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Short Communication

# The selective breeding of the freshwater microalga *Chlamydomonas reinhardtii* for growth in salinity

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## HIGHLIGHTS

- The first successful demonstration of genome shuffling with microalgae.
- Improved *C. reinhardtii* capable of growth in salinity exceeding seawater.
- Sexual development is shown to be significantly better than asexual development.

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## ABSTRACT

The potential for *Chlamydomonas reinhardtii* to be utilized for biofuel production was strengthened by developing it for growth in elevated salinity via the selective breeding method of genome shuffling. A population was constructed via random mutagenesis and subjected to multiple rounds of sex and growth in increasing salinity. This sexual line was capable of growth in up to 700 mM NaCl, unlike its progenitor, which could only grow in 300 mM NaCl. An asexual control line was capable of growth in 500 mM NaCl. Palmelloid aggregations increased in size and the concentration of final biomass decreased as a function of NaCl concentration, which poses considerations for future strain development. The sexual line maintained sexual efficiencies of up to 50% over the course of selection. This investigation achieved significant strain improvement of *C. reinhardtii* and demonstrated the clear advantage of its ability to participate in laboratory controlled and reproducible high efficiency sex.



## 1. Introduction

The economic large-scale outdoor cultivation of microalgae has long been recognized as a potential source of renewable energy and nutritional products, although there are considerable challenges in making required improvements in both the production and processing of microalgal biomass. While such improvements are important, the central component of these processes is the specific algal strain itself, together with its growth performance and its attributes in relation to downstream processing (Spiden et al., 2013).

To be considered a good candidate for commercial production, an algal strain must have key characteristics such as a high solar to biomass conversion ratio, high productivity of the targeted product (e.g. triacylglycerides for biodiesel production), the ability to outperform potential contaminants, and resistance to environmental stresses (Day et al., 2012). Over the years, considerable effort has been given to the bioprospecting of wild strains that possess such characteristics (Georgianna and Mayfield, 2012). With a number of capable candidate biofuel producers now being identified (Mata et al., 2010), a focus has also been the optimization of growth conditions and strain improvement for these strains (Larkum et al., 2012). Unlike growth optimization, research into strain improvement has had little success which can be attributed to the lack of genetic tools and general lack of understanding of their biology (Qin et al., 2012; Rasala et al., 2014). For example, most biofuel candidates cannot be stably transformed, which is a fundamental genetic technique.

The model microalga *Chlamydomonas reinhardtii* is well suited for strain improvement due to it having well developed and reliable genetic tools (Rasala et al., 2014) and extensive knowledge about its biology and genome (Blaby et al., 2014). A premier feature, which is lacking from other biofuel candidates, is its ability to participate in laboratory controlled and reproducible high efficiency sex, with a large body of literature describing this process.

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Through this ability arises the opportunity to selectively breed new or enhanced traits, which is a major advantage for industrial implementation (Georgianna and Mayfield, 2012) as this can increase its growth performance under conditions encountered during outdoor cultivation and also enables improvements made in the laboratory to be bred into existing production strains. Notwithstanding, *C. reinhardtii* is not considered a forerunner due to significant shortcomings in attributes such as the lack of products of industrial interest and lack of desirable growth characteristics. Some efforts have been made to overcome some of these limitations through strain development. For instance, mutants have been isolated to produce lipid under nitrogen deprivation (Wang et al., 2009), a key improvement toward biodiesel production. Other significant developments include lower light absorbance, increased hydrogen production and heterologous protein production.

The ability to grow microalgae in brackish water or seawater is imperative, as it greatly reduces our dependence on limited freshwater and reduces the severity of contamination. There has been some previous success with developing salt tolerance in *C. reinhardtii*, with wild type growth rates reported at 200 mM NaCl (Perrineau et al., 2014) and cells capable of survival at 550 mM NaCl (Lachapelle and Bell, 2012). We sought to investigate the improvement potential of *C. reinhardtii* to grow in elevated salinity by utilizing the proven selective breeding method of genome shuffling, which to date had not been demonstrated with microalgae (Biot-Pelletier and Martin, 2014). This resulted in a previously unreported achievement of growth in salinity exceeding seawater, along with the potential for further improvements to be made. This study significantly enhances the prospect of *C. reinhardtii* to be mass-cultured in brackish or seawater to economically produce biofuels or other biochemicals of interest.

