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Physiological and biochemical responses of *Chlorella vulgaris* to Congo red

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ABSTRACT

Extensive use of synthetic dyes in many industrial applications releases large volumes of wastewater. Wastewaters from dying industries are considered hazardous and require careful treatment prior to discharge into receiving water bodies. Dyes can affect photosynthetic activities of aquatic flora and decrease dissolved oxygen in water. The aim of this study was to evaluate the effect of Congo red on growth and metabolic activity of *Chlorella vulgaris* after 96 h exposure. Exposure of the microalga to Congo red reduced growth rate, photosynthesis and respiration. Analysis of chlorophyll *a* fluorescence emission showed that the donor side of photosystem II was affected at high concentrations of Congo red. The quantum yield for electron transport (φE_o), the electron transport rate (ETR) and the performance index (PI) also decreased. The reduction in the ability to absorb and use the quantum energy increased non-photochemical (NPQ) mechanisms for thermal dissipation. Overall, Congo red affects growth and metabolic activity in photosynthetic organisms in aquatic environments.

1. Introduction

Azo compounds constitute the largest and most diverse group of synthetic dyes and are widely used by the textile, food, cosmetic, and pulp and paper industries (El-Sheekh et al., 2009). At the global level, 280,000 t of textile dyes are discharged into industrial effluent each year (Jin et al., 2007). It is estimated that the quantity of dye that does not fix to textile fibers depends on the application method, varying from 2 percent when basic dyes are used to 50 percent when reactive dyes are used (Hai et al., 2007; Pearce et al., 2003). The discharge of azo dyes into the environment is a serious problem and should be avoided not only for esthetic reasons but also because of the threat it poses to public health and ecosystems (Golka et al., 2004). In aquatic environments, the colors produced by azo dyes impede plant development by decreasing the passage of light, thereby affecting the photosynthetic process (Annuar et al., 2009). The resulting changes in the concentration of dissolved O_2 damage the aquatic biota present and increase the biochemical oxygen demand of the surrounding water (Ali, 2010). In addition, some azo dyes, such as Congo red, Direct Blue 15 and Direct Red 2, have been reported to be toxic, mutagenic, and carcinogenic to aquatic life (Bafana et al., 2008; Golka et al., 2004; Saratale et al., 2011).

In aquatic ecosystems the green algae are the primary producers and are key indicator organisms (Wen et al., 2011) when used to evaluate water quality and the ecotoxicity of contaminants (Xu et al., 2013), such as metals, herbicides, insecticides, and other xenobiotic compounds (Jena et al., 2012; Levy et al., 2007; Qian et al., 2008). On the other hand, the chlorophyll *a* fluorescence kinetic has been used to indicate alterations of the photosynthetic capacity when damage is induced by pollutants or by environmental conditions (Jena et al., 2012; Kalaji et al., 2012). The advantage of such a method is that it is non-invasive and reliable (Muller et al., 2008; Xia and Tian 2009). Furthermore, studies have shown that changes in photosystem II (PSII) can be quantified using the JIP-test (Jena et al., 2012; Strasser et al., 2000). However, as of yet, no information is available regarding the impact of azo dyes on the photosynthetic apparatus of

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algae. Such information is important because it would permit us to determine the toxicity to the aquatic environment caused by this type of dye. For this reason, the objective of the current study is to evaluate the toxic effects of the azo dye Congo red on the growth and photosynthetic metabolism of the microalga *Chlorella vulgaris*.

2. Materials and methods

2.1. Dye used

Congo red (Sigma-Aldrich), also called Direct Red 28, was used for the experiments described herein. Its molecular form is $C_{32}H_{22}N_6O_6S_2Na_2$, and its molecular weight is 696.7 g mol^{-1}. All experiments employed a 100 mg L^{-1} stock solution of dye, which, prior to its addition in each experimental unit, was sterilized by filtration using Millipore® membranes of 0.22 μm pore diameter.

