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Development of activated sludge adapted to high concentrations of phenol and enhancement of its phenol removal ability by addition of a processed lignite

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Abstract: In this study, we successfully developed activated sludge (AS) adapted to a very high concentration (3 g/L) of phenol. A careful stepwise-adaptation process was considered important in the development of this AS. In addition, we found that processed lignite (PL) accelerated the removal of phenol which might lead to change in the dominant members of the microbial community. Phenol is an abundant industrial toxic contaminant in wastewater treatment processes.
Keywords: activated sludge, processed lignite, phenol removal.

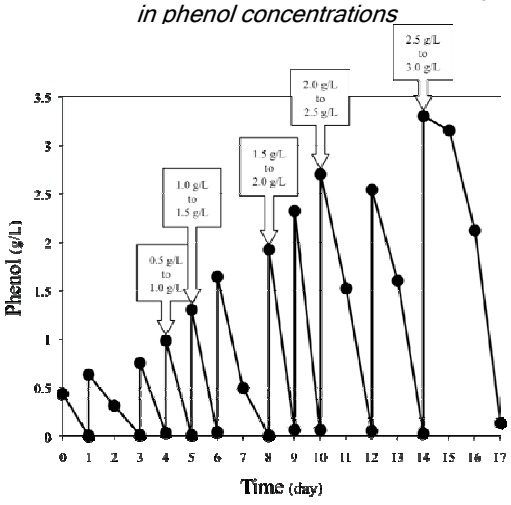
Introduction

The activated sludge (AS) process is widely utilized for the treatment of city sewage and industrial wastewater. Accelerating the removal of organic contaminants is important for increasing the treatment capacity. In wastewater treatment processes, phenol is an abundant organic industrial toxic contaminant, and there have been many studies of the removal of phenol in wastewater (Ibrahim *et al*., 2001; Shen, 2002; Pinto *et al*., 2003; D'Annibale *et al*., 2004; Veeresh *et al*., 2005). In addition, Khardenavis *et al*. (2008) have described that the careful exposure of AS to phenol would enhance treatment efficiency.

Although it has been shown that using AS enables efficient removal of phenol, a high concentration of phenol often affects the performance of AS (Hernández-Esparza *et al*., 2006). In general, phenol exerts toxic effects at concentrations of >1 g/L. In previous studies, Marrot *et al*. (2006) has reported the treatment of culture medium containing 3 g/L of phenol in a continuous immersed membrane bioreactor. However, the batch culture experiment showed that AS could not cope with phenol at concentrations of >2.5 g/L. In another side, hydrogels have been used for AS immobilization to overcome the inhibitory effects of high concentrations of phenol. Successful adaptations of AS with up to 2 g/L of phenol have been reported (Doria-Serrano *et al*., 2002; Hernández-Esparza *et al*., 2006). However, the use of AS-immobilizing hydrogels is not appropriate for practical purposes in conventional wastewater treatment, since the immobilization and maintenance processes require skilled technicians. Thus, we tried to develop AS adapted to a high concentration of phenol without using an immobilization technique.

Besides AS adaptation, shortening the time for phenol removal is also important for increasing the wastewater treatment capacity. Several studies showed that humic substances affect the biosorption and bioflocculation of AS (Choi *et al*., 2004; Esparza-Soto & Westerhoff, 2003; Moura *et al*., 2007). Lipczynska-Kochany and Kochany (2008a) reported that humic substances mitigated the inhibition of AS caused by phenol. In this study, we used a processed lignite (PL) to investigate the effect on phenol removal by AS. We obtained AS adapted to a high phenol concentration by stepwise batch subculture. We also found that a PL accelerated the removal of phenol from wastewater.

