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Removal of dichlorophenol by *Chlorella pyrenoidosa* through self-regulating mechanism in air-tight test environment

Feili Li[a,*], Liyuan Zhao[a], Yifei Jinxu[a], Wen Shi[a], Siqi Zhou[a,b], Kai Yuan[a], G. Daniel Sheng[b,*]

[a] College of Environment, Zhejiang University of Technology, Hangzhou 310032, China
[b] School of Environmental Science and Engineering, Suzhou University of Science and Technology, Suzhou 215009, China

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ABSTRACT

Microalgae are surprisingly efficient to remove pollutants in a hermetically closed environment, though its growth is inhibited in the absence of pollutants. The final pH, algal density, Chl-a content, and the removal efficiency of 2,4-dichlorophenol (2,4-DCP) by *Chlorellar pyrenoidosa* in a closed system were observed under different initial pH, lighting regimes, and various carbon sources. The optimal condition for 2,4-DCP removal was obtained, and adopted to observe the evolution of above items by domesticated and origin strains. The results showed that both respiration and photosynthesis participated in the degradation of 2,4-DCP, and caused the changes of pH. The photosynthesis seemed to increase the solution pH, while the respiration and the biodegradation of 2,4-DCP to decrease the solution pH. The domesticated strain achieved nearly 100% removal when initial concentrations of 2,4-DCP lower than 200 μg L^{-1}, due to providing a appropriate but narrow pH evolution range, mostly falling between 6.5 and 7.9. The research helps to understand the mechanism of biodegradation of chlorophenol compounds by green algae.

1. Introduction

Phenol pollutants have arisen as a serious problems in respect of its carcinogenicity and high toxicity in water resources (Gholizadeh et al., 2013). Chlorophenol toxicities increased as the number of chlorine atoms on the phenol ring increasing (Bayramoglu et al., 2009), while the difficulty of removal process was increased too.

Among lots of physical, chemical and biological methods of removing or degrading phenols in wastewaters, microalgae show more practical effects (Papazi and Kotzabasis, 2013; Paskuliakova et al., 2018; Wang et al., 2016; Zhou et al., 2013). For example, a maximal phenol degradation percentage of 97% was obtained at algae concentration of 4 g L^{-1}, phenol concentration of 0.8 g L^{-1} in 4-day reaction time (Priyadharshini and Bakthavatsalam, 2016). However, the treatment of alive microalgae for phenol compounds demands some specific and strict conditions, such as lighting regime, temperature and solution pH (Guedes et al., 2011). For example, the biodegradation of phenolic compounds by microalgae needs exogenous carbon and lighting (Papazi and Kotzabasis, 2007). In addition, suitable concentration ranges of algal mass and phenol, and sufficient reaction time were also important influencing factors for phenol degradation (Priyadharshini and Bakthavatsalam, 2016).

In order to overcome the low tolerance of microalgae for, and a low degradation rate to, high concentration of chlorophenol compounds, acclimation is now widely adopted for strain improvement of microalgae, especially the adaptive laboratory evolution (ALE) which had been successfully used to resist against the stress from salt and carbon dioxide (Perrineau et al., 2014), or to increase biomass productivity (Fu et al., 2012). The evolved populations stressed with salt had a rapid growth rate in high salt medium as progenitor ones after 1255 generations, but exhibited a quite different responses to salt stress in the genetic underpinnings (Perrineau et al., 2014). In environmental remediation, a resulting *Chlorella* sp. strain, obtained under the stress of 500 mg L^{-1} phenol for 95 d, gained the maximal biomass concentration of 3.40 g L^{-1} at day 8 exposure, which was more than twice those of the original strain (Wang et al., 2016). Such researches proposed one feasible way in the wastewater treatment for chlorophenol pollution through strain improvement of microalgae, however detailed information in optimum operating conditions of microalgae of ALE are still very limited, as well as algal adaptive mechanism. However, algae was 1.65–108 times more sensitive to the pollutant of volatile organic chemicals in an air-tight test than in an open test (Chen and Lin, 2006).

