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Continuous culture methodology for the screening of microalgae for oil

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ABSTRACT

A basic criterion in the selection of microalgae suitable as source of oil for biodiesel should be their actual capacity to produce lipids or, more properly, the fatty acid yield. Performance assessment of 10 preselected microalgae under both batch and continuous culture points to the latter approach as the most adequate for evaluating fatty acid productivity. Differences were patent in continuous culture among strains that otherwise had analogous oil accumulation potential under batch culture. Some promising strains under batch culture (like *Muriella aurantiaca* and *Monoraphidium braunii*) exhibited, however, values for actual fatty acid productivity lower than 40 mg L^{-1} d^{-1} in continuous regime. The analysis performed in photochemostat under continuous culture regime revealed the great potential of *Chlorococcum olefaciens*, *Pseudokirchneriella subcapitata* and *Scenedesmus almeriensis* as oil producing microalgae. Fatty acid productivity levels over 90 mg L^{-1} d^{-1} were recorded for the latter strains under moderate nitrogen limitation, conditions which led to an enrichment in saturated and monounsaturated fatty acids, a more suitable profile as raw material for biodiesel. The continuous culture methodology employed represents a sound procedure for screening microalgae for biofuel production, providing a reliable evaluation of their fatty acid production capacity, under conditions close to those of outdoor production systems.

1. Introduction

The use of microalgae as source of oil for biodiesel production is an issue of current general interest (Chisti, 2007; Hu et al., 2008). Since strain selection represents the first key step toward the development of an oil production process from microalgae (Griffiths et al., 2012; Rodolfi et al., 2009), the establishment of suitable selection criteria and appropriate experimental systems for screening are of outmost relevance.

Most of the information regarding lipid accumulation in microalgae is solely based on the lipid content and derives from studies performed in batch cultures, being highly diverse and heterogeneous with regard to both strains and experimental conditions used. Reported cellular lipid levels generally correspond to those found in the stationary phase, which is characterized by severe limitation in nutrients and light availability. Values as high as 70% of the dry biomass have been reported, although 20–50% are more common (Chisti, 2007).

The selection of algal strains for the purpose of oil production must consider not only the lipid content (more properly, that of fatty acids) but also the biomass generation capacity, as to define the oil yield or productivity (Griffiths et al., 2012; Pruvost et al., 2011; Rodolfi et al., 2009). Being essential the adoption of this parameter as key criterion, care has to be taken on how it is evaluated. A reliable and precise measurement of actual lipid/fatty acid productivity can be performed in the steady state situation of a continuous culture, in which the productivity of biomass and its composition (including lipid/fatty acid content) keep stable. Nevertheless, such a situation does not apply to the batch culture, in which growth rate and biomass composition change as growth progresses.

In the present work, a screening strategy for the selection of oil-producing microalgae is derived from experimentation with 10 strains, for which fatty acid productivity is evaluated. The continuous culture, especially operating under moderate N limitation, emerges clearly as the option of choice. The screening conditions

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include daylight cycle illumination, as to mimic those prevailing outdoors, in which production systems operate.

2. Materials and methods

2.1. Microalgae and culture conditions

The following 10 microalgae were used: *Chlorella fusca* (SAG 211-8b), *Chlorococcum oleofaciens* (SAG 213-11), *Monoraphidium braunii* (SAG 202-7d), *Muriella aurantiaca* (SAG 249-1), *Muriella decolor* (SAG 249-2), *Muriellopsis* sp. (Del Campo et al., 2000), *Neochloris oleoabundans* (UTEX 1185), *Pseudokirchneriella subcapitata* (SAG 61-81), *Scenedesmus almeriensis* (CCAP 276-24) and *Tetraselmis suecica* (ICMAN, Cádiz 03-0203).

Cells were grown photoautotrophically on the medium described by Arnon et al. (1974) modified to contain 4 mM K_2HPO_4, with the exception of *T. suecica*, for which f/2 medium (Guillard and Ryther, 1962) was used. The media were supplied with $NaNO_3$, as to reach the final nitrate concentrations indicated in each case.

Batch culture was performed at 25 °C, in 4.5 cm diameter 0.2 L capacity cylindrical flasks containing 0.15 L of culture, laterally illuminated with fluorescent lamps providing 115 $\mu E\,m^{-2}\,s^{-1}$ on the surface of the flasks. The culture was continuously sparged with air (40 L (L culture^{-1}) h^{-1}) supplemented with 1% CO_2. The culture medium and the vessels were sterilized in autoclave at 120 °C for 20 min.

Continuous culture was performed in 2.0 L capacity (0.07 m diameter, 0.50 m height) jacketed sterilized photochemostat (bubble columns) containing 1.8 L of cell suspension, continuously sparged with air (33 L (L culture^{-1}) h^{-1}). Temperature was maintained at 25 °C, and pH at 7.5 by on demand injection of CO_2 into the air stream entering the culture. The photochemostat was illuminated following a light cycle of 12 h light/12 h dark, with simulated solar daylight cycle during the light period, using six Phillips PL-32 W/840/4p white-light lamps, which provided a maximal incident irradiance of 2500 $\mu E\,m^{-2}\,s^{-1}$ on the reactor surface. Initially, the reactors were inoculated with batch-grown cells and operated on batch mode for about 3–4 d, with incident irradiance being progressively increased, until becoming close to stationary phase. Then, it was switched to operate on continuous mode, fresh medium being continuously fed during the light period at a flow rate of 45 mL h^{-1} (dilution rate (D), 0.3 d^{-1}), with withdrawal of culture at the same rate. Once steady state conditions were achieved, analytical determinations were performed. The data presented correspond to stabilized situations, being average values of 4 to 8 determinations of each steady state. Measurement of photosynthetically active irradiance was carried out using a 4π quantum scalar irradiance sensor QSL-100 (Biospherical Instrument, San Diego, CA), inside the vessel (without cells).

