Algal Research 18 (2016) 352–359

Contents lists available at ScienceDirect

Algal Research

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

Influence of temperature on *Chlorella vulgaris* growth and mortality rates in a photobioreactor

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ARTICLE INFO

Article history:
Received 24 December 2015
Received in revised form 10 May 2016
Accepted 15 June 2016
Available online xxxx

Keywords:
Microalgae
Photobioreactor
Temperature
Fluorescein diacetate
FDA viability
Cell mortality
Acclimation
Hinshelwood model

ABSTRACT

The ability of microalgae to fix carbon dioxide and convert it into biofuels, foods and other valuable products has drawn a lot of scientific attention in the last decades. In the last years a number of works aimed at understanding the influence of daily and seasonal temperature fluctuations that affect cell metabolism, and thus biomass production efficiency, have been carried out. However the impact of temperature on cell mortality has never been considered, while temperatures higher than the optimal growth temperature are often reached in summer for outdoor cultivation. This paper explores the effect of high temperatures both on mortality and growth for cultures of *Chlorella vulgaris* in a photobioreactor. Viability was measured with fluorescein diacetate (FDA), and thus mortality and growth rates were estimated, together with chlorophyll *a* and intracellular contents in carbon and nitrogen. While the fraction of viable cells decreased at higher temperatures, viable growth and mortality increased from 20 °C to 28 °C. Chl_a:C results suggest that temperature induced photoacclimation in the viable fraction of cells at higher temperatures. A Hinshelwood model was fitted to the data and appropriately described the mortality increase with temperature. Mechanisms affecting growth and mortality rates at high temperature are then discussed.

1. Introduction

The intensive use of fossil fuels is leading to the depletion of the world's current main energy source and intensifying climate change consequences [1–3]. The need for sustainable energy sources has gained significant importance within scientific community and microalgae have been proposed as a promising solution [1]. Microalgae are photosynthetic microorganisms able to fix carbon dioxide and convert it to organic matter that can be transformed into biofuels, foods, feeds and high-valuable products [4]. To produce microalgae photobioreactors or open ponds are generally used [5]. Microalgae show important advantages compared to plants such as, higher productivity and non-competition for agricultural lands. In addition they can grow in very different environments [6–9], such as extreme temperature, salinity and pH [10,11].

Light is the most studied parameter affecting photosynthetic efficiency [12], but temperature is also a key factor and far less understood [13]. Understanding the effect of temperature fluctuations on microalgae metabolism is crucial since the outdoor production systems – which are directly subject to the solar flux – can undergo very strong variations in temperature daily and seasonally [14,15]. Microalgae are typically able to develop over a wide range of temperatures between 15 and 35 °C [16], with a highly variable response between species. Temperature affects productivity at three different levels:

- From a physico-chemical point of view, it affects CO_2 and O_2 mass transfer rate and solubility in the medium, together with equilibrium/kinetics of the reactions [17],
- Strongly acting on all the cell enzymatic processes, it affects microalgae metabolism by modulating metabolic reactions, transport rate in the cell, cell regulation and finally shaping composition and structure,
- At high temperature, it also induces protein degradation, leading therefore to cell mortality.
- Temperature stress has been shown to strongly affect Reactive Oxygen Species (ROS) dynamics causing progressive oxidative damage and ultimately cell death [18,19].

Cell mortality takes place at temperatures in a range (depending on the species) between 20 °C and 45 °C [20]. Such temperatures can easily be reached in a microalgae cultivation device such as photobioreactors

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http://dx.doi.org/10.1016/j.algal.2016.06.016
2211-9264/

[15] or raceways [14] at midday in summer. In fact, temperature fluctuations from 10 to 45 °C may be easily observed in temperate regions [14], where the upper limit is indeed above the tolerated thresholds of most microalgae of interest [21]. It is therefore of key importance to unravel the effect of temperature on cell growth rate from its effect on mortality.

At moderate temperatures, growth rate is known to be enhanced by higher temperature, following the logic of an Arrhenius law [12]. But modifying the relative kinetic rates between the intracellular metabolic reactions, it also deeply affects cell composition. For example, De Oliveira et al. (1999) have found a decreasing trend in protein content together with increased amounts of carbohydrates and lipids in *Spirulina maxima* and *Spirulina platensis* when increasing temperatures from 20 up to 40 °C [22]. On the other hand *Nitzschia paleacea* produced the highest level of lipids at 10 °C while *Nitzschia clostridium* and commercial species of *Isochrysis* sp. produced their maximum amounts of lipids at 20 °C [23]. Thompson et al. [72] showed steady declines in lipid per cell as temperature increased from 10 to 25 °C in two *Chaetoceros* species. Another very interesting study was carried out by Maxwell et al. (1994) suggesting that at low temperature, growth mimicked high-light acclimation in *Chlorella vulgaris* cultures in terms of the ratio of chlorophyll *a*/*b*, total chlorophyll and xanthophyll contents and lower abundance in light-harvesting polypeptides [24].

The effect of temperature has been quantified with models describing microalgal net specific growth rate [25,26]. However, none of them explicitly include cell mortality at high temperature, and as a consequence, net growth rate is always positive. Not considering the effect of temperature in cell's viability when building a model may lead to inaccurate predictions of biomass productivities in outdoors facilities. As a matter of illustration, after an episode of extremely high temperature cells will die, while most existing models will only represent a zero growth rate, keeping the same biomass density for the next day. The models will then predict growth of this biomass the next day if more beneficial conditions are reached, while it is actually a dead biomass which will not be able to divide anymore. In order to improve their predictions, viable growth and mortality rates must be incorporated in the modeling approach.

