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Evaluation of the simultaneous production of lutein and lipids using a vertical alveolar panel bioreactor for three *Chlorella* species

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ABSTRACT

The concept of a biorefinery improves the economic efficiency of a biofuel production process from microalgae by recovering high value added compounds. Lutein is a carotenoid currently extracted from petals of *Tagetes erecta* with an established market in poultry and in human nutritional supplements. For the very first time, an extended study on the lipid and lutein production over three *Chlorella* species as well as cell disruption methods was performed. *Chlorella vulgaris*, *Chlorella zofingiensis* and *Chlorella protothecoides* were grown in an indoor vertical alveolar panel photobioreactor with continuous illumination, and two cell disruption methods were assessed at a laboratory scale: glass bead vortexing and ball mill grinding. For *C. vulgaris*, *C. zofingiensis* and *C. protothecoides* the intracellular lutein content was measured as: 3.86, 4.38 and 3.59 mg g^{-1} respectively. Lipid contents vary slightly among microalgae with a value close to 9% w/w. Biomass and lutein productivities were found to be higher for *C. vulgaris* (0.131 gL^{-1} d^{-1}, 0.51 mg L^{-1} d^{-1}) and for *C. zofingiensis* (0.122 gL^{-1} d^{-1}, 0.53 mg L^{-1} d^{-1}) compared to *C. protothecoides* (0.103 gL^{-1} d^{-1}, 0.37 mg L^{-1} d^{-1}).
C. vulgaris 1803 and *C. zofingiensis* B 32 were found to be promising organisms for simultaneous production of lutein and lipids. Although all the microalgae under study belong to the same genus, a species-specific response was observed for each of the cell grinding methods tested.

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1. Introduction

In the last decade, biofuel production from microalgae has been intensively investigated. This is evident by the rapidly increasing number of research articles published and patents issued [1]. A number of advantages of microalgae over conventional oilseed crops explain this research effort: fresh water consumption can be reduced using wastewater or seawater microalgae; its production does not require agricultural land and a high biomass and oil productivity per acre can be attained [2,3]. A theoretical ceiling of 94 to 155 m^3 oil ha^{-1} yr^{-1} was calculated for microalgae assuming oil contents of 25% or 50% respectively [4] and a 10% photosynthetic efficiency [5]. Although these figures represent a theoretical value, when a realistic lipid productivity is considered (cf. supplementary material in ref. [4]), oil productivity of microalgae culture is still 4.65 and 23.4 times higher when compared to palm or sunflower oil productivities [3]. However, using realistic productivities, biofuel production can only be economically feasible when high value products are concomitantly produced [4]. A biorefinery approach in which both fuels and multiple value-added compounds are produced, or even microalgae production is synergistically coupled with carbon sequestration and wastewater treatment [6], might support the development of the microalgae energy industry. The potential for valuable co-products in algae processing has been cited as one of the key reasons for exploring this source of biofuels [7]. Besides polar and non-polar lipids, microalgae produce pigments and sterols with established market values [1,8]. Among the colored compounds, carotenoids are lipid-soluble molecules that play essential roles in photosynthesis. They contribute to light harvesting, scavenge reactive oxygen species and dissipate excess energy [9].

Lutein, a carotenoid found in flowers, food and human serum [10] has also been reported to be available in microalgae [11], has an established market in poultry and human nutritional supplements with a world market valued at 233 million USD in 2010 [12]. Along with zeaxanthin, lutein is present in the macula lutea, a small area in the retina [13] and in the crystalline lens [14]. Oral intake of lutein has been linked to a reduced risk of diseases such as age related macular degeneration [13,15], cataracts [16–18] and retinal degeneration [19].

