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High-throughput screen for high performance microalgae strain selection and integrated media design

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ABSTRACT

Microalgae provide a powerful biotechnology platform for the production of renewable fuels, animal-feeds and high-value products. Here, a new high-throughput nutrient optimisation screen was used to maximise photoautotrophic growth rates for 100 newly bio-prospected microalgae isolates. The multidimensional statistical design enabled the optimisation of 12 critical nutrients (N, P, Ca, Mg, Fe, Cu, Mn, Zn, B, Se, V and Si) and identified the highest specific-growth rates, elemental main-effects (Ca, Mg and Zn accounted for 71% of all main effects under photoautotrophic conditions) and pair-wise nutrient interactions (e.g. Ca–Mg, Mg–Zn, B–Zn, B–Se, Mn–Zn and Zn–Si) for each of the 100 strains tested (25,100 trials, over 2000-fold data-compression compared to a full factorial design) as well as their ability to use different carbon sources. Photoautotrophic growth rates reaching 0.067 h^{-1} (0.289 h^{-1} at microwell plate scale) and 2.78 g L^{-1} (batch cultivation in flasks) are amongst the highest reported, promoting the capacity for a high-throughput optimisation of microalgae production processes using established and newly bio-prospected strains.

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1. Introduction

Single cell green algae (microalgae) are increasingly recognised as a powerful biotechnology platform for the production of a broad range of algae-derived products including renewable fuels (e.g. oil-based fuels, methane, alcohols and hydrogen), animal and aquaculture feeds as well as high value biologicals (e.g. pigments, unsaturated fatty acids and expressed proteins). Their ancient lineage (1.5–2 billion years since divergence from plants) and ability to colonise a broad range of ecological niches have led to the evolution of an estimated 350,000 species [1] and possibly more, providing a valuable genetic resource for breeding and genetic engineering. While to date, research has focussed on a limited number of production strains, rapid advances are being made in algae bio-prospecting [3,6,15,28,37], species purification [15], cryo-preservation [2] and genetic engineering [26,27,32]. However, given the diversity of microalgae, their specific production conditions must be individually optimised before their potential and handling requirements can be fully assessed. Here we have used a powerful high-throughput nutrient optimisation screen (100 species, 25,100 individual trials, 602,400 time resolved data points) to identify the best growth conditions. Conditions were sampled through the use of a large multidimensional statistical space matrix to identify improved production conditions in terms of N, P, Ca, Mg, B, Fe, Cu, Mn, Zn, Se, V and Si, to support the development of next generation high-efficiency microalgae production systems. Furthermore, this system is highly adaptable to a range of other high-throughput microalgal screening applications such as the enhancement of oil content or the identification of improved polyunsaturated fatty acids and carotenoids.

Until recently nutrient conditions used for algae production were largely based on the results of elemental analysis of biomass [17] or small complete factorial statistical screens of a few selected variable (e.g. N and P) [16,38]. These approaches are suboptimal for nutrient optimisation for two reasons. First, as the original biomass analysed, may itself have been produced under suboptimal conditions such as nutrient toxicity or limitation, basing media formulations on its elemental composition is no guarantee that optimal conditions will be identified. While macro-elements (e.g. N and P) are clearly required by all algae, their optimal ratios vary and for micronutrients both concentration-dependent nutrient sensitivities (e.g. resistance to metal ion toxicity) and biological and chemical interactions (both positive and negative) between nutrients can occur. Second, the use of complete factorial analysis of a few selected variables fails to identify the theoretical optimum of production, which is dependent on the optimisation of all necessary variables. While the use of a full factorial screen could be used to provide a statistically valid analysis, optimisation of the 21 most commonly used

Abbreviations: OD_{750}, optical density at 750 nm; TAP, Tris-acetate–phosphate medium; TP, Tris-phosphate medium; μ_{max}, maximum specific growth rate; CP, centre point; CBB cycle, Calvin–Benson–Bassham cycle; ATPase, adenosine triphosphatase; FACS, fluorescence-activated cell sorting; FTIR, Fourier transform infrared spectroscopy; NADH, nicotinamide adenine dinucleotide (reduced); Tris, Tris(hydroxymethyl) aminomethane; HAP, hydroxyapatite; Screen 1, nutrient screen part 1; Screen 2, nutrient screen part 2; GFP, green fluorescent protein; LED, light-emitting diode; NO_x, sum of nitrate and nitrite.

