Bioresource Technology 161 (2014) 297–303

Contents lists available at ScienceDirect

Bioresource Technology

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

Enhanced lipid production in *Chlorella pyrenoidosa* by continuous culture

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HIGHLIGHTS

- *Chlorella pyrenoidosa* XQ-20044 is able to accumulate lipids in growing cells.
- One step production of algal lipid was achieved in chemostat culture.
- Proper SNI was the key for simultaneous algal growth and lipid accumulation.
- Lipid productivity was significantly enhanced by continuous culture.

ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form 12 March 2014
Accepted 16 March 2014
Available online 25 March 2014

Keywords:
Chlorella
Growth
Lipid productivity
Continuous culture
Chemostat

ABSTRACT

Usually microalgae growth and lipid accumulation do not run in parallel throughout cultivation, which necessarily lowers overall lipid productivity. However, we show through batch and feed-batch studies of *Chlorella pyrenoidosa* XQ-20044 that by varying the nitrate concentration, conditions which produce fairly high lipid content could be achieved without sacrificing algal growth. Simultaneous microalgae growth and lipid production was achieved in continuous chemostat culture when the specific nitrate input rate was in the range of 0.78–4.56 mmol g^{-1} d^{-1}. Moreover, the maximum lipid productivity (144.93 mg L^{-1} d^{-1}) in the continuous culture was significantly higher than in batch culture (96.28 mg L^{-1} d^{-1}), thus indicating the feasibility and great advantage of one-step production of microalgal lipids.

1. Introduction

Aquatic microalgae have a high capacity for photosynthesis, and many are able to use excess photosynthetically-fixed carbon for synthesis of neutral lipids, which are also known as triacylglycerols (TAGs) (Chisti, 2007). Oleaginous microalgae are thus considered to be ideal raw materials for biodiesel production, especially if their growth is coupled to the direct bio-fixation of waste CO_2 (Kwak et al., 2006). Over the past 50 years the concept and feasibility of microalgal biodiesel have been discussed extensively (Wijffels and Barbosa, 2010), but this renewable energy source has yet to be exploited. Limiting factors include the lack of appropriate microalgal strains, less-than optimal lipid productivity and ineffective culturing techniques for lipid accumulation.

To date, several hundred oleaginous species have been isolated and characterized (Gouveia et al., 2009). A common thread in these studies is that cell growth and lipid accumulation do not happen at the same time during cultivation (Lourenco et al., 2002; Merzlyak et al., 2007), which results in lower overall lipid productivity. In order to overcome this, researchers have explored two-stage cultivation strategies to enhance microalgal lipid production. In such strategies, which have been used mainly with batch cultures, the microalgal cells first grow rapidly under growth-optimized conditions, and then are transferred to conditions where light irradiance (Zhang et al., 2009), nutrition (Su et al., 2011), culture pH (Han et al., 2013), as well as other factors (Das et al., 2011; Liu et al., 2008) are adjusted to promote lipid accumulation at the expense of cell growth.

However, detailed study of the stress response of oleaginous microalgae under nitrogen deficiency may provide a foundation for the production of biomass and lipids in one step. Recent data showed that while *Neochloris oleoabundans* UTEX #1185 accumulated lipid under relatively high nitrogen stress conditions (i.e., low nitrogen levels), its growth was not severely limited (Adams

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http://dx.doi.org/10.1016/j.biortech.2014.03.077
0960-8524/

et al., 2013). In contrast, growth of *Chlorella vulgaris* UTEX #265 was severely limited under high nitrogen stress, but lipid accumulation was triggered ahead of the growth limitation (Adams et al., 2013). These may or may not be species-specific characteristics, but the results have inspired the authors to explore the threshold values of nitrogen concentration and/or supply rate, in order to establish a continuous cultivation system for concurrent production of biomass and lipids. It is hypothesized that by properly regulating the nitrate concentration (e.g., severe stress or moderate stress) in continuous culture, the algal cells could grow at a reasonable rate and accumulate lipid simultaneously, thus leading to higher lipid productivity over batch culture.

Along this line, one-step astaxanthin production was achieved with continuous culture of *Haematococcus pluvialis*, where >0.8% (DW) astaxanthin accumulated in the green vegetative cells (Del Rio et al., 2005). However, only a few studies have been published on the continuous cultivation of microalgae for lipid production. Sobczuk and Chisti (2010) investigated the effects of dilution rate on lipid productivity of the freshwater microalga *Choricystis minor* in chemostat culture, and concluded that the lipid content did not change significantly with various dilution rates. Very similar results were reported for chemostat cultures of *Chlorella minutissima* and *Dunaliella tertiolecta* by Tang et al. (2012). In contrast, Klok et al. (2013) reported that excess light combined with a growth-limiting nitrogen supply resulted in TAG accumulation (up to 12.4%, w/w) and cell replication in turbidostat cultures of *Neochloris oleoabundans*. Given the quite different results of Klok et al., it is an open question how other microalgal species will respond in continuous culture.

In this report, batch and feed-batch cultures of *C. pyrenoidosa* were used to study its response, in terms of growth and lipid accumulation, to different nitrogen concentrations. The results were used to establish chemostat cultures with different dilution rates, in order to verify the assumption that microalgal cultures can be regulated to grow and accumulate lipids concurrently, thus enhancing lipid productivity.

