



Time of culture harvest affects lipid productivity of nitrogen-starved *Isochrysis galbana* U4 (Isochrysidales, Haptophyta)

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ABSTRACT

The effect of nitrogen concentration on lipid accumulation, biomass productivity and lipid productivity in *Isochrysis galbana* U4 (Isochrysidales, Haptophyta) was tested. Nitrogen limitation induces fluctuations in the lipid productivity in this species. The lipid productivities were influenced to a greater extent by the lipid yield rather than by biomass productivity. Lipid productivities were maximal during the early stationary phase when lipid accumulation was initiated and declined as the stationary phase progressed which was attributed to the cessation in lipid accumulation when the upper limit to the lipid storage capacity of *I. galbana* cells was reached. Hence, the cost of *I. galbana* mass culture for lipid production, as biofeed for biodiesel, can be reduced by harvesting the cells during the early stationary phase, when lipid productivity is at a maximum. This would maximize lipid yields and reduce the duration of photo-bioreactor runs resulting in reductions in energy costs.

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1. Introduction

Alterations in the growth environment have a significant effect on the chemical composition and growth characteristics of microalgal cells (Fidalgo et al., 1998). Factors that may be manipulated in an attempt to alter lipid composition and content include the source and concentration of major nutrients such as nitrogen and phosphorous, light intensity and the temperature at which the system is maintained (Rodolfi et al., 2009; Sharma et al., 2012; Tedesco and Duerr, 1989). Of these factors, the most widely reported inducer of lipid accumulation is nitrogen depletion (Li et al., 2008; Merzlyak et al., 2007).

Nitrogen is an essential component in genetic material, amino acids which make up peptides, proteins and enzymes, chlorophylls and energy transfer molecules such as ATP and ADP. It is therefore clearly evident that nitrogen is mandatory for cell growth and division (Barsanti and Gualtieri, 2006; Lavin and Lourenco, 2005; Li et al., 2008). Under nitrogen replete conditions, photosynthetically assimilated carbon is utilized for algal growth and reproduction. The accumulation of storage carbon compounds, upon nitrogen stress, is at the expense of algal growth. It may therefore be deduced that an inverse relationship exists between

algal cell growth and storage product accumulation in the form of lipids (Li et al., 2008). The microalgal species selected for biodiesel production should portray elevated growth rates and high intrinsic lipid yields (Doan et al., 2011).

Lipid productivity is the product of the lipid content (% dry weight) and the biomass productivity (grams dry weight per liter per day) and is a key characteristic when selecting a microalgal species for biodiesel production since it determines the eventual rate of lipid/oil production (Griffiths et al., 2009, 2012). Thus a balance needs to be established between both parameters (lipid yield and biomass productivity) in order for significant lipid yields to be realized. Numerous studies have investigated the effect of nitrogen limitation on the final lipid yield in microalgal species (Reitan et al., 1994; Sheehan et al., 1998; Shifrin and Chisholm, 1981) but the temporal aspect of any analysis of lipid production and lipid productivity have been neglected. The understanding of the temporal tradeoff between lipid accumulation and growth is essential if nitrogen stresses are to be used as a tool to maximize the final lipid yield obtained by algal cells (Adams et al., 2013).

This study was aimed at determining the effect of nitrogen limitation on lipid yield, biomass productivity and lipid productivity of *I. galbana*. *I. galbana* was investigated due to its ability to produce large amounts of lipid upon N stress making it a potential candidate for biodiesel production (Roopnarain et al., 2014). This study would aid in definitively verifying if nitrogen stresses have an effect on the lipid productivity of this species and how this effect varies over time.

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2. Materials and methods

2.1. Experimental setup

All culturing was conducted in modified 1 L Schott® bottles with glass tops equipped with quick fit gas inlet, gas exhaust and aeration tubes. All glasswares were thoroughly cleaned and autoclaved (121 °C for 30 min) prior to use. Fifteen vessels, containing 500 ml f/2 medium (Guillard and Ryther, 1962) each, were inoculated with approximately 3.0×10^6 cells of a healthy *I. galbana* culture. The cultures were left to grow for fourteen days (until the late exponential phase/early stationary phase was reached). Living cells were then harvested by centrifugation at 1000 rpm for five minutes, and the algal pellets were gently resuspended in 800 ml f/2 medium containing varying sodium nitrate concentrations. Each sodium nitrate concentration represented a percentage of that required to make up normal f/2 medium. The treatments included 0%, 25%, 50%, 75% and 100% sodium nitrate, where 100% refers to 8.82×10^{-2} M sodium nitrate in the medium (i.e. the total sodium nitrate in normal f/2 medium; Guillard and Ryther, 1962). A triplicate of each nitrate treatment was conducted. All cultures were sparged with filtered (Whatman® uniflo 0.2 µm) air and incubated at 23 ± 2 °C, under a photon flux density of $110 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a 10:14 h light–dark cycle.

2.2. Analytical methods

Samples, from all treatments, were extracted every third day to determine the nitrate concentration in the medium, lipid yield, biomass productivity and lipid productivity. Nitrate was determined using the salicylic acid method (Cataldo et al., 1975) and total lipid content determined indirectly by fluorescence using a flow cytometer after staining the cells with Nile red (da Silva et al., 2008; de la Jara et al., 2003) and relating it to lipid as measured by the Bligh and Dyer method (1959). This method is advantageous in that it allows for the rapid, *in situ*, accurate, routine determination of lipid content in the cells (da Silva et al., 2008; de la Jara et al., 2003). The sole use of the Bligh and Dyer method (1959) or Folch method (1957) of lipid quantification would be laborious and requires too many chemicals for routine analysis.

