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High Cell Density Cultures of Microalgae under Fed-batch and Continuous Growth

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Microalgae are pointed as one of the best alternatives for biofuels production due to high high lipid accumulation capacity. However, large scale production often results in low cell concentrations and low volumetric productivities. Heterotrophic cultures of microalgae can reach high biomass (over 100 g L^{-1} DCW - dry cell weight) and lipid (over 50 % m/m) concentrations reducing downstream costs and increasing volumetric productivities. In this work fed-batch and continuous systems were evaluated for heterotrophic high cell density (> 10 g L^{-1}) cultures of microalgae *Chlorella sp.* for biomass and lipids production. In fed-batch growth a two stage feeding strategy (nitrogen sufficient and nitrogen limited) was employed. For continuous cultures different dilution rates were evaluated. Fed-batch culture achieved maximum biomass concentration and productivity of 58.8 g L^{-1} DCW and 6.6 g L^{-1} d^{-1}, respectively. Under nitrogen limitation an increase of 38 % in lipid content (from 21 to 29 % m/m) and 26 % in overall lipid productivity (from 1.25 to 1.58 g L^{-1} d^{-1}) was observed. Continuous growth of microalgae started at a biomass concentration of 30 g L^{-1} DCW. At the dilution rate of 0.02 h^{-1} biomass concentration remained around 19 g · L^{-1} DCW with a lipid content of 18 % under steady state conditions, resulting in biomass and lipid productivities of 9.1 and 1.5 g L^{-1} d^{-1}, respectively. The results show that high biomass and lipid productivities can be attained in batch and continuous processes when high cell densities are applied.

1. Introduction

Most of the research on microalgae is concentrated on photosynthetic metabolism, which is limited by light irradiance and mutual shading of cells at high cell concentrations. Nevertheless, heterotrophic microalgae can be grown in the dark in the presence of an organic carbon source and potentially deliver higher yields of biomass and lipids (Liang, 2013).

Harvesting is considered to be the most costly and energy intensive stage in microalgae biomass production due to the low biomass concentrations achieved in traditional autotrophic cultivation (Zamalloa et al., 2011). Raceway ponds can typically achieve 0.5 - 1.0 g L^{-1} DCW (dry cell weight) while tubular photobioreactors will generally reach a maximum of 4 g L^{-1} DCW (Rawat et al., 2012). Higher concentrations produced under heterotrophic conditions can significantly reduce harvesting costs and energy use by reaching concentrations as high as 100 g L^{-1} DCW (Tabernero et al., 2012).

High cell density cultures are usually those with dry cell weight values from 10-20 g L^{-1} up to more than 100 g L^{-1}, however there is no clear definition for the term in literature (Shojaosadati et al., 2008, Bunch, 1994). For microalgae, in recent literature the term has been used for microalgae concentrations as high as 20 g L^{-1} (Bumbak et al., 2011).

High cell densities of microorganisms are typically achieved with fed batch cultivation methods. These are based on feeding highly concentrated nutrient solutions in bioreactors where the biomass is retained. The highest microalgal concentrations found in literature were attained using fed batch heterotrophic processes (Bumbak et al., 2011).

An alternative to fed batch operation is the continuous process, which is usually highly productive due to the reduced process downtime for cleaning, sterilization and setup when compared with batch and fed batch processes. Also, since it operates in steady state conditions, control of the process can be more simple and precise. Most studies involving continuous microalgae cultivation are done in photobioreactors

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in autotrophic conditions. Few studies are found in literature for heterotrophic continuous culturesof microalgae and even fewer operate at high cell densities.
The objective of this study is to study both fed batch and continuous cultivation mode for obtaining high cell densities of microalgae and high productivities of biomass and lipids.