## 2. Methods

### 2.1. Initial culture strains and growth media

The initial strains used were wild type $mt^-$ 137c and wild type $mt^+$ 137c sourced from the Chlamydomonas Resource Center. Tris Acetate Phosphate (TAP) liquid media (Gorman and Levine, 1965) was used for growth, with the NaCl concentration detailed below. Seawater medium was prepared by adding 5% (v/v) anaerobic digestate into seawater (Gunnamatta beach, Victoria, Australia) and passing through a 0.22 μm Millipore PVC syringe filter.

### 2.2. Genome shuffling strategy

The initial culture was exposed to ultraviolet (UV) mutagenesis followed by serial subculturing in elevated salinity, with three periodic sexual rounds (Fig. 1A) and a total of 491 generations. An asexual control line was cultured in parallel and did not participate in any sexual rounds. This line was subjected to 631 generations, with the additional generations accounting for the aggregate time the sexual line spent in the sexual phase.

### 2.3. Mutagenesis

The culture was incubated and grown in liquid TAP under 100 $\mu E\ m^{-2}\ s^{-1}$, 120 rpm at approximately 21 °C until early stationary phase. The cell concentration was adjusted to $5 \times 10^6$ cells per ml and 20 ml was UV irradiated in open Petri dishes, mixed at 120 rpm at a distance of 45 cm using a 30 W UV-C tube for approximately one minute. Overnight incubation in the dark followed, with gentle shaking (60 rpm) to prevent photo-reactivation.

### 2.4. Selection conditions

Serial sub-culturing in increasing salinity was performed with the concentration of NaCl adjusted so that the doubling time was between 15 and 25 h. Once the culture had adapted and the doubling time was consistently under 15 h, the NaCl concentration was increased. Each extended period of serial subculturing in elevated NaCl, either after UV mutagenesis or a sexual round was referred to as a growth series (GS).

### 2.5. Sexual reproduction

Sex was conducted as detailed by Harris (Harris, 2008). Cultures were grown in liquid TAP under illumination of 100 $\mu E\ m^{-2}\ s^{-1}$, 120 rpm at approximately 21 °C until late log phase. The cells were washed with TAP-N via centrifugation at 1000 *g* and re-suspended in TAP-N at a concentration of $1 \times 10^6$ cells per ml and incubated as described for a further 18 h and spread on TAP-N plates containing 4% agar. The plates were incubated under illumination for 24 h followed by 6 days of incubation in the dark. The zygotes were germinated by re-incubation in TAP under illumination.

### 2.6. Growth assays

Samples from the end of each growth series, denoted the initial population, population GS1, population GS2, population GS3, population GS4 and the asexual population or line, were taken out of stock and grown in small flasks in a volume of 40 ml liquid TAP under 100 $\mu E\ m^{-2}\ s^{-1}$, 140 rpm at approximately 25 °C. A sample for the initial culture (prior to UV mutagenesis) was also taken out of stock. Each culture was grown in triplicate at 0 mM, 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM and 700 mM NaCl until stationary phase. The optical density at 750 nm was measured every 24 h for all cultures during the entirety of each growth cycle. The growth rate and doubling time was determined by a line of best fit plotted during the log phase of growth. Samples with no growth or growth slower than 100 h doubling time were discarded and not further analyzed.

### 2.7. Dry weight and particle size measurements

All of the growth assays were also measured for their dry weight and particle size at stationary phase. Whatman GF/C 47 mm glass microfiber filters were weighed; 5–10 ml of sample culture was then passed through the filter via vacuum filtration. 10–20 ml of 0.5 M Ammonium formate was passed through to wash out salts, followed by incubation at 60 °C for 24 h, with the dried filters then weighed. 15–30 ml of sample was analyzed for particle size distribution using a Malvern Instruments Mastersizer 2000.

### 2.8. Sexual efficiency measurements

Sexual efficiency was determined using a light microscope and manual counting to determine the number of unmated cells, zygotes and non-germinated zygotes. Sex was performed in triplicate as detailed above in 2.5. However, prior to measurements, one round of obligate sex was conducted to equalize $mt^+$ and $mt^-$ so that the intrinsic sexual efficiency could be determined.

### 2.9. Statistical calculations

P-values and significance at the 95% confidence level were calculated using a two-sampled *t*-test. All tests were one-tailed, had 4 degrees of freedom and with equal variances assumed.