradation.

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2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents (m-cresol, pyridine, and inorganic salts) used in microbial growth in the study were of analytical grade, and were purchased from Sigma–Aldrich, Inc., St. Louis, MO.

2.2. Microorganism and culture conditions

L. cresolivorans originally isolated and enriched from the aerobic basin of a coking wastewater treatment plant located in Shaoguan, China. It was identified based on physiological and biochemical tests and 16S rRNA gene sequence (submitted to International Journal of Systematic and Evolutionary Microbiology, the accession number is EU043375).

A mineral salt medium (MSM) supplemented with m-cresol and/or pyridine was used for biodegradation studies containing the following ingredients (g/L): $(NH_4)_2SO_4$ 0.8, K_2HPO_4 1.5, KH_2PO_4 1.0, $MgCl_2{\cdot}2H_2O$ 0.2, NaCl 0.1, $FeCl_3{\cdot}6H_2O$ 0.02, CaCl 0.01, $MnSO_4{\cdot}H_2O$ 0.03. The initial pH value of the medium was adjusted to 7.0 before autoclaving. Growth media as well as all the solutions were autoclaved for 20 min at 121 °C and 10^5 Pa. All cultivation was conducted at 35 °C in a rotary shaker with a speed of 170 rpm.

2.3. m-Cresol and/or pyridine biodegradation experiments

All biodegradation experiments in this study were conducted in 250 ml Erlenmeyer flasks containing 100 ml of sterile MSM with various m-cresol and/or pyridine concentrations. Before using the acclimatized culture in the experiments, the microbial strain was inoculated into 50 ml LB medium and grown at 35 °C in a rotary shaker at 170 rpm. After 24 h of incubation, 5 ml of the cell culture was transferred to 100 ml fresh MSM medium. At late exponential growth phase (OD_{600} = 1.2), cells were harvested as inoculums.

When m-cresol or pyridine was used as the sole carbon and energy source, the biodegradation of m-cresol was studied for a concentration range of 0–1200 mg/L, and pyridine concentrations varied from 30 to 150 mg/L were investigated. Based on these results of single substrate biodegradation, batch experiments of the interactions between the mixed substrates were also performed in MSM. The used m-cresol concentrations were varying from 80 to 1200 mg/L with the values 80, 160, 240, 320, 400, 480, 560, 640, 720, 800, 880 and 1000 mg/L, and at each m-cresol concentration 30–120 mg/L (30, 60, 90, 120) pyridine were added to the medium. During the experiments, a sample volume was periodically removed from the medium for analysis of cell density and residual substrate concentrations.

2.4. Analytical methods

Cell density of the microbial culture was estimated with a UV-2800 spectrophotometer (Unico, Shanghai, China) by measuring its absorbance (OD) at the wavelength of 600 nm. Then OD_{600} was converted to dry cell weight by a calibration curve, which was prepared in terms of OD_{600} value versus dry weight of biomass per liter. Dry weight was measured by filtering a known volume of cell suspension then drying the cells at 105 °C to a constant weight. Immediately after measurements of optical density, samples of suspended culture were centrifuged at 10,000 rpm for 10 min. Then the supernatants were filtered through a 0.45 μm pore size filter for analysis. High performance liquid chromatography (HPLC) was employed to quantify the substrate concentrations in the cell free supernatants. HPLC was performed with C18 column (Agilent HC-C18, 5 μm, 4.6 mm × 250 mm) with a methanol/water (60/40, v/v) as the mobile phase at a flow rate of 0.7 ml/min, and detection was realized with a UV detector at 270 nm. The retention times for pyridine and m-cresol were 6.67 and 9.30 min, respectively.

