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Effect of moderate static electric field on the growth and metabolism of *Chlorella vulgaris*

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Abstract

An electric field (EF) generator device was fabricated and applied to the treatment of *Chlorella vulgaris* ISC33 at three distinct concentrations before cultivation. The EF of moderate intensity (2.7 $kVcm^{-1}$) has a hormetic effect on algal growth. The highest growth stimulation of 51% was observed after 50 min treatment of 0.4 g L^{-1} algal suspension. The influence of EF on the system was then studied from both theoretical and experimental perspectives. The growth rate increased with treatment time up to a maximum because of improved membrane permeability, and then declined afterwards due to peroxide accumulation in the medium. The contents of chlorophylls, carotenoids, soluble carbohydrates, lipids, and proteins were also measured to understand possible changes on algal metabolism. The EF treatment of algal suspension has no observable effect on the cell metabolism while both algal growth and metabolism was significantly affected by the inoculum size.

Keywords:

Cell metabolism; *Chlorella vulgaris*; electroporation; growth stimulation; inoculum size; peroxide accumulation

1. Introduction

Microalgae are industrially important microorganisms due to their capability in the production of a wide range of valuable bio-products for pharmaceutical, agro-food, and biodiesel industries (Harun, 2010). *Chlorella vulgaris* is one microalgae species that is mainly considered for mass cultivation as a valuable source of human nutrition and biodiesel feedstock (Safi, 2014). The annual world production of this algae is about 2000 tonnes, mainly devoted to the agro-food industry as a source of single cell protein (Kim, 2015). However, current production processes are expensive and restrict the full potential of microalgae.

The most common technology for microalgae production is the open raceway pond, which is used to produce 95% of all microalgae, as cited in (Kumar, 2015). Open ponds are relatively inexpensive to construct and to operate, but suffer from low yield of 0.12 - 0.48 g L^{-1} d^{-1} (Kumar, 2015). Although it is possible to improve the yield by injecting CO_2 into the aqueous media, the gas-liquid mass transfer rate is poor; the CO_2 uptake is limited and most of the CO_2 (about 90%) escapes to the atmosphere (Kumar, 2015). In addition, it is not possible to improve the yield by mixotrophic cultivation strategies (such as glucose injection) because this poses the risk of bacterial contamination. However, if a photobioreactor is used instead, the problems of CO_2 escape and bacterial contamination can be avoided. For example, Cheirsiple and Torpee (Cheirsilp, 2012) screened three mixotrophic, heterotrophic, and photoautotrophic cultivation conditions of *Chlorella sp.* in order to reach a high yield of biomass in a photobioreactor. They found that mixotrophic cultures of the algae containing 2 g L^{-1} glucose at a light intensity of 3000 lux produced about 90% higher yield of biomass than the heterotrophic and autotrophic cultures. Although bacterial contamination and CO_2 escape is not a problem in a photobioreactor, the operation and sterilization of numerous photobioreactors are laborious, time-consuming, and costly (Kumar, 2015). Several researchers have also reported that CO_2 gas-liquid mass transfer rates are still a factor inhibiting the growth of autotrophic microalgae in a photobioreactor (Fan, 2008; Langley, 2012; Pirouzi, 2014). Fan et al. (Fan, 2008) constructed a membrane sparged helical tubular photobioreactor in order to overcome the CO_2 mass transfer limitations during *C. vulgaris* cultivation. The biomass yield was improved by 85% (216 mg L^{-1} d^{-1}) compared to a helical tubular photobioreactor with a perforated pipe sparger (117 mg L^{-1} d^{-1}). Lv et al. (Lv, 2010) investigated the effect of cultivation conditions including nitrate and CO_2 concentration and irradiance on the growth of marine *C. vulgaris* in a membrane sparged photobioreactor. They reached an optimal biomass yield of 400 mg L^{-1} d^{-1} by adjusting the cultivation conditions at a nitrate concentration of 5 mM, an aeration rate of 1.2 L min^{-1} with 1% CO_2, and light intensity of 60 µmol m^{-2} s^{-1}. To improve both CO_2 gas-liquid mass transfer and light provision, Pirouzi et al. (Pirouzi, 2014) have constructed a novel triangular external airlift loop photobioreactor providing high mixing intensity and mass transfer rates at significantly low volumetric power consumption. However, despite these yield improvements over open pond systems, photobioreactors have ten times higher production cost, which limits their applicability to the production of nutrients and high value bio-products, which is only about 5% of the total microalgae market[1] (Kumar, 2015).