Fig. 1. Stepwise adaptation of the AS for phenol removal. The values indicate the concentration of phenol remaining in the AS, and the horizontal increases in phenol concentration indicate refreshments of synthetic wastewater. The boxed notes indicate the stepwise changes in phenol concentrations



Materials and methods

Development of AS adapted to phenol

AS adapted to high phenol concentrations was developed by subculturing sediment sludge from the wastewater treatment pond at the University of Yamanashi. Briefly, 2 L of seed sludge in a 2-L cylinder were aerated through a diffuser at its base at a flow rate of 1500 mL/min. For the batch experiment, at every 48 h, the sludge was sedimented for 30 min by stopping the aeration; 1400 mL of the supernatant was then removed and the same volume of fresh synthetic wastewater was added. Synthetic wastewater was prepared with a polypeptone (Wako Pure Chemical Industries, Osaka) concentration of 2 g/L and an appropriate concentration of phenol (Nacalai Tesque, Kyoto) in the culture. Adaptation of the culture to

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phenol was started at 0.5 g/L of phenol and increased stepwise: 1 g/L at 4 days, 1.5 g/L at 5 days, 2 g/L at 8 days, 2.5 g/L at 10 days, and finally 3 g/L at 14 days of subculture (Fig. 1).

Addition of processed lignite (PL)

The PL used in this study was magnesium humate stably containing 50% humate, which is produced from lignite by oxidization with nitrate and the following neutralization with magnesium hydroxide, manufactured by Denki Kagaku Kogyo Co. Ltd of Tokyo. Thirty-day subcultured AS (subcultured at 3 g/L of phenol in synthetic wastewater for 14 days after the stepwise adaptation) was used for the following experiment. Two 2-L volumes of AS were prepared (SV_{30} = 30%). After sedimentation of the sludge for 30 min, 1000 mL of each of the supernatants was replaced by fresh synthetic wastewater containing 4 g/L of polypeptone (a final concentration of 2 g/L) and 6 g/L of phenol (a final concentration of 3 g/L). The fine powder of PL was homogeneously suspended at a concentration of 100 g/L with distilled water by autoclaving and sonication, and 4 mL of the suspension was added to one of the AS (a final concentration of 0.2 g/L). During aeration, 10 mL of both the PL-containing and the PL-free AS were collected every 6 h for the following analyses.

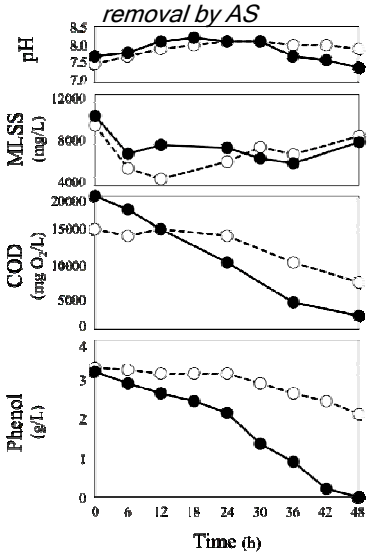
Analytical procedures

Ten milliliters of the collected samples were centrifuged at 2000 *g* for 10 min at room temperature, and the sediments were dried at 110 ˚C for 12 h to determine the MLSS (mg/L). The pH of the supernatants was determined using a B-212 pH meter (Horiba, Kyoto). The COD in the supernatants was determined using a Simple Pack COD kit in accordance with manufacturer's instructions (Shibata Scientific Technologies, Tokyo). The phenol concentrations in the supernatants were determined by following the method of the Japanese Standards Association (JSA, 1999).

