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# Highly efficient molecular delivery into *Chlamydomonas reinhardtii* by electroporation

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**Abstract**−Electroporation is a highly efficient delivery method for transformation in various cell types; however, in microalgae there is lack of optimized electroporation parameters due to cell wall, protoplast preparation and viability. Therefore, we optimized electroporation conditions for transforming microalgae using *Chlamydomonas reinhardtii* strains of wild type and mutant (cell wall deficient). To investigate the effects of molecule size, calcein (623 Da) and fluorescein isothiocyanate-dextran (FITC-dextran, 40 kDa) were used and various electroporation parameters were applied such as different voltage and pulse length and molecule uptake pattern and cell viability were observed. Cell wall is insignificant in case of small sized molecule uptake as noticed by 1.25 kV/cm and 30 ms for both strains, whereas for larger molecules by 1.5 and 2 kV/cm and 30 ms for mutant and wild type, respectively. In terms of viability, there was no significant difference in both the strains on applied electroporation parameters. The controlled parameters corresponding to 1.5 to 2.0 kV/cm and 20 to 30 ms could be used to deliver macromolecules (DNA, proteins) into cells effectively.

Key words: Electroporation, Microalgae, *Chlamydomonas reinhardtii*, Molecules Delivery, Viability

## INTRODUCTION

Lack of fossil fuel resources has led to the development of various types of biomass. The first generation biomass could be obtained from agricultural products, but it made way for the second generation cellulosic biomass due to its food security problem. However, the second generation biomass also revealed significant disadvantages such as low energy productivity caused by expensive pre-treatment process [1]. Therefore, the algal-based third and fourth generation biomass have been considered as a promising alternative, especially from microalgae [2,3]. Microalgae have many advantages as a biofuel resource, including faster growth rate, higher lipid content and higher photosynthetic efficiency in comparison to terrestrial crops [3,4].

Microalgae can provide several types of renewable biofuels, such as biodiesel from lipid, alcohols form starch and $H_2$ gas from photosynthesis [5]. Algal biodiesel is one of the most significant topics in biofuel production, as it performs similarly to petroleum diesel and possesses gross heating value (126,200 BTU/gal) comparable to that of diesel fuel (138,700 BTU/gal) [6], while being beneficial to the environment because it is biodegradable [7]. There are also several more advantages in comparison with diesel fuel: portability, ready availability, renewability, higher combustion efficiency, low sulfur and aromatic content [7,8], and low damage in existing engine performance [2].

Although microalgal fuel shows enormous potential, there still exist bottlenecks that should be overcome to make it economically feasible [3-5]. Genetic engineering has been regarded as a promising solution [2,9]. In this way, we can manipulate the characteristics of microalgae to improve the productivity, lipid yield and end products, and to secrete their lipids outside the cell [9]. Thus, if the genetic manipulation of microalgae succeeds, the overall algal-based productivity would be significantly improved. However, genome-based enhancement of microalgae is still in its infancy due to the lack of a genetically tractable model system despite successful gene delivery into microalgae with various genetic tools [9,10].

The green alga *Chlamydomonas reinhardtii* used in this study is currently the model eukaryotic system of choice [11,12], because its nuclear genome sequence is comprehensively annotated [13], and it reveals the possibility of extensive metabolic engineering in the algal system [14]. Genetic transformation of *C. reinhardtii* was reported as early as 1982 [15]; however, significant progress could not be achieved until genomics of this strain were discovered [13, 14] and transgenic tools were developed [8,12].

Electroporation has been commonly used as delivery method for over 30 years due to the simplicity of the procedure and high efficiency [16,17]. When short high-voltage pulses are applied on cells, the external electric field causes transient and reversible breakdown of cell wall [18]; this method has been used in genetic engineering of *Chlamydomonas* with high efficiency [19,20]. Most of the studies focused on finding the optimum conditions for electroporation to deliver DNA molecules and observed transformation efficiency. However, such an approach cannot reveal actual electroporation effects, as complex biological steps are involved in genetic manipulation after transferring genes into cells. There are a few reports which carried out quantitative studies [21,22].

