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Biodegradation of 2-naphthol and its metabolites by coupling *Aspergillus niger* with *Bacillus subtilis*

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Abstract

To explore biodegradation of 2-naphthol and its metabolites accumulated in wastewater treatment, a series of bio-degradation experiments were conducted. Two main metabolites of 2-naphthol, 1,2-naphthalene-diol and 1,2-naphthoquinone, were identified by high-performance liquid chromatography with standards. Combining fungus *Aspergillus niger* with bacterium *Bacillus subtilis* in the treatment enhanced 2-naphthol degradation efficiency, lowered the accumulation of the two toxic metabolites. There were two main phases during the degradation process by the kinetic analysis: 2-naphthol was first partly degraded by the fungus, producing labile and easily accumulated metabolites, and then the metabolites were mainly degraded by the bacterium, attested by the degradation processes of 1,2-naphthalene-diol and 1,2-naphthoquinone as sole source of carbon and energy. Sodium succinate, as a co-metabolic substrate, was the most suitable compound for the continuous degradation. The optimum concentration of 2-naphthol was 50 mg/L. The overall 2-naphthol degradation rate was 92%, and the COD_{Cr} removal rate was 80% on day 10. These results indicated that high degradation rate of 2-naphthol should not be considered as the sole desirable criterion for the bioremediation of 2-naphthol-contaminated soils/wastewater.

Key words: 2-naphthol; metabolites; identify; continuous degradation; accumulation
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Introduction

Phenols have been listed as priority pollutants by US Environmental Protection Agency and European Union (Terzian and Serpone, 1995). Naphthalene derivatives with substituents at position 2 are usually more toxic than those at position 1 (Zhang et al., 2008). 2-Naphthol is an organic pollutant presented in the environment as a result of dyestuffs manufactory, pharmaceutical production and some biogeochemical processes (Roch and Alexander, 1995). Because of its strong toxicity and low biodegradability, the removal of 2-naphthol is of great importance in water treatment or soil remediation. The metabolites of 2-naphthol, 1,2-naphthalenediol and 1,2-naphthoquinone in liver can circulate to lung to deplete glutathione in lung cells (Viravaidya et al., 2004). Although some 2-naphthol metabolites have been identified in recent study, the mechanism of continuous degradation process of both parent compound and its accumulated metabolites was still unclear (Matthews, 1986). Therefore, it is crucial to understand the continuous degradation of both parent compound-2-naphthol and its metabolites.

The main methods to remove and eliminate 2-naphthol from the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, extraction by organic reagents and so on (Li et al., 2007; Alcantara et al., 2008). However, those techniques have some limitations and disadvantages (Rengaraj et al., 2006). For example, photo-degradation of organic contaminants in water or soil usually be restricted to a certain depth (or thickness), and the efficiency depends on many factors such as light wavelength distribution, water (or soil) characteristics, and photodegradation mechanism (Miller et al., 1989; Balmer et al., 2000). The extraction of 2-naphthol by organic reagent is not effective, especially at low concentrations. Meanwhile, the great amount of organic reagent can lead to secondary pollution. Using Fe^{2+}/HClO and Fe^{2+}/H_2O_2 to oxidize 2-naphthol from the contaminated wastewater only receives degradation rate at 39% and 33.3%, respectively, for 500 mg/L 2-naphthol at pH 2.5 using 10 mmol/L Fe^{2+} and 4.5 mmol/L H_2O_2 (Liu et al., 2005). One possibly convenient and economic approach to eliminate naphthol is biodegradation by preponderant microorganisms (Lu et al., 2005; Xu et al., 2008).

Compared with bacterium, fungus is more effective in degrading organic compounds with more rings (Kadian et al., 2008). Fungus can usually produce extracellular

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enzymes that are able to degrade low bioavailable pollutants. However, it is almost impossible for one kind of fungi or bacteria alone to thoroughly mineralize hardly degradable contaminants (Brodkorb and Legge, 1992; Bouchez and Vandecasteele, 1995). Some researchers (Juhasz and Naidu, 2000) have found synergistic degradation of polycyclic aromatic hydrocarbons (PAHs) by the combination of fungus and bacterium in a PAHs-contaminated soil, this technique may be suitable for the mineralization of 2-naphthol.

