Journal of Hazardous Materials 166 (2009) 33–38

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Synergistic degradation of 2-naphthol by *Fusarium proliferatum* and *Bacillus subtilis* in wastewater

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ARTICLE INFO

Article history:
Received 2 July 2008
Received in revised form 31 October 2008
Accepted 31 October 2008
Available online 6 November 2008

Keywords:
2-Naphthol
Biodegradation
Enzyme
Response
Co-metabolism

ABSTRACT

2-Naphthol, which originates widely from various industrial activities, is toxic and thus harmful to human liver and kidney. A new compound biodegradation system was adopted to degrade 2-naphthol-contaminated wastewater. Enzymatic response to 2-naphthol biodegradation in the aqueous phase was also studied. As a co-metabolic substrate, salicylic acid could induce the two microorganisms to produce a large amount of degradation enzymes for 2-naphthol. The key enzymes were confirmed as polyphenol oxidase (PPO) and catechol 2,3-dioxygenase (C23O). The degradation extent of 2-naphthol, determined by high performance liquid chromatography (HPLC), was enhanced by nearly 15% on the 6th day after the addition of the co-metabolic substrate. The results obtained thus clearly indicated that the co-metabolic process was the most important factor affecting the degradation of the target contaminant. The optimal concentration of 2-naphthol was 150 mg L^{-1}, and the optimal pH value was 7.0. The degradation extent of 2-naphthol was further enhanced by nearly 10% after the addition of Tween 80, which increased the bioavailability of 2-naphthol. In a practical treatment of industrial wastewater from medical manufacture, the synergistic degradation system resulted in a high degradation efficiency of 2-naphthol although its lag time was a little long in the initial stage.

1. Introduction

In recent years, there has been an increasing concern about industrial wastewater containing phenolic compounds, which are harmful and toxic to marine life and human beings [1]. Naphthalene derivatives with substituents at position 2 are usually more toxic than those at position 1, therefore, 2-naphthol is listed among top priority contaminants [2]. This compound, which biodegrades very slowly, is usually present in drinking water and industrial wastewater. It is also present in the atmosphere through emissions from various industrial sources. Due to its toxicity, industrial wastewater containing 2-naphthol must be treated before it is reused or discharged to the environment.

More and more researchers are investigating suitable methods for removal or eliminating 2-naphthol since the contamination of biorefractory organic chemicals is a widespread problem [3]. The main methods to remove 2-naphthol from the environment include: volatilization, photo-oxidation, chemical oxidation, bioaccumulation and extraction by organic reagents [4,5]. The conventional method—extraction by organic reagent—cannot completely remove 2-naphthol, and the organic reagent itself will lead to environmental pollution [6]. Photo-degradation of organic contaminants in water or soil is usually restricted to a certain depth (or thickness), and the efficiency depends on many factors such as light wavelength distribution, water (or soil) characteristics, and photo-degradation mechanism. For example, the photo-degradation extent of 2-naphthol (2.0×10^{-5} M 2-naphthol, 4 h) was no more than 20% [7–9]. For chemical oxidation, the researchers used Fe^{2+}/HOCl and Fe^{2+}/H_2O_2 to treat 2-naphthol-contaminated wastewater, the degradation extent were 39% and 33.3%, respectively [10]. One possibly convenient and economic approach to eliminate 2-naphthol is biodegradation by preponderant microorganisms. However, one of the major problems encountered was that the biodegradation by fungus or bacterium alone was often ineffective or time-consuming owing to the inherent toxicity of the compound. Therefore, little has been reported on its biodegradation and enzyme response in the literatures.

Some researchers suggested that synergistic degradation by fungus and bacterium could occur in the bioremediation of polycyclic aromatic hydrocarbon (PAH)-contaminated soil [11]. A fungus, *Phanerochaete ehrysosporium*, has been successfully combined with *Bacillus subtilis* to degrade pyrene. The combination greatly speeded up the degradation of pyrene, much greater than that by the separate microorganisms [12]. Since the mutagenic and carcinogenic

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doi:10.1016/j.jhazmat.2008.10.117

PAHs could be degraded by combined microorganisms, this study was proposed to determine whether a similar method of biodegradation may be suitable for 2-naphthol.

