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Building a better Mousetrap I: Using Design of Experiments with unconfounded ions to discover superior media for growth and lipid production by *Chlorella* sp. EN1234

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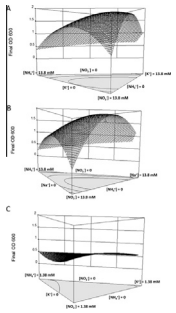
HIGHLIGHTS

- An unconfounded mix approach was used to probe important ions and their interactions.
- The major media anions PO_4^- and Cl^- negatively influence final cell densities.
- With *Chlorella* EN1234, maximal cell density is obtained with nitrate.
- Little correlation was found between nitrogen content and total lipid content.
- A composition space is defined for the best trade-off in lipid production.

GRAPHICAL ABSTRACT



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ABSTRACT

An unconfounded Scheffe Mix approach was used to probe important ions and their interactions in supporting biomass and lipid production by *Chlorella* sp. EN1234. Six major cations and anions; NH_4^+, NO_3^-, Na^+, K^+ PO_4^- and Cl^- were examined. Piepel plots and RSM analysis showed that in a number of cases, the major media anions PO_4^- and Cl^- negatively influence final cell densities, and that maximal cell density is obtained with nitrate over ammonium, with an optimal effect when mixed with equal molar potassium. As well, although it is commonly assumed that lipid content increases in nitrogen deficient media, here little correlation between nitrogen content and total lipid content was found with mixtures that supported high lipid productivity. Thus these mixtures define the composition space within which further R&D might produce the best trade-off between total biomass production and high cellular lipid content.

1. Introduction

Microalgae are a very diverse group of organisms which display a wide spectrum of potential ability to produce biofuels, neutraceuticals, or other valuable chemicals. In particular, they are under intense study recently for their potential for the sustainable production of biofuels (Abdelaziz et al., 2013a,b; Fields et al., 2014; Leite and Hallenbeck, 2011; Harwood and Guschina, 2009; Leite et al., 2013; Moody et al., 2014; Lee et al., 2013; Li et al., 2010; Schenk et al., 2008; Sheehan et al., 1998). Variation in microalgal capabilities is linked to the presence and diversity of metabolic pathways. In addition, nutrient composition and ratios might also determine where the cellular metabolism is directed. For example, key nutrients that reportedly affect lipid yield include carbon (in the form of carbon dioxide), nitrogen and phosphorus (Converti et al., 2009; Gordillo et al., 1998; Griffiths et al., 2014a,b), and iron (Morel et al., 1991). Algal culture media are composed of mineral nutrients in the form of salts and a great deal of effort over the past decades has been devoted to finding the optimal concentrations of

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salts for different species, resulting in a vast number of different media recipes.

A number of studies have shown that microalgal cellular lipid accumulation, generally in the form of triacylglycerides, is in many cases positively affected by stress conditions such as nutrient deficiency or high irradiance (Hu et al., 2008; Roessler, 1990; Volkman et al., 1989). Due to the wide diversity of microalgal species and the complexity of cellular biochemical processes involved in carbon flux, it might be expected that there are general mechanisms for regulation of carbon flux that apply to all algae, as well as other, more specific regulatory processes that only apply to specific subgroups. Although many studies have focused on identifying the regulatory mechanisms for lipid accumulation under stress, still relatively little is known about the correlation between lipid productivity of microalgae and general growth conditions. Until now, most strains of microalgae have been grown in cultivation media that were developed several decades ago for the general growth of larger groups of algae.

Changes in growth conditions; light intensity (Converti et al., 2009; Evens et al., 2008; Xia et al., 2013), temperature (Roleda et al., 2013), pH (Skrupski et al., 2013) and/or nutrient composition, can impact both lipid quality and quantity (positively and negatively). For example, a decrease in temperature, while not necessarily affecting the overall lipid yield, changes the lipid composition towards polyunsaturated fatty acids that are less suitable for biodiesel production (Converti et al., 2009). While irradiance can affect lipid production, high irradiance can actually reduce algal growth rate by photoinhibition (Radakovits et al., 2010) and thereby reduce lipid productivity overall. These cellular responses to irradiance and temperature changes are largely species-dependent (Guschina and Harwood, 2006). Most culture media in common use were developed many years ago for general algal culture, potentially leaving room for improvement in terms of individual species or specific needs. Thus, a recent study which examined the partial ionome of *Chlamydomonas reinhardtii* proposed a revised trace element supplement which supported faster growth rates and maximum cell densities (Kropat et al., 2011).