2. Materials and Methods

2.1 Microalgae strain and cultivation methods

The *Chlorella sp.* strain was obtained from Canadian Phycological Culture Centre. Stock cultures were maintained axenically on synthetic modified BBM medium with the following composition (mg L^{-1}): Na_2EDTA (50), KOH (3.1), $CaCl_2.2H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), MoO_3 (0.71), $Fe_2SO_4.7H_2O$ (4.98), H_2SO_4 (1 µL/L), H_3BO_3 (11.42), $ZnSO_4.7H_2O$ (8.82), $MnCl_2.4H_2O$ (1.44), $CuSO_4.5H_2O$ (1.57), $Co(NO_3)_2.6H_2O$ (0.49) (Stein-Taylor, 1973). Glucose was added as carbon source at concentration of 10 $g.L^{-1}$. $NaNO_3$ was added as nitrogen source as a function of carbon source to achieve the C/N ratio of 20). pH was adjusted at 6.8.

2.2 Fed-batch cultivation

Fed-batch cultivation was carried out in 3 L bioreactor (BioFlo III - New Brunswick Scientific-Edison N.J. U.S.A.) with 2 L of culture medium. The culture was inoculated with an initial *C. vulgaris* biomass concentration of 2 g L^{-1} grown heterotrophically. The initial glucose concentration was 10 g L^{-1}. The experiments were carried at 26 ºC, aeration rate of 1 vvm (volume of gases per volume of culture per minute), in dark and at initial agitation rate of 150 rpm.
Whenever high oxygen intake was observed, the agitation rate varied between 150-500 rpm to ensure that DO (dissolved oxygen concentration) remained above 20 % of saturation.
Feeding strategy was divided in two phases. In the first phase (nitrogen sufficient) substrate concentrations were maintained within inhibition ranges (below 10 g L^{-1} glucose) by feeding medium with the same composition described in item 2.1, but with all components concentrated 30 fold to allow higher final biomass concentrations. An increase in DO over 40% saturation indicated substrate depletion. Feeding pump was then activated to provide substrate (DO-stat strategy). When DCW reached approximately 50 g L^{-1}, nitrogen limited feeding started. Feeding medium composition was changed to a glucose solution at concentration of 500 g L^{-1} without any other nutrients to induce nitrogen depletion and carbon excess state.

2.3 Continuous cultivation

Continuous cultures started in a fed batch mode to concentrate biomass up to 30 g L^{-1} DCW following the process described in item 2.2. Once the culture reached this concentration, fresh sterile medium feeding started while broth containing biomass was harvested at the same flow rate to maintain constant volume. Feeding flow was adjusted according to the desired dilution rate. Feeding medium was prepared according to the composition described in item 2.1 but with all components concentrated 6 fold. Steady state was considered after 3 residence times. Four different dilution rates were evaluated: 0.005, 0.01, 0.015 and 0.02 h^{-1}.

2.4 Analysis

Glucose content in the broth was analyzed using a commercial enzymatic glucose-oxidase assay kit by Bioliquid®.
Biomass dry cell weight was measured by filtering a 5 mL sample of the culture broth through a 0.22 µm porosity membrane and drying of the filter in an oven at 50 °C until constant weight.
For lipid content determination, cells were harvested by centrifugation at 8000 g for 10 min, frozen at -80 °C and freeze-dried at -30 °C and 50 mmHg. 400 mg of lyophilized biomass were digested in 5 mL of HCl 2 $mol.L^{-1}$ at 80 °C during 1h. After digestion, extraction process started with the centrifugation (500 g for 2 min) of the digested biomass and addition of 4 mL of methanol, 2 mL of chloroform, and 3.6 mL of distilled water to the biomass pellet. The mixture was agitated (2 min) and centrifuged (500 g for 2 min). The lower phase containing chloroform and lipids was transferred to a test tube. Re-extraction was carried out in upper phase by addition of 4 mL of a 10% v/v methanol:chloroform solution, followed by agitation and centrifugation (500 g for 2 min). The lower phase was added to the test tube and the chloroform was removed in a rotary vacuum evaporator. The remaining material (total lipids) was left in the oven at 50 °C for 24 h, cooled down to room temperature and weighed.