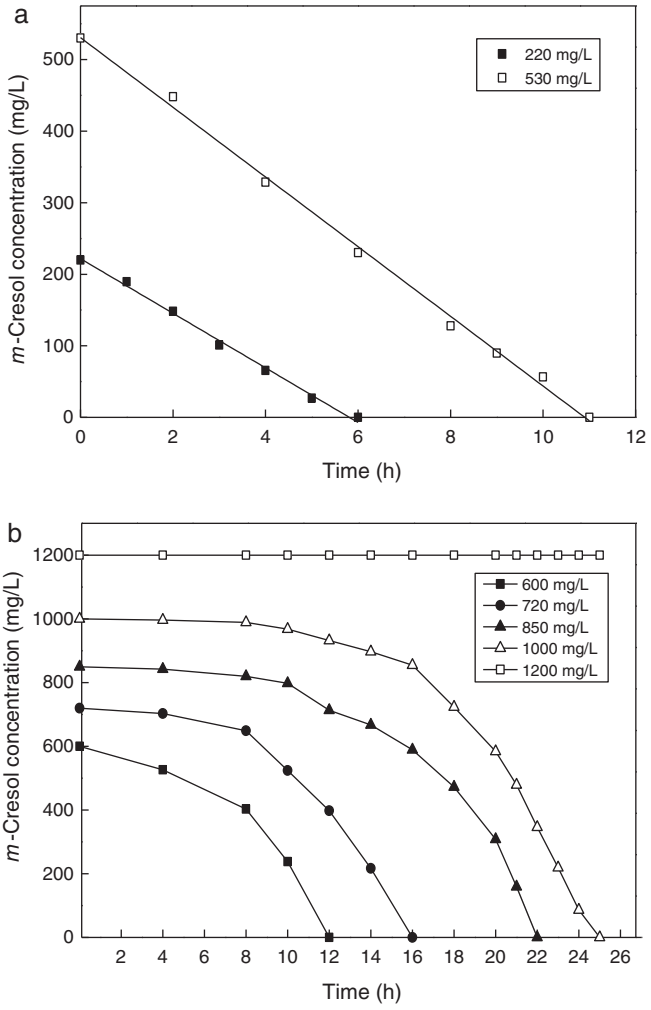

Fig. 1. Batch tests of m-cresol biodegradation in low (a) and high (b) initial concentrations.

All experiments and measurements were in duplicate and the arithmetic averages were taken for calculations and data analysis.

3. Results and discussion

3.1. Biodegradation of single substrate

In our previous studies, the m-cresol biodegradation capacity of *L. cresolivorans* from 0 to 500 mg/L has been examined [14]. Biodegradation kinetics followed closely a zero-order kinetics with no time delay. The specific degradation rate of m-cresol was 36.7 mg/(L h) and 48.2 mg/(L h) at 220 mg/L and 530 mg/L of m-cresol, respectively. The biodegradation capacity up to 1200 mg/L was shown in Fig. 1. With the increase of initial m-cresol concentration, a time lag was noticeable in tests. 12 h was required to completely degrade 600 mg/L m-cresol. When m-cresol concentration reached to 1000 mg/L, total biodegradation needed 25 h. *L. cresolivorans* failed to degrade 1200 mg/L m-cresol within 25 h, manifesting a strong substrate inhibition (see Fig. 1).

The biodegradation of pyridine and cell growth by *L. cresolivorans* is shown in Fig. 2. It is evident that the times required for complete biodegradation of pyridine are comparable at 120 mg/L. With the augmentation of initial pyridine concentration, the time needed for pyridine degradation increased

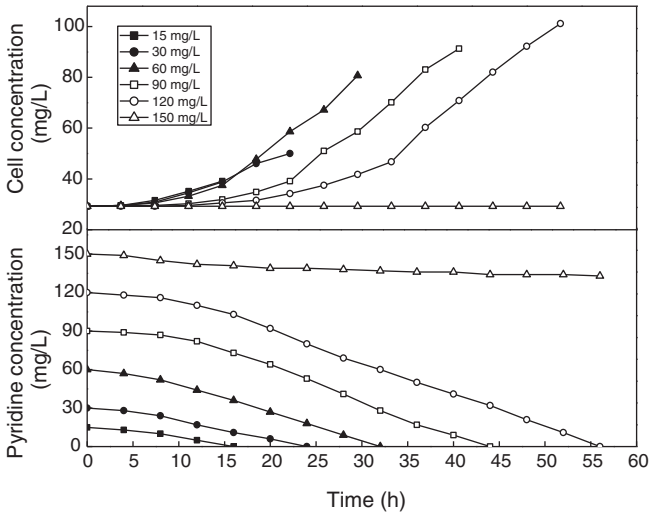

Fig. 2. The cell growth and pyridine biodegradation with different initial pyridine concentrations.

gradually, and the final biomass increased slightly. The ultimate pyridine-degrading concentration was lower than that of m-cresol alone. This may be attributed to the fact that pyridine exhibited stronger inhibitory effects than m-cresol on the substrate biodegradation.