We conducted an experiment on electroporation to directly measure and quantify the effects of a wide range of parameters to optimize the conditions for electroporation to microalgae, based on viability and molecular uptake pattern. To achieve this, we investigated two factors: the existence of cell wall and the size of molecules.

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‡This paper is dedicated to commemorate Prof. Ji-Won Yang (KAIST) for his retirement.

For the first factor, two kinds of *Chlamydomonas reinhardtii* strains were selected; one is a mutant strain which does not have cell wall, and another is wild type which has cell wall. For the second, we used two molecules with different sizes: calcein and fluorescein isothiocyanate-dextran (FITC-dextran). These optimized conditions can be used for delivering DNA fragments or other macromolecules into microalgae for high-efficiency biodiesel production in the future.

## MATERIALS AND METHODS

### 1. Algae Culture and Sample Preparation

*C. reinhardtii* strains, wild-type CC-124 and mutant CC-4348 (sta6-1 mt+), were grown in 200 ml of tris-acetate-phosphate (TAP) medium under continuous illumination (82 μmol photons/$m^2$ s) at 25 °C with shaking at 180 rpm. At the exponential phase, cells were counted ($5\times10^6$-$8\times10^6$ cells/ml) using a haemocytometer [22] and harvested at 4,000 rpm for 5 min.

The collected cells were resuspended in TAP media to meet the final concentration of $1.5\times10^8$ cells/ml in TAP. For the molecules uptake, two different fluorescent molecules such as calcein (Sigma, 623 Da) and FITC dextran (Sigma, 40 kDa) were used at the final concentrations of 100 μM and 50 μM, respectively. Both molecules can emit green fluorescence, and it is possible to measure the degree of intracellular delivery.

### 2. Electroporation

125 μl of the final mixture comprising cells and molecules was transferred to 2 mm gap electroporation cuvette (Model 620, BTX, USA), and applied to different electric fields and pulse lengths via electroporation system (ECM 830, BTX, USA). After an electric shock was applied, the cells in the cuvettes were allowed to recover themselves by incubating for 30 min at 25 °C and added to 300 μl of TAP media, and the mixture was transferred to 1.5 ml amber color tube to avoid fluorescence degradation and to allow continued recovery for an hour. All these steps were performed similarly following the method described previously [23].

### 3. Viability and Fluorescence Analysis Methods

The percentage of live cells after electroporation could be assayed by utilizing the fact that microalgae are photosynthetic unicellular eukaryotes. Quantum yield (QY) of chlorophyll photosynthetic efficiency was measured via AquaPen-C AP 100 (Photon Systems Instruments, Czech Republic) [24]. QY is defined as a value indicating the maximum rate of $CO_2$ fixed or $O_2$ evolved per mole of photons absorbed by oxygenic photoautotrophic organisms, which correlates to the photosynthetic efficiency [25]. Accordingly, the more the cells are alive, the higher QY can be detected and the relative viability estimated with control sample QY value.

To assess the amount of fluorescent dye in cells, the cells were washed twice with TAP media to remove cells attached to fluorochrome calcein, and for FITC-dextran, the cells were trypsin treated (1 mg $mL^{-1}$) for 15 min at 37 °C [26]. Finally, the cells were washed and resuspended in 400 μl of TAP media and were measured for fluorescence intensity spectrum by spectrofluorophotometer (RF-5301pc, Shimadzu, Japan). Excitation wavelengths for calcein and FITC-dextran were 488 nm and 490 nm, respectively, with slit width at 5 nm for both excitation and emission. The highest peaks at a certain emission wavelength for calcein and FITC-dextran (511 nm and 520 nm, respectively) were calculated for the degree of uptake. Relative fluorescence intensity was revealed as subtracting the intensity of control from that of each sample.

Electroporated cells were observed under fluorescence microscope (Leica DM 2500, Germany) and confocal laser scanning microscope (C1si, Nikon, Japan and Zeiss LSM 510, Germany) for the delivery of fluorescence molecules.

