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Dual-mode cultivation of *Chlorella protothecoides* applying inter-reactors gas transfer improves microalgae biodiesel production

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ABSTRACT

Chlorella protothecoides, a lipid-producing microalga, was grown heterotrophically and autotrophically in separate reactors, the off-gases exiting the former being used to aerate the latter.

Autotrophic biomass productivity with the two-reactor association, 0.0249 g L^{-1} h^{-1}, was 2.2-fold the value obtained in a control autotrophic culture, aerated with ambient air. Fatty acid productivity was 1.7-fold the control value.

C. protothecoides heterotrophic biomass productivity was 0.229 g L^{-1} h^{-1}. This biomass' fatty acid content was 34.5% (w/w) with a profile suitable for biodiesel production, according to European Standards.

The carbon dioxide fixed by the autotrophic biomass was 45 mg CO_2 L^{-1} h^{-1} in the symbiotic arrangement, 2.1 times the control reactor value.

The avoided CO_2 atmospheric emission represented 30% of the CO_2 produced in the heterotrophic stage, while the released O_2 represented 49% of the oxygen demand in that stage.

Thus, an increased efficiency in the glucose carbon source use and a higher environmental sustainability were achieved in microalgal biodiesel production using the proposed assembly.

1. Introduction

Microalgae are a promising, alternative source of oil for biodiesel production, showing many advantages over the present sources, mainly higher plants. The main advantages offered by microalgae are higher photosynthetic efficiency and biomass productivity when compared to plants. Additionally, microalgae cultures can use non-arable land and non-potable water, do not compete with food, and their production is not seasonal, allowing daily harvesting. The oil yield from algae is estimated to be 9-fold higher than the highest oil yield in plants, reported for palm (Chisti, 2007).

However, microalgal biomass production is, at present, still more expensive than growing plants. The key factors affecting profitability in this industry are capital costs, directly affected by biomass productivity, together with the market price of the products, presently only high-value products (Stephens et al., 2010).

Therefore, further research is necessary before economic viability of biodiesel production from algae can be attained.

To improve microalgae productivity, the use of fully controlled closed bioreactors is a necessary tool. When compared to open reactors, closed photobioreactors generally provide higher biomass concentrations, higher growth rates and reduced risk of microbial contamination (Tredici, 2004). Moreover, heterotrophic growth in fermenters can reduce the production cost of microalgal biomass by a factor of ten (Lee, 2004). The microalga *Chlorella protothecoides* is an excellent candidate for biodiesel production, since it can achieve high biomass productivity and high lipid content values, with a suitable fatty acid profile for biodiesel production, when grown under selected conditions. Furthermore, it is able to grow both photoautotrophically and heterotrophically, thus offering the alternative of photobioreactors and fermenters for its growth.

Several studies have been reported using *C. protothecoides* for a variety of purposes. In earlier studies the *C. protothecoides* strain CS-41 from the CSIR Marine Laboratory, Australia (Shi et al., 1999), was heterotrophically cultured in glucose-limited batch fermentation (3.7 L), at 28 °C, pH 6.6, agitation speed 480 rpm and dissolved oxygen above 50% of saturation, to produce lutein-rich biomass. Using an initial glucose concentration of 40 g L^{-1} the culture attained a maximum biomass concentration of 18.4 g L^{-1} after 178 h, with a productivity of 0.103 g L^{-1} h^{-1}; after glucose depletion, this *Chlorella* strain went on to attain a maximum lutein content of 4.4 mg g^{-1} biomass dry weight. The specific growth rate of this strain was 0.0417 h^{-1} and the yields on consumed

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glucose were, respectively, 0.48 g biomass dry weight per g and 1.9 mg lutein per g.

In another study, a *C. protothecoides* strain from the Algae Collection of UTEX (Xu et al., 2006), also cultured in heterotrophic conditions, attained a lipid content of 55% (w/w) (Bligh and Dyer extraction), which was 4-fold higher than the lipid content of the same algae cultured in photoautotrophic conditions. These authors attained, in a 5-L fermenter, 15.5 g L^{-1} biomass concentration after 184 h, using glucose in fed-batch operation. The biomass productivity achieved was 0.0842 g L^{-1} h^{-1} and the specific growth rate was 0.0405 h^{-1}. The obtained *C. protothecoides* biomass had a lipid content of 41.6% (w/w) containing, as major fatty acids, oleic acid (61%), linoleic acid (17%), palmitic acid (13%) and stearic acid (3%). This oil was then converted to biodiesel through acid transesterification and the final product proved to comply with all but one of the specifications established by the US standard (Xu et al., 2006).

Following these studies, several strategies were tested to improve the obtained results, aiming at the economic viability of lipid production for biodiesel with this microalga.

In a recent study, an integrated Photosynthetic Fermentation Methodology (PFM) was used consisting of two, sequential growth phases, namely, photosynthetic growth under excess of nitrogen source, followed by a glucose-limited, fed-batch fermentation (Xiong et al., 2010). Greatly improved biomass and lipid productivity levels were achieved, reaching 1.21 g L^{-1} h^{-1} and 0.7 g L^{-1} h^{-1}, respectively. In this study, however, CO_2 release into the atmosphere was not mitigated.