3.2. Kinetics on single substrate biodegradation

Biodegradation experiments of *L. cresolivorans* were conducted with m-cresol concentrations ranging from 0 to 1200 mg/L and pyridine concentrations ranging from 0 to 150 mg/L, respectively. The experimental data on the single substrate biodegradation were utilized for calculating the specific growth rate and culture biomass yield according to the following equation:

$$\mu_x = \frac{\gamma_x}{C_x} = \frac{dC_x}{dt}\frac{1}{C_x} \quad (1)$$

where μ_x is the specific growth rate (h^{-1}), γ_x the cell growth rate, and C_x is the cell concentration (mg/L).

Because of the substrate inhibition on the cell growth, the Haldane's equation was selected here due to its wide applicability for assessing the growth kinetics of inhibitory substrate (Haldane, 1965).

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

where S is the substrate concentration (mg/L), μ_{max} is the maximum growth rate (h^{-1}), K_s is the substrate saturation coefficient and K_i is the substrate inhibition coefficient. Table 1 lists the kinetic parameters for m-cresol and pyridine biodegradation, which are derived using a non-linear least-square regression method of Origin 7.5 based on the experimental data obtained in the tests. The residual sum of squares of m-cresol and pyridine were very small, which indicated that the regression curve fit the experimental data very well.

Table 1
The kinetic parameters of *Lysinibacillus cresolivorans* in single substrate systems.

Substrate	μ_{max} (h^{-1})	K_s (mg/L)	K_i (mg/L)	Residual sum of squares	R^2
m-Cresol	0.89	426.25	51.26	3.0×10^{-4}	0.9772
Pyridine	0.0925	60.28	16.17	1.18×10^{-6}	0.9907

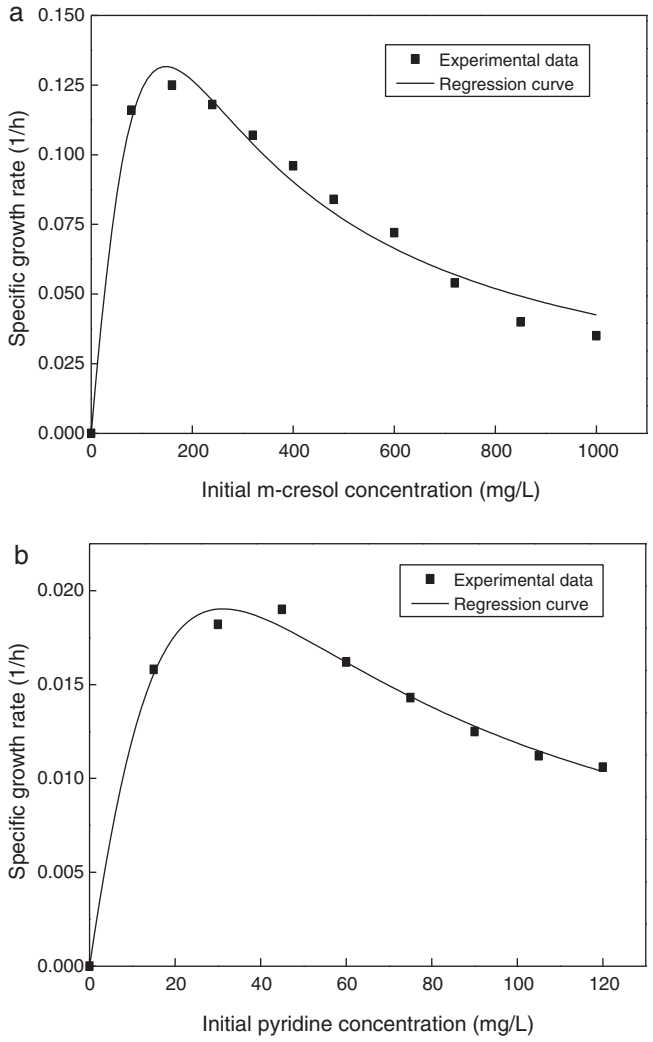

Fig. 3. Dependence of specific growth rate on the concentration of (a) m-cresol and (b) pyridine.