DNA extraction and PCR-DGGE

To compare the microbial communities of the PL-containing and PL-free AS, batch subculturing at 3 g/L concentrations of phenol was continued for 3 months. In the PL-containing AS, PL was added just once at the beginning of the subculture process; no further PL was added thereafter. For sampling, 1 mL of AS was collected during aeration every 6 h from the time of refreshment of the synthetic wastewater. Total DNAs were extracted using the methods of Zhu *et al.* (1993) from the sediments obtained by centrifugation of 1 mL of the collected samples. DNA fragments encoding the 16S rRNA gene were amplified by PCR. Amplification was performed using the PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, USA) with the Ampdirect Plus kit (Shimadzu, Kyoto) and a primer set of 357F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG- GGGGCCTACGGGAGGCAGCAG-3') and 517R (5'-ATTAC- CGCGGCTGCTGG-3') under the following conditions: 95 ˚C for 10 min; 10 cycles at 93 ˚C for 30 s, 65 ˚C for 30 s, and 72 ˚C for 1 min; 10 cycles at 93 ˚C for 30 s, 60 ˚C for 30 s, and 72 ˚C for 1 min; and 10 cycles at 93 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 5 min (Muyzer *et al.*, 1993). DGGE was performed using the Dcode system (Bio-Rad Laboratories, USA). Five microliters of PCR products were separated in 0.5 x TAE buffer with a denaturing gradient gel; the composition of the gel (made of polyacrylamide, urea, and deionized formamide) ranged from 6% polyacrylamide-1.4 M urea-8% deionized formamide to 12% polyacrylamide-4.2 M urea-24% deionized formamide. Electrophoresis was performed at 150 V for 6 h at 60 ˚C. The gel was stained with SYBR Green I and visualized on an UV transilluminator; the analysis results were recorded using the PrintGraph FX system (ATTO Corp., Tokyo).

Fig. 2. Changes in pH, MLSS, COD, and remaining phenol concentration in PL-containing (closed circle) and PL-free (open circle) AS. Addition of PL had an effect on acceleration of phenol removal by AS



Results and discussion

Development of AS adapted to a high concentration of phenol

By a stepwise adaptation to a high phenol concentration, we successfully developed AS with the ability to remove 3 g/L of phenol. The changes in the concentration of phenol remaining in the wastewater are shown in Fig. 1. In the subculture, as long as the phenol concentration in the refreshed wastewater was less than 2.5 g/L, the AS removed the phenol completely in about 1 day. Complete removal of phenol was achieved in 2 days for 2.5 g/L and in 3 days for 3 g/L (Fig. 1). It is noteworthy that this adaptation of the AS succeeded in a very short period (17 days), whereas the usual development of AS requires 30 to 60 days. Our attempt to subculture 3 g/L-adapted AS with 4 g/L of phenol led to deflocculation; therefore, the limit of this AS was considered to be around 3 g/L of phenol. Another attempt to subculture the seed sludge with 3 g/L of phenol (without stepwise adaptation) also failed, suggesting that the adaptation process is important for the development of the AS.

The AS adapted to 3 g/L of phenol was used to study the effects of the PL on phenol removal. Fig. 2 shows the changes in the pH, MLSS, COD, and residual phenol

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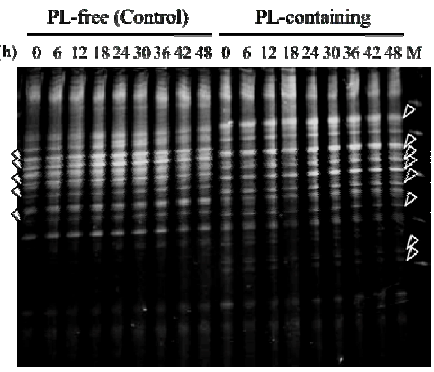
concentration in PL-AS and PL-free AS. In both AS samples, the pH did not changed drastically and remained at around 7.5. The MLSS values were both decreased during the initial 6 h, suggesting that the microorganisms were damaged by high concentrations of phenol. In the PL-free AS, this decrease continued up to a duration of 12 h, but in the PL-containing AS, it ceased after 6 h. It is remarkable that phenol was removed more rapidly from the PL-containing AS than from the PL-free AS (Fig. 2). The concentration of phenol remaining in the PL-containing AS became negligible after 48 h, whereas 64% (2.12 g/L) of the added phenol remained in the PL-free AS. Given this result, we suspected that phenol had been absorbed by the PL because Xing (2001) reported that PL absorbs various compounds. However, no decrease in phenol concentration was observed in the PL-containing synthetic wastewater without AS, clearly indicating that the PL used in this study did not absorb phenol. Therefore, it is strongly suggested that the acceleration of phenol removal by PL addition depends on activation of some function of the members of the AS community or a change in the AS community members themselves.