2.2. Experimental units

Experiments were conducted under sterile conditions with axenic cultures of the green microalga *C. vulgaris* donated by the Laboratorio de Hidrobiología Experimental of the Escuela Nacional de Ciencias Biológicas of the Instituto Politécnico Nacional. The strain was collected and isolated from temporary ponds in the State of Mexico (Atlacomulco). The registration number is LHE-Chl 01. The experimental culture units were glass bottles with flat surfaces, each of which had a total capacity of 0.5 L and a working volume of 0.25 L, that were inoculated with 15 ml of *C. vulgaris* culture in the exponential phase (11 mg L^{-1} dry biomass) Bold's Basal mineral medium (Stein, 1973) and different concentrations of dye (5, 10, 15, 20, and 25 mg L^{-1} of Congo red), the control used contained only the inoculum and the culture medium. The culture conditions were as follows: temperature, 25 ± 3 °C; illumination, 120 μmoles photons m^{-2} s^{-1}; photoperiod, 12/12 h (light/darkness); and air supply, 200 ml min^{-1}.

2.3. Growth and photosynthetic pigments

Growth was determined using the dry-weight values obtained at the beginning (0 h) and at the end (96 h) of the experiment. Each treatment used 5-ml aliquots that were vacuum-filtered using Millipore® membranes of 5.0-μm pore diameter, pre-treated for a constant weight. Later, the samples were dried at 80 °C for 48 h.

Growth rate of algal culture is a measure of the increase in biomass over time and it was determined by the following equation:

$$K = \ln (N_2/N_1)/(T_2 - T_1)$$

where, K is growth rate, N_1 and N_2, biomass content at time (T_1) and the time (T_2), respectively chlorophyll *a* and *b* and total carotenoids were determined using 3 ml of each sample, after the sample was centrifuged for 10 min. Subsequently, the pellet was added 2 ml of methanol and stirred for 1 min. Previously the pellet was washed using Bold´s Basal mineral medium to remove adsorbed dye completely. This procedure was done to prevent the absorption spectrum of Congo red which interferes with the absorption spectrum of photosynthetic pigments, especially carotenoids. The sample was kept in water bath at 60 °C for 10 min. Again, each sample was centrifuged at room temperature. The supernatant was separated and the concentrations of Chl-*a*, Chl-*b*, and total carotenoids were determined with a UV–Vis spectrophotometer (Genesys 10UV Thermo Electron Corporation) according to the equations of Wellburn (1994).

2.4. Chlorophyll fluorescence

For all treatments, the Chl-*a* fluorescence emitted via photosystem II was measured at 96 h in samples conditioned in the dark using a portable fluorometer, model HANDY-PEA (Hansatech Instruments Ltd., Norfolk, UK) coupled to the chamber for liquid phase HPEA/LPA (Hansatech, UK). For each of the experimental units, samples were corrected to the same optical density ($0.3_{680\ nm}$) and these were washed using Bold´s Basal mineral medium to remove adsorbed dye completely. After, 2 ml samples of each treatment ($n=4$) were taken and incubated at dark for 20 min at room temperature (25 °C), then the samples were irradiated for 1 s with red light (660 nm) saturating (3000 μmol photons m^{-2} $s-1$).

The equipment automatically recorded the following values: (i) minimal fluorescence (F_o) at 50 μs, (ii) maximum fluorescence (F_m) between 200 and 500 ms, (iii) variable fluorescence at 2 ms ($V_j=F_j-F_o/F_m-F_o$) and (iv) the slope at the beginning of fluorescence $M_o=4\ (F_K-F_o)/(F_M-F_o)$. These data were used to calculate the following parameters (Strasser et al., 2004):

1. The maximum quantum yield of primary photochemistry; $\varphi P_o=[1-(F_o/F_m)]=F_v/F_m$
2. The probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; $\Psi_o=1-V_j$
3. The quantum yield of electron transport; $\varphi E_o=[1-(F_o/F_m)\ (1-V_j)]=(\varphi P_o)\ (\Psi_o)$
4. Reaction centers per excited cross section; $(RC/ABS)=\ [(M_o/V_j)\ (1-(F_o/F_m))]$
5. The performance index; $PI_{ABS}=(RC/ABS)\ [\varphi P_o/(1-\varphi P_o)]\ [\Psi_o/(1-\Psi_o)]$

Data were interpreted using the Handy-PEA software developed by Hansatech Instruments Ltd., U.K., and Biolyzer-HP3 software designed at the Bioenergetics Laboratory of the University of Geneva, Switzerland (van Heerden et al., 2003).