The dependences of *L. cresolivorans* specific growth rate on the initial concentrations of m-cresol and pyridine alone are shown in Fig. 3. It could be seen that the maximum specific growth rate occurred at very low substrate concentration. When the initial concentration of m-cresol and pyridine were 151.5 mg/L and 30.3 mg/L, the maximum specific growth rates were 0.1316 h^{-1} and 0.0191 h^{-1}, respectively.

Table 1 and Fig. 3 were the comparisons between the prediction of cell growth kinetics and the experimentally determined specific growth rates of *L. cresolivorans* at different initial m-cresol concentration from 0 to 1200 mg/L and pyridine concentrations from 0 to 120 mg/L, respectively. With the increase of initial substrate concentration, the specific growth rate decreased, which may be resulting from an intense substrate inhibition. A higher substrate concentration demonstrated a stronger substrate inhibitory response.

Comparing the specific growth rate of m-cresol with pyridine, it can be found that the specific growth rates of m-cresol were much higher than pyridine, which indicated that pyridine played stronger inhibitory effect on the cell growth than m-cresol and the strain utilized m-cresol more easily than pyridine.

Bai et al. [3] studied the kinetic modeling of growth of phenol and m-cresol using a phenol-degrading microorganism, *A. faecalis*. The single-substrate kinetics was described well using the Haldane-type kinetic model, with model constant of $\mu_{max} = 0.15\ h^{-1}$ for cell

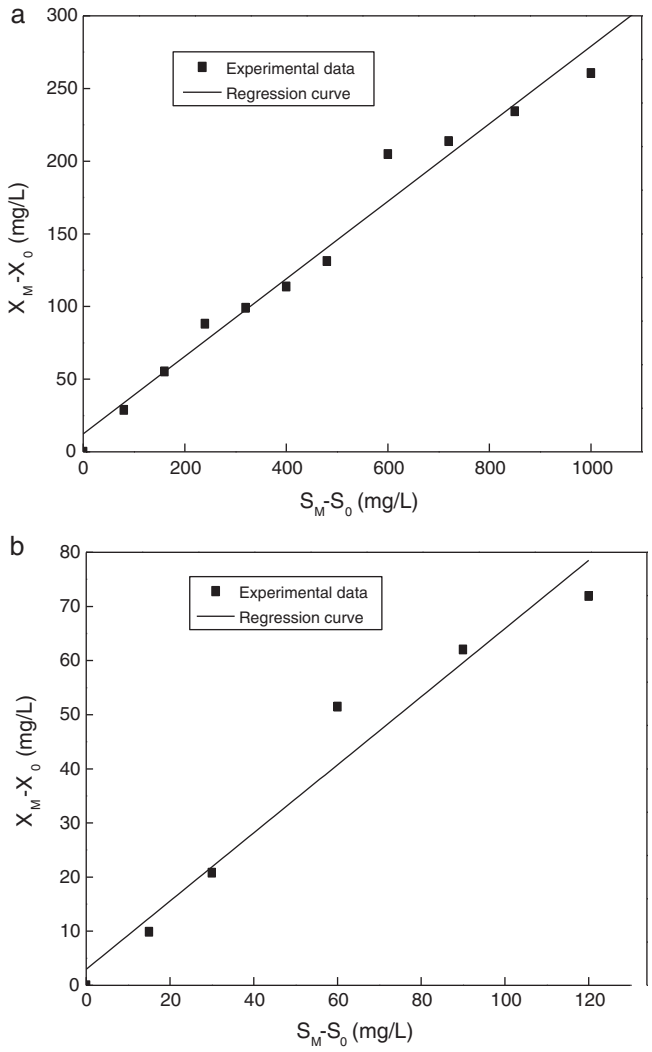

Fig. 4. Determination of yield values of (a) m-cresol and (b) pyridine.

growth on phenol and $\mu_{max} = 0.0782\ h^{-1}$ for cell growth on m-cresol, which means m-cresol was more toxic than phenol, m-cresol exhibited larger inhibitory effects on the cell growth behaviors. It is similar to the phenomenon of *L. cresolivorans* in our study. Because *L. cresolivorans* is a m-cresol-degrading microorganism, it degraded m-cresol more quickly than pyridine.