## RESULTS AND DISCUSSION

Electroporation has been recently used as a powerful method for genetic engineering of microalgae [27,28]. There are still only a few studies investigating the electric effect of the method without biological intervention [21,22]. Here, we optimized electroporation parameters on algal cells by applying various voltages and pulse lengths for manipulating its viability and uptake pattern of different sized molecules. To optimize the electroporation on algae, we assessed the existence of cell wall. Therefore, two different strains of *C. reinhardtii* were selected-the cell wall-deficient mutant (CC-4348 sta6-1 mt+) and wild type (CC-124)-and different sized molecules such as calcein (623 Da) and FITC-dextran (40 kDa) were used to investigate the effects. Most studies have examined gene transformation efficiency involving complex biological steps in cells, which causes the indirect measurement of electrical effects [19,20]. In this study, we observed the effects of electroporation by checking the photo-

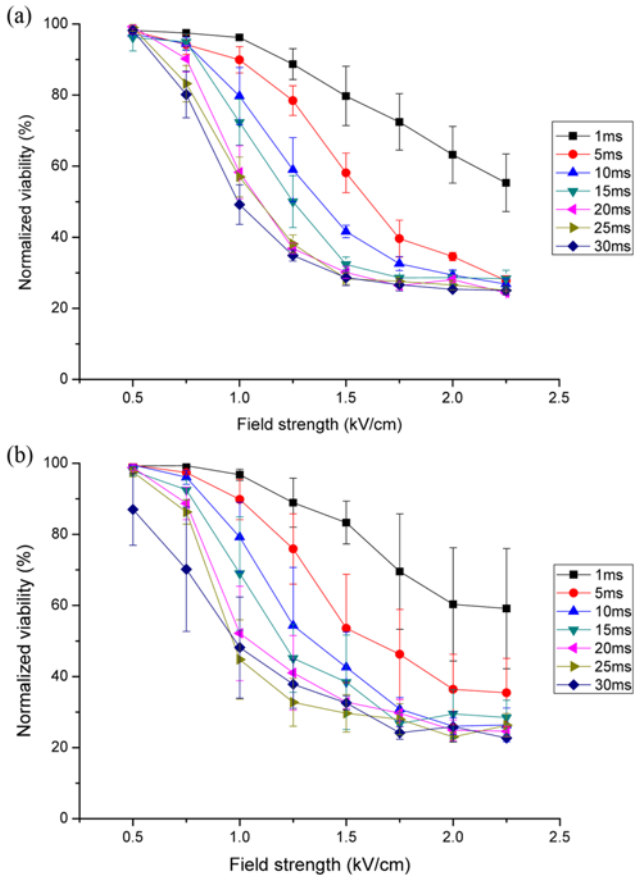


**Fig. 1. Normalized viability on different field strength and pulse length for *C. reinhardtii*. (a) Cell-wall-deficient mutant (b) Wild-type. The data points represent the average of samples and error bars indicate standard error (n=3).**

synthetic efficiency as viability and fluorescence intensity as indicators of molecules delivery in electroporated cells.

**1. Viability Assay**

It is critical to maintain a high proportion of viable cells after applying electric shock because many transformants must survive and propagate again for high transformation efficiency. Accordingly, the viability of cells was first investigated. There is a possibility that the kinds of additives used affected cell viability, but the effect was reported to be small [22], and thus we did not measure viability dependency on the delivered molecules. Fig. 1 shows that cell viability varies depending on field strengths and pulse lengths. It was clearly observed that the higher voltage and longer pulse showed more reduction in viability as expected. However, wild-type cells were initially expected to have higher survivability than mutant cells due to the presence of cell wall, but the extent of revival from the electroporation-caused injury in the two strains were almost same regardless of cell wall. Thus, the existence of cell wall seems to exercise low efficacy as a barrier against electroporation for delivery purposes in algae.

