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Enhanced phenol removal by floating fungal populations in a high concentration phenol-fed membrane bioreactor

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Abstract

Sludge bulking and washing-out phenomena may inhibit the efficiency and stability of treatment performances when activated sludge processes are used to treat phenol contamination at high concentration levels. In this study, a membrane bioreactor (MBR), as a modified activated sludge process, was applied to enhance the removal of high-loading phenol contamination by capturing non-flocculating degradatative populations in a bioreactor. The specific objectives were (i) to evaluate the validity of use of MBRs in enhancing the removal of highly-loaded (~1000 mg/L) phenol contamination, and (ii) to characterize the community structure and phenol degradation rate of non-flocculating populations that could tolerate phenol-mediated stresses. When fed with phenol at a non-toxic level (~100 mg/L), phenol was efficiently removed with maintaining stable and steady MBR performance. No shift in cellular and population morphologies was observed. When fed with phenol at a toxic level (~1000 mg/L), however, a microbial community shift was observed. Milky floating populations appeared in the bioreactor after an acclimation period of 3 days. Interestingly, the appearance of the non-flocculating populations resulted in an enhanced removal of phenol in the high phenol fed MBR. Small subunit rDNA analysis showed that most of the non-flocculating populations were fungal members. The specific phenol degradation rate for the floating fungal populations was approximately five times greater than that for settling microbes in the high phenol fed reactor. These findings suggest that the efficient containment of degradation-active floating populations by the MBR process led to an enhanced removal of phenol in the high phenol fed bioreactor.

Keywords: Membrane bioreactor (MBR); Filamentous microorganisms; Fungal phenol degradation; High concentration toxicant stress

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1. Introduction

Phenol and its related compounds are known to be major pollutants in wastewater from industrial plants, such as oil refineries, petrochemical plants, coking plants, and phenol resin industry plants [1,2]. Even in low concentration, phenol related compounds easily cause odor and taste problems in water, particularly in chlorinated drinking water [3]. Because of its corrosiveness and skin toxicity, systemic absorption by phenol through burn sites may result in cardiac arrhythmias, renal disease, and death [4]. In addition, phenol may cause to skin cancer in human [4]. It is possible that phenol and its related aromatic compounds may disrupt the health and stability of our ecosystems [5–7]. Because of such negative effects in public health and ecosystems, phenol and its related compounds have been in a high priority in governmental regulations in many countries.

A conventional activated sludge process has been one of the popular methods in biologically treating phenol contamination in industrial wastewaters. However, other studies previously reported that, when high concentrations of phenol (above 750 mg/L) was fed, floating (non-flocculating) biodegradative populations appeared dominant, and in turn were washed out, leading to the breakdown of phenol treating activated sludge processes [8,9]. To circumvent this problem, a modification of activated sludge processes is required for capturing floating degradative populations. One approach is to immobilize filamentous degradative populations on solid surfaces in bioreactors, e.g., a pulsed plate bioreactor [10] and biofilm attached membranes [11,12]. A potential draw back of such biofilm associated approach is that, if non-immobilizing populations have a strong degradation capability, a wash-out of such degradative microbes would not be resolved. An alternative approach is to contain the wash-out of floating populations in a bioreactor using a forced membrane filtration process, i.e., MBR (membrane bioreactor). However, very little is known about the applicability of MBR, as a modified activated sludge process, in treating highly loaded phenol contamination. Herein, we explored phenol removal performance in MBRs when a high concentration of phenol (~1000 mg/L) was fed, and also characterized the community structure and phenol degradation rate of non-settling populations that could tolerate phenol-mediated stresses.

