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Effect of nitrogen regime on microalgal lipid production during mixotrophic growth with glycerol

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HIGHLIGHTS

- Two different *N* limiting strategies boost lipid production with glycerol.
- The two strains examined had different responses to nitrogen limitation.
- Limiting nitrate supported high levels of total lipid production over one to two weeks.
- Glycerol boosted lipid production by strain PCH02 throughout the range of nitrate tested.
- Mixotrophic growth with glycerol may allow the industrial production of biodiesel.

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ABSTRACT

Mixotrophic growth of microalgae to boost lipid production is currently under active investigation. Such a process could be of practical importance if a cheap source of organic carbon, such as waste glycerol from biodiesel production, could be used. Several previous studies have already demonstrated that this carbon source can be used by different indigenous strains of microalgae. In this study it is shown that different nitrogen limitation strategies can be applied to further increase lipid production during growth with glycerol. In one strategy, cultures were grown in nitrogen replete medium and then resuspended in nitrogen free medium. In a second strategy, cultures were grown with different initial concentrations of nitrate. Lipid production by the two microalgal strains used, *Chlorella sorokiniana* (PCH02) and *Chlorella vulgaris* (PCH05), was shown to be boosted by strategies of nitrogen limitation, but they responded differently to how nitrogen limitation was imposed.

1. Introduction

Biofuel production using microalgae has many promising characteristics, but at the same time there are many obstacles that must be overcome before this could be a practical process (Leite et al., 2013; Abdelaziz et al., 2013a,b). One of the challenges to the practical development of algal biofuels is that growth conditions are suboptimal in many parts of the globe. For example, for countries like Canada in Northern latitudes (>45 N), temperatures and solar flux would likely limit productivities over much of the year. Various strategies can be suggested for overcoming these challenges. To compensate for low temperatures, growth facilities could be located next to fossil fuel burning power plants to access the waste heat and carbon dioxide for enhancing algal growth. In addition, it is likely that indigenous algal strains, already adapted to the local climate, could show good growth, and perhaps lipid production, at lower temperatures than well studied strains. In fact, several recent studies have reported this to be the case, with a large survey of local algae showing that a wide range of growth rates at various temperatures can be obtained relatively easily (Abdelaziz et al., 2014; Hallenbeck et al., 2014; Wang et al., 2016).

Different strategies are required to overcome the challenges posed by lower solar fluxes. One idea that is being explored is the use of heterotrophic growth of microalgae, or mixotrophic growth, where carbon dioxide fixation driven by photosynthesis is supplemented with organic carbon uptake and use. Organic carbon use by microalgae is species dependent, but as a whole, microalgae are capable of using a wide range of organic carbon compounds (Wang et al., 2014; Zhang et al., 2014). Of course, this kind of supplementation would be too costly, given the relatively low value of fuel, unless some form of waste stream were to be

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used. Fortunately, a number of possible waste streams have been proposed (Dubey et al., 2015; Lowrey et al., 2015; Mitra et al., 2012). Heterotrophic cultivation is capable of producing relatively high biomass concentrations, and can be used as a second stage after a first stage of photoautotrophic growth (Rismani-Yazdi et al., 2015). However, high density bacterial or yeast fermentations are probably a better choice for using these substrates to make biofuels than a single stage heterotrophic process with algae (Qiao et al., 2015). In addition, two-stage algal systems require an intermediate stage of harvesting that add both complexity and cost to the process.

Hence, it would appear that the best use of low or no cost organic carbon substrates would be their use in supporting mixotrophic growth of microalgae. In at least some cases there appears to be a synergistic effect, with higher growth, biomass yields, and lipid productivities being obtained with mixotrophic growth than with photoautotrophic growth (Heredia-Arroyo et al., 2011; Lin and Wu, 2015; Woodworth et al., 2015). In fact, mixotrophic growth on glycerol can even exceed that seen with heterotrophic growth (Leite et al., 2015; Yeh and Chang, 2012). Crude glycerol is a byproduct of biodiesel production, and, given the present scale of biodiesel manufacture, so much glycerol is generated that it has become a waste disposal problem. Thus, glycerol could be an ideal substrate for mixotrophic growth.

Indeed, a number of recent studies have examined microalgal mixotrophic growth using glycerol as substrate (Leite et al., 2015; Lin and Wu, 2015; Paranjape et al., 2016; Skorupskaite et al., 2015; Wang et al., 2016). Various reports with different strains and species of *Chlorella* have found that the addition of glycerol can enhance final biomass or lipid yields or both. One study found up to a threefold increase in dry weight with *Chorella prototothecoides* growing with glycerol and limiting nitrogen (Skorupskaite et al., 2015). In another report, the addition of glycerol to an indigenous strain identified as *Chorella* gave a significant increase in both dry cell weight and lipid content (Lin and Wu, 2015). Similarly, it has been reported that mixotrophic growth of *Chorella pyrenoidosa* on glycerol gave a sevenfold increase in lipid content over that seen with photoautotrophic growth (Rai et al., 2013). On the other hand, two studies with *Chlorella vulgaris* found that although mixotrophic growth with glycerol did not increase the cellular lipid content, lipid productivity was almost eightfold higher than photoautotrophic growth due to a large increase in biomass productivity (Kong et al., 2013; Liang et al., 2009). In our laboratory we have been investigating the mixotrophic growth of indigenous algae on glycerol and xylose, available in large quantities as a waste stream of the pulp and paper industry (Leite et al., 2015, 2016; Paranjape et al., 2016; Wang et al., 2016). We have found that many different strains are capable of using glycerol and that, in general, glycerol, when assimilated by a particular strain, boosts both cell biomass and lipid content.

