Algal Research 33 (2018) 448–455

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Improved DNA/protein delivery in microalgae – A simple and reliable method for the prediction of optimal electroporation settings

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ARTICLE INFO

Keywords:
Electroporation
Green microalgae
Labelled DNA
Labelled protein
Sytox Green
Propidium iodide

ABSTRACT

Genetic transformation of microalgae remains a challenge due to poor intracellular delivery of exogenous molecules. This limitation is caused by the structure and composition of the cell wall and cell membrane of each species. Moreover, successful delivery of proteins or nucleic acids cannot be assessed by determining transformability since their functionality is not always known in the studied microorganism. We propose a quick and effective screening tool for the prediction and optimization of electroporation settings by monitoring cell permeability and viability using Sytox Green and propidium iodide respectively. We determined voltage settings for the microalgae *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Neochloris oleoabundans* and *Acutodesmus obliquus*. To evaluate the predicted settings, we delivered labelled DNA and proteins into the cells. We demonstrated that high transformation efficiencies can be accomplished when predicted values were applied with functional plasmids. Additionally, we increased transformation efficiencies by testing cell concentrations, light intensities and fragment sizes. This method can be used to determine suitable transformation conditions for non-transformed microalgae species and to increase the insight on established transformation protocols.

1. Introduction

Microalgae provide a promising platform for the production of high value products such as nutraceuticals, commodity chemicals, health beneficial products, lipids and proteins for food and feed applications [1–7]. Wijffels and Barbosa [6] described the physiological and cellular characteristics of an "ideal microalgal cell factory" and even though extensive screening studies of microalgal isolates have been done, it is unlikely that single species can meet all required characteristics, such as high growth rate, high productivity of valuable compounds, product excretion and robustness. Genetic modification and metabolic engineering are valuable tools to speed up the development of microalgae derived products and are necessary to decrease the high production costs that are currently involved.

Methods have been developed to enable delivery of exogenous DNA into microalgal cells, such as particle bombardment, *Agrobacterium tumefaciens* mediated transformation, conjugation, electroporation, and methods involving glass beads and carbon whiskers [8–10]. Additionally, delivery of preassembled recombinant Cas9/Cpf1 ribonucleoproteins (RNPs) via electroporation has enabled genome editing in microalgae [11]. Most advances are restricted to the species *Chlamydomonas reinhardtii* which has been a model for photosynthesis and metabolic engineering research for many decades [12]. Although *Chlamydomonas* species have been well characterized, they are not well suited for producing high value compounds. Industrially relevant oleaginous microalgae such as *Chlorella vulgaris*, *Nannochloropsis* sp., *Neochloris oleoabundans* and *Acutodesmus obliquus*, have a great potential to become part of bio-based production platforms [13,14]. However, these promising species still need to be further optimized, for which a genetic engineering toolbox that can be applied to a wide range of microalgal species is required.

The most widely used method to deliver exogenous molecules such as DNA and proteins into cells is electroporation [15–17]. This physical method is based on electropermeabilization, in which an electric pulse generates temporary micro-pores in the cell membrane enabling the passage of molecules into the cells. This passage can be obstructed by the cell wall, or shape and size of the cells [18]. Since the main cause for unsuccessful delivery of molecules into microalgae is related to the

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https://doi.org/10.1016/j.algal.2018.06.021
Received 20 March 2018; Received in revised form 17 May 2018; Accepted 20 June 2018
Available online 04 July 2018

morphology of a specific species [19,20], all electroporation conditions such as electric field strength, electroporation buffer, capacitance, number of pulses or the use of enhancers have to be optimized for each species individually. However, optimal settings cannot be investigated by determining the amount of transformants obtained after electroporation since it is not always known if the expression vector to be delivered is functional in the investigated microorganism. We therefore propose a quick and effective screening tool that can be used for the optimization of electroporation settings needed for the delivery of exogenous molecules in poorly transformable strains or less well-studied microalgal species. We optimized the electroporation conditions and give guidelines for measuring cell permeability and viability by using two fluorescent dyes: Sytox Green and propidium iodide respectively. Furthermore, labelled DNA and labelled proteins were delivered into the cells to corroborate the results obtained during permeabilization experiments. Finally, we demonstrate successful microalgal transformation by using an optimized electroporation protocol. To generate a generic genetic engineering toolbox that can be applied to a wide range of microalgal species, we tested four industrially relevant microalgae species with different physiological and cellular characteristics: *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Neochloris oleoabundans* and *Acutodesmus obliquus*.

This method not only offers guidance for finding optimal electroporation settings in species that are less prone to take up extracellular molecules, but it can also be used to improve performance and increase insight in established protocols.

2. Materials and methods

2.1. Strains, medium and culture conditions

Chlamydomonas reinhardtii (CC1690, *Chlamydomonas* research center), *Acutodesmus obliquus* (UTEX 393, University of Texas), *Chlorella vulgaris* (UTEX 259, University of Texas) and *Neochloris oleoabundans* (UTEX 1185, University of Texas) were used in this study. *C. reinhardtii* was grown on Tris-Acetate-Phosphate (TAP) medium [36]. *Acutodesmus obliquus*, *Chlorella vulgaris*, and *Neochloris oleoabundans* were grown in fresh water medium [37]. Agar plates were made by supplementing 15 g/L agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). Cultures were kept in exponential growth phase under day:night cycle regime (16:8) at 25 °C at around 60 μmol $m^{-2} s^{-1}$ light intensity (Grolux fluorescent tubes, Sylvania F36W/GRO) at 125 rpm and 2.5% CO_2.