2. Materials and methods

2.1. Laboratory MBR set-up and operation

A Pyrex bottle (2.2 L) installed with a hollow-type microfiltration membrane (0.4 μm of a mean pore size; 14 cm^2 of effective area; PVDF material) was used as an MBR. A pH meter (Lab-215, Sechang Inc.) and a dissolved oxygen (DO) probe (YSI Inc.) were also installed in the MBR, so that pH and DO in the reactor could be monitored. The reactor was operated at 25°C in a steady-state continuous flow mode as described in Fig. 1. The effective reaction volume of each MBR was 1.8 L. The reactor was inoculated with activated sludge (~2000 mg VSS/L) from a municipal wastewater treatment plant in Seoul.

To maintain aerobic conditions (above 1.5 mg DO/L), air was continuously supplied at a flow rate of 3 L/min through a 0.2 μm-pore diffuser installed on the bottom of the reactor. For minimizing abiotic losses of phenol (sorption), Teflon and glass materials were used for all the fittings and tubes in the experimental set up. A mixture of phenol with a mineral medium was prepared in an influent tank, and then the phenol containing mixture was supplied to each MBR using a HPLC (high-performance liquid chromatograph) pump. A steady-state flow rate was set at 3.75 mL/min, resulting in a hydraulic retention time of 8 h. The mineral medium was prepared as described by Ayala-del-Rio et al. [13], containing 2.13 g Na_2HPO_4, 2.04 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.067 g $CaCl_2 \cdot 2H_2O$, 0.248 g $MgCl_2 \cdot 6H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 0.4 mg $ZnSO_4 \cdot 7H_2O$, 0.002 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg

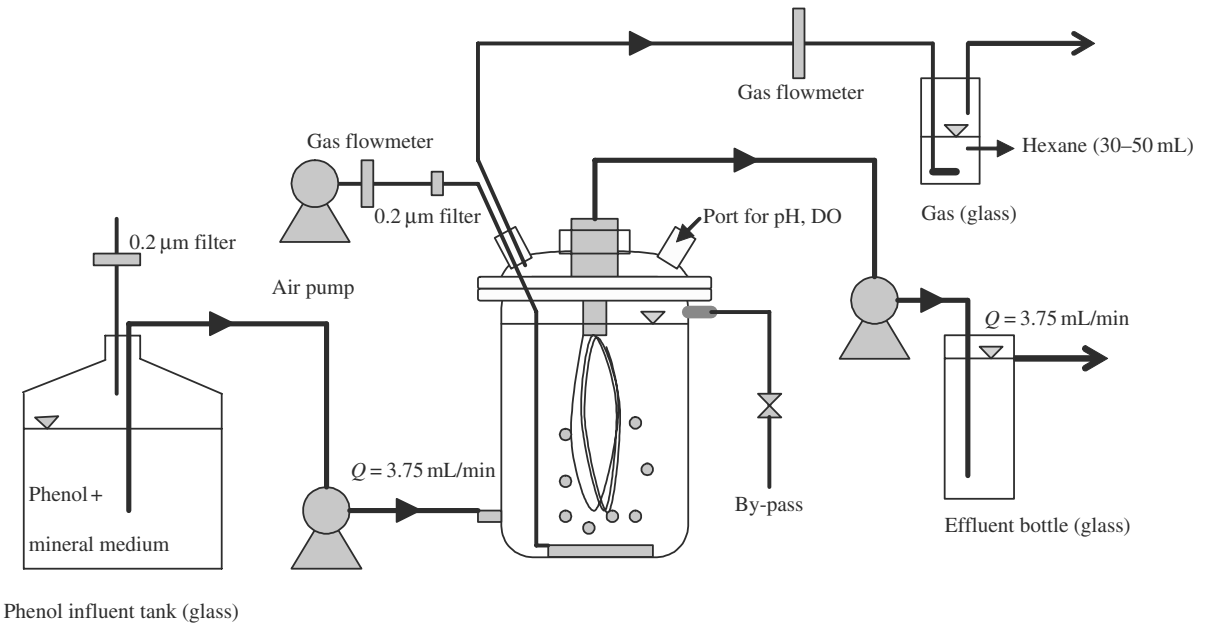

Fig. 1. MBR (membrane bioreactor) experimental system used in this work.