**2. Molecules Uptake Pattern**

Fig. 2 shows the levels of calcein uptake in mutant and wild-type strains of *C. reinhardtii* with different electroporation parameters. As higher electric shock is applied to cells, bigger pores are generated on the surface of the cells and lead to more uptake of extracellular molecules by the cells. Accordingly, more calcein should be delivered into the cells with increasing field strength and pulse length, and this study confirms the anticipated results. The relative fluorescence intensity of calcein was increased with increasing electroporation parameters in both strains. Moreover, small calcein molecules were delivered to a similar extent in both the strains. On the other hand, the extent of calcein uptake in wild type was still lower (0.96 times) than cell-wall-deficient cells in most samples. The delivery of dimensionally bulkier FITC-dextran was accomplished in a manner qualitatively similar to that for calcein (Fig. 3). In contrast to calcein delivery, the degree of FITC-dextran uptake in both cell strains was significantly different in that mutant strains revealed 24% more uptake. Cell wall blocks the insertion of large-size molecules into the cell, as shown by the result that wild type incorporated 0.76 times lesser amount of the fluorescent dye. Thus, it seemed logically apparent that we would experience measurable difficulty in transporting genes into wild-type cells, as has been mentioned in previous reports [19,20]. Furthermore, even the mutant cells could not take in similar amounts of FITC-dextran in comparison to the amounts of calcein despite the absence of cell wall. The basic principle of electroporation is to disrupt the lipid bilayer of cell membranes and help extracellular molecules to enter the cells [29]. Although the mutant strain was a genetically manipulated *C. reinhardtii* strain that is unable to synthesize cell wall due to the lack of essential glycoprotein, still

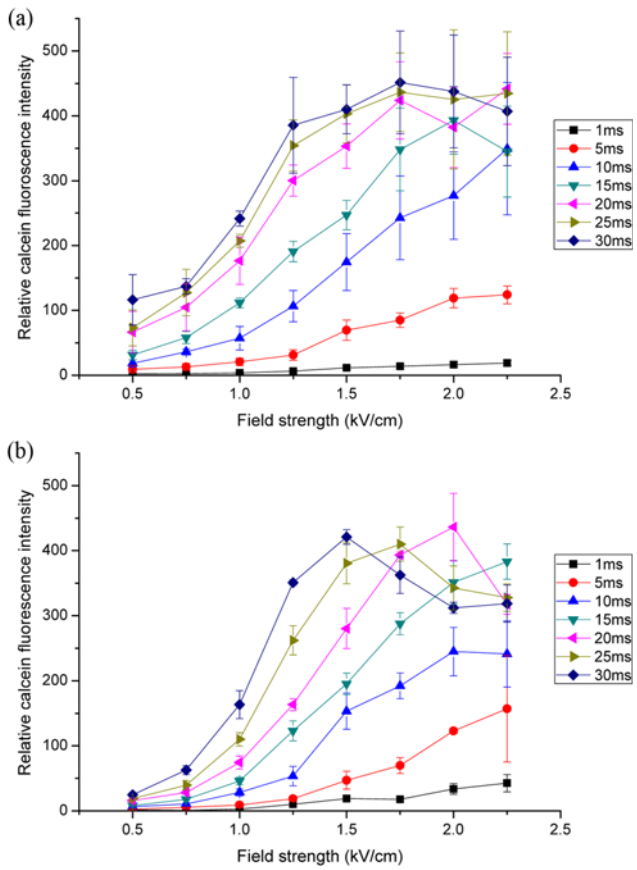


**Fig. 2. Calcein uptake pattern in *C. reinhardtii* on different field strength and pulse length (a) Cell-wall-deficient mutant (b) Wild-type. The data points represent the average of samples and error bars indicate standard error (n=4).**