For those organic compounds with low bioavailability, co-metabolic processes may be a main process. Co-metabolic substrates could make microorganisms produce some enzymes that have the potential to degrade the co-metabolic substrates themselves and other compounds with similar chemical structures [13]. In uninduced cultures of *Pseudomonas saccharophilia* strain P15, no significant mineralization of benzo(a)pyrene was observed in 48 h [14]. Low solubility is another limiting factor for biodegradation. Numerous studies [15–17] have dealt with the enhancement of solubility of hydrophobic contaminants by adding surfactants to keep above their critical micelle concentrations, which will necessarily result in subsequent degradation. Tween 80 is known as an earth-friendly surfactant with very low (or no) toxicity [18–20]. To investigate this point of view, Tween 80 was included in this study.

In order to effectively remediate and control 2-naphthol-contaminated industrial wastewater, several biodegradation systems were compared, and the response of microbial enzymes to 2-naphthol was also studied. In the new biodegradation system, several parameters were optimized.

2. Materials and methods

2.1. Chemicals and micro-organisms

All solvents except CH_2Cl_2 were of HPLC grade; K_2HPO_4, KH_2PO_4, NH_4NO_3, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, $Na_2HPO_4{\cdot}12H_2O$, $MnSO_4{\cdot}H_2O$, $(NH_4)_6MO_7O_{24}$, salicylic acid, Tween 80, glucose and CH_2Cl_2 were all of analytical grade. 2-Naphthol was purchased from the Xinxi Chemicals Company of Shenyang, China (>99% purity). Fungus *Fusarium proliferatum* and bacterium *Bacillus subtilis* were screened, isolated and identified from crude oil-contaminated soil in the Liaohe Oil Field in China [21]. The fungus and bacterium were capable of utilizing 2-naphthol as the sole carbon and energy source. Before the start of the experiments, the two organisms were domesticated with a mixture of phenolic compounds for four weeks. Mycelia were stored on agar slants at 4 °C, and were transferred to fresh slants every three months. The degradation mineral medium consisted of (in g L^{-1} distilled water): 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; $Na_2HPO_4{\cdot}12H_2O$, 0.6; $MnSO_4{\cdot}H_2O$, 0.001; $(NH_4)_6MO_7O_{24}$, 0.014. The culture method for the bacterium and the fungus was according to Su et al. [22].

2.2. Degradation experiments

2.2.1. Comparison of different biodegradation systems

All procedures were conducted in dim yellow light in order to avoid photodegradation of 2-naphthol.

Three biodegradation systems were setup as follows:

System 1: 2-Naphthol (at 100 mg L^{-1}) was added to the degradation culture medium described in Section 2.1 in Erlenmeyer flasks (125 mL) and the culture was inoculated with 10% v/v *B. subtilis* (the bacterial cells were pre-grown to an optical density of 1.2 at 590 nm). The final volume of the culture was 30 mL. The cultures were incubated on a rotary shaker at 30 °C and 130 revolutions per minute (rpm).

System 2: As in System 1 except that the culture was inoculated with both *B. subtilis* 5% (v/v) and *F. proliferatum* 5% (v/v; mycelium on agar slants were taken out as a 0.5 cm^3 block for inoculating into seed medium). Preliminary experiments showed that the dry weight of the cells was 0.2 g 30 mL^{-1} when the cells were pre-grown for 12 h, at 30 °C and 130 rpm).

System 3: The culture was inoculated with 10% *F. proliferatum* alone.

All control experiments were done under the same conditions except without inoculant. The residual concentrations of 2-naphthol were measured at different time intervals (0, 2, 4, 6, 8, 10 d) by HPLC. Three replicates were performed for each treatment.