$NiCl_2 \cdot 6H_2O$, 0.015 mg H_3BO_3, and 0.25 mg EDTA per 1 L. Two MBRs were operated simultaneously; one for low concentration phenol feeding (100 mg/L), and the other for high concentration phenol feeding (1000 mg/L). No microbe control experiment was conducted for 7 days to confirm insignificant abiotic losses during the experimental period (less than 5% of influent concentrations). For microbiological analysis and biomass quantification, mixed liquid samples (50 mL each) were daily taken from each reactor for 27 days. For phenol and TOC measurements, 5 mL of an influent sample was daily taken using a Hamilton syringe from a sampling port installed with a 15 mL-bottle capped with a Teflon-lined butyl septum, minimizing volatilization losses during sampling.

2.2. Chemical analysis

Phenol was measured in influent and effluent samples. For the phenol measurement, a reverse-phased HPLC analysis was performed with using a Hypersil $5C_{18}$ column and a UV detector (254 nm). The elution carrier was a methanol–water (40:60) mixture, and continuously pumped at a constant flow rate of 1 mL/min. TOC (total organic carbon) was also measured in influent and effluent samples using a TOC analyzer (Shimadzu). For biomass quantification, VSS (volatile suspended solids) was measured. Cellular protein was quantified by Bradford method (Bio-Rad, Inc.).

2.3. Morphology and small subunit rDNA analysis

Population morphology was examined using an optical microscope. For cellular morphology examination, SEM (scanned electron microscopy) analysis was conducted in Advanced Center for Microscopy in Yonsei University.

Microbial community analysis was conducted to identify dominant populations. For this, genomic DNA extraction was done with 2 mL of each sample using Fast DNA SPIN Kit (Bio 101 System).

The samples were concentrated using a bench-top microcentrifuge, by spinning down at 14,000 rpm for 5 min. From the pellet 500 μL of concentrated samples equivalent to 0.5 g VSS were added into each Lysing-Matrix-E-Tube, which was provided from the kit. About 978 μL of sodium phosphate buffer and 122 μL of a lysis buffer (MT buffer from the kit) were added to the Lysing-Matrix-E-Tube, and the mixture was vortexed for 10 min in order to remove DNA from cells in each sample. The rest of DNA extraction steps were performed according to the manufacturer's protocol. Bacterial 16S rDNA and fungal 18S rDNA were amplified from the extracted DNA using universal bacterial (27F/1492R) primers and fungal (ITS1/ITS4 and LROR/LR5) primers (see the primer sequences in Table 1). For 16S rDNA 25 μL PCR reaction volume contained 1× PCR buffer, 2 mM $MgCl_2$, 200 μM concentration of each deoxynucleoside triphosphate (dNTPs), 100 pM concentration of each primer, 0.025 U of *Taq* enzyme, 0.1 ng of DNA extract as template and rest of the volume was adjusted with sterile Millipore. The thermo-cycling conditions were starting with an initial denaturation step consisting of 94°C for 3 min, and this was followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and with an extension at 72°C for 2 min. Fungal 18S rDNA, 25 μL reaction volume containing 1× PCR buffer, 2 mM $MgCl_2$, 200 μM concentration of each deoxynucleoside triphosphate (dNTPs), 40 pM concentration of each primer, 2 U of *Taq* enzyme, 1 μL of DNA extract as template and rest of the volume was adjusted with sterile Millipore water. The amplifications started with an initial denaturation step consisting of 94°C for 4 min; this was followed by 30 cycles consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and finished with a final extension at 72°C for 4 min. All the PCR products were purified using a Qiagen DNA purification spin columns (Qiagen Inc., Valencia, CA). From the purified PCR products 18S rDNA clone libraries were constructed using TOPO TA cloning kit (Invitrogen, Inc., Carlsbad, CA). Plasmids containing 18S rDNA clones were extracted and sequenced (Macrogen Inc., Seoul, Korea). The sequenced samples of 18S rDNA were analyzed with the Sequence Match (version 2.7) program of the RDP database and the Blast 2 Advanced Blast program at the National Center for Biotechnology Information (NCBI).