E. coli S17 and DH5α were grown in LB and LB agar at 37 °C supplemented with 100 μg mL^{-1} of ampicillin or 50 μg mL^{-1} of kanamycin when needed.

2.2. Fluorescent dye delivery

Microalgal cells in the exponential phase (5×10^6–1×10^7 cells mL^{-1}) were collected by centrifugation at 2500 ×*g* for 10 min at 4 °C. The pellet was washed with electroporation buffer (5.0 mM KCl, 5.0 mM $CaCl_2$, 0.01 M HEPES, 0.1 M mannitol and 0.1 M sorbitol), and resuspended to a final concentration of 10^8 cells mL^{-1} in cold electroporation buffer. From this cell suspension 250 μL was transferred to a 2-mm electroporation cuvette. Sytox Green (Life Technologies, Grand Island, NY, USA; S7020) was added to a final concentration of 1 μM and incubated in the dark for 5 min on ice. This dye was used to assess the level of permeability as a result of the electric pulse. Electroporation was carried out using a Bio-Rad Gene-Pulser apparatus set at 25 μF and infinite resistance and varying yield strengths (0–10.5 kV cm^{-1}). After electroporation, the cell suspension was recovered in the dark for 5 min on ice. 1 mL of electroporation buffer was added and the cells were incubated for 10 min in the dark at room temperature. Cells that were assessed for cell viability were not exposed to Sytox Green but were supplemented with propidium iodide (Life Technologies, Inc., Carlsbad, CA) at a final concentration of 60 μM, after the recovery phase, before analysis by fluorescent microscopy or flow cytometry.

2.3. Labelled DNA and labelled protein delivery

The plasmid pOpt-Clover-Hyg [38] was extracted from *E. coli* DH5α (*Chlamydomonas* Resource Center) using the Gene Jet Plasmid Miniprep kit (Thermo Scientific). 5 μg of purified plasmid was labelled at a 1:1 (v:w) ratio of Label IT® Fluorescein to nucleic acid according to manufacturer instructions (Mirus, Madison, WI, USA). Fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) was delivered into the cells at a final concentration of 10 μM (Thermo Fisher). Labelled DNA and labelled BSA were delivered into *Chlamydomonas reinhardtii*, *Acutodesmus obliquus*, *Chlorella vulgaris* and *Neochloris oleoabundans* with electroporation conditions mentioned above and cellular uptake was analysed by fluorescent microscopy or flow cytometry.

2.4. Fluorescence screening

Cells were visually inspected with an Olympus IX71S8F-3 fluorescence microscope with 460–495 nm excitation filter, 510–550 nm Emission filter and a 505-nm dichromatic mirror for Sytox Green. High throughput quantification of the dye uptake was done by using a BD Accuri C6 flow cytometry (BD Biosciences, San Jose, CA, USA). The electroporated cells were diluted 1:200 in electroporation buffer, before flow cytometry analysis to obtain an optimal flow rate for the detectors. Per condition 20.000 cells were measured with an argon-ion excitation laser (488 nm) and detected using three different fluorescent channels: green (FL1 530 ± 15 nm) for Sytox Green, labelled DNA and labelled BSA, orange (FL2 585 ± 20 nm) for propidium iodide, and red (FL3 670 ± 25 nm) for chlorophyll autofluorescence using a flowrate of 14 μL min^{-1}. BD Accuri C6 Software 264.21 was used for data acquisition and analysis. Forward scatter and side scatter plots were used to remove background noise and cellular debris. Sytox Green uptake was analysed by plotting log autofluorescence (FL3) versus log green fluorescence (FL1), to discriminate between cells that did and did not take up Sytox Green, labelled DNA and labelled BSA. Propidium iodide uptake was analysed by plotting log autofluorescence (FL3) versus log orange fluorescence (FL2).

2.5. Microalgal transformation

Chlamydomonas reinhardtii, *Acutodesmus obliquus*, *Chlorella vulgaris* and *Neochloris oleoabundans* cells were harvested, washed, and re-suspended as mentioned before. Linearized plasmid pOpt-Clover-Hyg at a final concentration of 2 μg mL^{-1} and 25 μg mL^{-1} of boiled salmon sperm DNA (D1626, Sigma) were added to the cell suspension and kept on ice for 15 min. From this transformation mixture 250 μL was transferred into a 2-mm electroporation cuvette, followed by electroporation at 2.25, 5, 6 and 7.5 kV cm^{-1} respectively. The cells were recovered in the dark for 10 min, then transferred into a sterile 15 mL tube containing 10 mL of freshwater medium and kept in the dark overnight at 25 °C with continuous agitation at 125 rpm. After recovery, cells were collected by centrifugation at 2500 ×*g* for 10 min at room temperature. The cell pellet was re-suspended in 200 μL cultivation medium and 1×10^8 cells were plated onto selective agar plates with the selected hygromycin B concentrations. The antibiotic concentrations were determined for each microalgae species using an antibiotic sensitivity test. All selective plates were incubated under a day:night cycle regime (16:8) at 25 °C at 60 μmol $m^{-2} s^{-1}$ light intensity (Grolux fluorescent tubes, Sylvania F36W/GRO) at 125 rpm and 2.5% CO_2.

2.6. Antibiotic sensitivity test

The sensitivity of all microalgae species to hygromycin B was tested.