Biomass productivity was determined with the aid of Eq. (1):

$$P_{\text{Biomass}} (\text{g/l/day}) = (\text{DCW}_{\text{start}} (\text{g/l}) - \text{DCW}_{\text{end}} (\text{g/l})) / \text{Time (days)} \quad (1)$$

where $\text{DCW}_{\text{start}}$ refers to the dry cell weight at the beginning of the three day time interval, DCW_{end} refers to the dry cell weight at the end of the three day time interval and time refers to the duration of the experiment (i.e. three days; Rodolfi et al., 2009). To obtain the dry cell weight 100 ml of the cell suspension was centrifuged at 5000 rpm for ten minutes. The resulting algal pellet was rinsed with 0.5 M ammonium bicarbonate to remove salt crystals (Zhu and Lee, 1997), left to dry in a dessicator containing silica crystals and weighed.

Lipid productivity was determined using Eq. (2):

$$P_{\text{Lipid}} (\text{g/l/day}) = (C_{\text{Lipid}} (\text{g/g}) \times \text{DCW} (\text{g/l})) / \text{Time (days)} \quad (2)$$

where P_{Lipid} refers to the lipid productivity, DCW refers to the dry cell weight, C_{Lipid} refers to the total lipid content of the cells (indirectly determined from fluorescence readings as described above) and time refers to the algal cultivation period (Li et al., 2008).

Statistical analyses were conducted using SPSS 20 (Statistical Program for Social Sciences 20) software. Differences in various measurements were analyzed using one-way analysis of variance (ANOVA). Tukey's multiple comparison, post hoc tests were conducted to compare the different means if the ANOVA effects were significant ($\alpha = 0.05$).

3. Results and discussion

The luxury uptake of nitrogen was clearly demonstrated by *I. galbana* (Figs. 1, 2). The complete uptake of all measurable levels of nitrogen from the medium did not correspond with a decrease in the biomass productivity in any of the nitrogen treatments. This was expected since luxury uptake of nitrogen from the medium and gradual utilization of intracellular nitrogen by this microalgal species has been previously demonstrated (Roopnarain et al., 2014).

The biomass productivity in treatments supplied with nitrogen initially increased, but gradually decreased in the nitrogen starved treatment (0%) throughout the experiment (Fig. 2). This is expected since nitrogen is mandatory for growth. However, the absolute halt in algal growth should be represented by biomass productivities closely bordering on zero since biomass productivity measures the change in biomass yield over a time period. Biomass productivities that were greater than zero in the nitrogen starved treatment, in spite of growth inhibiting conditions (no nitrogen), implies that the dry weight measurements are influenced by other factors excluding the concentration of cells.

An elevated biomass yield in the early stages of nitrogen starvation in microalgal species is not new (da Silva et al., 2009; Li et al., 2008; Msanne et al., 2012; Pruvost et al., 2009, 2011). Some investigators attribute this phenomenon to the *de novo* synthesis of fatty acids which contributes to the weight of the cells (da Silva et al., 2009). The cells cultured in the nitrogen starved medium (0%) would indeed be in the stationary phase of growth since late lag/early stationary phase cells were used as the inoculum and these cells were cultured under stressful condition (N starvation). The stationary phase, in the species of *Isochrysis* used, is associated with the rapid accumulation of storage lipids (Roopnarain et al., 2014). The elevated lipid yield in the nitrogen starved cells in comparison to all other treatments is shown in Fig. 3. Hence, the biomass productivity measurements in this study were most probably influenced by the cell growth stage and concomitant increase in intracellular lipid. The increase in the cell weight due to lipid accumulation is verified by the biomass productivity (in the nitrogen starved treatment – 0% sodium nitrate) approaching zero towards day 15, when lipid accumulation started to cease (Figs. 2, 3).

A trend was noted in the cultures that were supplemented with nitrogen where the biomass productivity increased and thereafter declined. This observation was pronounced in the 100% sodium nitrate treatment where the biomass productivity reached a peak on day 12 (Fig. 2). Similar results (peaks in biomass productivities) with *I. galbana* have been observed elsewhere but have not been explained (Breuer et al., 2012). A possible reason for the peak in the biomass productivity could be the initiation of lipid synthesis accompanied by the increase in the cell yield

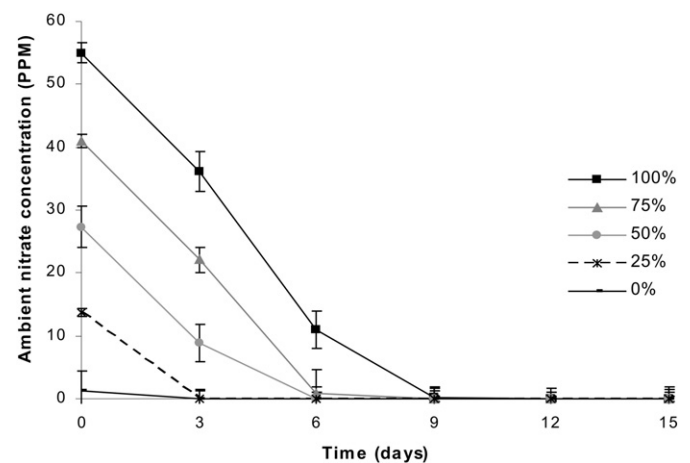


Fig. 1. A decrease in the ambient nitrate concentration with respect to time in *I. galbana* cultures containing varying sodium nitrate levels. Error bars represent standard deviation ($n = 3$).

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