Bioresource Technology 205 (2016) 264–268

Contents lists available at ScienceDirect

Bioresource Technology

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

Short Communication

Strain improvement of *Chlorella* sp. for phenol biodegradation by adaptive laboratory evolution

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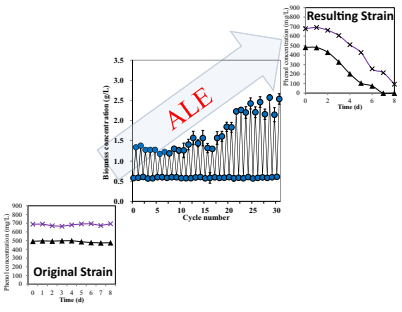
HIGHLIGHTS

- Adaptive evolution was performed for *Chlorella* sp. to remove phenol in wastewater.
- The resulting strain was obtained after 31 cycles for about 95 d.
- The resulting strain could grow under 500 mg/L and 700 mg/L phenol.
- The growth rates of resulting strain were more than twice of those of original one.
- 500 mg/L phenol could be fully removed in 7 d by the resulting strain.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 11 January 2016
Accepted 12 January 2016
Available online 19 January 2016

Keywords:
Phenol biodegradation
Adaptive laboratory evolution
Microalgae
Wastewater treatment

ABSTRACT

Microalgae are highly efficient photosynthesis cell factories for CO_2 capture, biofuel productions and wastewater treatment. Phenol is a typical environmental contaminant. Microalgae normally have a low tolerance for, and a low degradation rate to, high concentration of phenol. Adaptive laboratory evolution was performed for phenolic wastewater treatment by *Chlorella* sp. The resulting strain was obtained after 31 cycles (about 95 d) under 500 mg/L phenol as environmental stress. It could grow under 500 mg/L and 700 mg/L phenol without significant inhibition. The maximal biomass concentrations of the resulting strain at day 8 were 3.40 g/L under 500 mg/L phenol and 2.70 g/L under 700 mg/L phenol, respectively. They were more than two times of those of the original strain. In addition, 500 mg/L phenol was fully removed by the resulting strain in 7 d when the initial cell density was 0.6 g/L.

1. Introduction

Microalgae are highly efficient microbial cell factories for CO_2 capture, biofuels production and wastewater treatment. The metabolic phenotype of microalgae could be enhanced through genetic modification, mutations or adaptive laboratory evolution (ALE) (Anandarajah et al., 2012). ALE is widely utilized for strain improvement of yeast, bacteria and microalgae against stress conditions (Fu et al., 2012, 2013; Li et al., 2015; Perrineau et al., 2014). Phenols are considered to be typical environmental contaminants. A number of microalgae including *Ochromonas Danica* (Semple and Cain, 1997), *Chlorella* sp., *Scenedesmus obliquus* and *Spirulina maxima* (Klekner and Kosaric, 1992), *Chlorella vulgaris* and *Chlorella* VT-1 (Scragg, 2006), and *Chlorella pyrenoidosa* (Das et al., 2015) have been investigated to degrade phenols. Phenol is a possible substrate for *Chlorella* sp. but high concentrations of phenol is also

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http://dx.doi.org/10.1016/j.biortech.2016.01.022
0960-8524/

toxic to microalgae. *C. pyrenoidosa* could tolerate and degrade up to 200 mg/L of phenol when the initial cell density was about 0.22 g/L (Das et al., 2015). When the initial phenol concentration was 400 mg/L, 44% growth inhibition for *Chlorella* VT-1 and almost 100% for *C. vulgaris* were observed (Scragg, 2006). In addition, the phenol was scarcely degraded under light conditions after two weeks while 100–300 mg/L phenol could be removed under dark conditions for 20 d by *Chlorella* VT-1. High initial cell density was helpful in allowing microalgae to tolerate phenol. Klekner (Klekner and Kosaric, 1992) found that 700–810 mg/L of phenol was degraded by *Chlorella* sp. in less than 7 d when the initial cell densities were 3.4 g/L and 6.3 g/L. The initial cell density was so high that much more nutrients and operating costs were needed. High concentration phenol could be degarded in a lower initial cell density if a powerful microalgae strain could be screend or improved. It is critical to carry out strain improvement for enhancing high concentration phenol tolerance and improving the biodegradation rate of *Chlorella* sp. This study proposes an approach based on ALE to improve its metabolic phenotype.