2. Methods

2.1. Strains and pre-culture conditions

C. pyrenoidosa XQ-20044 was used in this study. It was provided by the Algae Culture Collection of Wuhan Botanical Garden, Chinese Academy of Sciences. This fast growing strain was originally isolated from a *Spirulina* culture pond in Sichuan province, China. Its lipid content in a column photobioreactor was >45% of dry biomass (unpublished results), thus showing its potential as a biodiesel feedstock.

For seed cultures, the algal cells were grown photoautotrophically in Erlenmeyer flasks at 25 °C. The flasks contained 600 mL of medium, and were placed on a shaker (100 rpm) for 48 h of cultivation before further experiments. Continuous illumination was provided by fluorescent lamps, and the light intensity on the surface of the flask was adjusted to 50 μmol m^{-2} s^{-1}. The basal growth medium for seed culture and culture experiments was a modified BG11 medium which had the following composition (per liter): $NaNO_3$, 100 mg; $K_2HPO_4{\cdot}3H_2O$, 40 mg; $MgSO_4{\cdot}7H_2O$, 75 mg; $CaCl_2{\cdot}2H_2O$, 36 mg; Citric acid, 6 mg; Fe-Ammonium citrate, 6 mg; EDTA·Na_2, 1 mg; Na_2CO_3, 20 mg; H_3BO_3, 2.86 μg; $MnCl_2{\cdot}4H_2O$ 1.8 μg; $ZnSO_4{\cdot}7H_2O$, 0.22 μg; $CuSO_4{\cdot}5H_2O$, 0.08 μg; $Na_2MoO_4{\cdot}2H_2O$, 0.391 μg; $Co(NO_3)_2{\cdot}6H_2O$, 0.0494 μg (Grobbelaar, 2007). Sodium nitrate concentration was modified as indicated in the text.

2.2. Batch cultures

Batch cultures of *C. pyrenoidosa* XQ-20044 were grown in aerated-column photobioreactors. The seed cultures were centrifuged, rinsed, and resuspended in nitrate-free BG-11 medium to an optical density of 0.5 ± 0.05 at 540 nm. Then aliquots of this suspension were transferred to the glass columns (inner diameter 3 cm), which were submerged in a water tank. A thermostatic water circulator was used to provide 30 °C water bath for the culture columns. CO_2-enriched (1%, v/v) air was passed through a sterile filter and then bubbled into the bottom of each column at a flow rate of 250 ml min^{-1}. Light (300 μmol m^{-2} s^{-1} irradiance impinging on the surface of each column) was continuously provided by ten Phillips 36 W cool white fluorescent lamps. A concentrated sodium nitrate stock solution (autoclaved and stored at 4 °C) was used to adjust the columns to initial nitrate concentrations that ranged from 0.24 mM to 17.65 mM. And for each nitrate concentration, three replicate cultures were conducted in parallel. After 8 d of cultivation, cells were collected by centrifugation (5000 rpm at 15 °C for 5 min) and lyophilized (−56 °C cryotrapping, 10–14 Pa vacuum) for further analysis.

2.3. Feed-batch cultures

Feed-batch culturing of *C. pyrenoidosa* XQ-20044 was carried out similar to the batch cultures, except nitrate (in concentrations of 0.24, 0.48, 0.72, 0.96, 1.18, 2.35 and 3.53 mM) was added to the photobioreactors at 0, 24, 48, 72, and 96 h of cultivation. In the batch cultures with an initial nitrate concentration >3.53 mM, the residual nitrate level after 24 h was >50% of the initial value. Thus, only nitrate concentrations ranging from 0.24 mM to 3.53 mM were investigated in these feed-batch cultures.

2.4. Chemostat cultures

A peristaltic pump with a ten-roller pump head was used for the continuous cultures. It delivered a maximum flow rate of 32 mL min^{-1} and a minimum flow rate of 0.1 mL min^{-1}. Exactly 2000 mL of algae were grown in a closed column bioreactor with an inner diameter of 10 cm. A silicone tube was connected to the bottom of the column to form a U-shaped overflow tube. Air enriched with CO_2 (1%, v/v) was passed through a sterile Millex syringe filter (0.22 μm) and then bubbled into the bottom of the culture at a flow rate of 2 L min^{-1}; this continuously agitated the cells, so they received equivalent light. The culture pH was maintained in the range of 7–8, and continuous illumination (600 μmol m^{-2} s^{-1} photon flux) was provided by four fluorescent lamps (Philips MASTER PL-L 36 W) on one side of the bioreactor. The whole cultivation unit was placed in a thermostatic room to maintain the culture temperature at 30 °C.

Cultivations (in triplicate) were carried out initially in batch mode by inoculating the seed culture into BG-11 medium to an optical density of ~0.1 (540 nm). After 30 h of cultivation, when the optical density of the algal suspension had increased to 1.0 or more, the bioreactor was switched to continuous mode by feeding BG-11 medium (0.71 mM sodium nitrate). Cultivations with different dilution rates (ranged from 0.24 d^{-1} to 2.4 d^{-1}) were carried out successively. Achievement of steady-state condition at each dilution rate was monitored by daily measuring of the optical density, biomass dry weight, and residual nitrate concentration. The steady state was maintained at least for three days before further determinations.

2.5. Analytical methods

Biomass dry weight was measured to evaluate microalgal growth. About 10 mL algal suspension was filtered through a pre-dried GF/C glass microfiber filter paper (0.45 μm), which was dried at 80 °C under vacuum for 4 h (Lee et al., 1996) and re-weighed to