More recently, aiming at a more sustainable and economic process, a symbiotic strategy consisting of growing this algae in two separate bioreactors under two different nutritional modes was shown to improve CO_2 and O_2 utilization in photosynthesis and respiration (Santos et al., 2011). In this symbiotic bioreactor association, dissolved CO_2 required for photoautotrophic growth of the microalgae was supplied by the off-gas from the heterotrophic microalgae growth stage. No measurements for CO_2 quantification were done in this study, but the photoautotrophic biomass productivity was enhanced by 55% in a 5-day growth run, compared to a control run supplied with ambient air.

In the present work the two-bioreactor association presented earlier (Santos et al., 2011) was scaled-up in order to improve the biomass and lipid productivities. Namely, reactor configurations closer to full-scale production reactors were used and process control was improved. The tested assembly consisted of a stirred fermenter supplying its CO_2-enriched off-gas to a vertical alveolar panel (VAP) photobioreactor. The fermenter was operated in fed-batch mode in order to provide an extended supply of CO_2 to the batch-operated VAP, to support the slower biomass growth in the latter.

The microalgal lipid content and cell viability were monitored by multi-parameter flow cytometry during the cultivations, for a better understanding of the process in near real time. Such information would allow optimal actions to be taken during the biological processes.

The aim of this work was to demonstrate the benefits of growing the microalgae *C. protothecoides* in such a two-reactor association, to obtain single cell oil for biodiesel and a high-value product (carotenoids) to approach economic viability.

2. Methods

2.1. Microalgae

The microalga *C. protothecoides* strain 25 was purchased from the UTEX Collection (Texas University of Austin, USA). The strain was maintained on two different standard liquid media: an inorganic medium (Section 2.2) at room temperature (22 °C) under continuous illumination and an organic medium (Section 2.2) at room temperature under normal daylight.

2.2. Culture media

C. protothecoides was cultivated autotrophically in an inorganic medium containing, per litre: 1.25 g KNO_3, 1.25 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 0.11 g $CaCl_2{\cdot}2H_2O$, 0.5 g $NaHCO_3$, 0.1 mg FeEDTA·$3H_2O$, and 10 mL trace elements solution (Vonshak, 1986). The trace elements solution contained, per litre: 286 mg H_3BO_3, 154 mg $MnSO_4{\cdot}H_2O$, 22 mg $ZnSO_4{\cdot}7H_2O$, 5 mg $CuSO_4$, 6 mg $Na_2MoO_4{\cdot}2H_2O$ and 8 mg $CoSO_4{\cdot}6H_2O$.

C. protothecoides was heterotrophically maintained in a complete organic medium containing, per litre: 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4{\cdot}H_2O$, 3 mg $FeSO_4{\cdot}7H_2O$, 10 g glucose, 0.1 g glycine, 10 μg vitamin B1 and 1 mL trace elements solution (Xiong et al., 2010). The trace elements solution was Arnon A5, with the following composition, per litre: 0.222 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, 0.015 g MoO_3, 2.86 g H_3BO_3, and 1.81 g $MnCl_2{\cdot}4H_2O$.

C. protothecoides fermentation was carried out in simple organic medium containing, per litre (Santos et al., 2011): 20 g dextrose monohydrated, 5 g yeast extract and 2 g Red Sea salt.

2.3. Experimental

2.3.1. C. protothecoides growth in a fermenter

A classical fermenter (SGI Set 002, Toulouse, France) of 2-L capacity (1.3 L working volume), with pH and temperature controls, and stirring speed (1 turbine agitator) and dissolved oxygen (DO) monitoring, was used to grow *C. protothecoides* in aerobic heterotrophic mode. Aeration rate was kept at 2 vvm, temperature at 28 °C, and the initial pH value in the medium was set to 6.5 through the addition of NaOH 2N or HCl 2N.

The organic medium (Section 2.2), without glucose, was introduced in the fermenter and sterilized for 30 min at 121 °C. Glucose was heat sterilized separately and added after cooling.

The inoculum was an exponentially growing culture (3 days growth) on the standard organic medium used for culture maintenance. A 300-mL portion of inoculum was used to inoculate the fermenter. Polypropylene glycol (PPG, 200 μL) as anti-foam agent and 1 mL of antibiotics solution (Santos et al., 2011) were added to the culture, after one day of growth. The dissolved oxygen level was kept above 40% of air saturation by acting manually on the stirring speed. The sole fed-batch feed, comprised of a pulse of 20 g glucose in 100 mL distilled water and another of 5 g yeast extract in 100 mL distilled water, added in this order, was introduced at the end of the first exponential growth phase (after 48 h).

2.3.2. C. protothecoides growth in a photobioreactor

The vertical alveolar panel (VAP) configuration was used for the photobioreactor to grow *C. protothecoides* in autotrophic mode. The VAP was made of Plexiglas 173 cm in height, 13 cm in width and 1.2 cm in thickness (2.6 L working volume). The photobioreactor had an s/v ratio equal to 166 m^{-1}, s being the surface area of the two illuminated lateral panels area and v the working volume. The VAP was specified with 4 alveoli and two gas injectors at the bottom of alternate alveoli to promote air-lift circulation. A dissolved oxygen probe (dissolved oxygen galvanic type plus ATC/Pt1000, model SZ12T, connected to a multi-parameter data logger, model 3021, both from Consort, Belgium) was inserted at the top of the VAP to measure dissolved oxygen and temperature in the culture.

The VAP operated with an aeration rate of 1 vvm, illuminated continuously with 4 cool white lights (Philips 58W) assembled in pairs on each lateral side of the two VAPs, supplying an average