2.2.2. Influence of 2-naphthol of different concentrations on enzyme activities

In order to examine the effect of initial concentration of 2-naphthol on the enzyme activities of the combining microorganisms, a series of concentration experiments (0, 50, 100, 150, 200 and 250 mg L^{-1} of 2-naphthol) were selected and repeated. The enzymes produced by the microorganisms in degradation experiments were extracted for assaying. The extraction method of enzymes was similar to that of Kalme et al. [23]. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg^{-1} of enzyme under the test conditions. The residual concentrations of 2-naphthol and the enzyme activities of the bacterium were measured on the 2nd day (since pilot experiments had shown that the enzyme activities were the greatest on the 2nd day). Abiotic controls (without microorganisms) were always included. Three replicates were performed for degradation extent, and five replicates were performed for enzyme activities. In the experiments, several enzymes were measured at different time intervals, and only catechol 1,2-dioxygenase (C12O), C23O, and PPO gave strong responses to changing conditions during the degradation process of 2-naphthol. Therefore, C12O, C23O, and PPO were selected for the following study.

2.2.3. pH value of the culture

A degradation experiment was conducted with pH change. The initial pH value of the culture solution was adjusted by adding either 0.1 M HCl or NaOH, and measured by a pHs-29A acidity meter. Initial pH values of the different groups were 6.6, 7.0 and 7.4, respectively. All the other conditions were as those described in Section 2.2.1.

2.2.4. Effect of salicylic acid on enzyme activities and degradation extent

As a co-metabolic substrate, salicylic acid (50 mg L^{-1}) was added to the above cultures in which the other parameters (such as pH and concentrations of 2-naphthol) were set at the optimum values determined from experiments described above. Control experiments were done under the same conditions but without addition of salicylic acid. Both the residual concentrations of 2-naphthol and the enzyme activities of the microorganisms were measured.

2.2.5. Influence of surfactant Tween 80 on the degradation of 2-naphthol

Culture flasks were prepared with 2-naphthol (150 mg L^{-1}), Tween 80 (500 mg L^{-1}) and salicylic acid (50 mg L^{-1}) as well as the nutrients and inoculant to a final volume of 30 mL. Experimental controls were carried out under the same conditions except for the absence of Tween 80. The residual concentrations of both 2-naphthol and Tween 80 were measured at different time intervals (0, 2, 4, 6, 8, 10 d), by HPLC and spectrophotometry, respectively.

2.2.6. Practical application of the biodegradation system

Wastewater was sampled from a medical manufacturing company (Meng J-L, Jilin Province, China) and diluted to reach the optimal 2-naphthol concentration as determined above. Parameters of the wastewater after dilution by a factor of 1.77 were as

follows: 2-naphthol 150 mg L^{-1}, COD_{cr} 4786.6 mg L^{-1}, pH 7.26, SS 126.1 mg L^{-1}. In order to get the optimal applied effect, suitable concentration of Tween 80 and salicylic acid (decided by above experiments) were added into the wastewater, and pH was also adjusted. The wastewater sample (both sterilized and unsterilized) was used to conduct the degradation experiment in the laboratory by above experimental method, where a 30 mL sample was used in place of the 2-naphthol solution. Three replicates were performed for each treatment. Control experiments for both sterilized and unsterilized samples (control 1 and control 2) were done under the same conditions except without inoculant.

2.2.7. Analytical methods

2.2.7.1. Extraction and analysis of 2-naphthol. The extraction of sample medium was described [24,25] as follows: the samples were extracted three times with 8 mL CH_2Cl_2. The organic extracts were combined after the separation of organic and water phases, and cleaned by passing through a chromatographic column (SiO_2 overlain with Na_2SO_4). The extracts were concentrated at 50 °C by means of a rotary evaporator, dried by nitrogen gas and finally dissolved in methanol (1 mL) for further analysis. The concentrations were determined after filtration of the suspension through membrane filters (Millipore, 0.45 μm).

2-Naphthol in the samples was analyzed on a quantitative and qualitative basis by HPLC equipped with a gradient pump (KNAUER K1001), an Autosampler (TSP AS100), a reverse-phase C-18 column (length 170 mm, diameter 8 mm) and variable wavelength UV detector (LC-10AV). Elution was isocratic with 3:1 (v:v) mixture of methanol and water (containing 0.005 M KH_2PO_4) as the eluent at a flow rate of 0.8 mL min^{-1}. In all cases, 10 μL of sample was injected to the HPLC by the Autosampler. 2-Naphthol was identified based on its retention time. At the wavelength of 280 nm, the retention time of 2-naphthol was 7.238 min.