2.5 Calculations

Biomass productivity (P_X) is the relation of the variation of biomass concentration in time:

$$P_X = \frac{X_{final} - X_{initial}}{t} \quad (1)$$

In continuous processes (substrate limited) where the growth is kept exponential at specific growth rate (μ) equal to the dilution rate (D), biomass productivity can also be expressed as:

$$P_X = \mu \cdot X = D \cdot X \quad (2)$$

The lipid content of the microalgae was calculated by the ratio between lipid weight (W_L) and dry biomass weight (W_X).

$$\%\text{Lipid} = \frac{W_L}{W_X} \quad (3)$$

Lipid concentration [Lipids] is calculated as the product of biomass concentration (X) and Lipid content (%Lipid):

$$[Lipids] = X \cdot \%Lipid \quad (4)$$

Lipid productivity (P_L) was determined as a function of biomass productivity and lipid content:

$$P_L = P_X \cdot \%Lipid \quad (5)$$

Defatted biomass is calculated as the difference of X minus L:

$$DefX = X - L \quad (6)$$

3. Results and Discussion

3.1 Fed batch cultivation

Figure 1 shows biomass, substrate and lipids (during nitrogen limitation phase) profile during fed batch cultivation of *C. vulgaris*.Maximum specific growth rate (μmax) was observed during batch phase in the first 48 hours reaching the value of 0.035 h^{-1}. DO controlled feeding started after substrate depletion around 48 hours. During DO-stat mode the residual concentration of glucose inside the bioreactor remained at values lower than the analysis sensibility. After 7 days of cultivation DCW reached 50 g L^{-1} and feeding medium was changed for sole glucose (500 g L^{-1}) to induce nitrogen exhaustion. Two feeding pulses of glucose were applied in the lipid accumulation phase.

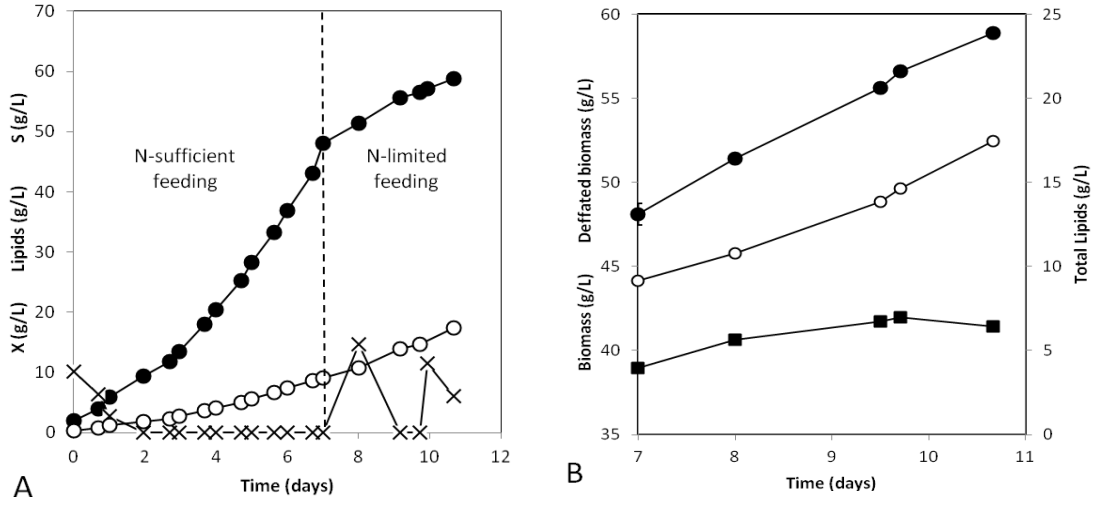

Figure 1. A : Biomass, Substrate and Lipid profiles under fed batch heterotrophic growth of C. vulgaris. -●- DCW; -○- Lipids; -x- Glucose. B: Composition of biomass under second feeding phase (Nitrogen limited). -●- Biomass; -○- Lipids; -■- Defatted biomass.

