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The removal of ρ-chlorophenol in aqueous cultures with free and alginate-immobilized cells of the microalga *Tetraselmis suecica*

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Abstract The present study aimed at evaluating the ability of some isolated cyanobacterial and microalgal strains for the removal of ρ-chlorophenol (ρ-CP), an environmentally harmful contaminant. To identify the most efficient species, a screening program was carried out using 15 microalgal and cyanobacterial strains. Among them, *Tetraselmis suecica* was able to remove 67 % of the ρ-chlorophenol at an initial concentration of 20 mg L^{-1} from the medium within a 10-day period. The efficacy of the process was dependent on the ρ-chlorophenol concentration. At concentrations above 60 mg L^{-1} of the pollutant, no removal was observed due to the inhibitory effect of ρ-chlorophenol on the *T. suecica* cells. The effect of cell immobilization in alginate beads on *T. suecica* removal capacity was also examined. Using this technique, the removal efficacy for the initial ρ-CP concentration of 20 mg L^{-1} increased up to 94 %.

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Introduction

The contamination of aquatic ecosystems by phenolic compounds is a serious environmental problem (Scragg 2006). In aqueous environments, these pollutants affect the community composition of phytoplanktons, which in turn may result in harmful effects on higher levels of the food chain (Jensen 1996). While the level of chlorophenols in a marine environment should be below 10 μg L^{-1}, there are reports of the presence of concentrations of 0.5–10,000 μg L^{-1} of these hazardous pollutants in industrial effluents (Petroutsos et al. 2007). The US Environmental Protection Agency (EPA) lowered the allowable amounts of chlorophenol concentration from several mg L^{-1} to <1 mg L^{-1} (Denizli et al. 2004). ρ-Chlorophenol (ρ-CP) is a halogenated phenolic compound that is used as an intermediate during the synthesis of insecticides, herbicides, preservatives, antiseptics, and disinfectants (Petroutsos et al. 2008). It is also used in the manufacture of pharmaceuticals, dyes, aromatic compounds, and other organic materials (Petroutsos et al. 2008). Due to this widespread usage, as well as its abundance in oil refineries and coke manufacturing effluents (Anirudhan et al. 2009), ρ-CP is considered to be a priority environmental pollutant (Olivier et al. 2003; Scragg 2006). Although some physicochemical methods, such as TiO_2 photocatalysis, electron-beam irradiation (Shi et al. 2009), UV/Fenton oxidation (Du et al. 2005), electrochemical oxidation (Zhou et al. 2005), carbon adsorption (Anirudhan et

al. 2009), solvent extraction, and chemical oxidation (Gonzalez et al. 2010), have been proposed for the removal of ρ-CP, the advantages of the biological degradation of pollutants, such as lower costs, higher efficiency, and less toxicity, have persuaded researchers to apply this environmentally friendly method. The ability of bacteria (Yao et al. 2006; Carvalho et al. 2001; Caldeira et al. 1999), fungi, especially laccase producing fungi (Forootanfar et al. 2012), and cyanobacteria (Pavlostathis and Jackson 2002; Shashirekha et al. 1997) to degrade phenolic pollutants has been extensively studied, whereas a limited number of reports have focused on the degradation potency of photosynthetic microalgae (Lima et al. 2004; Hirooka et al. 2003; Semple et al. 1999), which are able to either eliminate aromatic and azo dye compounds or utilize them as carbon or nitrogen sources. For example, golden unicellular alga, *Ochromonas danica*, can completely degrade phenolic compounds and utilize them as a carbon source (Pinto et al. 2002; Semple et al. 1999). Furthermore, the special degradation pathways of xenobiotics make the use of microalgae, which perform the ortho- and meta-cleavage of the aromatic rings and conjugation (Lovell et al. 2002; Petroutsos et al. 2007), more preferable than bacterial strains.

The advantages of cell immobilization, such as long-term stabilization, higher cell density, and increased specific yield, encouraged researchers to apply this technique (Arabi et al. 2010). Despite the widespread use of bacterial and fungal immobilization for the biotransformation of xenobiotics (Kutney et al. 1985; Manosroi et al. 2003), immobilized microalgae have rarely been employed for this purpose (Arabi et al. 2010).