[1] Algatechnologies Ltd. http://algatech.com/technology.asp

Therefore, to realize the full potential of microalgae, new strategies need to be developed for the improvement of algal growth in a cost effective manner. In particular, physicochemical pretreatments have the potential to promote algal growth in open pond systems and to stimulate the synthesis of specific metabolites, without causing bacterial contamination. For example, Wang et al. (Wang, 2008) studied the effect of static magnetic fields on the growth of *C. vulgaris*. They observed an increase of up to 68% in the growth rate of algal cells exposed to 10-35 mT for 12 h compared to a control. Small et al. (Small, 2012) found that the growth rate of *C. kessleri* cells almost doubled during cultivation both in open raceway ponds and in flask cultures when they periodically exposed to a uniform 10 mT static magnetic field throughout cultivation. A few studies also report the stimulatory effects of electric fields (EF) on the growth and development of non-microalgae plant cells. For example, Eing et al. (Eing, 2009) observed positive growth stimulation in *Arabidopsis thaliana* after exposure to nanosecond pulse electric fields of 5 kV cm^{-1}. The EF systems are simpler in operation than the magnetic ones due to lower energy consumption which avoids the need to use complicated cooling systems.

These results are very promising, but to the best of our knowledge, experiments using EF pretreatment has never been conducted with microalgae species. Therefore, in this preliminary study, the interaction of static EF with *C. vulgaris* ISC33 was comprehensively examined. An electric field generator was designed, fabricated, and applied to the treatment of the microalgae at various time intervals. The algal growth was monitored after exposure to moderate static electric fields and the effects of the treatment on the growth rate and metabolism were studied from both theoretical and experimental perspectives. Further research regarding the application of this novel strategy of growth stimulation to large scale cultivation systems will be considered in the future.

2. Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and purchased from Merck (Darmstadt, Germany). Deionized water was prepared in the laboratory by a reverse osmosis system (HastaranTeb Co., Tehran, Iran).

2.2 Microalgae and culture conditions

The freshwater microalgae *C. vulgaris* ISC33 was obtained from the Research Institute of Science, Iranian Academic Center for Education, Culture & Research (Shahid Beheshti University, Tehran, Iran). The autotrophic cultivation of microalgae was done in BG11 medium. The pH of the medium was adjusted to 7.0±0.1 prior to autoclaving. All solutions and culture media were prepared with deionized water. To prepare algal stock cultures, *C. vulgaris* ISC33 cells were batchwise cultivated in 250 mL Erlenmeyer flasks on a shaker operating at 120 rpm. The cultures were incubated at 25±1°C under 16/8 light/dark cycle illumination using cool white fluorescent lamps at photosynthetic photon flux density of 27 µmol m^{-2} s^{-1}. The carbon source was inorganic carbon dioxide supplied by air (composed of about 0.04 mol% CO_2).

2.3 Electric field treatment

Generation of EF was performed by a device designed and built in-house consisting of a pulse generator and a treatment chamber. The chamber was made of two stainless steel concentric cylinders fixed on a silicon sheet and the culture media was confined

in between the cylinders. The outer diameter of the inner cylinder and inner diameter of the outer cylinder were 4.8 cm and 6.3 cm, respectively. The inner and outer cylinders were respectively connected to negative and positive poles of the device in order to generate a uniform constant electric field with amplitude of 2 kV corresponding to field strength of 2.77 kV cm^{-1}.

Constant EF with amplitude of 2 kV was generated by a television (TV) high voltage generator, Figure 1. The TV high voltage generator was coupled with a 100 MΩ resistor and then switched onto the output line and the treatment chamber. Impulse voltage waveforms were acquired by a 100 MHz Oscilloscope (DS 1102, RIGOL, China). The voltage was measured by a calibrated voltage divider circuit made in house.

Figure 1

20 mL of microalgae starter cultures containing 0.25, 0.4, and 1.0 g cell dry weight (CDW) per liter were prepared from the stock culture at the end of their logarithmic growth stage. The algae samples were then exposed to a static electric field for 0 (control), 10, 30, 50, and 70 min under aseptic conditions. The samples under treatment were at approximately room temperature, which was maintained by an air conditioner at 25±1°C. After EF treatment, 20 mL of the microalgae samples were diluted with 170 mL of the fresh culture media and batchwise cultivated for four days in graduated cylinders of 28 cm in length and 4 cm in diameter. The samples were exposed to continuous illumination of 27 μmol m^{-2} s^{-1} at the top surface. There was no need for pH maintenance as only a slight steady decline from 7.0±0.1 to about 6.8±0.15 was observed for all cultures. Filtered humidified air was sparged into the reactor to avoid volume loss of the aqueous media. The cells were harvested after 4 days by centrifugation at 5000 rpm for 5 min, thoroughly washed with deionized water, and freeze dried for further analysis.