Biomass yield for m-cresol and pyridine can be calculated using the following equation:

$$Y_{X/S} = \frac{X_M - X_0}{S_M - S_0} \quad (3)$$

In the above expression X_M and X_0 are the maximum and initial dry cell concentration, S_M and S_0 are substrate concentrations at the maximum cell concentration and initial substrate concentration. The calculated biomass yield values of m-cresol and pyridine are presented in Fig. 4. The values for m-cresol and pyridine were 0.26 ($R^2 = 0.971$), 0.6 ($R^2 = 0.94$), respectively.

Under the same condition, *L. cresolivorans* can biodegradate more m-cresol than pyridine. And the value of $(S_M - S_0)$ of m-cresol was much greater than that of pyridine. At the same time, the value of $(S_M - S_0)$ increased more quickly than the value of $(X_M - X_0)$. So the biomass yield for m-cresol and pyridine were different.

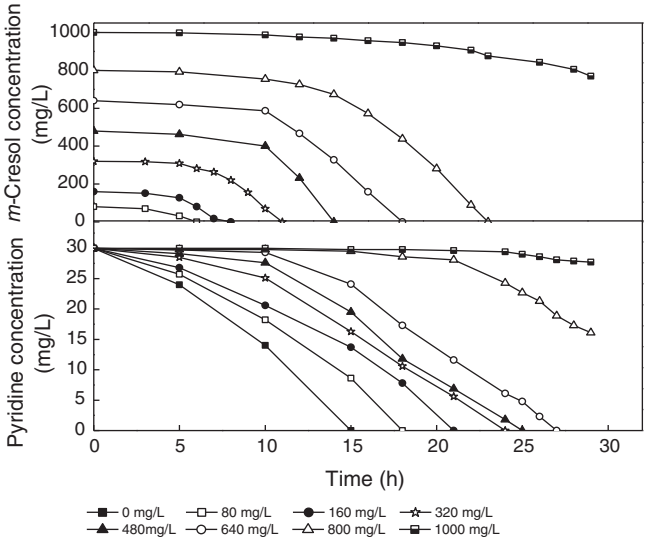

Fig. 5. Effect of m-cresol on pyridine biodegradation in dual substrates with 30 mg/L pyridine and m-cresol concentration from 0 to 1000 mg/L.

3.3. Biodegradation of dual substrates

A series of biodegradation experiments containing dual substrates were conducted. The cell growth and biodegradation on dual substrates were different from the single substrate system. Fig. 5 illustrates the biodegradation of dual substrates with initial m-cresol concentration varying from 0 to 1000 mg/L and the fixed pyridine concentration of 30 mg/L. It can be seen that the existence of m-cresol inhibits the pyridine biodegradation even at very low m-cresol concentration. The complete biodegradation of 30 mg/L pyridine in the absence of m-cresol was seen within 15 h. However, with the increase of initial m-cresol concentration in dual substrates system, the lag phase was prolonged, whereas more time was needed to degrade pyridine. It can be concluded that m-cresol, as a growth substrate, supplied carbon and energy source for *L. cresolivorans* in addition to being much easier to utilize than pyridine.

Fig. 6 represents m-cresol biodegradation with different concentrations of pyridine. It shows that the strain was also able to degrade pyridine, although more time was required for degradation. *L. cresolivorans* was enriched from m-cresol, and when a new

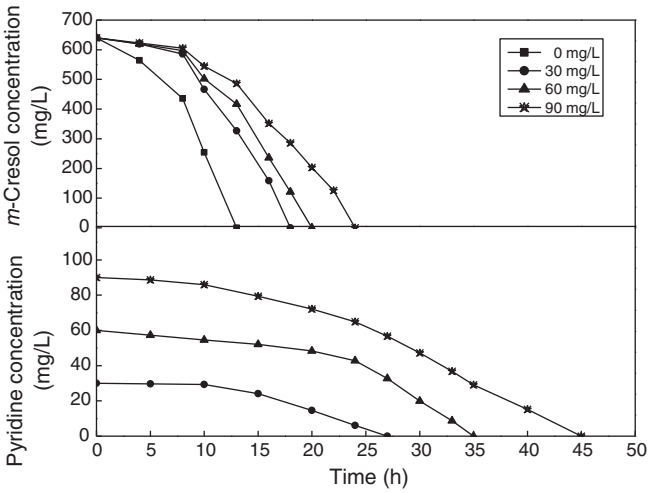

Fig. 6. Effect of pyridine on m-cresol biodegradation in dual substrates with 600 mg/L m-cresol and pyridine concentration from 0 to 90 mg/L.