The aim of the present study was to screen microalgal and cyanobacterial strains from our laboratory's local collection (Faramarzi et al. 2008; Hajimahmoodi et al. 2010) that are capable of removing ρ-chlorophenol, a hazardous pollutant, in order to introduce an efficient microalga for ρ-CP removal in aqueous cultures. Further investigation was performed on the removal by immobilizing the selected microalga in alginate beads.

Materials and methods

Axenic cultures of 15 microalgae and cyanobacteria strains were obtained from various sources and screened for p-chlorophenol degradation efficiency (Table 1). The algal strains were maintained on sterile BG-11 (Arabi et al. 2010; Borowitzka 1988) agar slants at 4°C and freshly subcultured before being used. A loopful of each strain was individually inoculated into 100 mL of f/2 artificial seawater (for *Tetraselmis suecica*) (Borowitzka 1988) or BG-11 (Borowitzka 1988) medium (for other strains) in a 500-mL Erlenmeyer flask and incubated at 25°C in a shaker (100 rpm) under continuous illumination of 60 μmol photons m^{-2} s^{-1} supplied by three fluorescent tubes for at least 48 h. The initial pH of the cultivation flasks was adjusted at 7.2 using Tris–HCl (40 mM) prior to sterilization by autoclave (Petroutsos et al. 2007).

Screening for microalgae with ρ-CP removal ability

In order to obtain tolerant strains, each microalga was adapted to the ρ-chlorophenol in a stepwise procedure prior to screening. Thereafter, the tolerant microalgae were cultivated in f/2 artificial seawater or BG-11 for 48 h, and then the ρ-CP [from a stock solution of ρ-CP in high-performance liquid chromatography (HPLC) grade methanol] was added to the culture medium under aseptic conditions to a final concentration of 20 mg L^{-1} (Petroutsos et al. 2007), followed by the incubation of the bottles in an incubator shaker (25°C, 100 rpm) under continuous illumination. The final concentration of methanol in the medium was 0.006 % (*v*/*v*). Samples of 1 mL were taken every other day, cells were removed by centrifugation at 8,000×*g* for 6 min, and the concentration of ρ-CP in the supernatant was determined by HPLC. Similarly, the pellets were washed with methanol and centrifuged at 8,000×*g* for 6 min, and the methanol extract was analyzed by HPLC to estimate the amount of ρ-CP that entered into or adsorbed on the cells. Each experiment was performed in triplicate. The control bottles were designed to investigate abiotic elimination by introducing ρ-CP to the algae-free culture media.

HPLC analysis

Quantitative studies were performed by HPLC (Knauer, Germany). The data were acquired and processed by means of ChromGate software (version 3.3.1), also from Knauer. Chromatographic separation was achieved on a Lichrospher 100 RP & EC C_8 reverse phase column (C8, 25×0.46 cm i d, 5 μm particle size; Teknokroma, Spain). Detection was at 280 nm. The isocratic solvent used was water/methanol/acetic acid (49:50:1, *v*/*v*/*v*) with a flow rate of 2 mL min^{-1}. The retention time for ρ-CP was 7 min. The ρ-CP concentration was determined using a standard curve obtained from known concentrations of ρ-CP in which the limit of detection was found to be 0.085 mg L^{-1}. 1,2-Dichlorobenzene was used as an internal standard (Angerer and Schaller 2001; Sadeghi-aliabadi et al. 2009). Cell numbers were determined using a Neubauer hemocytometer.

The effect of the initial ρ-CP concentration on the removal of ρ-CP by *T. suecica*

The *T. suecica* cells were grown in the f/2 artificial seawater medium to a final concentration of 0.5–1×10^5 cells mL^{-1}.