For those organic compounds with low bioavailability, their degradation mainly depends on co-metabolic degradation processes, especially in initial phase. Co-metabolic substrates can induce microorganisms to produce some kind of oxygenases that have the potential to degrade those compounds with similar chemical structures to the co-metabolic substrates. Usually, bacterium produces dioxygenase, while fungus produces monooxygenase (Gallert and Winter, 2008). Our pre-experiment revealed that no significant mineralization of 2-naphthol was observed after 36 hr in cultures in absence of co-metabolic substrates.

No information is available on the bioremediation of both 2-naphthol and its metabolites accumulated from soil or wastewater. The objectives of our study were to enhance the degradation of 2-naphthol and reduce the accumulation of the metabolites using preponderant *A. niger* combining with *B. subtilis*. The kinetics of 2-naphthol biodegradation was analyzed, and several important parameters were optimized. The relationship between degradation rate and the mineralization rate of 2-naphthol was also evaluated.

1 Materials and methods

1.1 Chemicals

2-Naphthol was purchased from the Xinxi Chemicals Company of Shenyang, China (> 99% purity), and 1,2-naphthalene-diol and 1,2-naphthoquinone (> 95.0% purity) were purchased from Aladdin Reagent Inc (Shanghai, China). Except that methanol was of HPLC grade, all other common reagents were of analytical grade, and were used without further treatment.

1.2 Microorganisms

A fungal strain of *A. niger* and a bacterial strain of *B. subtilis* were isolated from a crude oil-contaminated soil in Liaohe, China, and has been confirmed by molecular methods (16S DNA sequence analysis). The isolating medium contained (g/L) K_2HPO_4 1.0, KH_2PO_4 0.2, NH_4NO_3 1.0, $MgSO_4{\cdot}7H_2O$ 0.4, $CaCl_2{\cdot}2H_2O$ 0.02, $FeSO_4{\cdot}7H_2O$ 0.001, $Na_2HPO_4{\cdot}12H_2O$ 0.001, $MnSO_4{\cdot}H_2O$ 0.001, $(NH_4)_6MO_7O_{24}$ 0.014, 2-naphthol 0.05 and agar 18 in 1 L distilled water, and the medium was autoclaved at 115°C for 30 min. The seed media of the bacterium and the fungus were the same as described by Su et al. (2006). The degrading medium was the same as the isolating medium except that the latter was agar-free and 2-naphthol was added after autoclaved with different quantities.

1.3 Degradation experiments

1.3.1 Comparison of different degradation methods

Three biodegradation systems were setup.

Fungal method: 2-naphthol was degraded by *A. niger* alone. In details, 30 mL degrading medium with 100 mg/L 2-naphthol in 125-mL Erlenmeyer flasks was inoculated with 10% (*V/V*) *A. niger* seed medium. The seed medium was the same with the reference (Zang and Lian, 2009). Bacterial method: the culture was inoculated with 10% (*V/V*) *B. subtilis* alone. Combined method: the culture was inoculated with both 5% (*V/V*) of the fungus and 5% (*V/V*) of the bacterium seed culture, of which the bacteria were pre-grown to an optical density of 1.2 at 590 nm and the fungus was pre-grown for 12–18 hr. All the control experiments were conducted under the same conditions except for inoculant. The residual 2-naphthol was measured at different time intervals (0, 2, 4, 6, 8, 10 day) by high-performance liquid chromatography (HPLC) (model 5500, Palo Alto, CA). Calculation of the residue rate was similar to the reference (Lu et al., 2005). Three replicates were performed for each treatment. The degradation experimental conditions of 1,2-naphthalene-diol and 1,2-naphthoquinone were the same as above description. The best degradation method among the three was adopted by following experiments. All procedures were conducted in dim yellow light to avoid photo-degradation of 2-naphthol.

1.3.2 Effect of co-metabolic substrates

Co-metabolic substrates can induce microorganisms to produce some kinds of oxygenase that have the potential to degrade target contaminants. Glucose, salicylic acid, sodium succinate, and phthalic acid were chosen as co-metabolic substrates of 2-naphthol by pre-experiments. In co-metabolic substrate experiments, Erlenmeyer flasks (125-mL) were divided into 4 groups, and each had 50 mg/L glucose, salicylic acid, sodium succinate, or phthalic acid. Three replicates were applied to each group. The control experiments were conducted under the same conditions without addition of co-metabolic substrate. The residual 2-naphthol and the two metabolites were measured by HPLC on day 10. All other conditions were the same as above description.

