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# The use of Design of Experiments and Response Surface Methodology to optimize biomass and lipid production by the oleaginous marine green alga, *Nannochloropsis gaditana* in response to light intensity, inoculum size and $CO_2$

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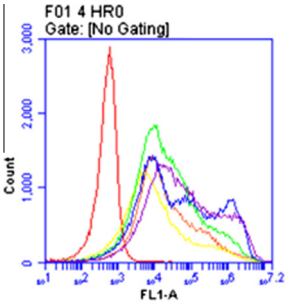
## HIGHLIGHTS

- A RSM–DOE study of [$CO_2$], light intensity and inoculum was made with *N. gaditana*.
- Culture conditions for maximal lipid productivity are defined.
- Culture conditions that allow the accumulation of biomass up to 15 g/L are given.
- Flow cytometry shows that cells enlarge as they age.
- Lipid production could be increased if cells were shifted into a high FLl state.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Biodiesel produced from microalgal lipids is being considered as a potential source of renewable energy. However, a number of hurdles will have to be overcome if such a process is to become practical. One important factor is the volumetric production of biomass and lipid that can be achieved. The marine alga *Nannochloropsis gaditana* is under study since it is known to be highly oleaginous and has a number of other attractive properties. Factors that might be important in biomass and lipid production by this alga are light intensity, inoculum size and $CO_2$. Here we have carried out for the first time a RSM–DOE study of the influence of these important culture variables and define conditions that maximize biomass production, lipid content (BODIPY® fluorescence) and total lipid production. Moreover, flow cytometry allowed the examination on a cellular level of changes that occur in cellular populations as they age and accumulate lipids.



## 1. Introduction

The need for sustainable replacement fuels in both meeting energy demand and climate change challenges has been widely discussed and drives intense R&D efforts in a variety of disciplines. One potential solution that has already been deployed at large scale is the production of biofuels. As pointed out elsewhere, and is apparent from the current special issue, microalgae are perhaps uniquely capable of producing various liquid replacement fuels that in many cases can be direct, "drop-in" replacements for petroleum-based fuels (Hu et al., 2008) while at the same time responding fairly well to the various sustainability issues faced by large-scale fuel production (Abdelaziz et al., 2013a,b). Large-scale

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practical production of algal-based fuels will not be possible until a number of significant challenges have been met (Leite et al., 2013).

One critical factor to consider is the algal strain to be used. A wide variety of microalgae can be isolated from the environment (Abdelaziz et al., 2014) but few will have the requisite properties for a large-scale production strain (Leite et al., 2013). Various *Nannochloropsis* species are under active investigation for biodiesel production due to a number of very attractive properties. They grow optimally on brackish water and thus their large-scale cultivation would not threaten limited freshwater resources. They appear naturally resistant to predation and to being overrun by competing algal species. Finally, as a genus they are naturally oleaginous, with cultures of six different *Nannochloropsis* strains reported to vary from 23% to 48% lipid by dry weight under normal exponential growth conditions (Wang et al., 2014; Radakovits et al., 2012). A number of studies on scale-up and large-scale growth of different strains have already been carried out (Olofsson et al., 2012; Quinn et al., 2012; Sforza et al., 2012; Silva et al., 2014).

In addition, much is known about this genus on the genomic level (Corteggiani Carpinelli et al., 2014; Jinkerson et al., 2013; Radakovits et al., 2012; Liang et al., 2013) and, although mutants have been available through classic means (Anandarajah et al., 2012), reports of transformation (Radakovits et al., 2012) and high rates of nuclear homologous recombination (Kilian et al., 2011) offer the short-term promise of strain improvement though metabolic engineering.

Among the various bioprocess parameters affecting lipid productivity, culture conditions are known to play major roles in growth, biomass production, and lipid accumulation. There have been a number of studies reported regarding the effects of different cultivation conditions, in particular nitrogen sources and levels, on growth (biomass) and lipid production (Chi et al., 2013; Huang et al., 2013; Lin et al., 2012; Ren et al., 2013; Ren and Ogden, 2014). Nitrogen is a key factor since its depletion can lead to drastic metabolic remodeling and the increased production of lipids relevant to fuel production (Simionato et al., 2013).