In addition, Tween 80 was measured by spectrophotometry at 620 nm using standard methods [26].

2.2.7.2. Extraction and analysis of enzymes. Determination of PPO, C12O and C23O was carried out using reported methods [27].

2.2.8. Statistical analyses

The statistical analysis of variance (ANOVA) and multivariate ANOVA (MANOVA) was performed using Excel and SPSS programs [28].

3. Results and discussion

3.1. Comparison of different biodegradation systems

It is a necessary step and also a challenge to design a practical environment-friendly degradation system for toxic contaminants. Among the three degradation systems in this study, the degradation of 2-naphthol by System 2 (combining fungus *F. proliferatum* with bacterium *B. subtilis*) was the fastest at all time intervals (Fig. 1). 2-Naphthol was rapidly degraded by System 2 after 2 days of incubation and reached its maximum degradation on the 6th day. After day 6, the three degradation curves all reached a relatively slow decay rate. The residue 2-naphthol was approximately 40% on the 6th day in the System 2, but 60% and 67% for System 1 and 3 respectively. In addition, the degradation effect of the System 1 was better than that of System 3 before the 4th day, and after that time, the position was reversed.

Compared with the fungus, chemical processing by the bacterium is usually less reliable due to the inability of the target compound to pass through bacterial cell walls [29]. However, the fungus could overcome this shortcoming, for it could usually produce extracellular enzymes which are significant for pollutants of low bioavailability [30]. The study of microbial activity in mixed substrate systems is therefore important for biotechnological applications and wastewater treatment [31–33].

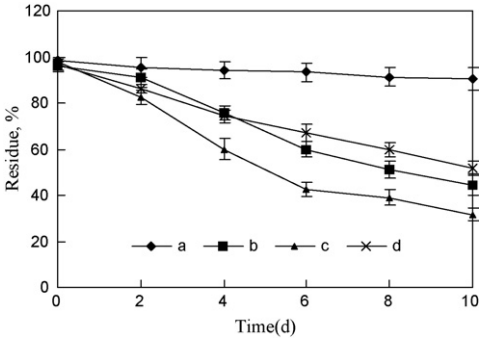

Fig. 1. Comparison of different biodegradation systems. a, Control; b, degradation by bacteria *Bacillus* sp.; c, degradation by both fungus *Fusarium* sp. and bacteria *Bacillus* sp., d, degradation by fungus *Fusarium* sp. Error bars represent the standard deviation from the mean value of triplicate experiments.

Synergistic degradation by fungus and bacterium (System 2) was found to be suitable for the degradation of 2-naphthol in the experiment. Therefore, this degradation system was adopted in all the subsequent experiments.

3.2. Enzyme response

The relationship between enzyme activity, degradation effect and the initial concentration of 2-naphthol is presented in Fig. 2. When the concentrations of 2-naphthol were less than 200 mg L^{-1}, enzyme activities of C12O, C23O and PPO increased with increasing concentration of 2-naphthol. However, the response of C12O with increasing the concentration of 2-naphthol was only small.

When the concentrations of 2-naphthol were above 200 mg L^{-1}, the concentrations of residual 2-naphthol tended to reach a limiting value. When enzyme activities of C12O, C23O and PPO was decreased, degradation extent were all decreased (that is, the residual concentration after two days was higher). For C12O and PPO enzyme activities, the most suitable concentration of 2-naphthol was 150 mg L^{-1}, while that for C23O, the most suitable concentration was 200 mg L^{-1}. In order to avoid excess compound toxicity and to maximize enzyme activities, 150 mg L^{-1} was chosen as the optimum concentration for further experiments. Fig. 2 also shows that PPO and C23O were the main enzymes responsible for the