The initial COD values were high (15000 in PL-free AS and 20000 in PL-containing AS) and were considered to primarily reflect the total concentration of phenol and components of polypeptone. It was considered that half of the initial COD (7000–8000) could be attributed to oxidation of phenol, since phenolic compounds are known to show high COD values even at low concentrations (Veeresh *et al*., 2005). The initial COD value in the PL-containing AS was greater than that in the PL-free AS by 5000 mg/L. This increased value corresponded to the COD value obtained in PL-suspended water at a concentration of 0.2 g/L; thus, it was considered that the increase in COD in the PL-containing AS could be attributed to the oxidation of PL. A comparison of the changes in phenol concentration and COD suggests that phenol and polypeptone were completely assimilated in the PL-containing AS and that the remaining COD at 48 h can be attributed to oxidation of the remaining PL. In fact, the very dark brown color of PL was retained with AS flocs for as long as 3 months, suggesting that the added PL was adsorbed or incorporated with the flocs.

Comparison of microbial communities in developed AS

Both the PL-containing and PL-free AS were subcultured for 3 months after adaptation to a high concentration of phenol. The ability of the PL-containing AS to remove high concentrations of phenol was consistently maintained, although both types of AS were able to remove phenol for up to 3 months (data not shown). This observation strongly suggests that one-time addition of PL would promote the transition of the microbial community from original members to phenol-adapted members. Fig. 3 shows the chronological DGGE patterns of the PL-containing and PL-free AS. The major DNA bands in the patterns of the PL-containing AS clearly differ from those of the PL-free AS, indicating that dominant members in the AS underwent considerable transformations when PL was added. Although the population of each species could not be quantified by DGGE analysis, it is noteworthy that the diversity of the microbial community was not changed, thus implying that the stability of the AS was maintained by constant existence of all the community members during the entire batch culture period. Lipczynska-Kochany and Kochany (2008a, b) reported that PL activated the ability of municipal sewage to uptake oxygen. The relationship between PL addition and oxygen uptake is not yet known for our AS; further information in this regard can be obtained by correlating the results of population analysis and respirometry analysis in future studies.

Fig. 3. Comparison of the microbial communities of PL-containing and PL-free AS (subcultured for 3 months), as analyzed using 16S rDNA fragment patterns. The numbers indicate sampling times (h) from refreshment of synthetic wastewater. The letter 'M' indicates that the lane was applied with 5 µl of DGGE marker I (Wako Pure Chemical Industries). The positions that are characteristic of DNA patterns in the PL-free AS are indicated by open arrowheads, while those in the PL-containing AS are indicated by closed arrowheads



Conclusions

In this study, we successfully developed AS adapted to a high concentration of phenol. By this stepwise adaptation, we also succeeded to develop several AS adapted to high concentrations of phenol (3 g/L) using other seed sludge obtained from riverbeds in the nature (data not shown). Therefore, the stepwise adaptation to phenol is considered to be applicable to the development of AS for phenol removal in many cases. It is also remarkable that the removal of phenol was considerably accelerated by the addition of PL, and almost all the phenol was removed within 48 h. Furthermore, phenol removal could be continued for at least 3 months by one-time addition of PL. The PL used in this study can be supplied in a stable form through an industrial process. It is reasonable to expect that other types of AS will exert similar effects and that controlling the AS conditions will be simple. Our results strongly suggest that changes in the microbial community in the AS, which begin with the addition of PL, significantly affect the phenol-removal ability. A study is currently

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underway to investigate the correlation between the phenol-removal ability and the function of each community member.

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