1.3.3 Effect of initial concentration of 2-naphthol

To determine optimum concentration of 2-naphthol, a series of concentrations (150, 100, 50, and 20 mg/L) were employed. Thereafter, the same procedures as above description were followed. The residual 2-naphthol and the two metabolites were measured under the optimum conditions of above experiments. To determine COD_{Cr}, the above experimental samples were centrifuged at 6000 r/min for 10 min at room temperature to minimize the influence of thalli cell. The supernatant was collected.

1.3.4 Analytical method

The above samples were extracted three times with 8 mL CH_2Cl_2. The organic extracts were combined after the separation of organic and water phases. The organic phase was then cleaned in a chromatography column (length

170 mm, diameter 8 mm) filled with 3 g of deactivated aluminum oxide (deactivated by 15% water addition), 5 g of activated silica gel (70–230 mesh, activated by placing in an oven at 130°C for 16 hr and cooled in a desiccator at least for 10 min before use), and 1 g of anhydrous sodium sulfate. The chromatography column was eluted with 10 mL 4:1 hexane:dichloromethane (*V/V*). Other operating conditions were the same as described in the literature (Kadian et al., 2008).

The analysis was carried out by HPLC equipped with a gradient pump and a reverse-phase C18 column. Elution conditions were presented as follows: 3:1 (*V/V*) mixture of methanol and water (containing 0.005 mol/L KH_2PO_4) was used as the solvent at a flow rate of 0.8 mL/min. In all cases, 10 mL of sample was injected to the HPLC by autosampler. Average extraction recoveries of 2-naphthol, 1,2-naphthalene-diol and 1,2-naphthoquinone were greater than 92% under the given conditions.

Formulas of COD_{Cr}, degradation and accumulation were all based on the references (Kim et al., 2000; Somasiri et al., 2008).

1.3.5 Statistical analysis

Statistical significance was evaluated using the software Statistical Package for Social Sciences (SPSS11.5 for Windows) with one-way ANOVA and least significant difference (LSD) for comparison of treatment means with $p < 0.05$ (Abdullah et al., 2007).

2 Results and discussion

2.1 Identification of metabolites of 2-naphthol

With 2-naphthol degraded, some labile and accumulatable metabolites of 2-naphthol were gradually produced. The accumulation of metabolites indicated that the speed of metabolites degradation was lower than the production speed of those metabolites. 2-Naphthol, 1,2-naphthalene-diol and 1,2-naphthoquinone were identified based on the retention time of their standards using reversed phase HPLC. At wavelength of 280 nm, the retention time of 2-naphthol, 1,2-naphthalene-diol and 1,2-naphthoquinone were 7.238, 5.537, and 4.823 min, respectively. Under our experimental conditions, the main metabolites of 2-naphthol by *A. niger* were 1,2-naphthalene-diol and 1,2-naphthoquinone (Fig. 1). The other metabolites were not identified because their concentrations were under

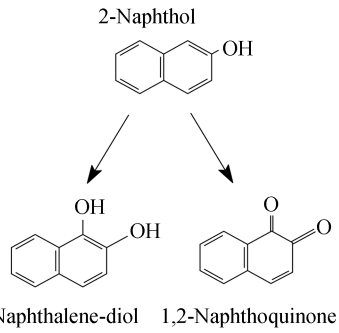

Fig. 1 Chemical structure of 2-naphthol and its metabolites.

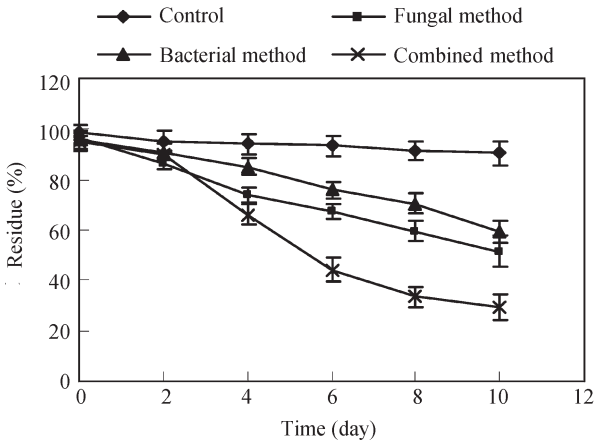

Fig. 2 Comparison of different methods for 2-naphthol degradation.

detection limits of the HPLC method.