2.4 Determination of total chlorophylls and carotenoids

Total carotenoids and chlorophylls were extracted from microalgae with acetone (aqueous, 80 vol%) and the absorbance was read at 470, 647, and 663 nm by a UV-vis spectrophotometer (Cintra 6, GBS, Australia). The total contents of carotenoids and chlorophylls were calculated according to the following correlations (Lichtenthaler, 1987).

$$C_{a+b} = 7.15 \times OD_{663} + 18.71 \times OD_{647} \quad (1)$$

$$C_{a+x} = 5.05 \times OD_{470} + 2.077 \times OD_{663} - 9.206 \times OD_{647} \quad (2)$$

Where, C_{a+b} and C_{a+x} show the concentration of total chlorophylls and carotenoids in mg L^{-1}, respectively.

2.5 Determination of biochemical composition

The biochemical composition of the microalgae was determined after four days of cultivation. Proteins were extracted from freeze dried algal biomass by ultrasonication for 5 min at 50 W in 60 mM phosphate buffer solution and the content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. The soluble carbohydrates were extracted in 0.1 M phosphate buffer

solution prior to analysis and the content was determined by the phenol-sulfuric acid method (Dubois, 1956), using glucose as standard. The lipid of the lyophilized sample was extracted by ultrasonication for 10 min at 60 W in chloroform/methanol (2:1 by volume) mixture and quantified according to the modified Bligh and Dyer method (Zhu, 2002). All the extraction processes were repeated three times in order to have complete extraction of the biochemicals.

2.6 Determination of cell electrolyte leakage

To monitor electropermeabilization of the plasma membrane, the leakage of electrolytes through algal membranes was recorded by observing changes in the electrical conductivity (EC) of the medium. The harvested microalgae cells at the end of the logarithmic growth phase were first washed three times with deionized water to remove any extracellular electrolytes. The microalgae cells were then used in the preparation of three stock cultures with the concentrations of 0.25, 0.4, and 1.0 gCDW L^{-1}. Each stock culture was divided into 8 aliquots of 20 mL algal suspension and exposed to EF either for 10, 30, 50, or 70 min. Every treated sample was then shaken at 150 rpm for 10 min in order to provide suitable conditions for permeation of intracellular electrolytes. A control algal suspension was concurrently subjected to the same manipulations except for EF exposure as well. Both control and treated algal suspensions were centrifuged at 5000 rpm for 5 min and the electrical conductivities of the aqueous supernatants were read by a conductivity meter (SARTORIUS Mechatronics PT-20/C1.0, USA).

2.7 Monitoring of algal growth and contamination

Algal growth was monitored by daily removal of 1 mL aliquots from microalgae culture under aseptic conditions. The turbidity at 750 nm and pH of the samples were measured by a UV-visible spectrophotometer and pH meter, respectively. The biomass optical absorption was correlated with the gravimetrically measured algal dry weight and cell counts according to the following correlations:

$$CDW = 0.56 \times OD_{750} - 0.07 \quad (3)$$

$$N_{cell} = (2347 \times OD_{750} - 748.2) \times 10^{7} \quad (4)$$

Where, N_{cell} stands for the number of cells in one milliliter of solution, and CDW is the cell dry weight in g L^{-1}. To derive correlation 3, the algal cells were first washed thoroughly with deionized water to remove any extracellular electrolytes. The algal cells were then dried at 60 °C in a hot air oven and weighed after reaching a constant mass. To derive correlation 4, the cells were loaded on a hemocytometer and then placed on an optical microscope stage for cell counting. The whole process of these two experiments was done in triplicates and the mean values were used for the development of the correlations.

The growth rate of the microalgae was calculated by the following equation:

$$\mu = \frac{\ln\left(OD_{750,f} / OD_{750,i}\right)}{\Delta t} \quad (5)$$

Where, μ, OD_{750}, and Δt show the specific growth rate in h^{-1}, light absorption at 750 nm, and cultivation time in h, respectively. The indexes i and f represent the initial and final time of cultivation.

To examine the possibility of bacterial contamination, the optical absorption of the samples at peak absorbance of chlorophyll (680 nm) was measured as well. The ratio of chlorophyll absorbance at 680 nm to turbidity at 750 nm is a criterion for judging the purity of the algal culture during cultivation (Small, 2012).