Currently, the lutein production process involves the extraction of this compound from dried *Tagetes* sp. petals with organic solvents, saponification of the extract to remove waxes and fatty acids, and finally crystallization [20]. The product contains lutein as the major component and a smaller proportion of zeaxanthin. Lutein production from petals is

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a labor-intensive and land-demanding process. Moreover, the lutein content of *Tagetes* sp. petals is variable, ranging between 0.01% w/w and 0.31% w/w as free lutein[1] [21]. On the other hand, the following advantages can be ascribed to certain microalgae as a source of lutein: (i) high lutein content compared to marigold petals, from 2.28 mg g^{-1} in *Spirulina platensis* [22] to 10.13 mg g^{-1} in *Chlamydomonas acidophila* [23]; (ii) potentially homogeneous lutein content and productivity throughout the year regardless of weather, leading to precisely designed and optimized extraction processes; (iii) whole biomass processing methods that reduce labor-intensity compared to marigold processing; and (iv) besides lutein, valuable products such as fine lipids or proteins for food or feed can be obtained [11]. Despite the above mentioned advantages, due to the use of photobioreactors and extraction and purification processes, the process is capital intensive when compared to lutein production from marigold. This is why the selection of highly productive microalgae strains and optimization of the lutein content and volumetric productivity are key factors. Moreover, the control of operational factors such as temperature, irradiance and/or media composition should be assessed. In this regard, Sánchez et al. report the effect of irradiance and temperature on the continuous culture of *Scenedesmus almeriensis* [24]. They report a high sensitivity of the specific maximum growth rate (μ_{max}) towards temperature with an optimum value of 35 °C and a hyperbolic behavior of μ_{max} with respect to the average irradiance. In batch cultures, *Muriellopsis* sp. was selected among several chlorophycean microalgae as a potential source of lutein because of its short doubling time and high volumetric biomass productivity [25]. *Chlorella zofingiensis* also shows promising results [26]. Unfortunately, the lipid content of the microalgae was not reported in any of the aforementioned studies.

Since lipids and lutein are intracellular metabolites, cell disruption is necessary in order to recover them. Lutein and lipid recoveries vary considerably depending on the grinding method employed. In *Chlorella vulgaris*, lipid recovery was improved by a factor of 2.6 using a combination of conventional grinding, microwaves and sonication [27]. On the other hand, lutein recovery from *Chlorella* sp. was shown to be higher when grinding was used compared to sonication or microwave assisted cell disruption [28]. In *Scenedesmus obliquus*, lutein recovery was 5.4 times higher using a bead-beater compared to a sonicator or autoclave, and this difference was shown to be related to the extent of cell disruption [29].

This study focused on establishing the possible differences in productivity and content of lutein and lipids among the different species of *Chlorella*, and determining whether there is any difference in the recovery of lutein depending on cell disruption methods. Considering that the microalgae used in this study belong to the same genus, one would expect only minor differences in the studied parameters. However, this was not observed.

2. Materials and methods

2.1. Microalgae and culture conditions

C. vulgaris 1803, *C. zofingiensis* B 32 and *Chlorella protothecoides* 25 were purchased from the UTEX Collection (University of Texas at Austin, USA). The strains were maintained in a standard liquid inorganic medium (described below) at 21 °C under continuous light. Microalgae were cultivated in a BG-11 medium, containing per liter: 1500 mg $NaNO_3$, 3.05 mg KH_2PO_4, 6 mg ferric ammonium citrate, 1.81 mg $MnCl_2 \cdot 4H_2O$, 75 mg $MgSO_4 \cdot 7H_2O$, 0.079 mg $CuSO_4 \cdot 5H_2O$, 36 mg $CaCl_2 \cdot 2H_2O$, 0.222 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CoCl_2 \cdot 6H_2O$, 2.86 mg H_3BO_3, 6 mg citric acid $\cdot 1H_2O$ and 20 mg Na_2CO_3[30]. Air supply was not supplemented with CO_2, hence pH was controlled by means of a phosphate buffer containing per liter: 6.67 g K_2HPO_4 and 3.21 g KH_2PO_4. The pH was adjusted to 7.5 before sterilization at 121 °C for 15 min.