In our study, we focus on *C. vulgaris* because of its biotechnological interests. It has been used in several areas such as bioremediation of textile wastewater [27] as well as production of biofuel [26], medicinal food [28] and nutritional supplements [29]. This microorganism is for example able to take up heavy metals from aqueous environments and was shown to help in cancer prevention and immune system support [30,31].

The goal of this work is to gain understanding in the effect of temperature on cell composition and kinetics of *C. vulgaris* cultures and to propose a model accounting for temperature induced mortality. For that, batch experiments of *C. vulgaris* grown at different temperatures were performed. The study was done in photobioreactors in order to better characterize cell growth and composition in realistic conditions accounting for the realistic stress conditions (agitation, high oxygen concentration, *etc.*) which may have a synergic effect with temperature. For each run, the cell composition was evaluated in terms of chlorophyll *a* and major chemical elements, and kinetics of growth were determined. The first step consisted of the validation of the FDA staining method with cultures of *C. vulgaris* to determine cell viability over the course of the experiments. Afterwards, growth (net and viable) and mortality rates were determined for the tested temperatures. The biochemical composition, *i.e.* chlorophyll and intracellular carbon and nitrogen contents are also presented. Finally the Hinshelwood model [32] is proposed to represent the growth and mortality processes.

2. Materials and methods

In order to assess the effect of temperature on *C. vulgaris* cultures, cells were grown at 20, 25, 28 and 30 °C in sterile conditions, maintaining all other environmental conditions constant, *i.e.* aeration rate, light intensity and CO_2 supply. The influence of temperature was assessed in terms of viability, growth (net and viable) and mortality rates and physiological contents in chlorophyll *a* and *b*, carbon and nitrogen. Replicate photobioreactors were carried out for each temperature (duplicates at 20 °C and 28 °C and triplicates at 25 °C).

2.1. Inoculum

The strain *C. vulgaris* CCAP 211/11B (Trebouxiophyceae) was obtained from the culture of algae and protozoa, CCAP (UK). The maintenance of the strain was done by frequent inoculations of 100 mL in 500 mL Erlenmeyer flask containing 3N-Bristol culture medium [33] at 25 °C, under continuous agitation, light intensity (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and an atmosphere of air fortified to 1.4 ± 0.5% CO_2 (v/v).

2.2. Photobioreactor and batch culture conditions

Experiments were carried out in a cylindroconical bubble-column photobioreactor, with a volume of 2.4 L and surface area of 0.1096 m^2. The light intensity was maintained constant at 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by 2 white and 2 pink ORAM fluorescent tubes (Biolux L36W/965 and Fluora L36W/77 respectively).

A continuous flow of air at 200 $mL \cdot min^{-1}$ (0.083 vvm) containing 2% CO_2 was used to supply CO_2 and ensure culture-mixing. The air stream passed through a 0.22 μm pore glass sinter filter before entering the reactor.

The photobioreactor (PBR) was inoculated with 20 mL (1% of PBR total working volume) of a culture in the exponential phase of growth (cell concentration at time zero was approximately 3×10^5 cells/mL). The culture medium was the same as that of the inoculums.

The temperature in the PBR was controlled at the target temperature by circulating water through the bioreactor's double envelope.

All cultures were grown for 3 days. Aliquots of 10 mL were withdrawn three times a day for total cell concentration, viability and pH measurements. At the end of the assay, on day 3, contents in carbon, nitrogen, chlorophyll *a* and *b* were determined.

2.3. Multicultivator experiments

Complementary experiments have been carried out in the PSI MC-1000 multicultivator (Photon Systems Instruments, Czech Republic), with 70 mL tubes. The cultivation vessel was inoculated with *C. vulgaris* suspension, in exponential growth phase, to establish a cell concentration of 3×10^5 cells/mL at time zero. The LED illumination system generated an incident light of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. A continuous flow of air at 220 $mL \cdot min^{-1}$ (3.14 vvm) was added to the vessel allowing mixing and supplying atmospheric CO_2 to the culture.

Assays were performed at 25 °C. In the reanimation experiments, cells were kept at 35 °C or 30 °C during 3 days and afterwards temperature was decreased down to 25 °C. Three replicates were carried out. Cell concentration and viability were measured over time. On the contrary to the photobioreactor experiments, a low cell density (lower than 2×10^6 $cells \cdot mL^{-1}$) was maintained by regular dilution of the medium.

2.4. Measurements

2.4.1. Sample staining with fluorescein diacetate (FDA) and viability analysis

Samples containing 1 mL of culture were first centrifuged at 12,000 rpm for 1 min and the supernatant was discarded. The pellet was then washed twice with PBS and finally dispersed in 200 μL of PBS added with 4 μL 2% FDA (Sigma-Aldrich, dissolved in acetone). The sample was kept in the dark for 5 min at room temperature. Ten microliters were then loaded onto the hemacytometer Thoma cell (2×128 spaces with $0.1 \times 0.1 \times 0.1$ mm of dimension, Grosseron) and a 10 min