At the end of nitrogen sufficient feeding stage, DCW reached 48 g L^{-1} in 7 days cultivation with a global productivity of 6.9 ± g L^{-1} d^{-1}. Lipid content at this stage was determined as 18.9 % (w/w) with a global lipid productivity of 1.3 g L^{-1} d^{-1}.

Final lipid content at the end of the nitrogen limited stage reached 30 ± 1 % (w/w) of a final biomass of 58.9 ± 0.4 g L^{-1}. Data are summarized in Table 1. It is noteworthy that defatted biomass (Total biomass - Lipids) remained practically constant throughout nitrogen limited phase. This behavior was expected since nitrogen depleted conditions favour lipid accumulation in detriment of amino acids and proteins synthesis (Xiong et al., 2010), which constitute a large portion of lipid-free biomass. Lipid productivity and total lipid concentration almost doubled during second stage (1.8 and 1.9 fold, respectively).

Table 1. Summary of parameters from fed batch heterotrophic growth of C. vulgaris.

Parameter	Final N-sufficient stage	Final N-limited stage	Global
X (g L^{-1})	48.2 ± 0.5	58.9 ± 0.5	-
Defatted Biomass (g L^{-1})	38.9 ± 0.4	41.4 ± 0.3	-
Lipid concentration (g L^{-1})	9.1 ± 0.3	17.5 ± 0.6	-
Px (g L^{-1} d^{-1})	6.9 ± 0.2	2.9 ± 0.1	5.5 ± 0.1
Pp (g L^{-1} d^{-1})	1.3 ± 0.1	2.3 ± 0.1	1.6 ± 0.1

X = biomass concentration in dry cell weight, Defatted biomass = X - Lipid concentration, Px = biomass productivity, P_L = Lipids productivity

Global productivity of biomass and lipids reached 5.5 and 1.6 g L^{-1} d^{-1}, respectively. It is noteworthy the impact of nitrogen limited phase on overall lipid productivity. The optimization of the duration of each phase for maximum biomass and lipid productivity may allow for even higher values.

3.2 Continuous cultivation

All experiments in continuous mode started at 30 g L^{-1} DCW as initial concentration. DCW at steady state decreased for higher dilution rates, as well as the lipid content. The average biomass concentration and lipid content are shown in Figure 2. Although biomass concentration decreased, the global productivity increased significantly.

Similar behavior was observed in literature for different microorganisms. When the microalgae *Schizochytrium limacinum* was grown in continuous cultures for DHA production both biomass and total fatty acids concentrations decreased with increasing dilution rates (Ethier et al., 2011). For the yeast *Pichia angusta* DL-1 a significant decrease in biomass concentration was observed with increasing dilution rates (Aguirre-Ezkauriatza et al., 2010).