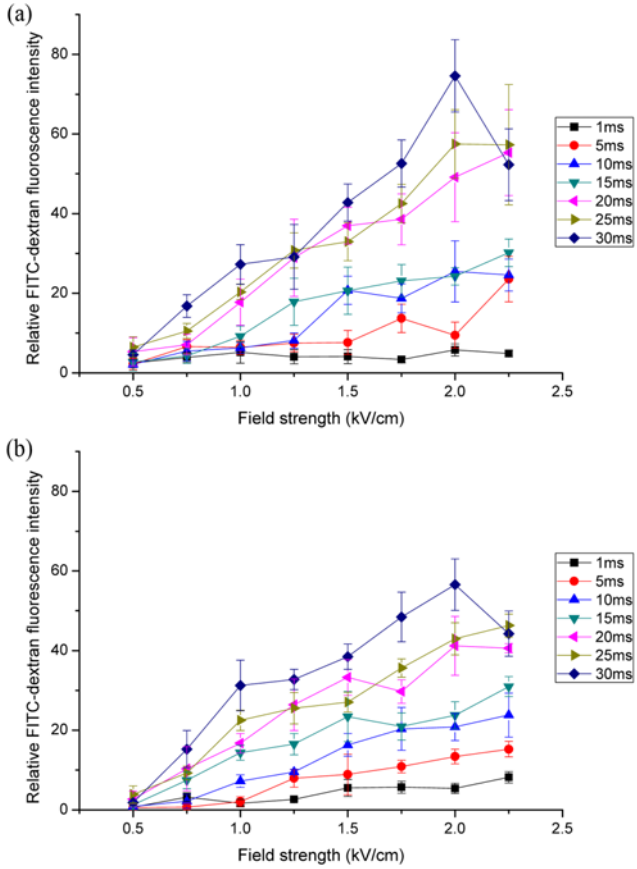


**Fig. 3. FITC-dextran uptake pattern in *C. reinhardtii* on different field strength and pulse length (a) Cell-wall-deficient mutant and (b) Wild-type. The data points represent the average of samples and error bars indicate standard error (n=6).**

other proteins exist on the surface of the algal cells [30] and may have disrupted the insertion of extracellular molecules. Thus, the degree of FITC-dextran incorporation was lower than that of calcein in both cell strains. Finally, electroporation parameters for high-efficiency molecular delivery should be optimized due to the opposite tendency of viability and molecular uptake. Optimum electroporation conditions for calcein and FITC-dextran delivery were obtained by combining viability and each uptake result. In the case of small-sized calcein, the conditions of 1.25 kV/cm and 30 ms can be identically applied to mutant and wild-type cells because of the similar levels of viability and calcein uptake in both strains. Higher electric field was required for bigger-size FITC-dextran delivery as the change of cell strains, so the condition of 1.5 and 2.0 kV/cm at 30 ms is considered appropriate for mutant and wild-type strains, respectively. Fig. 4 illustrates the confocal images of electroporation-mediated calcein and FITC-dextran delivery into mutant and wild-type *C. reinhardtii*, with some variations in parameters for molecular delivery, in order to qualitatively compare the delivery efficiency depending on the strains and the sizes of molecules. It was clearly manifested that green fluorescence from calcein was greater than the FITC-dextran, which led to the establishment that small molecules were delivered more efficiently than larger molecules. In addition, the cell wall-deficient mutant observed more fluorescence cells than wild type, as cell wall prevents the uptake of molecules into the cytoplasm of cells. However, in terms of viability, there was no significant difference in both the strains. Therefore, we concluded that the increased amount of delivered molecules inevitably accompanied the low level of surviving cells.

According to our results, the controlled parameters corresponding to 1.5 to 2.0 kV/cm and 20 to 30 ms were effective in deliver-