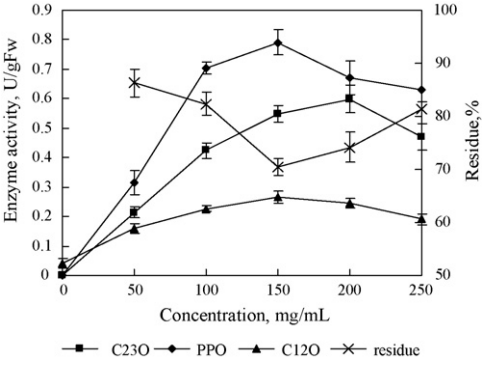

Fig. 2. Enzyme activities in different concentrations of 2-naphthol. Error bars represent the standard deviation of the mean ($n = 5$).

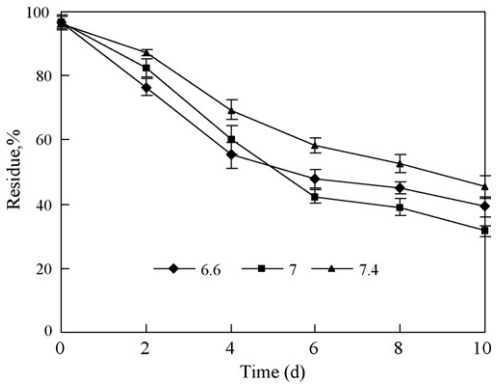

Fig. 3. Effect of initial pH value of the culture. Error bars represent the standard deviation of the mean ($n = 3$).

degradation of 2-naphthol due to their strong response with concentration change of 2-naphthol.

3.3. Effect of the initial acidity

Acidity plays an important role at different phases in synergistic degradation. Because the fungus grows better in mildly acid conditions (pH 5.0–7.5), while the bacterium grows better in mildly alkaline conditions (pH 6.5–8.0), therefore, the pH range was chosen 6.5–7.5 in the experiments. During the initial 4 days of incubation, the degradation extent of 2-naphthol was the highest in the culture with pH 6.6 (Fig. 3). After that time, the degradation extent in the culture with pH 6.6 was lower than that with pH 7.0. The degradation extent in the culture with pH 7.4 was the lowest throughout the degradation processes. Therefore, the optimal pH for the biodegradation was pH 7.0 from the long run.

3.4. Effect of salicylic acid on enzyme activities and degradation extent

Co-metabolic substrates are of crucial importance for the degradation extent of 2-naphthol and enzyme response. A specific microorganism will have different responses to different kinds of contaminants and co-metabolic substrates [34]. From its chemical structure and properties, salicylic acid has broadly similar structure to 2-naphthol, and much lower toxicity. Compared with 2-naphthol, salicylic acid is liable to preferential degradation in the same culture; in the processes, mixed-function enzymes were produced which could also degrade 2-naphthol. The degradation extent of 2-naphthol was low in the first 24 h in the presence of salicylic acid compared with the control group (without salicylic acid). But after that time, the degradation extent of 2-naphthol was rapidly enhanced (Fig. 4B). When multiple substrates are present, microbial-substrate degradation may be affected by the presence of other substrates. During the induction period (24 h), the combined microorganisms utilized salicylic acid as a main carbon and energy source, which would lead to a lower degradation extent of 2-naphthol. After 24 h, the salicylic acid induced activity of the enzymes in the mixed-function oxidase system (PPO, C23O and C12O) could then degrade 2-naphthol quickly.

The activities of PPO and C23O were significantly affected by salicylic acid (Fig. 4A). The extent of induction of enzyme activity follows the response order of C12O < C23O < PPO. Compared with PPO and C23O, the enzyme activity of C12O was still lower after induction. The characteristic feature, in combination with little change in the residual concentrations of 2-naphthol (as noted in 3.2 above), indicated that C12O was not the main enzyme involved in 2-naphthol degradation. During the degradation process of 2-naphthol, PPO and C23O were sensitive to changes conditions. Therefore, the key enzymes for the degradation of 2-naphthol were PPO and C23O.