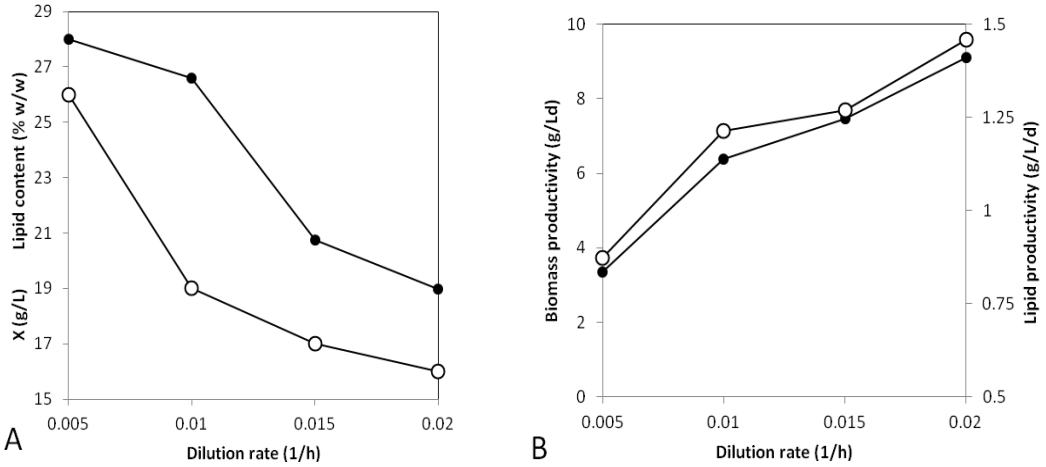

Figure 2. A: Biomass and lipid content profiles for different dilution rates during continuous heterotrophic growth of C. vulgaris -●- DCW; -○- %Lipids (w/w). B: Biomass and lipid productivities for different dilution rates. -●- Biomass productivity; -○- Lipid productivity.

Lipid content decreased significantly with increasing dilution rates. Data for continuous experiments are summarized in Table 2. Changes of lipid concentrations as function of dilution rate were observed for different microorganisms as well. Papanikolaou and Aggelis (2002) observed a reduction in lipid content with increasing dilution rates when growing the yeast *Yarrowia lipolytica* in chemostats for lipids production. The authors noted that the high growth rates forced by higher dilution rates may change the metabolic state of the cells favoring the flux of carbon to lipid free materials, such as amino acids and nucleic acids.

Table 2. Summary of steady state parameters of continuous heterotrophic cultivation of C. vulgaris.

	Dilution rate (h^{-1})			
Parameter	**0.005**	**0.01**	**0.015**	**0.02**
X (g L^{-1})	28 ± 1	26.6 ± 0.6	20.7 ± 0.5	18.9 ± 0.9
Lipids conc. (g L^{-1})	7.3 ± 0.2	5.1 ± 0.2	3.5 ± 0.1	3.0 ± 0.2
Px (g L^{-1} d^{-1})	3.4 ± 0.1	6.4 ± 0.1	7.5 ± 0.1	9.1 ± 0.1
Pp (g L^{-1} d^{-1})	0.92 ± 0.03	1.22 ± 0.05	1.3 ± 0.1	1.5 ± 0.1

X = biomass concentration in dry cell weight, Px = biomass productivity, P_L = Lipids productivity

Comparable productivities of lipids and even higher biomass productivities were achieved in continuous cultivation when compared with fed batch. However, the highest productivities were obtained at the condition of lowest biomass and lipid titers. This may seem contradictory but even at lower concentrations of biomass and lipids, the higher dilution rate results in an overall larger production. The downsize of this condition is that, as cited before, lower concentrations lead to higher downstream costs and energy use. This shows how complex the selection of the production regimen may be.

4. Conclusion

Both fed batch and continuous cultivation methods could yield high productivities at high cell densities. Fed batch cultivation achieved the highest biomass and lipids concentrations (58.9 and 17.5 g L^{-1}, respectively). High biomass concentrations are desirable for reducing costs and energy use on cell harvesting. Similarly, higher lipid contents helps reducing downstream extraction costs.

Continuous growth, on the other hand, resulted in higher biomass productivity (9.1 g $L^{-1}\cdot d^{-1}$ at D = 0.02 h^{-1} against 6.9 g L^{-1} d^{-1} in fed batch) and similar lipid productivity (1.5 g $L^{-1}d^{-1}$ against 1.6 g $L^{-1}\cdot d^{-1}$) when compared with the fed batch experiment. Although the lower biomass titer and lipid content may increase downstream costs, continuous production usually results in lower upstream costs due to the reduced downtime for cleaning, sterilization and setup. Upstream and downstream conditions may also vary if other co-products besides lipids are recovered from the biomass, such as chemicals for pharmaceutical and nutraceutical industry (Mata et al., 2013). If lipids are not the most valuable product, growth conditions can

be designed to reduce lipid content favoring non lipidic material synthesis. From the economical point of view all these variables should be considered during process design.

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