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macro- (C, N, P, K, Ca, Mg, S, Na, Cl) and micro- (Fe, Mn, Zn, Cu, B, Mo, Si, Co, Se, V, Ni, I) elements (excluding additional commonly required amino acids, vitamins and/or other additives) at even 3 different concentrations would require 3^{21} experiments (=10,460,353,203).

An entirely different approach is to use theoretical nutrient uptake and growth models to explore these nutrient relationships (e.g. nutrient uptake, storage and growth models) [7,10,39] but the use of these, requires an understanding of the biology of regulation of uptake receptors and the measurement of specific kinetic parameters, which is ideal for an intensively studied species but too detailed a programme for a high-throughput bio-prospecting screening application.

Here we have employed an advanced high-throughput robotic screen designed to identify the best nutrient conditions for a diverse set of 100 new isolated algae strains [15] within this complex multi-dimensional statistical space [25]. The screen included the above 21 mineral elements as well as vitamins B_1 and B_{12}, and focuses on the statistical optimisation of the 12 most important of these. These include 12 macro- (N [i.e. NO_3^-· NH_4^+ & urea], P, Ca, Mg) and micro- (Fe, Mn, Zn, Cu, B, Si, Se, V) elements with the remaining nutrients provided in reportedly replete levels at 1% CO_2 concentration (near optimal, though this can be adjusted). This nutrient screen was designed to identify the best production conditions within the broad multidimensional statistical space sampled, avoiding local minima and enabling the identification of significant nutrient interactions. This in turn enables more detailed studies of the optimal nutrient space to maximise growth rate (Figs. 1, 2 & 5) and minimise nutrient wastage (Figs. 2A & 5) which help to accomplish economic and environmentally sustainable commercial microalgae production systems. Furthermore this approach ranks the statistical significance of each element tested in terms of its effect on growth rate (Fig. 2A) and for the first time identifies complex pairwise interactions of nutrients and their effect on growth rate (Figs. 2B & 4). This method enables the rapid optimisation of strain-specific cultivation media both for established production as well as uncharacterised candidates on the path towards the efficient development of commercial microalgae production systems. The approach and experimental progression are summarised in Supplementary Fig. 1.

2. Materials and methods

2.1. Algae strains

100 algae isolates were obtained from an in-house established strain library holding a broad range of indigenous Australian species sampled from the east and south of Australia as described in [15]. Algae isolates were identified using morphological investigation and molecular classification by 18S and 16S rDNA analysis [15]. Sequences were aligned using nucleotide BLAST (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the 'nucleotide collection (nr/nt)' database. The eukaryote specific 18S primer sequences were 5′-ACCAGACTTGCCCTCC and 3′-CCTC CCGTTCAGACCA. The 16S universal primer sequences were 5′ (Uf: GAGAGTTTGATCCTGGTCAG) and 3′ (Ur: ACGGYTACCTTGTTACGACTT) [33]. The top 10 candidate strains (Table 1) were identified as *Chlorella pyrenoidosa* (10_B9), *Chlorella* sp. (11_H5), *Micractinium pusillum* (5_H4), *Chlorella sorokiniana* (7_B6), *Chlorococcum* sp. (12_02), *Chlorella sorokiniana* (12_A9), *Chlorella sorokiniana* (15_E4) (brackish water isolate), *Chlorella* sp. (20_G10), *Chlorella sorokiniana* (8_C4) and *Desmodesmus intermedius* (1_C4) and were selected based on their high growth rate, strain diversity (see Fig. 6: rDNA, morphology, geographic sampling site).