2.2 Comparison of different degradation methods

To enhance the degradation of 2-naphthol and reduce the accumulation of its metabolites, it is crucial to select a suitable method. From Fig. 2, it is evident that the fungal method because the most effective for 2-naphthol degradation among the three methods in first 2 days. After that time, the combined method became the best, while the bacterial method was the worst at all the time, despite of the strong endurance of *B. subtilis* to 2-naphthol. The residue rate of 2-naphthol on the tenth day for the fungal, bacterial and combined methods were 51%, 59% and 29%, respectively. Overall, the combined method was the most suitable for 2-naphthol degradation from a long run.

According to the experiment, the biodegradation of 2-naphthol could be well described by a first-order kinetic model. The kinetic analysis of 2-naphthol biodegradation of above three different methods was summarized in Table 1. The longer the half life ($t_{1/2}$, day) was, the worse the degradation efficiency was. Half life of the above three methods was 11.2 days, 14.84 days and 5.33 days, respectively, further suggesting that the combined method (combining fungus with bacterium) was the best.

On the basis of these observations, we propose that the parent compound and its metabolites were degraded in two different phases (Fig. 2). The initial degradation of 2-naphthol mainly involves the fungus, while the further mineralization may rely on the participation of the bacterium. In order to further prove this hypothesis, we used standards of 1,2-naphthalene-diol and 1,2-naphthoquinone as main contaminants to be degraded by the three methods (Fig. 3). The results were consistent that the two metabo-

Table 1 Kinetic parameters of 2-naphthol biodegradation in different treatments

Treatment	Kinetic equations	$t_{1/2}$ (day)	Correlation coefficient (r^2)
Control	$C = 97.79e^{-0.0082t}$	84.5	0.9502
Aspergillus niger	$C = 96.57e^{-0.061t}$	11.2	0.9976
Bacillus subtilis	$C = 99.20e^{-0.046t}$	14.8	0.9600
Combined method	$C = 104.21e^{-0.13t}$	5.33	0.9684

C is concentration; $t_{1/2}$ is half life.

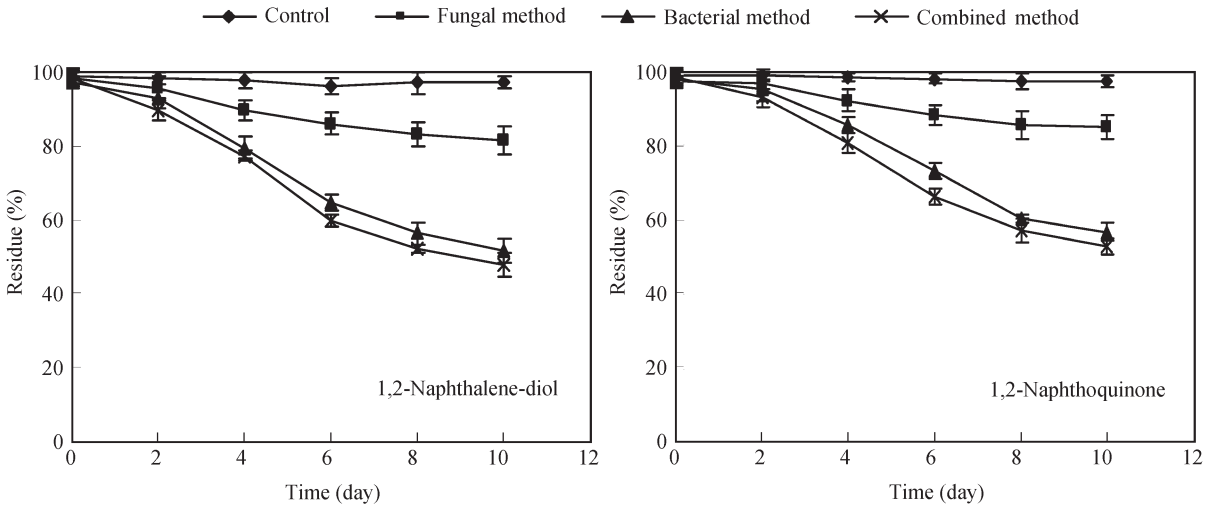

Fig. 3 Degradation of the two metabolites by the three methods.

lites were more liable to be degraded by *B. subtilis* than by *A. niger*. In addition, there was a distinct synergistic degradation between the preponderant fungus and bacterium throughout the degradation processes.