2.2. Experimental

2.2.1. Photoautotrophic growth of Chlorella sp. in a photobioreactor

A vertical alveolar panel (VAP) photobioreactor was used for the autotrophic growth of microalgae in this study. The VAP was made of Plexiglas with height, width and thickness of 173, 13 and 1.2 cm, respectively (2.6 L working volume). This photobioreactor has an illuminated surface/volume ratio equal to 166 m^{-1}. The VAP was built with four alveoli and two gas injectors at the bottom of the alternate alveolus to promote airlift circulation [31]. The VAP operated during 188 h at 21 °C with an aeration rate of 1.22 L min^{-1}. It was continuously illuminated with 2 cool white fluorescent light tubes (Philips 58 W) at the surface of the VAP, supplying a total light intensity of 4.2 klx (measured at the outside surface of the VAP with a Phywe Lux-Meter, Germany). The inoculum was grown in 1 L Schott bottles with a light intensity of 3 klx measured at its surface. Culture media were already described and the culture temperature was 21 °C. At the end of the culture, microalgal biomass was harvested by centrifugation at 10,000 rpm, at 15 °C for 15 min (Beckman, Avanti J-25l). The wet biomass pellet without further washing step was frozen at −18 °C and then freeze-dried. Samples were stored at −18 °C and then grounded.

2.3. Analytical methods

2.3.1. Biomass concentration

Samples were aseptically collected daily. Biomass concentrations were measured spectrophotometrically at 540 nm, in duplicate against distillate water in a GENESYS 20 Visible Spectrophotometer (Thermo scientific, USA). A linear correlation of dry cell weight concentration (X, gL^{-1}) versus optical density ($OD_{540\ nm}$) was previously obtained by collecting samples during a batch culture, yielding the following slopes (m), intercepts (b) and correlation coefficients: *C. vulgaris*, 0.3601, 0.0012, 0.999; *C. zofingiensis*, 0.5613, 0.0044, 0.998; and *C. protothecoides*, 0.5938, 0.0019, 0.999. Where m and b are the coefficients for the linear equation: $X\ (gL^{-1}) = m \cdot OD_{540\ nm} - b$.

2.3.2. Cell disruption

Two methods were tested in this study for cell disruption. Glass bead vortexing (GBV) was performed in 50 mL Falcon plastic centrifuge tubes with 0.12 g of freeze-dried sample, 1 mL of distilled water, 5 mL of acetone, 10 mL of 0.5 mm diameter glass beads and alumina (Type A-5, Sigma-Aldrich) as the abrasion agent, added in a 1:1 biomass to alumina ratio. Vortexing was conducted in a laboratory vortex close to its maximum speed for 30 s and repeated three times. Samples were allowed to cool down at ambient temperature for 30 s between each vortexing. 5 mL of acetone was added to the grounded sample, and this was centrifuged at 2000 *g* for 5 min to remove the alumina. The supernatant was collected and the pellet was resuspended with 5 mL of acetone and centrifuged again. This procedure (serial extraction) was repeated until the supernatant and the biomass were colorless.

Ball mill grinding (BMG) was performed with Retsch® Mixer Mill MM 400, using 50 mL stainless steel jar, and eight stainless steel balls (10 mm diameter). An amount of 0.5 g of freeze-dried biomass was placed inside the jar together with the balls. Further, the jar was placed on the mill for 3.5 min at the speed of 25 Hz (modified from Ref. [32]). An amount of 0.12 g of grounded sample was collected and mixed with 5 mL of acetone. The mixture was centrifuged at 2000 *g* for 5 min and a serial extraction procedure was applied until the biomass and supernatant were colorless (approximately 10 times).

After biomass disruption, extraction was performed in duplicate and results were analyzed by Student's *t*-test using MS-Excel™. A P value of less than 0.05 was taken as significant unless otherwise stated.

[1] As non-esterified lutein. For calculation purposes, molecular weight of "unknown compounds" in Ref. [21] was taken as the average value of those known.

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