It has been reported that salicylate (at 90 mg L^{-1}) could be completely degraded in 14 h [35]. When the co-metabolic substrates were exhausted, the continuous degradation of 2-naphthol depended mainly on the metabolites themselves, which were also better co-metabolic substrates with low molecular weight and were easily degraded than the parent compound. The results were similar to some reports concerning PAH degradation [36].

3.5. Influence of surfactant Tween 80 on the degradation of 2-naphthol

Bioremediation of organic contaminants is highly challenging because of the low solubility [37], which is one of the problems for low bioavailability pollutants. Solubility of our 2-naphthol (were purchased from the Xinxi Chemicals Company of Shenyang, China) was about 66 μg mL^{-1}. After the addition of Tween 80, the capability of 2-naphthol dissolving in cell walls would be greatly enhanced, and the chance for microorganisms to process 2-naphthol remarkably increased. Hence, bioavailability would be simultaneously improved.

It could be seen that the degradation extent of 2-naphthol (on the 6th day) was enhanced by nearly 10% compared with that in the absence of Tween 80 (Fig. 5). The degradation of Tween 80 was nearly 95% on the 6th day under the experimental conditions, indicating that the addition of non-ionic surfactants would not bring about further contamination to the environment.

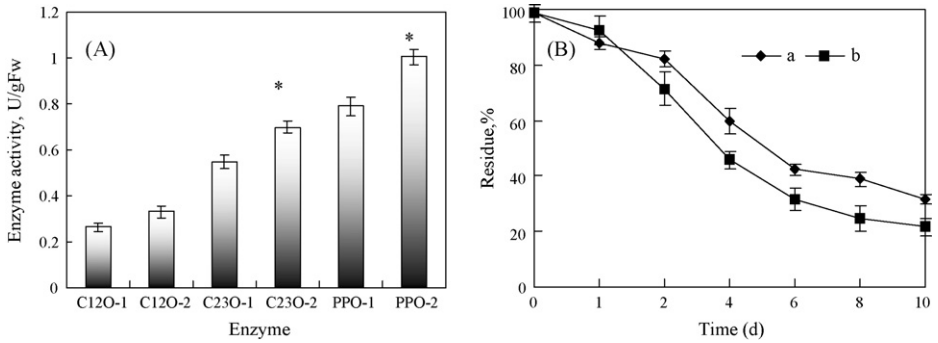

Fig. 4. Effect of salicylic acid on enzyme activities and residue rates. (A) influence of salicylic acid on enzyme activities; (B) effect of salicylic acid on 2-naphthol; a, degradation of 2-naphthol in the absence of co-metabolite; b, degradation of 2-naphthol in the presence of co-metabolite. Error bars represent the standard deviation of the mean ($n = 5$). The asterisk symbol (*) denotes significantly different when compared to the control group ($p < 0.05$).

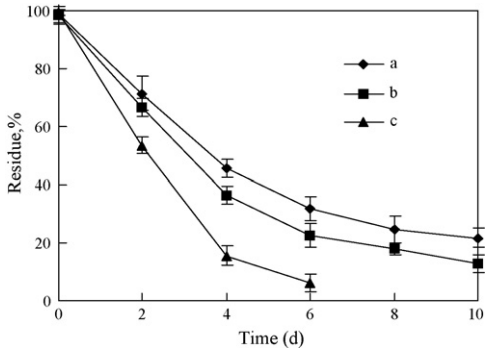

Fig. 5. Influence of surfactant Tween 80 on degradation of 2-naphthol. a, Degradation of 2-naphthol in the absence of Tween 80; b, degradation of 2-naphthol in the presence of Tween 80; c, degradation of Tween 80. Error bars represent the standard deviation of the mean ($n = 3$).

3.6. Practical application of the biodegradation system

In order to test the practical application effect of the synergistic degradation system, three treatments of the real wastewater were designed (1): the real wastewater unsterilized; (2): the real wastewater sterilized; and (3): a distilled water with 2-naphthol 150 mg L^{-1}. The results are compared in Fig. 6. It can be seen that the degradation effect of the treatment (1) was the best on the 10th day, while that of the treatment (2) was the worst, and that of the treatment (3) was intermediate.