Thirty milliliters of this culture was used to inoculate 270 mL of f/2 medium containing 10, 20, 40, and 60 mg L^{-1} of ρ-CP in 1,000-mL culture bottles. The cultures were then incubated at 25°C under continuous illumination of 60 μmol photons m^{-2} s^{-1}, while the media were bubbled by sterile air. Three bottles were used for each concentration to achieve a mean for the ρ-CP removal. A control bottle was also designed to investigate abiotic pollutant removal. Samples were taken every other day and analyzed by HPLC after centrifugation, as previously described.

The effect of $NaHCO_3$ concentrations on *T. suecica* pollutant elimination

To investigate the biomass productivity and *T. suecica* removal of ρ-CP in the presence of bicarbonate as a carbon source, various concentrations (0.5, 1, 1.5, and 2 g L^{-1}) of $NaHCO_3$ were added to the pre-inoculated culture media (see above) and incubated at 25°C, while sterile air was bubbled through the media. This was followed by determining the mass production and pollutant elimination of the test bottles (in triplicates), as compared to the control bottle that did not have bicarbonate salt added.

Assay for laccase activity

To determine *T. suecica* intracellular laccase activity, the harvested biomass was washed three times by an acetate buffer of 100 mM pH 4.5, followed by cell disruption by grinding the frozen cells in liquid nitrogen with a mortar and pestle. The cell debris was discarded after centrifugation (8,000×*g*, 5 min) and the supernatant used for the laccase activity determination, according to Forootanfar et al. (2011).

The effect of cell immobilization in sodium alginate on ρ-CP elimination

After the cultivation of *T. suecica* in the air-bubbled bottle at 25°C under continuous illumination, the cell biomass was harvested in the logarithmic phase (1.2×10^6 cells mL^{-1}) by centrifugation ($2.0\times10^3\times g$ for 10 min at 4°C), in order to reach 5 mL packed cell volume (Arabi et al. 2010), and then followed by mixing it homogenously with the same volume of 2 % *w*/*v* sodium alginate solution. The mixture was passed through a needle into a cold calcium chloride solution of 0.2 M and left for 1 h. The alginate beads were washed with distilled water before being used (Ramachandra Rao et al. 1999). The beads were added to 20 mL of an f/2 medium containing 20, 40, and 60 mg L^{-1} of ρ-CP, followed by incubation at 25°C and continuous illumination, in order to investigate the ability of immobilized cells to remove ρ-CP. To investigate the ρ-CP adsorption or entrance into the alginate beads in a separate trial, a control bottle containing the same ρ-CP concentration to alginate beads but without cells was used. The control beads were extracted by methanol and subjected to HPLC analyses. All experiments were done in triplicate.

Results

Screening studies were carried out using a ρ-CP concentration of 20 mg L^{-1} (Petroutsos et al. 2007). As shown in Table 1, *T. suecica* was able to remove 67 % of the ρ-CP from the medium after 10 days, while *Nostoc muscorum* H1, *Chlorella vulgaris*, and *Fischerella ambigua* transformed 56, 51, and 46 % of the pollutant, respectively. During this period, no removal occurred in the uninoculated control media. Based on these results, *T. suecica* was selected for further studies.

Figure 1 shows the effect of ρ-CP concentration on *T. suecica* growth. Cell growth was considerably suppressed at concentrations above 40 mg L^{-1}, and at 60 mg L^{-1}, no growth was detected. Increasing the initial concentration of ρ-CP up to 20 mg L^{-1} led to an increase in the removal efficacy of *T. suecica* (Fig. 2). However, at higher concentrations, ρ-CP removal was lower due to its toxic effect. The absence of ρ-CP in the methanol extract of the microalgal pellets indicated no ρ-CP bioaccumulation.

Increasing the concentration of $NaHCO_3$ to 1 g L^{-1} led to more biomass production and consequently more pollutant elimination (Fig. 3). The concentration above 1 g L^{-1} decreased both the cell count and pollutant removal.

When immobilized cells were used, the efficacy of *T. suecica*'s ρ-CP removal (at a concentration of 20 mg L^{-1}) was increased to 94 % (Fig. 4). It is of interest to note that, after immobilization, the time required to remove 73 % of the ρ-CP from the medium was reduced to 4 days, which is less than half the time required to obtain the same results using free *T. suecica* cells. The entrapped cells were able to remove 34 and 26 % of the pollutant at the initial concentrations of 40 and 60 mg L^{-1}, respectively, after a 10-day period. The amount of ρ-CP absorbed into the alginate beads was negligible.