3. Theoretical description of electropermeabilization in algal suspension

The phenomenon of electropermeabilization was observed in the treatment of microorganisms and synthetic membranes by electric fields. Different theoretical models including electromechanical collapse, electrohydrodynamic instability, wave instability, and transient aqueous pore models were applied to describe the influence of EF on membrane systems (Weaver, 1996). The transient aqueous pore model is one class of theories which can account for the key features of electropermeabilization and describe all of the experimental findings (Weaver, 1996). Accordingly, it was used for theoretical descriptions of the experimental EF results. In this work, the model has been slightly modified for the present problem and a brief description of the key features is discussed in the following sections. A complete list of the notations and the values of the fixed parameters are presented in Table 1.

Table 1 Parameters of the electropermeabilization study in algal suspension

3.1 Mechanism of hydrophilic pore formation

According to the transient aqueous pore theory, the formation of hydrophilic pores in the cellular membrane is a two-step process. The thermal fluctuations of the membrane components, i.e. phospholipids, cause the formation of transient hydrophobic pores. The stochastic interaction and fluctuation of the components results in pores with the radius exceeding the critical size 0.3-0.5 nm (Glaser, 1988). These critical narrow pores leads to substantial deformation of molecular order and a reorientation contributing to the formation of hydrophilic pores. These hydrophilic pores are unstable due to strong repulsive forces between hydrophilic parts of the lipid components. The energy needed to create a hydrophilic pore is associated with the strain and surface energies. The former leads to an increase and the latter causes a decrease in the energy which respectively impedes and favors the further growth of the hydrophilic pores. The following equation can be used to calculate the energy of the hydrophilic pores:

$$\Delta U = 2\pi r\gamma - \pi r^2 \sigma_{eff} \tag{6}$$

Where, ΔU is the energy of the pore and r is the radius of the narrowest part of the channel in the membrane. γ and σ are the strain energy per unit length of the pore edge and the surface energy per unit area of the pore, respectively. The first term in the right hand side of equation 6 is much larger than the second one for narrow pores at the intermediate stages of the process which makes the growth of hydrophilic pores improbable. This energy barrier to pore formation and growth can be decreased through the induction of an electric field. Indeed, interfacial tension and capacitive energy contribute to the surface energy of the pore. This capacitive energy can be

calculated by modeling the pore as a parallel plane capacitor. Thus, the effective surface energy can be written as follows:

$$\sigma_{eff} = \sigma + \frac{(\varepsilon_w - \varepsilon_l)\Delta\phi_m^2}{2h\beta} \frac{\int_{r_{\min}}^{r} \alpha^2 r dr}{\int_0^r r dr} \tag{7}$$

Where, ε_l and ε_w are the dielectric constants of the lipid membrane and water, respectively. h, r, and β are the membrane thickness, pore radius, and a modifying parameter including the effects of cell membrane and wall interactions and EF-induced shrinkage in the model. $\Delta\phi_m$ is the average transmembrane potential. It was assumed uniform around the circumference of a cell and was calculated by the following approximate equation for the algal suspension (Weaver, 1996):

$$\Delta\phi_m = \frac{3}{2} E.r_{cell} \tag{8}$$

Where, E is the electric field strength and r_{cell} is the radius of the algae.

The local voltage across a pore is less than this average transmembrane potential due to the spreading resistance of ion fluxes near the pore entrances, Born energy repulsion of ions, and hindered permeation of ions within the pore. These effects can be combined into a voltage divider (α) given by (Weaver, 1996):

$$\alpha = \left(1 + \frac{\pi r k_p}{2h k_e}\right)^{-1} \tag{9}$$

Where, k_p and k_e are the conductivity of the pore and the bulk solution, respectively. The conductivity of the bulk solution, in $\mu S\, cm^{-1}$, was correlated with cell number for the specific case study as $k_e = 0.215\times10^{-7} N_{cell} + 0.258$; however, it can easily be calculated from the mobility and concentration of the ions in any solution (Weaver, 1996). The conductivity of the pore is given by (Weaver, 1996):

$$k_p = k_e H_i \exp\left(\frac{\mu_i^o}{kT}\right) \tag{10}$$

Where, H_i and μ_i^0 are the steric hindrance factor and standard chemical potential of ion i (modeled with K^+ in this study), respectively. kT is the product of Boltzmann constant and absolute temperature, equivalent to 4.11×10^{-21} J mol^{-1}. The hindrance factor can be calculated by (Barnett, 1991):

$$H_i = \left(1 - \left(\frac{r_i}{r}\right)^2\right)\left(1 - 2.1\left(\frac{r_i}{r}\right) + 2.09\left(\frac{r_i}{r}\right)^3 - 0.95\left(\frac{r_i}{r}\right)^5\right) \quad (11)$$

Where, r_i is the radius of the ion which equals 0.136 nm for K^+.