The degradation rate of 2-naphthol by bacterium was evidently lower than that by fungus, although the bacterium could also degrade 2-naphthol. However, the degradation rate of 1,2-naphthalene-diol and 1,2-naphthoquinone by fungus were evidently lower than that by bacterium, although *A. niger* could also degrade two metabolites. From this study, it can infer that one kind of microorganism alone might not be efficient enough to completely mineralize low bioavailable contaminants.

2.3 Effect of co-metabolic substrates

Co-metabolic substrates could induce microorganisms to produce degradation enzymes that have the potential to degrade both co-metabolic substrates and other compounds with similar chemical structure. Co-metabolism was the major degradation process for those contaminants with many rings and having low bioavailability. Sodium succinate, phthalic acid, biphenyl and salicylic acid all had a certain effect on the degradation of the target contaminants (Fig. 4). Among them, the effect of sodium succinate was the most evident ($p < 0.05$). The main difference between glucose and sodium succinate was that sodium succinate, as a co-metabolic substrate, could induce some enzymes (such as dioxygenase) that facilitate the degradation of our target contaminants, while glucose could not. However, glucose could shorten the delay in the initial stage of degradation (Tao et al., 2007). This is consistent with other findings (Schneider et al., 1996). Between the two metabolites, 1,2-naphthoquinone was more liable to be accumulated than 1,2-naphthalenediol in above cultures.

2.4 Effect of initial concentration of 2-naphthol

Four initial concentrations (20, 50, 100, and 150 mg/L) of 2-naphthol were examined in this experiment (Fig. 5). The results showed that decreasing concentration of 2-

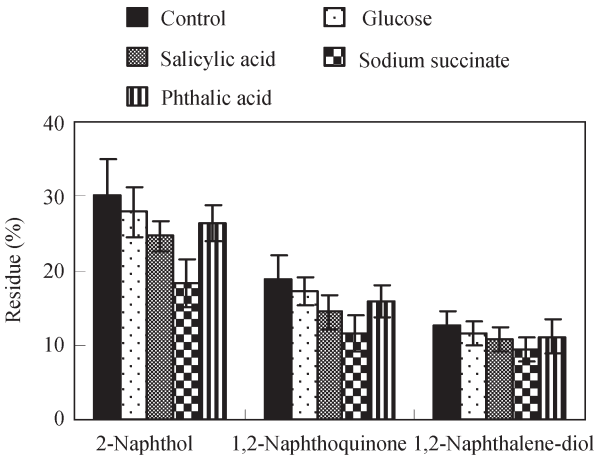

Fig. 4 Effect of different co-metabolites on the degradation of 2-naphthol and its metabolites.

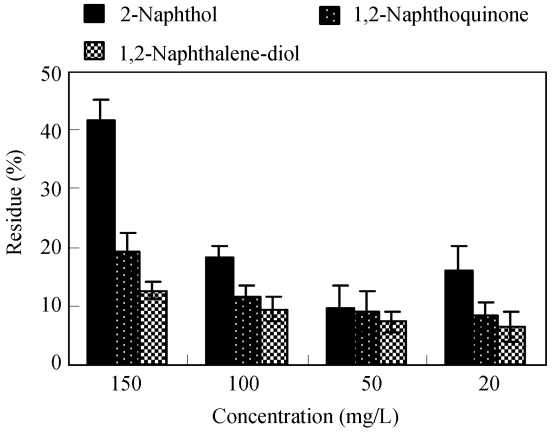

Fig. 5 Effect of initial concentration on the degradation of 2-naphthol and its metabolites.

naphthol in a certain range led to the enhancement of degradation rate and reduction accumulation rate of two metabolites which was similar to the results obtained by Su et al. (2006). 2-Naphthol and its metabolites could be well degraded when the initial concentration of 2-naphthol was between 20 and 100 mg/L. The integrative degradation

efficiency was the best when 2-naphthol concentration was 50 mg/L.

The environmental processes can also affect the concentration and physicochemical form of the contaminants. Because a higher concentration is normally accompanied with strong toxicity, which microorganisms need a longer time to adapt it. However, studies at low concentrations may become questionable about its practical usability. For different degradation systems, a suitable concentration range may be different, and it may be related to many parameters of environmental microorganisms and pretreatment extent by chemical reagents.

