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# Growth and photosynthetic utilization of inorganic carbon of the microalga *Chlamydomonas acidophila* isolated from Tinto river

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**Abstract**

Growth and utilization of inorganic carbon were investigated in the acidophilic microalga *Chlamydomonas acidophila*, isolated from Tinto river, in the spanish southwest mining area, in the province of Huelva. According to the chemical composition of its natural habitat, an artificial culture medium containing 4 g $l^{-1}$ $SO_4K_2$ was designed at pH 2.5 and used to grow the acidophilic microalgae in batch cultures. The cultures were bubbled with air enriched with 5% (v/v) $CO_2$ as unique carbon source. The culture reached the stationary growth phase 250 h after the experiment started up. The initial chlorophyll content was 5 μg $ml^{-1}$ (initial value, $t = 0$) and it increased up to 186 μg $ml^{-1}$ (maximum value). The growth rate was 0.52 $d^{-1}$, low value when compared to other non-extremophilic microalgae. Carotenoids to chlorophyll ratio continuously increased from 0.1 to 0.2 due to fast production of carotenoids. HPLC analysis of the accumulated carotenoids showed lutein to play a significant role in the antioxidant response of the microalgae. The photosynthetic utilization of inorganic carbon by *C. acidophila* was studied in liquid medium cultures growing with air enriched with 5% (v/v) $CO_2$. The maximum photosynthetic activity increased when increasing pH from 2.5 to 6. The apparent photosynthetic affinity constant for dissolved inorganic carbon ($K_{DIC}$) was 1.7 μM at pH 2.5 and it hardly changed at pH 4.0 and 6.0. These results show the acidophilic microalgae to have high affinity by $CO_2$ which seems not to depend on the presence of $HCO_3^-$. The growth of the acidophilic microalgae was found to be dependent on $CO_2$.


*Keywords:* *Chlamydomonas acidophila*; Extremophilic microalgae; Acidic stress; Carotenoids; Carbon utilization

## 1. Introduction

Over the last years cultivation of extremophilic microorganisms has gained interest due to their ability to accumulate and produce high value compounds, namely metabolites, enzymes, surfactants, etc. [1]. Microalgae have been widely recognized as producers of value compounds, carotenoids among them, having the advantage of growing in minimal culture media with an inorganic carbon source which prevents cultures from bacterial contamination [2].

Tinto river (Huelva, Spain), so-called "Red river" due to the high iron concentration of the water, is an acidic river that presents a constant pH value between 2 and 3 along 80 km. Such this low pH is due to sulphuric acid formation which appears as a consequence of both chemical and biological iron redox reactions-based activities [3]. The extreme oxidant conditions of Tinto river suggest that the microorganisms growing in the river should express different antioxidant mechanisms to defend themselves from oxidative stress. For that reason, our group intend to isolate microalgae living in this acidic environment and study their biotechnological potential as antioxidant producers.

Besides the advantage of acidophilic microalgae to selectively grow in extreme culture medium at low pH, it has been reported that growth rates are much lower than the so-called "common" microalgae [4]. The aim of the present work is to show that *Chlamydomonas acidophila* has potential for biomass production and accumulation of carotenoids of commercial interest, and therefore, it could become of biotechnological interest. In addition, this study was aimed to determine which pH

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conditions are best for *C. acidophila* to make better use of inorganic carbon and, consequently, to show faster growth and higher biomass production.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

*C. acidophila*, the algal material used in this study, was isolated from the acidic water of the Tinto river, which runs through a mining area in Huelva (Spain). In this river, the pH ranges during the year between 1.7 and 3.1.

An axenic culture of the alga was obtained by streaking it on basal agar medium at pH 2.5. Isolated colonies were transferred from the agar medium to the liquid medium. To prepare agar medium, 300 ml of 7% agar and 700 ml of modified K9 medium [5] (Table 1) were autoclaved separately and mixed before use. *C. acidophila* was maintained by periodic transfers in sterile modified K9 medium adjusted at pH 2.5 with concentrated $H_2SO_4$. Unless otherwise indicated, cultures were grown at 25 °C, bubbled with air containing 5% (v/v) $CO_2$ and continuously illuminated with fluorescent lamps (Philips TLD, 30 W, 200 μE $m^{-2}$ $s^{-1}$ at the surface of the flasks). The irradiance was measured with a photoradiometer Delta OHM (mod. HD9021).

### 2.2. Dry weight measurements

To measure the dry weight, 5 ml of each culture were taken and the liquid medium was removed by means of a vacuum pump using a cellulose acetate filter (weighed before using it) with 0.45 μm pore size from Sartorius (Goettingen, Germany) to separate the cells from the medium. The cells were then washed with demineralized water before drying the filters. The filters with the cells were dried and stoved at 70 °C. The filters with the dried cells were weighed after 24 h.