The standard chemical potential of an ion in the pore was approximated by the Born energy of a small charged sphere on the axis of a finite cylinder (Weaver, 1996):

$$\mu_i^0 = \frac{(z_i e)^2}{4\pi\varepsilon_l r} P\left(\frac{\varepsilon_l}{\varepsilon_w}\right)\frac{0.02h}{r} \quad (12)$$

Where, $e = 1.6\times10^{-19} C$ and z_i is the charge of the ion i. The function $P\left(\frac{\varepsilon_l}{\varepsilon_w}\right)$ has a value of 0.12 for the adopted dielectric constants of the water and lipid (Parsegian, 1969).

The pore energy distribution as a function of pore radius and transmembrane voltage is shown in Figure S1 of the supplementary information. The spontaneous formation of initial pores due to thermal fluctuations in the absence of an electric field is possible but the high energy barrier makes the continuous growth of the pores improbable. In the absence of an electric field, the transmembrane voltage is 0.3 V, with the large positive pore energy shown in Fig. S1 of the supplementary material. Although pores above 20 nm are thermodynamically stable in the absence of electric field, the possibility of occurrence of pores above the critical radius of 20 nm is very small since cell death is likely to occur. The induction of an electric field to an algal suspension decreases the critical pore radius since the permittivity of the dielectric within the pore is larger than the permittivity of the lipid in the membrane. An electric voltage of 2.1 V is high enough to produce stable pores. Accordingly, the field strength of 2.7 kV cm^{-1} was chosen in this treatment study.

3.2 Nucleation and evolution of the pores

Nucleation of hydrophilic pores occurs stochastically even at low transmembrane voltages. The pores with a radius greater than the critical radius will continue to grow until membrane rupture. The rate of pore creation and growth is described by the following equation (Krassowska, 2007):

$$\frac{dN}{dt} = \exp\left(-\frac{\Delta U\left(r^*, \Delta\phi_m\right)}{kT}\right)\left(1 - \frac{N}{N_{eq}}\right) \quad (13)$$

Where, N is the number of pores per unit area, r^* is the minimum radius of a conductive pore which was taken to be 0.5 nm, and N_{eq} is the equilibrium distribution of pores which can be calculated by assuming that pores tend to adjust their radii to a minimum of about 0.76 nm.

$$N_{eq} = N_0 \exp\left(-\frac{\Delta U(r_{\min}, \Delta\phi_m)}{kT}\right) \tag{14}$$

Where, N_0 is the initial number of pores created by thermal fluctuations at a zero transmembrane voltage. This initial number of pores can be obtained by quasi-steady state solution of the Smoluchowski's equation (Weaver, 1996):

$$\frac{\partial n}{\partial t} = D_p\left(\frac{\partial^2 n}{\partial r^2} + \frac{\partial}{\partial r}\left(\frac{n}{kT}\frac{\partial \Delta U}{\partial r}\right)\right) \tag{15}$$

Where, $n(r,t)$ is the number of pores in unit membrane area with radius between r and $r+dr$ at time t. At the initial condition, we assume that the membrane is in equilibrium via the expression $\Delta\phi_m = 0$. At $r = r_{\min}$, the flux of pores from a unit volume is governed by the rate of creation and destruction (Weaver, 1996):

$$J = \nu \exp\left(-\frac{\delta_c - a\Delta\phi_m^2}{kT}\right) - \chi n(r_{\min}) \exp\left(-\frac{\delta_d}{kT}\right) \tag{16}$$

Where, δ_c and a are constants describing the energy barrier for pore formation, δ_d is a fixed energy barrier for pore destruction, ν is the attempt rate of pore creation, χ is the rate of pore destruction scaled to 298 K and to 300 g mol^{-1} (the molecular weight of a typical phospholipid). At the critical radius, there are no pores due to membrane rupture ($n = 0$). According to these assumptions, the following analytical solution can be found.

$$N_0 = \int_{r_{\min}}^{r_c} \frac{\nu}{\chi} \exp\left(\frac{\delta_d - \delta_c - 2\pi\gamma(r - r_{\min}) + \pi\sigma(r^2 - r_{\min}^2)}{kT}\right) dr \tag{17}$$

The total pore population of the algal membrane can be calculated as a function of time using this initial value of the pore density. In this work, equation 13 was solved numerically by the 4th order Runge-Katta method with a time step of 0.5 s.