Modulated fluorescence was measured using the fluorescence modulated system (FMS 2 Hansatech Instruments Ltd., Norfolk, UK) in accordance with Nielsen and Nielsen (2005) and Masojídek et al. (1999). All measurements were obtained at room temperature (25 °C) and in dark conditions. Prior to examining Chl-*a* fluorescence, samples of each experimental unit were adjusted to the same optical-density value ($0.3_{680\ nm}$) and these were washed using Bold´s Basal mineral medium to remove adsorbed dye completely.

Each cellular suspension was vacuum-filtered using Millipore® membranes of 5.0-μm pore diameter and were later adapted to darkness for 5 min. Signatures for Chl-*a* fluorescence were detected directly from the filter surface (Perales-Vela et al., 2007).

The value of F_o at room temperature was obtained by irradiating the sample with a modulated light of low intensity (0.1 μmol m^{-2} s^{-1}) and after 10 s the sample was superimposed with one saturating pulse of white light of 10,000 μmol m^{-2} s^{-1} for 0.8 s, to close all reaction centers and obtain the maximum fluorescence value of F_m in darkness. Immediately after the saturating pulse, the samples were irradiated with a non-saturating actinic white light 200 μmol m^{-2} s^{-1} for 5 min to achieve a steady state and then these were superimposed a saturating white light pulse of 10,000 μmol m^{-2} s^{-1} for 0.8 s to obtain the value of F_m'.

Finally the values were calculated as follows:

1. Relative electron transport rate ($_rETR$)$=[((F_m'-F_s)/F_m')]\ (0.5)(PAR)$. The value of 0.5 corresponds to the proportion of light that is transferred to each of the two photosystems (PSII and PSI) and the photosynthetically active radiation (PAR) utilized was of 162 μmol m^{-2} s^{-1}.
2. Non-photochemical quenching (NPQ)$=[(F_m-F_m')/F_m']$.

2.5. Measurement of photosynthesis and respiration

Measurements were made using an oximeter (Oxylab-Hansatech, UK) at a controlled temperature of 30 °C in exponentially growing cultures. Previously, the cells were washed using Bold´s Basal mineral medium to remove adsorbed dye completely. The samples of each experimental unit were adjusted to the same optical-density value ($0.3_{680\ nm}$) before the photosynthesis and respiration study. The oxygen release rate (photosynthesis) was obtained by illuminating each of the samples with an actinic red light of 400 μmol m^{-2} s^{-1} for 1 min. Immediately after turning off the light source, the oxygen consumption rate (respiration) was recorded for 2 min.

2.6. Statistical analysis

All experiments were performed in triplicate, and the results obtained were statistically analyzed via one-way analysis of variance ($P<0.05$) and Tukey's test for comparison of means using the statistical package Sigma-Plot (version 11.0).

3. Results and discussion

3.1. Effect of Congo red on growth

The microalga *C. vulgaris* was affected after 96 h of exposure to Congo red. A decrease in growth rate (μ) was observed in every experimental unit after increasing the concentration of dye in the culture medium; this decrease was significant ($P<0.05$) for concentration over 15 mg L^{-1} compared to the control (Fig. 1). The μ value was 0.657 day^{-1} for cells with no dye, while the values of 0.538 and 0.391 day^{-1} were obtained when cells were exposed to 5 and 10 mg L^{-1} of dye, respectively. However, the μ values remained at 0.201 day^{-1} when cells were exposed to dye concentrations between 15 and 25 mg L^{-1}. Chu et al. (2009) reported μ values of 0.06–0.35 day^{-1} in cells of *C. vulgaris* exposed separately to 30 mg L^{-1} of Supranol Red 3BW, Lanaset Red 2GA, and Levafix Navy Blue EBNA. The cultures grown in textile dyes