The degradation extent of 2-naphthol in the treatment (1) was much lower in the first 6 days, but after that time, the degradation extent was rapidly enhanced so that by day 10, it was the best. Main reasons were that, on the one hand, *F. proliferatum* and *B. subtilis* need some time to adapt to the new environment containing full of many kinds of nature microorganisms; on the other hand, the *F. proliferatum* and *B. subtilis* may first degrade those organic compounds with lower molecular weight and easier degradation in the initial stage. Thereafter, the synergistic degradation by many kind of microorganism together was effectual.

In addition, from curve control 2, we could infer that those natural microorganisms in the wastewater had little ability to degrade 2-naphthol although they had strong tolerance for 2-naphthol and may have played an important role for the mineralization of metabolites of 2-naphthol. Fig. 6 also showed that the practical wastewater sample was more complex than the degradation culture under laboratory conditions. Dissolved organic matter has a capacity for complexing hydrophobic organic compounds and trace metals, which may significantly increase their aqueous solubility [38,39].

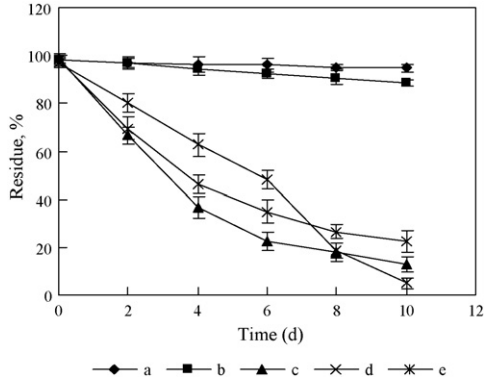

Fig. 6. Practical application of the biodegradation system on treatment of 2-naphthol-contaminated wastewater. a, Control 1; b, control 2; c, degradation of 2-naphthol in the pure culture; d, degradation of 2-naphthol in the wastewater sample sterilized; e, degradation of 2-naphthol in the wastewater sample unsterilized. Error bars represent the standard deviation of the mean ($n = 3$).

4. Conclusions

A close relationship between degradation extent of 2-naphthol and enzyme response has been established. A synergistic degradation system (*F. proliferatum* and *B. subtilis*), which is environment-friendly, was adopted to degrade 2-naphthol. This combined degradation system, compared with the conventional method of degradation by one domestic bacterium alone, enhanced the degradation extent of 2-naphthol by nearly 20% on the 6th day. Co-metabolic substrate salicylic acid could well make the two microorganisms produce degradation enzymes for 2-naphthol. After the addition of the co-metabolic substrate, the degradation extent of 2-naphthol was enhanced by nearly 15% on the 6th day. The results indicated that the co-metabolic substrates were the most important factor affecting the degradation of our target contaminant.

The key enzymes for the degradation of 2-naphthol were PPO and C23O. The optimal concentrations of 2-naphthol were 150 mg mL^{-1} and pH value was 7.0. The degradation extent of 2-naphthol was enhanced by nearly 10% compared with that in the absence of Tween 80. The degradation extent of Tween 80 itself was nearly 95% on the 6th day under the experimental conditions, which indicated that the addition of non-ionic surfactant could not bringing about further contamination to the environment again. The results which medical manufacture wastewater without sterilization was used, evidenced that the degradation extent of 2-naphthol was up to 95.2% by combination method on the 10th day. This study presents important information on the design of schemes for the treatment of more complicated industrial wastewaters.

In conclusion, it is important to further study the synergistic degradation system so as to more effectively degrade 2-naphthol with higher concentration. Photocatalysis/chemical oxidation combined with biodegradation would be a more potential method for 2-naphthol degradation in theory based on its chemical structure.

Acknowledgments

The research project was funded jointly by The National Science Fund for Innovative Research Group (no. 40721002), the National Science Fund (no. 40803037), the Guizhou Provincial Foundation for Science and Teaching Talents Program (2005–356). The authors appreciated helpful advice from Professor Tim Ford, Professor Yosef Steinberger and Professor X.Z. Xiong. Thanks are due to X.C. Yu, Y.J. Yang and Y. Zhang for their assistance in experimental procedures.

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