2.4. Specific phenol degradation rate determination

To characterize phenol degradation by floating and settling microbes, respectively, phenol degradation was monitored and the specific degradation rates were compared. A similar method for determining quantifying specific toluene degradation rates [17] was adopted here with minor modifications. A sample (25 mL) of a unit optical density (OD at 600 nm = 1.0) was added into a 120-mL

Table 1
16S rDNA, 18S rDNA and fungal ITS (inter-space) PCR primers

Primer pair ID	Target genomic region	Primer sequence (5′–3′)	Approx. fragment size (bp)	Target group	Ref.
27F 1492R	16S rDNA	AGAGTTTGATCATGGCTCAG(20) TACGGTTACCTTGTTACGACTT(22)	1492–1513	Bacteria	[14]
ITS1F ITS4R	ITS between 5S &18S	CTTGGTCATTTAGAGGAAGTAA(22) TCCTCCGCTTATTGATATGC(20)	450–750	Fungi	[15]
LROR LR5	18S rDNA	ACCCGCTGAACTTAAGC(17) TCCTGAGGGAAACTTCG(17)	550	Fungi	[16]

bottle. After phenol was added at the level of 10 mg/L, the bottles were sealed with Teflon-lined butyl-rubber septa. Phenol disappearances were monitored, and initial degradation rates (after a lag period) were estimated. To calculate specific degradation rates, the initial degradation rate was normalized by its corresponding protein concentration.

3. Results and discussion

3.1. Phenol removals by MBRs

When phenol was fed into the MBR at the level of low concentrations (~100 mg/L), phenol was completely removed after 8 days, and then a complete disappearance was continuously observed during the rest of the operational period (Fig. 2). Even during the first 8 days, the phenol removal efficiency was fairly high (~90%). The trend in TOC concentrations exactly followed the trend in phenol concentrations, indicating no accumulation of the intermediates of phenol degradation (data not shown). This indicates that the MBR process was able to completely mineralize the fed phenol contamination. The biomass in the reactor remained fairly constant (2000–2500 VSS mg/L) during the whole operational period. Since no biomass was washed out from the bioreactor, the fairly constant biomass indicates that the rate of actual growth on phenol was equal to the rate of cell decay during the experiment (no net growth). This no net biomass growth could result in an operational stability in the MBR, without causing overgrowth or undergrowth problems.

When phenol was fed at the high level of concentrations (~1000 mg/L), little phenol was removed for the first 3 days, and then completely disappeared (Fig. 3). After the 3-day acclimation period, apparent biomass increased up to ~9000 mg VSS/L. Inhibited performance in membrane filtration was observed when biomass concentration was higher than 8000 mg VSS/L. To circumvent such operational difficulty, after the 13th day, excessive sludge was manually removed from the reactor so that the sludge biomass concentration remained at ~7000 mg VSS/L. The complete phenol disappearance remained during the rest of the operational period. TOC concentration data followed the same trend in phenol concentrations, indicating no accumulation of the intermediates of phenol degradation (data not

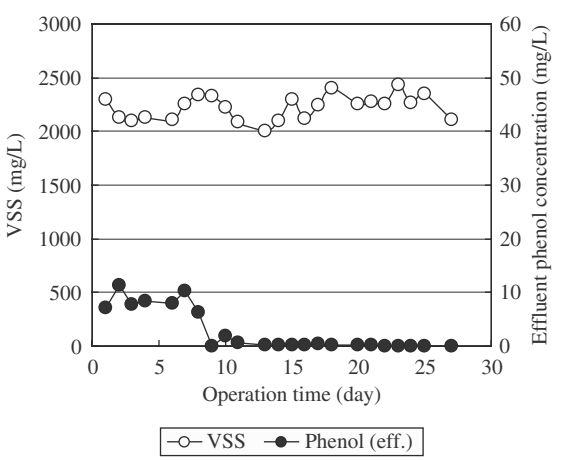

Fig. 2. Reactor biomass (VSS) and effluent phenol concentrations in the low phenol fed MBR.