2.2. Analytical procedures

Biomass concentration was determined by dry weight measurement. Ten milliliter aliquots of the cell suspension were filtered through pre-weighed filters (1.2 μm or 0.45 μm, depending on the cell size). Cells were washed with either distilled water or 1% ammonium formate, for freshwater or marine microalgae, respectively. The filters containing the algae were weighted after drying in an oven at 80 °C during 24 h.

Fatty acids were analyzed on 10 mg of freeze-dried biomass, after transesterification of the dry lipid fraction (Rodríguez-Ruiz et al., 1998). The lipid fraction was obtained by mechanical disruption of cells employing a Mini Bead Beater (Biospec Products), using chloroform:methanol (2:1, v/v) as extracting solvent and 0.5 mm diameter glass beads. Ten microliter of an internal standard, containing 10 mg of either heptadecanoic or nonadecanoic acid in 1 mL toluene, was included in the disruption step. For transesterification, 2 mL of methylation mixture (methanol:acetyl chloride, 20:1 v/v) and 1 mL hexane were added to the dry lipid fraction and heated at 100 °C for 20 min. After cooling to room temperature, 2 mL distilled water were added. Following centrifugation (5 min, 1500 × *g*), the upper hexane phase was injected into the gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector. A Suprawax-280 (15 m × 0.1 mm × 0.1 μm) capillary column (Teknokroma) was used, with He as carrier gas. Temperature was programmed to increase from 150 (hold 4 min) to 200 °C at 15 °C min^{-1} (hold 21 min), then to 250 °C at 15 °C min^{-1} (hold 7 min). Injector and detector were kept at 250 °C and 270 °C, respectively. For determination of fatty acid content (% of dry biomass), peaks between C14 and C24 were integrated. Retention times for individual fatty acids were previously calibrated with a mixture of methyl esters (SupelcoTM 37 Fame mix, no. 47885U; PUFA-3 Matreya LLC, no. 1177). The percentage of each fatty acid was expressed in relation to the total sample (% of total).

Table 1
Biomass concentration and fatty acid content of microalgae in batch culture (7th day, stationary phase).

Strain	Biomass concentration (g L^{-1})	Fatty acids (% of dry biomass)
Chlorella fusca	5.88 ± 0.17	11.67 ± 1.06
Chlorococcum oleofaciens	5.51 ± 0.22	33.62 ± 1.90
Monoraphidium braunii	4.08 ± 0.41	20.39 ± 0.86
Muriella aurantiaca	5.29 ± 0.19	21.96 ± 2.04
Muriella decolor	3.02 ± 0.06	11.21 ± 0.21
Muriellopsis sp.	6.34 ± 0.34	14.16 ± 0.33
Neochloris oleoabundans	1.74 ± 0.01	16.66 ± 3.10
Pseudokirchneriella subcapitata	2.42 ± 0.11	20.49 ± 0.91
Scenedesmus almeriensis	3.22 ± 0.09	20.43 ± 2.36
Tetraselmis suecica	2.76 ± 0.29	11.35 ± 0.96

2.3. Productivity calculation

Productivity of biomass (*Pb*) and that of fatty acids (P_{FA}) were estimated in steady state situations of continuous cultures: $Pb = Cb \times D$; and $P_{FA} = Pb \times X_{FA}$; where; *Cb* is the concentration of biomass; *D* is the dilution rate; and X_{FA} the mass fraction of fatty acid in the biomass.

3. Results and discussion

3.1. Batch culture

The potential of 10 preselected microalgae for lipid accumulation has initially been evaluated in 7-day old batch cultures, when nitrate in the culture (10 mM at $t = 0$ h) was exhausted (data not shown). At this stage of the batch culture, *C. oleofaciens*, *M. braunii*, *M. aurantiaca*, *P. subcapitata* and *S. almeriensis* exhibited a fatty acid content of at least 20% of the dry biomass, outstanding *C. oleofaciens*, with 34%. Under the same conditions, the fatty acid levels of *M. decolor*, *T. suecica*, *C. fusca*, *Muriellopsis* sp. and *N. oleoabundans* were between 11 and 17% (Table 1). According to the prevalent trend, the relative potential for fatty acid accumulation of the different strains could be predicted on the basis of the fatty acid content alone, or considering as an additional reference the biomass accumulation capacity. Total biomass accumulated in the stationary phase (day 7) was over 5 g L^{-1} for *C. oleofaciens* and *M. aurantiaca*, with the highest accumulation of biomass (around 6 g L^{-1}) corresponding to *C. fusca* and *Muriellopsis* sp. From the results of batch growth experiments, the group of most promising strains would thus include *C. oleofaciens*, *M. aurantiaca*, *Muriellopsis* sp. and *M. braunii*, whereas *T. suecica*, *M. decolor*, *P. subcapitata* or *N. oleoabundans* would appear