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Altered lipid accumulation in *Nannochloropsis salina* CCAP849/3 following EMS and UV induced mutagenesis

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ABSTRACT

Microalgae have potential as a chemical feed stock in a range of industrial applications. *Nannochloropsis salina* was subject to EMS mutagenesis and the highest lipid containing cells selected using fluorescence-activated cell sorting. Assessment of growth, lipid content and fatty acid composition identified mutant strains displaying a range of altered traits including changes in the PUFA content and a total FAME increase of up to 156% that of the wild type strain. Combined with a reduction in growth this demonstrated a productivity increase of up to 76%. Following UV mutagenesis, lipid accumulation of the mutant cultures was elevated to more than 3 fold that of the wild type strain, however reduced growth rates resulted in a reduction in overall productivity. Changes observed are indicative of alterations to the regulation of the omega 6 Kennedy pathway. The importance of these variations in physiology for industrial applications such as biofuel production is discussed.

1. Introduction

Despite a slowdown in growth, global energy demand rose 2.3% in 2013 with 32.9% of all consumption derived from oil [5]. Finding alternative sources of liquid transportation fuels to replace the diminishing sources of petroleum oil is crucial to economic stability and development as well as being vital to reduce CO_2 emissions [23]. In 2012 this translated to a production of biofuels equivalent to 60.22 million tonnes of petroleum oil, representing 1.45% of global oil consumption [5]. The need to find alternative sources of renewable bioenergy and chemical feedstock has in recent years spurred intense interest in the viability of biologically derived oils from sources such as microalgae [22]. Many eukaryotic microalgae have a natural potential to accumulate industrially relevant quantities of triacylglycerols (TAG), neutral lipids that can be converted to fatty acid methyl esters (FAMEs) which are the main component of biodiesel [15]. These photosynthetic micro-organisms have low input requirements, higher predicted energy yields per area than terrestrial crops, and the use of marine species would significantly reduce the fresh water impact [6,9].

In addition to biofuel production, microalgae show promise in production of oils for the aquaculture and healthcare industries since many strains are able to produce high value lipid components such as arachidonic acid (ARA) and eicosapentaenoic acid (EPA). This diversification could help mitigate the large and measurable impact of fish oil production on falling fish populations which is giving rise to both environmental concerns and unsustainable products [12].

Although many microalgae can naturally accumulate TAG in large quantities during the stationary phase of growth [8,30], actual productivity levels need to be increased in a reproducible manner to make industrial applications for low value products like biofuel production commercially viable [24]. In addition, the suitability of micro algal biomass as biofuel feedstock is closely related to its fatty acid profile with regard to length and degree of saturation which must comply with target ranges defined by the European norm. For many algal species the quantity of high value components such as omega 3 and 6 fatty acids are often at levels too high for fuel specifications but too low for commercial production [28].

Bioengineering offers the possibility of strain development via the introduction of foreign genes to increase lipid production or alter the profile of fatty acids accumulated, pushing towards increasing the poly unsaturated fatty acid (PUFA) content or conversely modifying to produce elevated levels of the saturated medium chain fatty acids (C8–C14) which have properties that mimic current diesel fuels [4]. In practice the number of microalgae species successfully transformed is very low [20,35] and routine methods for nuclear and organellar transformation severely limited [35]. Furthermore, the availability of multiple

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genome sequences for both *Nannochloropsis* [26,32,36] and *Emiliania huxleyi* [27] has revealed that many microalgae may be subject to extensive genomic variation reflecting their native environment. Consequently, the biochemical and physiological variation amongst strains of the same genus may vary significantly despite near-identical 18S rRNA genes as demonstrated for *Nannochloropsis* [36]. This can complicate targeted modification because considerable variation exists between strains and no two strains will be directly comparable, nor necessarily respond to standard molecular techniques (e.g. antibiotic selection) in the same way. Additionally with targeted modification also comes the need to use selective agents that may be detrimental to the environment and the lengthy and costly legislative requirements involved in GM control, containment and product labelling. Random mutagenesis techniques in contrast are subject to no such controls and over the past 75 years random mutagenesis has been used to create and release more than 2500 plant varieties in 175 plant species, both crop and decorative [29].

In the present study, chemical and physical mutagenesis techniques were used to generate random mutants by exposure to ethyl methane sulfonate (EMS) and UV radiation, respectively. Strain selection via florescence activated cell sorting (FACS) was then used to isolate new strains with enhanced lipid content. Analysis was undertaken to compare two key characteristics deemed to be most desirable: biomass productivity (fast doubling times coupled with growth to high cell densities) and the ability to accumulate a high percentage of biomass as lipid. Considerable variations in productivity were observed across isolated mutant populations of *Nannochloropsis salina* CCAP849/3 strains and the implication of these observations for high and low value oil production is discussed.