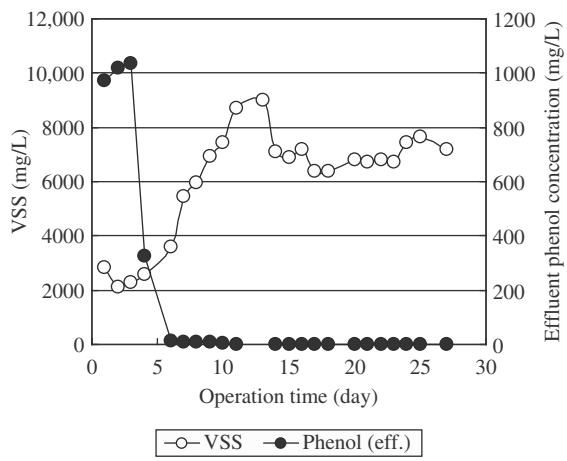

Fig. 3. Reactor biomass (VSS) and effluent phenol concentrations in the high phenol fed MBR.

shown). These results indicate that the high concentration phenol was completely mineralized.

After an acclimation period of 3 days, milky white populations appeared floating and accumulated over the surface of water in the reactor. Interestingly, immediately after the appearance of the milky non-settling populations, the phenol drastically disappeared. This suggests that the milky non-settling microbes may be involved in the removal of high concentration of phenol in the high phenol fed MBR.

3.2. Cellular and population morphologies

According to optical microscope analysis (Fig. 4), a filamentous structure was observed in the milky non-settling microbes from the high phenol fed MBR. The population morphology apparently differs from that of the microbes from the low phenol fed MBR. The microbial population morphology for the low phenol fed MBR was almost identical to that for the original activated sludge (data not shown), suggesting that the low phenol concentration did not result in a shift in microbial characteristics of the activated sludge. When further analyzed with SEM, a filamentous cellular morphology was also found from the milky non-settling microbes. These microscopic analysis results suggest that the filamentous formation probably led to making the milky populations floating.

3.3. Small subunit rDNA analysis

In the milky non-settling populations, bacterial 16S rDNA was not amplified while a significant amplification of bacterial 16S rDNA was observed from the original activated sludge or the microbes from the low phenol fed MBR. This indicates that bacterial concentrations were very low in the milky non-settling populations, while bacteria were present in the original activated sludge or the low phenol fed MBR. When fungal 18S rDNA was analyzed in the milky non-settling microbes, however, significant amplification was detected. This indicates that fungal members were dominant in the floating populations of the high phenol fed MBR.

According to the further community analysis of the milky non-settling microbes with cloning and sequencing, the dominant fungal populations were *Fusarium oxysporum* (37.4% of clone library), *Cryphonectria eucalypti* (27.5% of clone library), and *Symbiodinium* sp. (12.5% of clone library). The milky non-settling populations seem to consist of biodegradative and non-degradative fungi. *Cryphonectria eucalypti* has not been reported as a biodegradative species.