To better understand the interrelationships between abiotic and biotic variables and the removal efficiency of organic contaminants by freshwater algae helps to reveal the biodegradation mechanism of organic contaminants. A series of experiments were conducted to evaluate

* Corresponding authors.
E-mail addresses: lifeili@zjut.edu.cn (F. Li), dansheng@usts.edu.cn (G.D. Sheng).

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the effects of pH, lighting regime, and external carbon source in the culture solution on algal growth and on the removal of 2,4-dichlorophenol by *Chlorella pyrenoidosa* (*C. pyrenoidosa*) in a hermetically closed system. A 96-h acclimation were conducted for algal evolution, after then a series of detailed observation of above parameters was conducted with domesticated and original strains.

2. Materials and methods

2.1. Experimental organism and growth conditions

The standard material of 2,4-dichlorophenol (2,4-DCP, 99.7%) was purchased from Sigma-Aldrich (Madison, WI, USA), and the stock solution was prepared in HPLC grade methanol (Tedia, USA) at 1 mg L^{-1} stored at 4 °C in a freezer, and working solutions were obtained by appropriate dilution. HPLC-grade solvents (n-hexane) were purchased from Shield (Tianjin, China). All other chemicals used were of analytical grade. The ultrapure water was purified with a Milli-Q academic water purification system (Milllipore, USA).

The freshwater microalgae *C. pyrenoidosa* was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, and kept in sterile BG11 medium at the intensity of illumination of 4000 lx (light: dark of 12:12 h) at 25 ± 1 °C. The pH was adjusted to 7.0 by using solutions of 0.1 M HCl and 0.1 M NaOH. Before inoculation, the culture medium was heated at 121 °C for 20 min in a high-pressure sterilizing pot. The original algae were centrifuged at 2000 g for 8 mins in a low speed centrifuge. The supernatant was discarded, and the algae at the bottom of the tube were resuspended in fresh medium. About 500 mL algal liquid were cultured in 1000 mL conical bottle, with the bottle mouth covered with gauze.

2.2. Growth measurements

Cell density was measured by UV–visible spectrophotometer (Aoxi UV754N, Shanghai, China). The optical density (*OD*) of the algal culture was measured at 660 nm and converted into the mass of algae (dry weight) at the onset of experiment and the end of exposure by Eq. (1).

$$\text{Algaldensity } (\text{mgL}^{-1}) = 348.96 \times OD_{660} + 0.0043 \quad (1)$$

2.3. Determination of algal chlorophyll a (Chl-a) content

About 5 mL algae were first filtered, and then extracted with 5 mL of 90% acetone in a 10-mL glass centrifuge tube with a screw cap, at 4 °C for 24 h as reported. The tube was centrifuged at 3000 g for 8 min. The absorbance of Chl-a was measured with a spectrophotometer at 630, 645, 663 and 750 nm in the supernatant of the extracts. The content of Chl-a was calculated by the absorbance as the Eq. (2).

$$\text{Chl-a}(\mu\text{gL}^{1}) = \frac{[11.64\times(A_{663} - A_{750}) - 2.16\times(A_{645} - A_{750}) + 0.1\times(A_{630} - A_{750})]}{V_e \times d} \times V_s \quad (2)$$

Where V_e is the extract volume (mL), d is the thickness of the colorimetric cell (cm), V_s is algal sample volume (mL), and A_{663}, A_{750}, A_{645}, and A_{630} are the absorbance at 663, 750, 645 and 630 nm.

2.4. Determination of 2,4-DCP

When suspension was filtered with a 0.45 μm cellulose-acetate membrane filter (Millipore Co., Bedford, MA, USA), and 1 mL of the filtrate was mixed with 10 mL of 0.1 M sodium carbonate solution which provided a suitable medium for the derivatization reaction. Then, 0.4 mL of acetic anhydride was added as the derivatization reagent, and the mixture solution was shaken for 1 min at room temperature. Following this, the acetylated derivative (2,4-DCPA) was extracted twice by 5 mL of hexane, and the hexane solution was transferred to a sampling bottle and subsequent measurement of 2,4-DCPA.