Discussion

While the presence of chlorophenolic compounds at 0.5 μg L^{-1} (Jennings et al. 1996) is considered lethal for some marine organisms, the concentration of these substances in industrial wastes may be much higher (Ettala et al. 1992; Dimou et al. 2006). Incomplete degradation and the

Table 1 Percentage of eliminated p-CP following a 10-day period treatment by 15 individual microalgae at initial concentration of 20 mg L^{-1}

Algal strains	Percent of ρ-CP elimination[a] (% ±SD initial concentration of 20 mg L^{-1})	Algal source
Cyanobacterial strains		
Anabaena cylindrica	29.5±0.9	Hajimahmoodi et al. 2010
Calotrix sp.	25.5±0.7	Hajimahmoodi et al. 2010
Chroococcus dispersus	18.5±0.8	Ghasemi et al. 2007
Fischerella ambigua	46±0.7	Tabatabaei Yazdi et al. 2005
Fischerella muscicola	20±0.9	Hajimahmoodi et al. 2010
Microchaete tenera	27±0.6	Safiarian et al. 2012
Nostoc ellipsosporum	3.5±0.5	Moradpour et al. 2006
Nostoc muscorum strain A1	16.5±0.4	Faramarzi et al. 2006; Arabi et al. 2009
Nostoc muscorum strain H1	56±1	Hajimahmoodi et al. 2010
Nostoc piscinale	30.5±0.6	Gharaei-Fathabad et al. 2007; Kalbasi et al. 2009
Nostoc sp.	7.5±0.4	Hajimahmoodi et al. 2010
Tolypothrix tenuis	3.5±0.5	Hajimahmoodi et al. 2010
Eukaryotic algae		
Chlorella sp	12.5±0.9	Hajimahmoodi et al. 2010
Chlorella vulgaris	51±0.8	Hajimahmoodi et al. 2010
Tetraselmis suecica	67±0.4	Shakibaie et al. 2010

[a] Mean±SD, $n=3$

production of hazardous byproducts from using physico-chemical methods for the removal of chemical pollutants (Pera-Titus et al. 2004) persuaded researchers to use biological systems for the removal of such harmful compounds (Denizli et al. 2005). In this study, the ability of some microalgal and cyanobacterial strains to remove these pollutants was investigated by incorporating ρ-CP into the culture medium. Among them, *T. suecica* was the most efficient strain and removed 67 % of ρ-CP. *N. muscorum* H1, *C. vulgaris*, and *F. ambigua* were able to eliminate 56, 51, and 46 % of the pollutant, respectively. In a similar

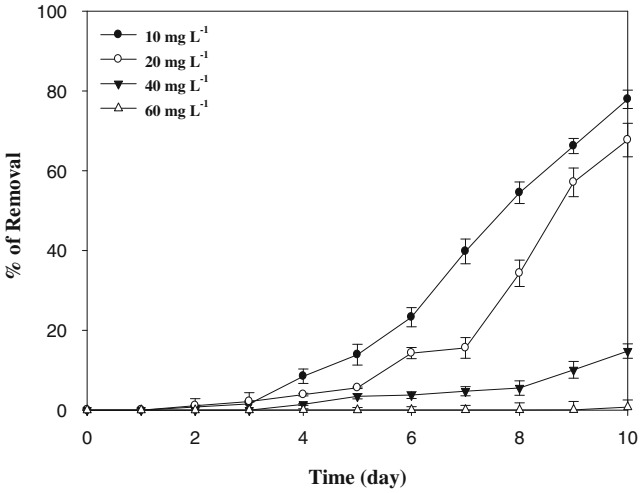

Fig. 2 ρ-CP removal curves for initial concentrations of 10, 20, 40, and 60 mg L^{-1} by free cells of *T. suecica* (mean±SD, $n=3$)

