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Diethylene glycol removal by *Echinodorus cordifolius* (L.): The role of plant–microbe interactions

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ABSTRACT

This work presents the use of the plant *Echinodorus cordifolius* for remediating diethylene glycol (DEG) contaminated waters. The potential of this plant for treating DEG wastewater in a remediation system was observed. We found that *E. cordifolius* was able to remove DEG from wastewater, decrease the pH to neutral and remove approximately 95% of the chemical oxygen demand within 12 days. The plants can grow well in DEG wastewater, as indicated by their root and leaf biomass, which was found to be statistically similar to control. Wilting, chlorosis and necrosis were observed in DEG-treated plants, but the relative water content was not significantly different between control and treated plants, suggesting that the plants were able to take up and tolerate DEG present in the wastewater. Plant roots changed to black colour during experimental period. The fluorescence in situ hybridisation and bacterial enrichment confirmed that 4.30×10^5 cells/g of sulphate reducing bacteria and 9.30×10^8 cells/g of acid-producing bacteria were found associated with the plant roots. Furthermore, volatile fatty acids were found in non-sterile soil treatments, indicating that soil microorganisms are associated with DEG remediation. These results demonstrated that plants and bacteria have the ability to form a relationship to remove the organic contaminant DEG.

1. Introduction

Diethylene glycol (DEG; CAS number 111-46-6) is an organic compound prepared by heating ethylene oxide and glycol to form an ether bond joining two ethylene glycol molecules. The compound is used as a coolant, as a building block in organic synthesis and as a solvent in many industries [1,2]. Environmental contamination by DEG is of worldwide concern because of its toxicity to living organisms, for example, mice and humans [3–6]. DEG environmental contamination has been reported, especially in water [7].

The conventional method that is mostly used for treating DEG wastewater is chemical precipitation. However, this method is not efficient enough since the chemical oxygen demand (COD) of treated wastewater still remains higher than the acceptable standard of around 120 mg l^{-1}. It might be due to high solubility of DEG in water that makes it difficult to precipitate or eliminate from wastewater. The other methods for treating DEG such as nanofiltration, biodegradation, chemical oxidation with ozone and modified Fenton processes have been reported in many literatures [8–10]. Although, these methods are effective but they need specific operational conditions and the intermediate products may occur. Under such problems, phytoremediation could be an alternative method for treatment of DEG contaminated wastewater. The use of phytoremediation to remove organic contaminant has been reported in several studies [11–13] with the goal of completely mineralising the contaminants into relatively non-toxic constituents, such as carbon dioxide, nitrate and ammonia [14]. For example, *Jatropha curcas*, grasses, legumes and their associated bacteria have been studied for treatment of organic contaminant [15,16]. However, the use of phytoremediation in the field is still limited by our incomplete knowledge of the biological processes involved in plants, microbes and soil. Thus, a better understanding of the basic biological mechanisms would lend more efficiency to the management of phytoremediation.

Echinodorus cordifolius, or burhead, is an aquatic plant with a fibrous root system that is easily cultivated and requires low growth maintenance. It has a high growth rate and can grow in a wide range of environmental conditions. It also consumes a large quantity of water in a short time [17], which makes it a good choice for phytoremediation. Furthermore, this plant has root nodules that might be associated with rhizosphere bacteria, which has never

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been reported in a phytoremediation system. Therefore, this work presents the potential treatment of DEG-contaminated wastewater by the plant *E. cordifolius*, and the bacterial communities associated with its roots were studied to better understand the use of this plant in remediation systems.

2. Materials and methods

2.1. Plant culture conditions

E. cordifolius was grown in a greenhouse at the Remediation Laboratory of King Mongkut's University of Technology Thonburi (KMUTT), Bangkhuntien campus. Plants at the same growth stage (8 leaves, 500 g of fresh weight) were selected and cultured in glass cylinders (30 cm × 50 cm) containing Hoagland's solution [18] for a week prior to the start of the experiments.

2.2. Experimental design

DEG wastewater was obtained from the effluent of a plasticisers and stationary materials factory at Samut Prakan, Thailand. The wastewater had been pre-treated by coagulation, but it was still high in COD content because DEG remained in the solution.

The experimental design was random with six treatments to study the efficiency of each factor in the treatment system: three experimental groups, three control groups and an additional control of DEG wastewater, without plants, to evaluate DEG photodegradation and water evaporation. The three experimental groups were DEG wastewater + *E. cordifolius* plants, DEG wastewater + *E. cordifolius* plants + non-sterile soil (1000 g) and DEG wastewater + non-sterile soil (1000 g). The three control groups were distilled water + *E. cordifolius* plants, distilled water + *E. cordifolius* plants + non-sterile soil (1000 g) and distilled water + non-sterile soil (1000 g).