In addition, other factors that have been shown to be important are: initial inoculum density (Chen et al., 2012), light intensity (Pal et al., 2011; Solovchenko et al., 2014; Tamburic et al., 2014; Van Wagenen et al., 2012; Wahidin et al., 2013) and the $CO_2$ concentration (Chiu et al., 2009). Here we have applied a Design of Experiments (DOE) driven Response Surface Methodology (RSM) approach to simultaneously studying all three of these parameters. The use of this method, in addition to allowing the assessment of any interaction between these important variables, also allows the development of a model which allows the prediction of both the obtainable biomass and the lipid productivity under any given combination of these variables. While this approach cannot provide details as to mechanisms involved, it will show which factors drive the largest responses and which ones interact, thus highlighting the areas of focus for other studies involving mechanisms. This is the first time that such a method has been applied to the optimization of cultivation of this microalga of potential industrial importance. The only other multifactorial optimization study carried out previously chose to optimize N, [Fe], and temperature through a less robust Taguchi procedure that does not provide a model allowing simulation (Wei et al., 2013). Moreover, the applicability of those results to large-scale cultivation is doubtful since obviously it would be impractical and costly to add large amounts of supplemental iron and difficult to finely control the temperatures of real world outdoor culture facilities.

Likewise, the utility of RSM analyses conducted in small volumes can be questioned since they are far from the scale of practical cultivation and the parameters obtained by RSM may change when the size of the photobioreactors is scaled up and the cultivation system is operated outdoors. However, it is obvious that conducting a DOE–RSM study with a large number of full scale pond systems or bioreactor systems would be exceeding impractical. Therefore, the next best thing is to carry out small-scale studies which will highlight the most important factors in terms of driving responses and which will determine the extent of interaction between the different factors. These aspects will be very helpful in eventual process scale up and are the focus of the DOE–RSM study reported here.

## 2. Methods

### 2.1. Strain and growth conditions

*Nannochloropsis gaditana* CCMP526 (obtained from Dr. Matt Posewitz, Department of Chemistry and Geochemistry, Colorado School of Mines) cultures were grown in modified saltwater medium, MMI (Table 1). The medium was made up using 920 mL of Salt Solution, 40 mL of 1 M Tris (pH 8.0), 20 mL of the Iron Solution, 0.5 mL of the Trace Metals Solution, and 20 mL 1 M $KNO_3$ and autoclaved. After autoclaving, 0.4 mL 0.5 M $KH_2PO_4$ was added.

Culture conditions were varied based on three different $CO_2$/air mixtures, light intensities, and inoculation concentrations, as indicated by a three-factor Box–Behnken design generated using DesignExpert™ 9 software. Media were sparged with the appropriate gas mixture for 24 h to achieve equilibration. After 24 h, the culture medium pH was brought to pH 8.0 with the addition of 50% NaOH. To achieve a concentrated inoculum, *N. gaditana* precultures were centrifuged at 3700*g* for 5 min, using a Thermo Centra8GPR centrifuge, in 50 mL conical bottom centrifuge tubes and subsequently decanted. The pelleted algae were then re-suspended in ultrapure water to achieve a concentrated inoculum. A BD Accuri™ C6 Flow Cytometer was used to establish the resulting inoculum cell concentration. The cultures were then inoculated with the appropriate volume to achieve the starting cell density indicated by the DesignExpert™ 9 software. Cultures were grown using LED light sources with a 16 h light/8 h dark cycle. The appropriate $CO_2$/air mixtures that were sparged into the cultures were made up using mass flow controllers. Cultures were grown in 25 mm × 200 mm glass round bottom test tubes (50 mL liquid volume) fitted with small bore 1 mL pipettes that served to introduce the filter sterilized humidified gas mixtures.

**Table 1**
MMI components.

| *Salt solution* | |
|---|---|
| Component | Amount (g/L) |
| NaCl | 15 |
| $NaHCO_3$ | 0.08 |
| $CaCl_2{\cdot}2H_2O$ | 0.5 |
| $MgSO_4{\cdot}7H_2O$ | 6.6 |
| $MgCl_2{\cdot}6H_2O$ | 5.6 |
| *Iron solution*[a] | |
| Component | Amount (g/100 mL) |
| $FeCl_3{\cdot}6H_2O$ | 0.2 |
| $Na_2$-EDTA | 0.7 |
| *Trace metals solution*[b] | |
| Component | Amount (g and mg) |
| $Na_2$-EDTA | 3.9 g |
| $MnCl_2{\cdot}4H_2O$ | 360 mg |
| $CoCl_2{\cdot}6H_2O$ | 20 mg |
| $Na_2MoO_4{\cdot}2H_2O$ | 36 mg |
| $ZnSO_4{\cdot}7H_2O$ | 44 mg |
| $CuSO_4{\cdot}5H_2O$ | 20 mg |

[a] Dissolve $Na_2$-EDTA in 80 mL of DI water, bring to pH 8 with NaOH, then add $FeCl_3{\cdot}6H_2O$ and bring volume to 100 mL.
[b] Dissolve $Na_2$-EDTA in 200 mL DI water; bring to pH 8.0 with NaOH. Dissolve each metal individually and bring final volume to 250 mL.

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