2. Methods

2.1. Strains and culture conditions

N. salina algal strain CCAP 849/3 was obtained from the Culture Collection of Algae and Protozoa (Scottish Association for Marine Science, Oban, Scotland, U.K.). Experimental cultures were grown in F/2 medium [13], bubbled with air and maintained under 7400 lux (10.83 W m^2) irradiance on a 16 h:8 h light:dark cycle at 25 °C. Cells at mid log phase were treated with an antibiotic cocktail of ampicillin (sodium salt) 100 μg mL^{-1}, kanamycin monosulphate 100 μg mL^{-1}, gentamycin sulphate 50 μg mL^{-1} and streptomycin sulphate salt 100 μg mL^{-1} for 48 h to obtain axenic cultures.

2.2. Growth rate determination

Culture density was determined via light microscope cell enumeration in a haemocytometer following staining with Lugols iodine solution (2%). Specific growth rates (K) were calculated according to the following equation: $K = \ln(N2/N1)/(t2 - t1)$, where N2 and N1 are the total cells mL^{-1} at time point (t2) and time point (t1) respectively, and where t2 > t1. Exponential growth refers to cultures ~5–17 days old. Stationary cultures were >20 days old.

2.3. Mutagenesis

For EMS mutagenesis, a method modified from Chaturvedi et al. [7] was used: cells were washed and resuspended at 1×10^8 cells mL^{-1} in fresh sterile sea water with glycerol at 0.1% as a carrier. Cells were subject to EMS (Sigma–Aldrich) exposure at a range of concentrations (0.24 mol L^{-1}, 0.42 mol L^{-1} and 0.63 mol L^{-1}) over 0.5, 1, 2 and 4 h in darkness at room temperature. EMS was inactivated by addition of sodium thiosulphate solution to a final concentration of 5%. Cultures were pelleted at 8000 g and washed twice with sterile sea water and resuspended in F/2 media. For UV mutagenesis, axenic cultures of wild type 849/3 and two EMS mutagenised lines previously selected for elevated lipid content (mutants NBF6-7 and NBF7-10) were re-suspended at 2×10^7 cells mL^{-1} in fresh F/2 medium. Excess culture (6 mL) was added to a 50 mm petri dish, from which 3 mL was then removed to enable surface tension to form a micro layer of culture spread evenly over the base of the plate. Cells were exposed to UV for 5, 10, 30, 60, 120, 180 and 240 s using the UVP CX-2000 cross linker with a short wave UV source (245 nm) fixed at 89 mm from the sample. Samples from each condition as well as a control were serial diluted and plated for single colonies for determination of cell mortality.

2.4. Fluorescence activated cell sorting

Two weeks after EMS treatment and one month after UV treatment, cells were stained with the neutral lipid stain Bodipy 505/515 (Invitrogen) using 0.1% glycerol as a carrier and subject to cell sorting on a Beckman Dickinson FACSAria MkII. Cells were gated on histogram using a blue laser (488 nm) and 530/30 emission filter for the top 5% highest florescence at a rate of 15–30 events per second. EMS cultures were subjected to 3 rounds of sorting, UV cultures to a single round. For EMS mutants only, a final round of sorting was used to initiate monocultures: individual cells were sorted into 96-well plates primed with F/2 media under the same conditions as before but gating limited to the top 1% highest florescence.

2.5. Lipid analyses

Fatty acid concentrations and profiles in microalga cells were determined post conversion to fatty acids methyl esters (FAMEs) and analysed by GC–MS (Agilent 7890A GC and 5975C inert MSD, Agilent Technologies Ltd., Edinburgh, UK). Samples were centrifuged (10,000 g), washed in distilled water and resulting pellets lyophilised. Nonadecanoic acid (C19:0) was added as an internal standard and cellular fatty acids were converted directly to FAMEs by adding 1 mL of transesterification mix (consisting a volume to volume ratio of 95% methanolic HCl (3 mol L^{-1}) to 5% 2,2-dimethoxypropane) followed by incubation at 90 °C for 1 h. After cooling, FAMEs were recovered by addition of 1 mL NaCl (1%) solution and *n*-hexane (1 mL) followed by vortexing. The upper hexane layer was injected directly onto the GC–MS system and FAMEs were separated on a fused silica capillary column (30 m × 0.2 mm × 0.25 μm; Omegawax™ 250, Supelco, Sigma–Aldrich, Gillingham, Dorset, UK) using an oven temperature initiation of 75 °C for 5 min followed by a gradient increase of 4 °C min^{-1} to 240 °C followed by a hold time of 15 min. Helium was used as a carrier gas (0.4 mL min^{-1}) and the injector and detector inlet temperatures were maintained at 280 °C and 230 °C, respectively. FAMEs were identified using retention times and qualifier ion response and quantified using respective target ion responses. All parameters were derived from calibration curves generated from a FAME standard mix (Supelco, Sigma–Aldrich, Gillingham, Dorset, UK).

2.6. Statistical analysis

Data was assessed for normality and then subject to ANOVA and 2-sample T testing. P values of less than 0.05 were considered to be significant.

3. Results

Previous work [3] indicated that *N. salina* has a good combination of high oil accumulation and cell proliferation