All treatments were done in triplicate in separate glass cylinders under a static system, in which the 3000 ml of DEG wastewater was not refreshed during the 20-day experimental period, but water was added to maintain a constant volume despite evaporation. In addition, sterile soil was used to study the effects of soil microbes in the remediation system. The experiment was conducted at an average temperature of 32 ± 5 °C, with 60.51 ± 8% relative humidity and 12-h light/dark cycles.

2.3. Water sample analysis

Water samples of 30 ml each were taken by autopipette from different depths and points in the glass cylinders over a period of 20 days. The system pH, total phosphorus (P) and COD were analysed following standard methods [19]. The DEG concentration was analysed by capillary gas chromatography (GC). The DEG removal efficiency was calculated by the following equation:

$$\text{DEG removal efficiency } (\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (1)$$

where C_0 is the initial DEG concentration (mg l^{-1}) and C_t is the DEG concentration at the time indicated (mg l^{-1}).

2.4. Plant sample analysis

Tissue samples of *E. cordifolius*, including roots and leaves, were taken. The plant tissue samples were immediately frozen in liquid nitrogen and stored at −20 °C for subsequent analysis. One gram of sample was ground and DEG was extracted by MilliQ water. Then the DEG was quantified by GC.

Signs of stress, such as leaf and root appearance, were observed, the number of roots and leaves were counted for dry weight and the relative water content (RWC) was calculated to determine the toxicity of DEG to the plant. The RWC calculation was performed as described in Barr and Weatherley [20].

2.5. DEG analysis using GC

The DEG concentration was analysed by GC using the following parameters: inlet split/splitless (split mode), flame ionisation detector (FID) and 30 m × 0.32 mm, Rtx-200 capillary column (internal diameter 0.32 μm). The experimental conditions for GC–FID were 1 μl injection volume, 250 °C inlet temperature, 250 °C column oven temperature and 280 °C detector temperature.

For analysis, the external standard technique was used. Stock standards were prepared by diluting DEG (GC grade, Sigma–Aldrich) in distilled water at a concentration of 3000 mg l^{-1}. DEG concentrations in the standard solutions for the calibration curve were 0, 100, 500, 1000, 1500, 2000, 2500, and 3000 mg l^{-1}.

2.6. Fluorescence in situ hybridisation (FISH)

The FISH technique enables both the isolation and determination of specific bacteria by specific probes, and FISH has been widely applied in the analysis of SRB communities [21]. FISH technique includes four steps: the fixation and permeabilisation of samples, hybridisation, washing away the unbound probe and the detection of labelled cells by microscopy [22]. *E. cordifolius* roots were washed with tap water and rinsed three or four times with sterile distilled water to remove the dirt. Then, the roots were ground and extracted by sterile distilled water for bacterial analysis.

The bacteria in *E. cordifolius* roots were fixed with 1 ml of 4% paraformaldehyde at 4 °C. All fixation steps were done overnight, which ensured that the gram-positive bacteria could also become permeable to the oligonucleotide probe. Subsequently, the samples were washed 3 times with 750 μl of phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) to remove the fixative and then kept at 4 °C in a mixture of 1:1 ethanol and PBS.

The following Cy3-labelled oligonucleotide probes were used: EUB338 for the domain Eubacteria and SRB385 for most SRB. In addition, *Escherichia coli* and *Desulfovibrio desulfuricans* strain ESSEX were used as positive controls for EUB338-Cy3 and SRB385-Cy3, respectively. The formamide concentrations used for optimum stringency are shown in Table 1. In situ hybridisation was performed at 46 °C for 1.5 h. After washing away the excess oligonucleotide probes, the slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (6.26 μg ml^{-1} in 0.1 M Tris–HCl and 0.9 M NaCl, pH 7.2) for 5 min and rinsed with distilled water. The slides were mounted with an anti-fade agent (molecular

Table 1
Oligonucleotide probes used in this study with target groups and optimized formamide concentrations.

Probe	Specificity	Position[a]	Probe sequence (from 5′ to 3′)	Formamide[b] (%)
EUB338 (S-D-Bact-0338-a-A-18)	Most bacteria	338–355	GCTGCCTCCCGTAGGAGT	15
SRB385 (S-*-Srb-0385-a-A-18)	SRB of the δ-proteobacteria	385–402	CGGCGTCGCTGCGTCAGG	35

[a] Position in the 16S rRNA of *Escherichia coli* [23].
[b] Formamide concentration in the hybridization buffer [24,25].