### 2.3. Oxygen evolution

The biological activity used to test cell viability was photosynthetic activity. For photosynthetic activity determinations 1 ml cell culture of the microalgae was placed into a Clark-type electrode (Hansatech, UK) to measure $O_2$-evolution. Measurements were made at 25 °C under saturating white light (1500 μE $m^{-2}$ $s^{-1}$) or darkness (endogenous respiration).

### 2.4. Photosynthetic activity (oxygen liberation) kinetics

The use of inorganic carbon by *C. acidophila* was studied by photosynthetic activity (oxygen liberation) kinetics in cultures growing at different pH values (2.5, 4.0 and 6.0). The oxygen liberation kinetics for each one of the cultures were studied placing algal samples into the Clark electrode under saturating PAR (photosynthetically active radiations, 1500 μE $m^{-2}$ $s^{-1}$) and registering the oxygen liberation as a function of the inorganic carbon concentration added into the electrode. The inorganic carbon was added in the form of $NaHCO_3$, partly converted into $CO_2$ as a function of the pH according to the chemical equilibrium $NaHCO_3/CO_2$ in water. The initial oxygen liberation rate was registered for each $NaHCO_3$ concentration added.

Table 1
Culture medium composition for *C. acidophila* growth

| Composition | Concentration |
|---|---|
| $K_2SO_4$ | 3.95 g $l^{-1}$ |
| KCl | 0.1 g $l^{-1}$ |
| $K_2HPO_4$ | 0.5 g $l^{-1}$ |
| $MgCl_2$ | 0.41 g $l^{-1}$ |
| $KNO_3$ | 2.29 g $l^{-1}$ |
| $CaCl_2$ | 0.01 g $l^{-1}$ |
| Hutner solution | 5 ml $l^{-1}$ |

### 2.5. Calculation of apparent affinity constants ($K_{DIC}$) for inorganic carbon

The apparent affinity constants ($K_{DIC}$) for inorganic carbon were calculated from data in Fig. 2. To calculate them, 1/PA (PA = photosynthetic activity) was plotted versus 1/[DIC]. $K_{DIC}$ were determined from linear regressions of the obtained curves, according to the equation:

$$1/PA = m(1/[DIC]) + 1/K_{DIC} \quad (m = \text{slope}).$$

### 2.6. Analytical determinations

Aliquots (1 ml) of the cultures were spinned down for 10 min at 5000 rpm. The supernatant was discarded and the pellet obtained was placed for 1 min in boiling water. After that the pellet was resuspended in 4 ml of absolute methanol to extract pigments. The suspension was shaken vigorously for 1 min and centrifuged for 10 min at 5000 rpm. Chlorophyll and total carotenoid concentrations were determined spectrophotometrically in the supernatant, using the equations proposed by Wellburn [6] or by HPLC analysis (see below).

Protein content was determined following the method described by Bradford [7]. The cell concentration was determined by measuring the optical density of the culture at 680 nm in a spectrophotometer. Nitrate was determined spectrophotometrically as described by Cawse [8].

### 2.7. HPLC analysis of carotenoids

Separation and chromatographic analysis of pigments was performed in a Merck Hitachi HPLC equipped with a UV–vis detector as described by Young et al. [9], using a RP-18 column and a flow rate of 1 ml $min^{-1}$. The mobile phase consisted on ethyl acetate (solvent A) and acetonitrile/water (9:1 v/v) (solvent B), and the gradient programme applied was: 0–16 min, 0–60% A; 16–30 min, 60% A; 30–35 min, 100%. Pigments detection was carried out at 450 nm, and their identification and quantification was achieved by injecting known amounts of pigment standards supplied by DHI-Water and Environment (Denmark).

### 2.8. Cell counting

The number of cells was determined by microscopy Olympus CX41 in a Neubauer chamber.

### 2.9. Statistics

Unless otherwise indicated, figures show means and standard deviations of three independent experiments.

## 3. Results and discussion

### 3.1. Growth kinetic of C. acidophila

The growth of *C. acidophila* in terms of dry weight is shown in Fig. 1. As shown, no adaptation period was observed. The pH was maintained between 2.2 and 3.4 by addition of sulphuric acid when required. The pH varies rapidly. *C. acidophila* may also grow at a higher pH (e.g. 4.0) with similar biological activity than that shown at pH 2.5 (Fig. 2). Therefore, one unit pH variation (2.2–3.4), the same variation that naturally occurs in the habitat of the microalgae (Tinto river), does not influence cell viability. After 350 h growth, an amount of 10 g biomass dry weight per liter was reached. Therefore, a biomass production yield of *C. acidophila* up to 10 kg/$m^3$ can be achieved under standard culture conditions, the biomass production of the acidophile strain being about two-fold that amount achieved in *Chlamydomonas*