The pore distribution at each time step can be described approximately by:

$$n(r,t) = N(r,t) \frac{\exp\left(\frac{\delta_d - \delta_c - 2\pi\gamma(r - r_{\min}) + \pi\sigma(r^2 - r_{\min}^2)}{kT}\right)}{\int_{r_{\min}}^{r_c} \exp\left(\frac{\delta_d - \delta_c - 2\pi\gamma(r - r_{\min}) + \pi\sigma(r^2 - r_{\min}^2)}{kT}\right) dr} \tag{18}$$

Pore creation and evolution leads to a reduction in membrane resistance which subsequently affects the transmembrane voltage. The voltage is calculated at any instant by the balance of current across the membrane:

$$-\hat{n}.(k_i \nabla\phi_i) = -\hat{n}.(k_e \nabla\phi_e) = C_m \frac{\partial \Delta\phi_m}{\partial t} + I_{ion} + I_{ep} \tag{19}$$

Where, k_i and k_e are respectively the intracellular and extracellular conductivities, C_m is the specific membrane capacitance taken 0.95 μF cm^{-2}, and the symbols ϕ_e and ϕ_i represent the extracellular and intracellular potentials, respectively. The external potential is related to the uniform external electric field by $\nabla\phi_e = -E$.

The ionic current through the membrane is described by $I_{ion} = g\left(\Delta\phi_m - E_r\right)$. Where, g is the specific membrane conductance and E_r is the reversal potential of the current. The electroporation current is described by (Weaver, 1996):

$$I_{ep} = \Delta\phi_m \int_{r_m}^{\infty} \frac{n(r,t)}{R_s(r) + R_p(r)} dr \tag{20}$$

Where, $R_s(r) = \frac{1}{2k_e r}$ and $R_p(r) = \frac{h}{\pi r^2 k_p}$ which are the spreading and pore resistances acting in series and hindering the current flow through the pores, respectively.

The reversible pores created during EF treatment can be resealed in the absence of the EF ($\Delta\phi_m = 0$). The number of pores and their distribution is described by Eq. 13 to 18. After the EF is stopped, the resealing process begins, and the number of pores decreases exponentially with time:

$$N = N_{eq}(\Delta\phi_m = 0) + \left(N_t - N_{eq}(\Delta\phi_m = 0)\right)\exp\left(-t/\tau\right) \tag{21}$$

Where, $\tau = N_{eq}(\Delta\phi_m = 0) / \exp\left(\frac{\Delta U\left(r^*, 0\right)}{kT}\right)$ is the resealing time constant, and N_t is the total number of pores created after t min exposure to EF.

4. Results and discussion

4.1 Effect of EF treatment on algal growth

The microalgae cells were prepared in three starter cultures of approximately 120 mL each at densities of 0.25, 0.40, and 1.0 g L^{-1}. Each of these three starter cultures were divided into five samples and each sample was treated with an EF for one of five exposure times (0, 10, 30, 50, or 70 min). These ranges of algal concentrations and treatment times were chosen by an initial experimental trial. The treated microalgae cells were then cultivated by inoculating 20 mL of each of the fifteen samples into 170 mL BG11 media. Fig. 2(A), (B), and (C) show the growth curve of the microalgae cells during 4 days batch cultivation. The continuous increase in turbidity of the medium is proportional to an increase in the number of microalgae cells. To eliminate any possibility that bacterial contamination could be responsible for this continuous increase in turbidity, the ratio of chlorophyll absorbance at 680 nm to turbidity at 750 nm was measured and is shown in Fig. 2S of the supplementary material. The initial ratio of chlorophyll absorbance to turbidity for all control and treated samples was around 1.05±0.05, while the final ratio of chlorophyll absorbance to turbidity for all control tests was around 1.25±0.2 and that of EF treated ones was

around 1.6±0.2. Since this ratio increased from the beginning to the end of the test instead of decreasing for all cases, the increase in turbidity is most likely related to the algal growth and not to bacterial contamination (the presence of chlorophyll free bacteria would decrease the ratio due to an increase in turbidity).

Similar to other microorganisms, the growth pattern of microalgae cells started with an initial lag phase followed by an exponential growth phase. The algal cells were harvested at the end of the exponential growth stage for biochemical analysis.