However, nutrient manipulations aimed at changing the concentration of a particular ion have previously been achieved mainly by changing the amounts of particular salt constituents of the growth medium, neglecting the effect of the corresponding counter ion (Niedz and Evens, 2007). This leads to a confounding effect where it is not possible to quantitatively separate the effects due to the two different ions. As well, the total ion concentration is often increased in such studies, creating yet another series of confounded variables. In a previous study, an experimental design approach was used to investigate the effects of five different ions on specific growth rates using a DOE (Design of Experiments) approach with an unconfounded ion matrix (Evens and Niedz, 2010). This type pf experimental design was shown to produce a much richer data set than possible with a one-factor-at-a-time approach. The optimization of biofuel production is essential to making renewable fuel sources economically viable. Here this approach was used to examine the effects of media composition and other environmental manipulations on biomass and lipid accumulation with the ultimate goal of formulating a model that identifies the drivers for growth and lipid production and predicts the optimal media composition for desired productivity indices.

2. Methods

2.1. Strains and growth conditions

Chlorella sp. EN1234 was obtained from Dr. Juergen Polle, Brooklyn College, and cultures were grown in 3 mL volumes in 12 well covered plates without agitation or CO_2 supplementation at 25 °C, with a light intensity of 150 μE on a 16 h light-8 h dark cycle. Each 3 mL culture was inoculated with 3000 algae cells/μL from a single actively growing mother culture which had been grown to early stationary phase in BBM minimal medium.

2.2. Growth, lipid and biomass measurements

Optical density (OD) measurements were taken every few days over a two week period at 600 nm with a BioTek μQuant® microplate spectrophotometer. Prior to measurement of OD measurement, cultures were agitated on a microtiter plate shaker for 2 min to re-suspend any settled algal cells. Variation between runs was ±25% and variation between biological duplicates within the same run were ±20.5%. Cell counts and neutral lipid productivity (Elsey et al., 2007) were determined using flow cytometry with a BD Accuri® C6 Flow Cytometer. Flow cytometry samples were prepared in 1.5 mL conical bottom tubes with 50 μL of culture and 150 μL of deionized water (final sample volume of 200 μL). Samples were measured with (1 μL of 200X BODIPY® (200 ng/μL) in DMSO) and without BODIPY® 493/503 (Life Technologies, D3922) dye. Dyed samples were vortexed and incubated for 10 min before measuring. Undyed samples were vortexed and measured immediately.

2.3. Design of experiments study of operational parameters

Using experimental design software (Design Expert 8) and the mixture and optimal design functions a matrix was developed to optimize freshwater media. Such mixture designs give response surface designs that are constrained such that all the component proportions add up to 1. Here, a Scheffe Mix Model with a quadratic process order, special cubic mix order and a sixth combined order limit was used. 210 media variations were generated and used to assess algal biomass and neutral lipid production. The 210 solutions included 69 pairs of duplicates and one triplicate. This allowed the determination of replication within the same experimental run, which gave a standard deviation of 20.5%. As noted above, the entire experiment was repeated twice, giving a variation between runs of ±25%.

Each medium consisted of a basal medium to which were added varying amounts of H_3PO_4, HCl, HNO_3, KOH, NaOH, NH_4OH, $Mg(NO_3)_2 \times 6H_2O$, $MgCl_2 \times 6\ H_2O$, and $Mg(OH)_2$ (Table S1).

2.4. Phylogenetic analysis of EN1234

EN1234 is a natural isolate that has been little characterized or described until now. To better understand its relationship to previously described microalgae, portions of its DNA encoding 18S rRNA and 23S rRNA were amplified by PCR and sequenced. Briefly, samples were held at 94 °C for 5 min, subjected to 30 cycles of 94° for 40 s, 61 °C for 40 s, 72 °C for 90 s, and then held at 72 °C for 5 min before storing at 4 °C. PCR reactions were run on an Eppendorf 6325 Mastercylcer Pro S PCR machine using an Ambion AgPath-ID One-Step RT-PCR Kit (catalog # AM1005). PCR solution components included: 12.5 μL of 2X RT-PCR Buffer, 1 μL 5X RT-PCR Enzyme Mix, 1 μL of forward primer, 1 μL of reverse primer, 3 μL of algal culture, and 6.5 μL of nuclease free water (total volume 25 μL). The sequences obtained have been deposited in GenBank (accession numbers: KM213393, 23S, KM213394, 18S). Basic BLAST (NCBI) was used to obtain sequences showing significant homology. These were aligned online using CLUSTALW (http://www.genome.jp/tools/clustalw/) and the FASTA output was then used as input to tree construction using the Maximum Likelihood function of MEGA6 (http://www.megasoftware.net/). En1234 was originally supplied to us as a potential candidate for lipid