2.2. Algal cultivation for high-throughput screens

Microalgae cells cultivated on agar plates (TAP + 0.3% yeast extract + 1.5% agar, 50 μmol photons m^{-2} s^{-1}) were inoculated into 50 mL Tris-acetate–phosphate medium (TAP, pH 7, 23 °C, 120 μmol photons m^{-2} s^{-1}, ~120 rpm orbital shaking) [12] in 150 mL flasks. Algae strains that did not tolerate acetate were grown in Tris phosphate (TP, pH 7) media. Algal strains isolated from brackish water were cultivated in TAP media supplemented with 250 mM NaCl. Cell densities were measured (OD_{750} — a proxy for biomass) using a microwell plate reader (Infinite M200 PRO, Tecan Group Ltd, Switzerland). During log-phase growth the algal cells were collected by centrifugation (500 *g*, 10 min, 25 °C). The pelleted cells were washed once in 100 mM Tris buffer (pH 7.4) before being inoculated (to $OD_{750} = 0.1$) into sterile 96-well plates for screen 1 & 2 nutrient trials. All algae strains were grown in 150 μL of medium in 96-well plates (5 mm culture depth, 580 rpm mixing, 120 μmol photons m^{-2} s^{-1} continuous light, 23 ± 0.5 °C and 1% ± 0.3% CO_2 atmosphere; for detailed equipment and control descriptions see [25]). OD_{750} measurements were recorded at 3-hour intervals using a spectrophotometric microwell plate reader (Infinite M200 PRO, Tecan Group Ltd., Switzerland). The maximum specific growth rate (μ_{max}) was determined according to Eq. (1).

$$\mu_{max} = \left(\ln OD_{750,\ n+1} - \ln OD_{750,\ n}\right) * \left(t_{n+1} - t_n\right)^{-1} \tag{1}$$

The cell doubling time (or generation time, t_d) is described as [$t_d = \ln 2 * \mu_{max}^{-1}$].

2.3. Flask cultivation

Algal cells of the 10 selected candidate strains were collected during log-phase growth by centrifugation (500 *g*, 10 min, 25 °C), and washed once with 10 mL Tris buffer (50 mM, pH 7.4). The candidate strains were then inoculated in 200 mL (134 fold volume increase compared to high-throughput screen) of individually optimised media (Table 1) using a starting concentration of 10^6 cells mL^{-1}. The algae strains were grown for 2 weeks (~30 mm light path — 6 fold increase compared to high-throughput screen), 150 rpm on orbital shaker, continuous light at 120 μmol photons m^{-2} s^{-1}, 23 ± 0.5 °C and 1% ± 0.3% CO_2 atmosphere. Culture growth was determined in 24-hour intervals.

2.4. Microscopy

Cell morphology and culture behaviour were monitored microscopically using an Olympus BX 41 or a Nikon Ti-U inverted microscope (200, 400 and 1000× magnifications). Cell count was performed using a Neubauer cell counting chamber (0.1 mm depth, 0.0025 mm^2).

2.5. Volumetric biomass yield Y_V (g L^{-1})

Volumetric biomass yield Y_V (g L^{-1}) was estimated using flask cultures by collecting 3 × 10 mL in sterile 15 mL Falcon tubes (Becton Dickinson) every 4 days. Each sample was centrifuged (4000 ×g, 10 min), and the cell pellet washed with 10 mL MilliQ water to remove medium salts, before being centrifuged again (4000 ×g, 10 min). The initial culture supernatant after the first centrifugation step was carefully removed and used for nutrient analysis. For biomass dry weight analysis the pellet was dried until the weight remained constant (60 °C, typically 3 days). The tubes were then weighed on a precision balance (Shimadzu AUW220D) and biomass dry weight determined by subtracting the predetermined weight of the empty tube. Volumetric productivity P_V (g L^{-1} d^{-1}) of the flask cultivations (batch) was determined using the quotient of the biomass yield over the cultivation time (Δt) [$P_V = Y_V * \Delta t^{-1}$].

2.6. Nutrient screen

A total of 100 isolated algae strains were analysed using the high-throughput nutrient screen approach described in [25]. The first stage of the screen (screen 1) optimises phosphate concentration (0, 2, 10 mM KH_2PO_4) as well as four N-type ($NaNO_3$, NH_4Cl, $(NH_2)_2CO$ and NH_4NO_3) at five concentration levels (0–30 mM) in a full factorial

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