study, it was shown that *Tetraselmis marina* can eliminate 65 % of ρ-CP when this compound is added to the medium at an initial concentration of 20 mg L^{-1} (Petroutsos et al. 2007). The lag phase of growth of *T. suecica* increased with increasing the pollutant concentration from 10 to 60 mg L^{-1} (Fig. 1). This toxic property decreased the ability of *T. suecica* to remove ρ-CP when the pollutant level was increased in the culture medium (Fig. 2). According to the profiles of ρ-CP removal (Figs. 1 and 2), it seems that ρ-CP was not used as a primary growth substrate by the algae because ρ-CP elimination increased by enhancing the cell numbers during the 10-day period. Besides cell adsorption and the entrance of such organic pollutants (bioaccumulation), biodegradation/biotransformation is another biological pathway for removing organic contaminants. The degradation of substituted phenols by purified laccase (benzenediol/oxygen oxidoreductase: EC 1.10.3.2) and laccase producing microorganisms is an environmental application

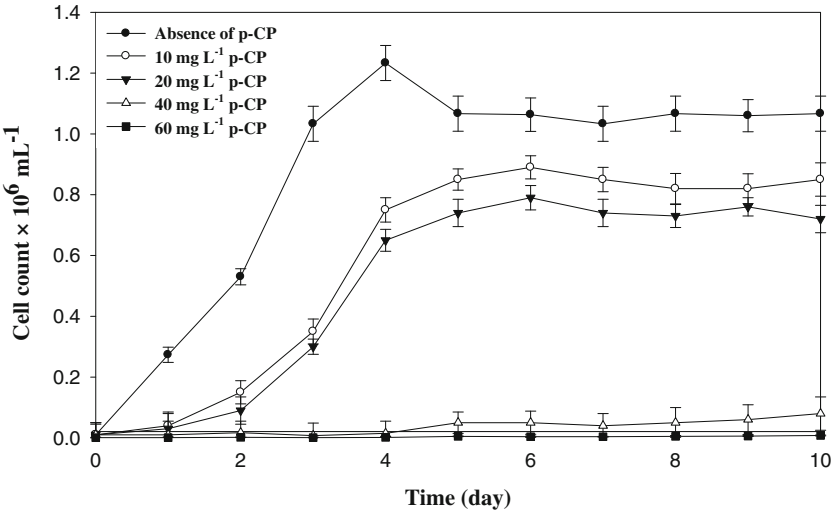

Fig. 1 The growth curve of *T. suecica* in absence and presence of initial concentrations (10, 20, 40, and 60 mg L^{-1}) of ρ-CP (mean±SD, $n=3$)

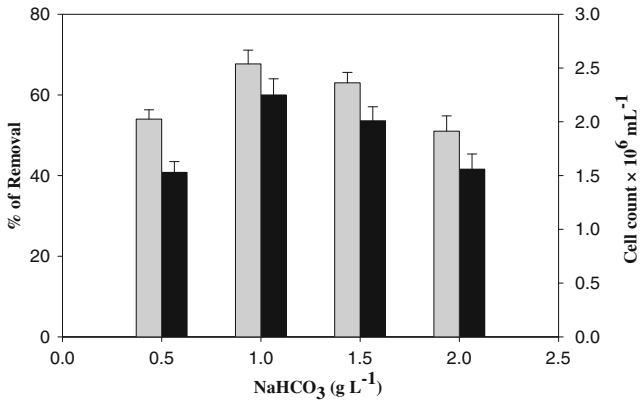

Fig. 3 The effect of $NaHCO_3$ concentrations on ρ-CP (initial concentration of 20 mg L^{-1}) elimination (*gray*) and biomass production (*black*) of *T. suecica* (mean±SD, *n*=3)