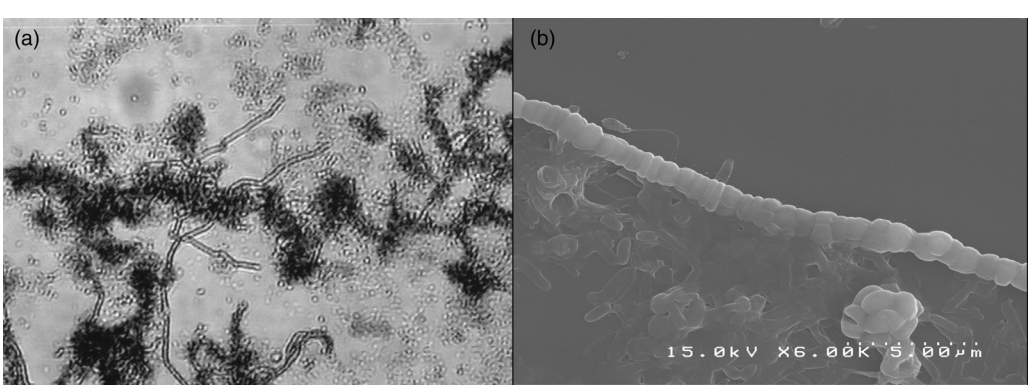

Fig. 4. A micrograph (×400) (a) and SEM (×6000) (b) of the microorganisms in the high phenol fed MBR.

Meanwhile, *Fusarium oxysporum* species is known to degrade nonylphenol surfactants [18], and some *Symbiodinium* species have been reported to contain oxygenase activity [19]. These suggest a possibility that *Fusarium oxysporum* and *Symbiodinium* populations might have been involved in phenol degradation in the high phenol fed MBR.

3.4. Phenol degradation rate for non-settling fungal populations

To examine whether the non-settling fungal populations were the major phenol degraders in the high phenol fed MBR, the settling microbes and non-settling microbes were separated from the reactor, and their specific phenol degradation rates were compared. As seen in Fig. 5, non-settling (floating) fungal populations exhibited faster phenol degradation than the settling populations did. The specific phenol degradation rate for the non-settling microbes was 4.1 ± 0.5 μg-phenol/mg-protein/min, which was approximately 5 times greater than that for the settling microbes (0.8 ± 0.2 μg-phenol/mg-protein/min). These results provide evidence that the removal of phenol in the high phenol fed MBR was mainly due to phenol degradation by the non-settling fungal populations.

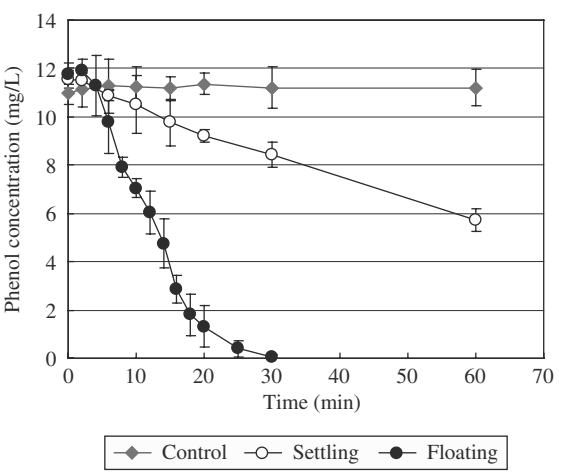

Fig. 5. Phenol concentration profiles in specific degradation rate assays.

The specific phenol degradation rate for the non-settling fungal populations is smaller than those for previously-known phenol degrading fugal species such as white rot fungus species (33 μg-phenol/mg-protein/min [20]), and *Candida albicans* (62 μg-phenol/mg-protein/min [21]). In addition, the phenol degradation rate for the non-settling fungal populations is seemingly greater than or comparable to those for *Pseudomonas* strains, well-characterized phenol degrading bacteria (0.8–4.5 μg-phenol/mg-protein/min [22–23]).

4. Conclusions

Because of the use of the MBR process, this study could provide evidence that the floating fungal populations were able to tolerate phenol-mediated stress and to efficiently degrade phenol at a high level of concentration. The non-settling fungal populations were not strong phenol degraders compared to other phenol-degrading fungi. Nevertheless, this study could provide novel information that floating fungal species such as *Fusarium oxysporum* and/or *Symbiodinium* sp. are able to degrade phenol of high concentrations (~1000 mg/L). These findings from this work demonstrated that MBR processes have potentials to use in enhancing the treatment of high concentration phenol contamination, by capturing floating phenol-degrading microbes.

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