substrate was added to the medium, the strain needed a longer period of time to adapt to the environment. This demonstrated that the strain might have the potential to degrade other N-heterocyclic compounds. Compared with the control experiments on single substrate of m-cresol, the speed of m-cresol biodegradation was slower. 13 h was required to completely degrade 600 mg/L m-cresol, whereas 18 h was needed to degrade the same amount of m-cresol in the presence of 30 mg/L pyridine, 20 and 24 h were required in the presence of 60 and 90 mg/L pyridine, respectively. When in dual substrates system, the m-cresol biodegradation was slower than that of the control. Pyridine inhibited the biodegradation of m-cresol.

Other researchers have studied the biodegradation of multiple substrates. Adav et al. [15] used aerobic granules to degrade pyridine in the presence of phenol. They found phenol concentrations of 500–2000 mg/L limited pyridine degradation in a competitive inhibition pattern. Kim et al. [12] studied the effect of a co-contaminant (phenol) on the biodegradation of pyridine by freely suspended and calcium alginate immobilized bacteria. When the concentration of phenol reached 380 mg/L, pyridine degradation was inhibited and the increased inhibition with the higher phenol levels was apparent in increased lag times. Pyridine degradation was essentially completely inhibited at 500 mg/L of phenol.

3.4. Kinetics on dual substrates biodegradation

For dual substrates system, on the basis of the experimental results, the interactions parameters were determined using the sum kinetics equation:

$$\mu = \frac{\mu_{\max,1} S_{1L}}{K_{S,1} + S_{1L} + S_{1L}^2/K_{1i} + I_{2,1} S_{1L}} + \frac{\mu_{\max,2} S_{2L}}{K_{S,2} + S_{2L} + S_{2L}^2/K_{2i} + S_{1L} I_{1,2}} \quad (4)$$

where the interaction parameter $I_{i,j}$ indicates the degree to which substrate i affects the biodegradation of substrate j, large value of the parameters indicate stronger inhibition on the substrates [16]. The other kinetic parameters μ_{max}, K_s and K_i in the equation are the same as those in the single substrate system.

Based on the experimental data and Eq. (4) above, the specific growth rate in mixed substrates could be obtained as:

$$\mu = \frac{0.89 S_m}{426.25 + S_m + S_{1L}^2/51.26 + 0.11 S_p} + \frac{0.0925 S_p}{60.28 + S_p + S_p^2/16.17 + 0.76 S_m} \quad R^2 = 0.9736$$

where m means m-cresol and p means pyridine. $I_{p,m}$ means the inhibition coefficiency of pyrine on m-cresol, and $I_{m,p}$ means the inhibition coefficiency of pyrine on m-cresol. It can be seen that $I_{m,p}$ (0.11) is less than $I_{m,p}$ (0.76), which meant that m-cresol inhibited the utilization of pyridine much more than pyridine inhibited the utilization of m-cresol. As mentioned earlier, *L. cresolivorans* has stronger potential to degrade m-cresol than pyridine. As a growth substrate, m-cresol was more easily utilized to synthesize the new cells. Pyridine biodegradation in the presence of m-cresol was greatly inhibited that L. cresolivorans inclined to utilize m-cresol first. To some extend, inhibition occurred in the mixed substrates, but m-cresol inhibited pyridine biodegradation more than pyridine inhibited m-cresol biodegradation. According to R^2, it was concluded that the regression curve was well consistent with the experimental data.

4. Conclusions

Biodegradation of m-cresol and pyridine as single and dual substrates by *L. cresolivorans* was investigated. *L. cresolivorans*, a bacterium obtained from a coking wastewater treatment plant, was able to degrade m-cresol and pyridine to a maximum concentration of 1000 and 120 mg/L, respectively. The kinetic models for the specific growth rate of m-cresol and pyridine as the single and mixed substrates were proposed, and the simulated values agreed well with the experimental data. When cells grew on the mixture of m-cresol and pyridine, substrates inhibition was observed, but the inhibitory effect of m-cresol on pyridine biodegradation was much more than that of pyridine on m-cresol degradation. *L. cresolivorans* may be a potential source for degradation of m-cresol in industrial wastewater containing pyridine. Based on the results, multi-substrates with pure or mixed microbial culture will be carried out in our future work.

Acknowledgements

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