of this oxidase (Forootanfar et al. 2012). The lack of laccase activity, as well as the absence of ρ-CP in the microalgal pellet methanol extract (bioaccumulation) in our investigation, indicated that *T. suecica* utilized another pathway to eliminate ρ-CP from the culture medium. Petroutsos et al. (2008) also used *T. suecica* and found that 2,4-dichlorophenol was conjugated to β-D-glucopyranoside and β-D-(6-*O*-malonyl)-glucopyranoside, which reduced the toxicity of this hazardous compound. Such conjugation reactions may also be the likely mechanism of ρ-CP removal. However, the HPLC chromatogram done 1 h after ρ-CP elution (data not shown) revealed no possible metabolite, which was probably due to an inappropriate mobile phase and/or masking of the metabolite peak by the ρ-CP. The best concentration of $NaHCO_3$, in which maximum elimination and biomass production were observed, was 1 g L^{-1}. The decrease of both ρ-CP removal and cell count above this critical concentration might be ascribed to the toxic effect of produced CO_2. This finding is in agreement with Petroutsos et al. (2007), indicating a straight correlation between cell count and pollutant removal to the addition of bicarbonate. Yan and Pan (2004) also determined the significant effect of $NaHCO_3$ addition on the biodegradation of dimethyl phthalate using *Closterium lunula*. In order to improve their removal capacity, the *T. suecica* cells were immobilized in alginate beads. This method not only increased removal efficacy to 94 % but also decreased the time required to achieve 73 % removal from 10 to 4 days. Furthermore, the encapsulated cells had the ability to remove more ρ-CP compared to suspended cells at higher toxic concentrations (40 and 60 mg L^{-1}). It seems that higher reaction rates due to the presence of higher cell densities in the immobilized form, compared to a free suspension culture (Arabi et al. 2010), increased the ability of *T. suecica* to remove more pollutants. These results are in agreement with other studies, which also utilized immobilization techniques. Santos-Rosa et al. (1989) studied the improvement of ammonium production from nitrite by immobilizing *Chlamydomonas reinhardtii* in alginate beads. In addition, phosphorous uptake (Robinson 1995) and heavy metal removal capacity of algae were found to be increased by entrapping cells in alginate beads (Wilkinson et al. 1990). It seems that immobilized cells are less sensitive to inhibition by ρ-chlorophenol (Arabi et al. 2010).

In conclusion, the ease of growing and culturing *T. suecica* with only a few simple salts and the possibilities of applying immobilization techniques are the main advantages for employing *T. suecica* in further studies of removing pollutants such as chlorophenols. In addition, *T. suecica* is a marine microalga and could be used to remove ρ-CP in seawater.

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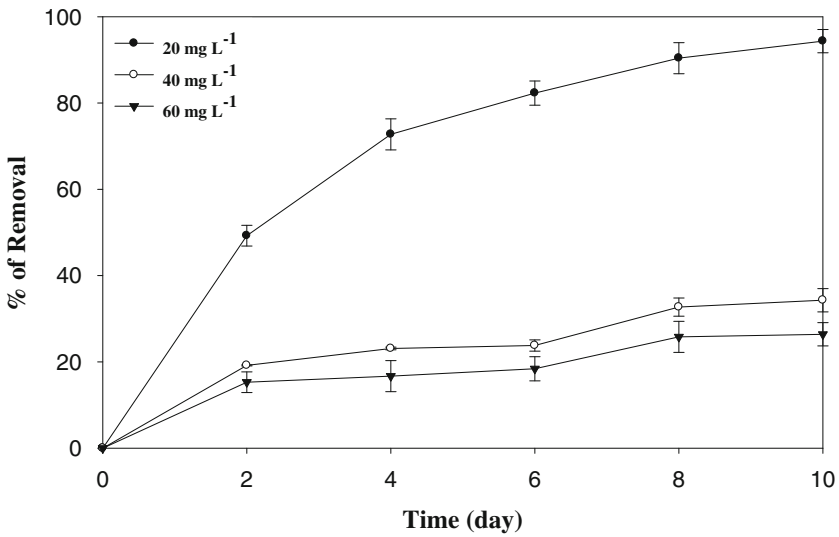

Fig. 4 ρ-CP removal profiles at initial concentrations of 20, 40, and 60 mg L^{-1} by immobilized cells of *T. suecica* (mean±SD, *n*=3)

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