2. Methods

2.1. Experimental organism and materials

This study was conducted using a freshwater *Chlorella* sp., which was isolated from a local water sample (Shanghai, China). It was chosen for improving CO_2 tolerance as the original strain in ALE process previously (Li et al., 2015). Phenol and other reagents used in the study were AR grade with high purity, and purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2. Effect of initial cell density and phenol concentrations on phenol removal

In the ALE process, the selections of both the initial phenol concentration as an environmental stress and initial cell density are necessary. Microalgae should keep growing under this stress condition using the selected initial cell density. The growth of microalgae inoculated with low initial cell density will be inhibited with high concentration of phenol. Before the ALE experiment, the effects of initial cell density and initial phenol concentration were evaluated in 250 mL glass flasks. The initial phenol concentrations were 0 mg/L, 100 mg/L, 200 mg/L, 300 mg/L or 500 mg/L for each initial algal cell densities (0.2 g/L, 0.4 g/L, 0.6 g/L or 1 g/L). *Chlorella* sp. was kept in sterile TAP medium at 28 ± 1 °C and the initial pH was 7. White LEDs were used for continuous light during the cultivations. The average light intensity of the white LEDs was 117.67 ± 6.89 μmol/m^2/s which was monitored by a quantum sensor described in previous study (Li et al., 2015). The flasks were incubated in an orbital shaker set at 150 rpm with 100 mL medium. Samples were collected every 24 h and centrifuged at 6000 rpm for 5 min. Supernatant was used to detect residual phenol concentrations. The controls were TAP medium with different concentrations of phenol that did not contain microalgae in it. All experiments were carried out in triplicate.

2.3. Adaptive laboratory evolution with high phenol concentrations

ALE was performed with the *Chlorella* sp. in exponential growth phase. For each new cycle, the initial cell density in fresh TAP medium was all maintained 0.6 g/L. *Chlorella* sp. were cultivated in 250 mL glass flasks containing 100 mL sterile TAP medium. A phenol concentration of 500 mg/L was selected as the environmental stress. At the beginning of ALE process, the microalgae cells had a long lag phase under the high concentration of phenol. Therefore, the first cycle was adjusted to 5 d so that enough biomass of *Chlorella* sp. cells could be obtained for the next cycle. All subsequent cycles were 3 d. At the end of each cycle, the medium containing the microalgae in each glass flask was centrifuged at 6000 rpm for 5 min to obtain algal pellets, and then the pellets were washed with sterilized water twice. Finally, the obtained algal pellets were re-suspended in fresh TAP medium and the re-suspension was used as the inoculum in new TAP medium in order to start a new cycle of ALE. Samples were then collected at the beginning of the ALE and at the end of each cycle to measure both the biomass and residual phenol concentrations in the medium. This process was repeated for 31 cycles (about 95 d) and the resulting strain was obtained.

2.4. Evaluation of growth rate and biomass component

In order to characterize the phenol tolerance and phenol removal capability, the resulting strain and the original strain of *Chlorella* sp. were each cultivated for 8 d. The initial phenol concentrations were 0 mg/L, 500 mg/L or 700 mg/L and initial cell density was 0.6 g/L. The growth rates of the original and resulting strains were determined by the dry weight (Li et al., 2015). At the 8th day, the contents of total carbohydrate, total protein and total lipid of the original strain and the resulting strain were extracted and analyzed by the method described previously (Li et al., 2015).

2.5. Determination of residual phenol

Before measuring the concentration of phenol, a 5 mL sample was withdrawn from each glass flask and centrifuged at 6000 rpm for 5 min at room temperature. The supernatants were used to monitor the residual phenol concentration by 4-AAP spectrophotometric method (Emerson, 1943). The phenol concentration was detected spectrophotometrically by measuring the absorbance at 510 nm.

3. Results and discussion

3.1. Effects of initial cell density and phenol concentration

Firstly, the suitable initial cell density and initial phenol concentration used in ALE process were determined. The selected initial phenol concentration should be an environmental stress for microalgae and a lower concentration of phenol should be fully degraded under the selected initial cell density. The growth rates of the original strain of *Chlorella* sp. are shown in Fig. S1. For each initial phenol concentration from 0 mg/L to 500 mg/L, the higher initial cell density, then the higher final biomass concentration if the initial cell density was from 0.2 g/L to 0.6 g/L. When the initial cell density was less than 1.0 g/L, 500 mg/L phenol will lead to serious growth inhibition and the final biomass concentrations were almost 50% of those of the control. It was obvious that 500 mg/L phenol was selected as the environmental stress in the ALE process. The residual phenol concentrations in the medium under different initial cell densities are shown in Fig. S2. It should be mentioned that the volatile phenols in the controls were deducted. When phenol concentration was 100 mg/L, the original strain can remove all of the phenol in the medium within 3–4 d when the initial cell density was between 0.2 g/L and 0.6 g/L. As the initial phenol concentration was 200 mg/L, it was fully degraded by the original strain within 4 d when the initial cell density was 0.6 g/L. Although the original strain could grow under 300 mg/L of phenol (Fig. S1C), the removal of phenol was very limited shown in Fig. S2. When the initial cell density was 1.0 g/L, there were no significant differences for the microalgae growth under all initial phe-