A variety of previous studies have demonstrated that the lipid content of photoautotrophically grown microalgae can be increased through some form of stress (Leite et al., 2013; Breuer et al., 2013). In most cases, nitrogen limitation has been chosen as the stress used to induce lipid accumulation (Breuer et al., 2012; Negi et al., 2015; Griffiths et al., 2014). Fixed nitrogen is essential for the biosynthesis of a variety of required cellular components and hence cell growth is necessarily arrested in its absence. However, in the presence of light, photosynthetic metabolism continues to function and newly fixed carbon is accumulated as storage material since it cannot be used for cell growth in the absence of nitrogen. For example, resuspension of photoautotrophically grown cells of *Chlorella sorokiniana* in *N*-medium has been demonstrated to increase oil accumulation 20-fold (Negi et al., 2015). Several studies with *C. vulgaris* have shown that the degree and timing of nitrogen limitation influence greatly how it affects lipid production (Griffiths et al., 2014; Stephenson et al., 2010). Since the addition of glycerol by itself seems to increase the lipid production of some strains, we were interested to see if some type of nitrogen limitation strategy with mixotrophic cultures could increase lipid production even further. Thus, in the present study, we examined the effects of two different nitrogen limitation strategies on the lipid production of several strains that were chosen based on their lipid production patterns as previously determined (Paranjape et al., 2016).

2. Materials and methods

2.1. Algal cultivation

The algal strains used in this study were from the collection of the Laboratory of Advanced Biofuels, Département de microbiologie, infectiologie et immunology of the Université de Montréal. These are strains isolated in the region of Québec, Canada, and were previously described (Abdelaziz et al., 2014, Hallenbeck et al., 2014). Three strains were chosen for the present work; PCH02 (closely related to *C. sorokiniana*), PCH05 (closely related to *C. vulgaris*), and PCH 28 (closely related to *Hindakia fallax*). Bold's Basal medium (BBM) (Andersen, 2005) was used for photoautotrophic cultivation, and with 25 mM glycerol, for mixotrophic cultivation. Strains were grown in a shaker in 125 ml Erlenmeyer flasks containing 50 mL of BBM with and without 25 mM glycerol. The strains were grown in constant light at 40 W/m^2 intensity at room temperature and shaken at 160 rpm. Each day, 200 µl of culture were taken and placed in 96-well microplate for measurement of OD at 630 nm using an EL-800 universal microplate reader from Bio-Tek instruments, Inc. Maximal absorption for chlorophyll is at 675 nm, and the region between 600 and 630 nm is a minimum in a spectral scan of a microalgal culture. In fact, this is the wavelength recommended by BioTek, a highly regarded manufacturer of microtiter plate readers (http://www.biotek.com/resources/articles/monitoring-of-algal-growth-using-intrinsic-properties.html). For all experiments pre-inocula were grown under photoautotrophic conditions. Two different strategies to impose nitrogen limitation were used. In one strategy, cultures were first grown under photoautotrophic conditions in complete BBM for seven days, harvested by centrifugation, and then resuspended in medium lacking nitrogen with 25 mM glycerol (mixotrophic conditions) and without glycerol (photoautotrophic conditions) and incubated for three weeks. 25 mM glycerol was chosen since this is the concentration used in our previous study (Paranjape et al., 2016) and is similar to the concentration of glycerol used in several previous studies with this source of organic carbon (Lin and Wu, 2015; Skorupskaite et al., 2015). In a second strategy, nitrogen limitation was imposed by using BBM medium with varying amounts of nitrate. As indicated, no, or varying amounts of sodium nitrate were used, to give a final nitrate concentration of 0 mg L^{-1}, 20 mg L^{-1}, 40 mg L^{-1}, 60 mg L^{-1}, 80 mg L^{-1}, 100 mg L^{-1} and 250 mg L^{-1} (nitrogen-replete conditions).

2.2. Biomass and lipid quantification

Biomass (dry weight) was calculated using the relationship OD_{630} gm (dry wt)$^{-1}$ = 1.055 ± 0.12 previously determined (Abdelaziz et al., 2014). Nile red, a fluorescent dye capable of staining neutral lipids, was used to quantify intracellular lipids (Bertozzini et al., 2011) using a slight modification of previously used methods (Abdelaziz et al., 2014; Leite et al., 2015; Paranjape et al., 2016). Algal samples were diluted with BBM to obtain 0.06 OD for each sample and 143 µl was transferred into black flat-bottom 96 well plates. To this, 50 µl of DMSO was added