Figure 2

The EF treatment stimulated the growth of all samples. The enhancement of the growth rate depends on the treatment time and the algal concentration, as shown in Fig. 2D. For each of the three cases, a hormetic response was observed. The growth rate appears to experience a maximum value after a certain treatment time, with that treatment being longer for higher algal concentrations. For example, the maximum occurs between 10 to 30 min for the case of 0.25 g L^{-1}, with a peak specific growth rate (0.02 h^{-1}) of 25% higher than that of the control (0.016 h^{-1}) at all treatment times. Similarly, the peak for the case 0.4 g L^{-1} was about 50% (0.017 h^{-1}) higher than that of the control (0.012 h^{-1}), and the peak for the case 1.0 g L^{-1} was about 20% (0.013 h^{-1}) higher than that of the control (0.011 h^{-1}). The difference in the maximum percentage of increase in the specific growth rate compared to that of control at different algal concentrations might be attributed to the pore distribution on each individual cell, the number of electroporated cells, and to differences in membrane physiological characteristics. The number of electroporated cells decreases with the cultivation time because the next generation of cells were not subjected to EF treatment. The highest decrease would occur at the lowest algal concentration of 0.25 g L^{-1} because the number of initially electroporated cells is the least. These newly generated cells were tested for the second round of inoculation in order to check the possibility of adaptation. The same growth stimulation was also observed which shows the lack of any adaptation to EF treatment. The difference in the membrane physiological characteristics at different inoculum sizes was studied by Lu et al. (Lu, 2013) and a significant change in the biochemical composition was observed. Given the fundamental role of the membrane in different biological processes, there is a high possibility of distinct growth patterns under different inoculum sizes.

This hormetic response to EF was also observed during treatment of *C. kessleri* (Small, 2012) and *C. vulgaris* (Wang, 2008) cells with a static magnetic field. Gusbeth et al. (Gusbeth, 2013) observed an increase of about 10-20% in biomass yield of *C. vulgaris* during 30 min treatment with low intensity pulsed electric field. Unfortunately, these authors provided no information on the field strength and the concentration of algal suspension.

The algal concentration of 0.25 g L^{-1} has shown the highest specific growth rate compared to two other cases. The dependence of growth rate on algal concentration was shown in other studies as well. Lu et al. (Lu, 2012) and Wang et al. (Wang, 2013) reported higher specific growth rates for lower initial biomass densities of *C. sorokiniana* and *Haematococcus pluvialis*, respectively. These authors have shown that the inoculum size significantly affects the growth and the accumulation of intracellular and extracellular metabolites. There is a point of optimum concentration

for cell cultivation. On one hand, the algal growth rate increases with a decrease in inoculum size due to higher accessible nutrients and light (less self-shading effect). On the other hand, the rate of growth is in direct proportion to cell concentration and cellular reproduction is a normal outcome of this autocatalytic reaction. At very low initial biomass densities, there are increased possibilities of contamination and light-induced photooxidative cell death as well (Wang, 2013).

4.1.1 Algal growth stimulation through enhanced membrane permeabilization

A few studies have examined the effect of electric field treatments on microorganisms and various mechanisms were established by which electric fields interact with cells (Hunt, 2009). The permeabilization of cell membrane by EF might be the main possible mechanism for algal growth stimulation. An enhancement in transport of substrates into or products from the cytoplasm could lead to an increase in algal growth rate. For instance, McCabe et al. (McCabe, 1995) studied the effect of EF on the yeast and they reported an increase in substrate utilization efficiency of *Kluyveromyces marxianus* due to the facilitated entry of the substrates into the cell. The electric field exerts forces on intracellular and extracellular electrolytes and thereby regulates their concentration in a cell (Scarlett, 2009). The regulation of signaling chemicals such as Ca^{2+} could affect cell growth and development (Hepler, 2005).

These changes in permeability of algal membranes after EF treatment were determined through the measurement of EC. Table 2 presents the percentage of increase in the EC of the medium compared to that of the control. The increase in conductivity is a function of both algal concentration and treatment time. The highest increase in percentage of EC was observed at a moderate algal suspension of 0.4 g L^{-1} for any treatment time. At high algal suspensions, a local perturbation of the EF caused by neighboring cells hinders the electrolyte leakage. There is also a high potential for algal clump formation which can restrict or limit the diffusion of electrolytes. At low suspensions, there are a comparatively small number of pores for electrolyte leakage. This result is consistent with the above observation (Fig. 2D) that the EF treated culture with the moderate algal concentration has shown the maximum growth enhancement compared to the control.

Table 2 Percentage of increase in electrical conductivities of the algal suspension medium after EF treatment for different time intervals

The rate of change in EC is governed by the concentration of the ions permeated out from the intracellular space. These changes can be predicted by an ordinary differential equation with the following assumptions: (1) the extracellular concentration of ions (C_o) changes in a layer of thickness λ_x;(2) the exchange of ions between this layer and the surroundings is negligible;(3) the changes in ion fluxes of the membrane compared to the control are affected only by electroporation current;(4) the intracellular concentration of the ions does not change with time;(5) the ion fluxes were modeled with only the flux of K^+; and (6) the ion flux is only in the radial direction around a cell.