2.5 Relationship of degradation and mineralization

In order to understand the relationship between degradation rate and mineralization rate, the degradation of 2-naphthol, the accumulation of the two metabolites and COD_{Cr} levels were monitored under laboratory conditions. From the degradation curves of 2-naphthol and COD_{Cr} reduction curves (Fig. 6), it was apparent that the former was much higher than the latter, especially in the initial phase. Removal rate of COD_{Cr} was only 25% when degradation rate of 2-naphthol was 64% on day 6, while the accumulation rate of the two metabolites reached its maximum. The result revealed that a high degradation rate should not be considered as the only criterion for the bioremediation of 2-naphthol contaminated wastewater because of the potential health risk of accumulated metabolites to human. This result is similar to benzopyrene degradation. In that study, only 18.3% was detected as carbon dioxide when benzopyrene degradation rate was 97%, while 16.8% and 31.3% were detected as intermediates from aqueous phase and soil extract, respectively (Kyoungphile at el., 2001).

After the accumulation diminished, the mineralization rate was gradually enhanced (when the degradation rate of 2-naphthol was 92%, the COD_{Cr} removal rate was 80% on day 10). Between the two metabolites, 1,2-naphthoquinone was more liable and easily accumulated than 1,2-naphthalene-diol. Comparing HPLC and COD_{Cr} curves, it is apparent that the metabolites of 2-naphthol degrade at a much slower rate than 2-naphthol, and this phenomenon is similar to that of photo-degradation of 2-naphthol (Qourzal et al., 2008).

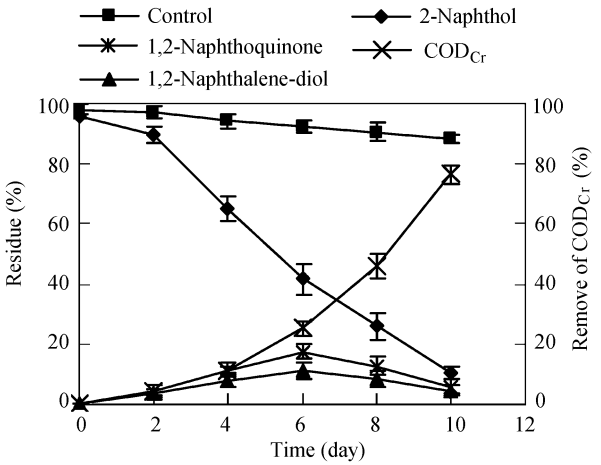

Fig. 6 Relationship of degradation and mineralization.

The degradation of 2-naphthol may be more complicated than we think. From the data of COD_{Cr}, it can be concluded that there must be other metabolites besides 1,2-naphthalene-diol and 1,2-naphthoquinone. The other intermediates were not identified mainly due to their low concentrations, instability or low sensitivity to the experimental conditions. Future researches are recommended to focus on the structure-activity relationship in metabolites of 2-naphthol, including more detailed characterization of accumulated metabolites. Because practical wastewater usually offers more contaminants than the ideal experimental conditions, an integrated photocatalytic and biodegradation system may be more effective. In addition, the distribution, elimination, and toxicity of 1,2-naphthalene-diol and 1,2-naphthoquinone in wastewater are not clear so far, and this brings additional difficulty to effectively evaluate a remediation scheme of 2-naphthol polluted wastewater or soil.

3 Conclusions

In this study, we have identified two main metabolites of 2-naphthol as 1,2-naphthalene-diol and 1,2-naphthoquinone. The results revealed a potential degradation mechanism. A synergistic degradation system of 2-naphthol can be achieved by combining environment-friendly fungus–*A. niger* with bacterium–*B. subtilis*. There were two main phases for 2-naphthol degradation by kinetic analysis. One was the degradation of parent compound by fungus *A. niger*, and the other was the degradation of the metabolites by bacterium *B. subtilis*. As a co-metabolic substrate, sodium succinate was the most suitable for the continuous degradation, and the optimum concentration of 2-naphthol was 50 mg/L. The degradation rate of 2-naphthol was 92%, and the removal rate of COD_{Cr} was 80% on day 10 under the optimum conditions. This suggests that the bioremediation of 2-naphthol contaminated wastewater using the combination of fungus and bacterium can potentially be practical application.

Acknowledgments

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