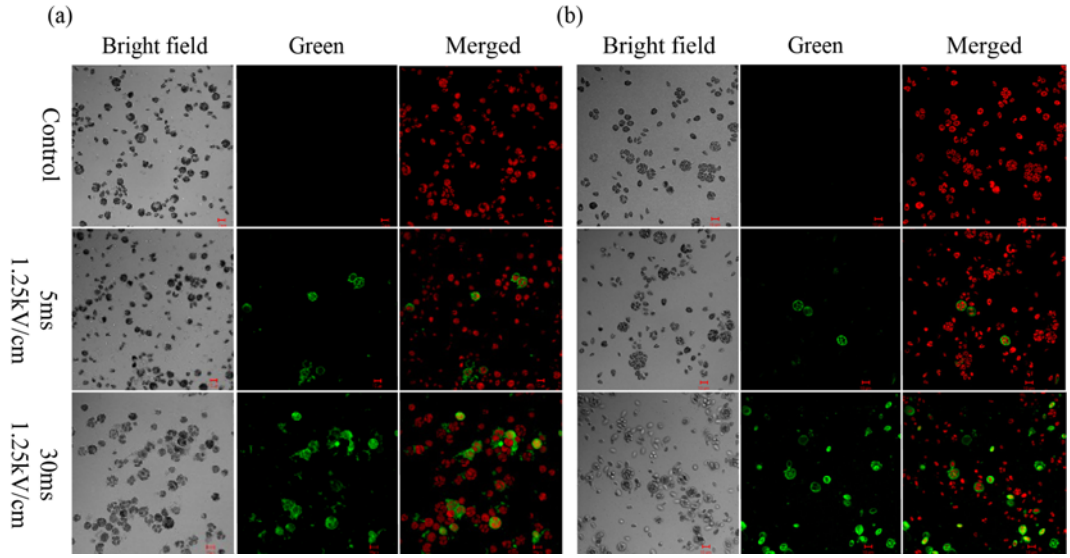


**Fig. 4. Confocal micrograph shows the comparison of intracellular uptake of Calcein in *C. reinhardtii* (a) Cell-wall-deficient mutant and (b) Wild-type.**

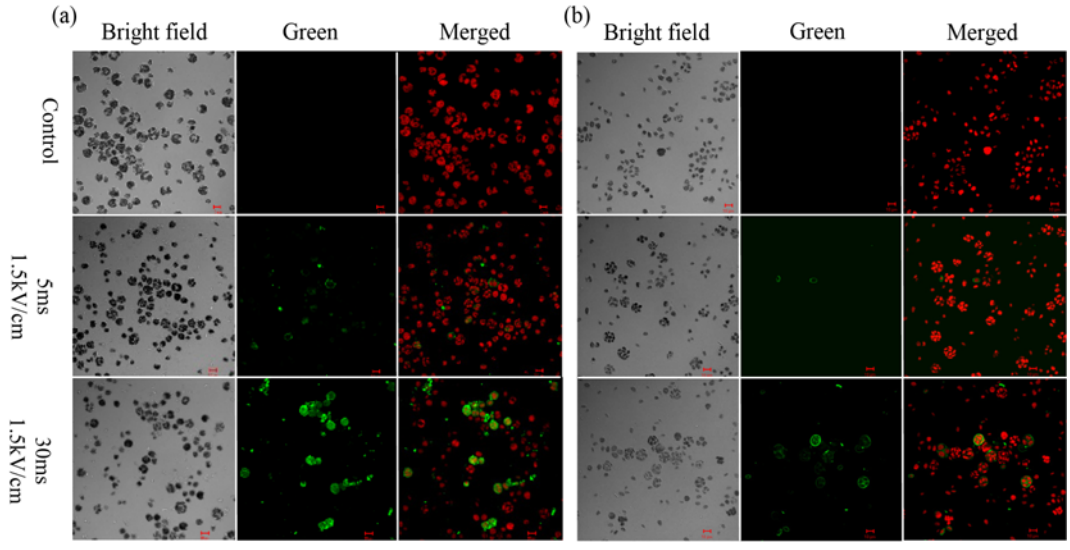


**Fig. 5. Confocal micrograph shows the comparison of intracellular uptake of FITC-dextran in *C. reinhardtii* (a) Cell-wall-deficient mutant and (b) Wild-type.**

ing macromolecules such as DNA into cells, since little FITC-dextran could be transferred to the inside of cells at lower electric field even though high viability was obtained. Therefore, we concluded that molecular uptake has a higher impact on the net effect rather than the viability.

## CONCLUSION

In this study, we intended to establish optimum electroporation parameters for the delivery of macromolecules (DNA fragments or proteins) into microalgae. It was possible to deliver external molecules into cells with the increase of field strength and pulse length of electroporation. However, high molecular uptake accompanied low viability. Thus, targeting only the high degree of delivery is not appropriate, for macromolecules must be stably delivered into viable cells and high transformation efficiency gained. We tried to screen the net efficiency of electroporation parameters and found the optimum for each case. Therefore, the optimum electroporation condition could be applied for gene transformation if the charge and structure of the molecules is not considered during its uptake into algal cell.

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