The measurement was performed on a GC-5973C MS spectrometer system (Agilent Technologies, CA, USA, 7890 A Palo Alto). Ultra-pure helium was used as the carrier gas at flow rate of 1.6 mL min^{-1}. The injection volume was 1 μL. The inlet temperature was maintained at 280 °C in the splitless mode. The analyses were carried out by using a fused silica HP-5 MS capillary column (30 m × 0.32 mm I.D., 0.25 μm film thickness, Agilent Technologies, Palo Alto, CA, USA). The column was maintained at an initial temperature of 95 °C for 0.5 min, then heated to 280 °C at 13 °C min^{-1}, and held for 4 min, and the transfer line set at 310 °C (Paredes et al., 2014). Detection was carried out using electron impact ionization in selective-ion monitoring mode, selecting the mass-to-charge ratio of 162 and 164. An external calibration solution was analyzed every five samples. The limit of detection (LOD for S/N = 3) was 0.39 μg L^{-1}. Linearity was observed in the range of 1 − 20 μg L^{-1}, and correlation coefficients (R^2) was 0.9995. The addition standard recovery was 97.7%. Moreover, there was no 2,4-DCP peak detected from the blanks of the filtrate.

A 40-mL sample of algal suspension was centrifuged at 2000 g for 8 min, then the supernatant was stored for analysis. The remaining pellet was ultrasonic disrupted (JY92-IIN; Scientz, Ningbo, China) in 5 mL of dichloromethane after completely washed, then re-centrifuged. The supernatant solution was transferred to a clean flask and the pellet was re-extracted with another 5 mL dichloromethane. The two extracts were merged and concentrated to 1–2 mL by a rotary evaporator (Buchi R210, Switzerland). A chromatographic column consisted 1 g Na_2SO_4, 2 g silica gel and 1 g Na_2SO_4 from top to bottom was used to clean-up the concentrated extract. The column was eluted with 30 mL dichloromethane, then evaporated to 1–2 mL, and mixed with 10 mL 0.1 M sodium carbonate solution. The content of 2,4-DCP in the solution was measured as mentioned earlier.

2.5. Exposure experiments of 2,4-chlorophenol to algae

All of the batch experiments were conducted in duplicates.

Preliminary experiments were in 6 h-exposure. The initial concentration of 2,4-DCP and algal density were 100 μg L^{-1} and 174 mg L^{-1}, respectively. Algae were cultivated in a closed and dark system in order to obtain a limited alga growth. About 30 mL cultural solution was added in a 40-mL TOC bottle with a screw cape. The bottles were shaken on a 25 °C constant temperature rocker at a speed of 100 r min^{-1} for 6 h under dark conditions. As a chemical control (DCP and culture medium without alga, same as follows), the algae solution was replaced by the culture medium with 2,4-DCP added. About 5 mL of the mixture solution was filtered by a 0.45 μm cellulose-acetate membrane filter, and transferred to a sampling bottle for GC-MS analysis, and the rest mixture solution for determination of pH.

Long term exposure experiments. Initial algal density was 34.9 mg L^{-1} and cultivated in a closed system at initial pH 7.0, light: dark ratio 12:12. The investigated factors included initial pH (5, 6, 7, 8, and 9), light: dark ratios (24:0, 16:8, 12:12, 8:16 and 0:24 h), carbon sources (Na_2CO_3, citric acid, Na_2CO_3 + citric acid, and no external carbon source added). The initial chlorophenol concentrations depend on exposure experiments. About 50 mL culture solution was added in a 100-mL serum bottle with a screw cap. Then, algae and different amount of 2,4-DCP were added. As an biological control (algae without DCP, same as follows), 2,4-DCP was not added. After 120 h, the solution pH was measured, and 5 mL of culture solution was filtered with 0.45 μm cellulose-acetate membrane filter and transferred to a sampling bottle for 2,4-DCP measurement, and the remaining algae together with the filter were put in a 10-mL centrifuge tube with acetone for Chl-a measurement. About 4 mL of remained culture solution was used for algal density measurement, and 40 mL solution were used for the measurement of 2,4-DCP in algae after centrifuged and washed.

In the course of the above experiments, the bottle was sealed with a