$$\frac{dC_o}{dt} = -\frac{N_{cell}}{z_{K^+}F}\Delta\phi_o \int_{r_{\min}}^{\infty} \frac{A_{cell}}{R_s(r)+R_p(r)+R_d(\lambda_x,r)} \frac{\partial n(r,t)}{\partial r} dr \tag{22}$$

Where, A_{cell} and N_{cell} are the surface area of a cell and the number of algal cells in 20 mL suspension culture, respectively. $R_d = \frac{\lambda_x}{\pi r^2 k_e}$ is the diffusive resistance of the ions from the outer cell wall up to a layer of thickness λ_x. This characteristic length scale can be calculated by $\lambda_x = \sqrt{6\hat{D}_x t}$ for ion diffusion (DeBruin, 1999). The differentiation of pore distribution vs. the pore radius, *i.e.* $\frac{\partial n(r,t)}{\partial r}$, can be obtained by finite difference method with a step size of 0.001 nm. The calculation of pore distribution at each time step, 0.5 s, was explained in section 3. $\Delta\phi_o$ stands for the electrochemical potential between the cell interior and the bulk solution. The potential gradient can be semi-empirically derived as a function of the number of cells by assuming direct proportion between the number of cells and total ionic concentration, as it affects the surrounding electrostatic charges of each individual cell. Indeed, the ions sense two types of forces: (1) the electrostatic force arising from surface charge of the cells and (2) the electrochemical force arising from the permeation of the ions from the neighboring cells. The following semi-empirical model for $\Delta\phi_o$ was used:

$$\Delta\phi_o = G_1 \times N_{cell} \exp(G_2 \times N_{cell}) + G_3 \times N_{cell} \tag{23}$$

Where, G_1, G_2, and G_3 are constant dummy parameters characteristic of this biological system, the adjustable correlating parameters.

The modifying parameter of membrane thickness (β) was obtained by electrodynamic theory as derived in the supplementary material:

$$h^2\beta(1-\beta) = \frac{\varepsilon_l \Delta\phi_o^2}{2Y_m} \tag{24}$$

Where, Y_m is the modulus of elasticity of the plasma membrane and is an adjustable correlating parameter characteristic of each microorganism. These four adjustable correlating parameters can be obtained for this biological system by minimizing the root mean square standard deviation (RMSD) of the extracellular concentration as an objective function:

$$RMSD = \sqrt{\frac{\sum \left(C_o^{\exp} - C_o^{cal}\right)^2}{NP}} \tag{26}$$

Where, NP is the number of experimental data points. The superscripts *exp* and *cal* denote the mean of the experimental data at a given treatment time, and the equivalent points calculated by Eq. 22, respectively. This optimization problem is convex and so the global optimal best fit was found using the fminsearch function in Matlab. The resulting parameters of the correlating functions are $G_1 = 3.095\times10^{-7}$, $G_2 = -5.052\times10^{-8}$, $G_3 = -4.27\times10^{-8}$, and $Y_m = 0.37$ Gpa.

Fig. 3 shows the correlation of experimental data points and the model. The model is in good agreement with the experimental data (RMSD of 6 μg L^{-1}). The RMSD of the model from the experimental data and the adjustable parameters are separately reported in Table 3 for each algal suspension. The sharp drop in the potential gradient in the medium with a high concentration of algae (1.0 g L^{-1}) is due to close proximity of the algal cells and the corresponding overlapping set of the boundary layers. This semi-empirical result provided evidence for the suggested local electric field perturbation at high algal concentrations. As expected, the modifying parameter (β) is nearly constant for all three cases showing a small decrease with an increase in potential gradient due to EF compression (Lu, 2013).

Figure 3

Table 3 Predictions of the model along with the root mean square standard deviation

To find out whether the ion permeation arises from new pores created on the cell membrane or rather by the already existing ones, the number of pores vs. treatment time was calculated for different algal suspensions. Fig. 4(A), (B), and (C) show the pore distribution of each individual cell with the total number of pores in Fig. 4D, E, and F, respectively. The moderate algal concentration of 1.305×10^7 cells mL^{-1} showed the highest number of pores while a low number of pores were observed at the highest algal concentration 3.19×10^7 cells mL^{-1}. This result is in agreement with our previous observation regarding the percentage of growth enhancement at different algal suspension, Fig. 2D. The change in EC at the highest algal concentration can be mainly attributed to the high number of cells rather than the generation of new pores. According to the present study, the high potential gradient (Table 3) created by passive permeation of ions through the cells might cause active transport of the ions back into the cell interior by the surface ion channels along the proton electrochemical gradient. This potential gradient can be used for the synthesis of high energy compounds such as adenosine triphosphate (ATP). The presence of plasma membrane ATPase and other oxidoreductases on the cell membrane of *C. vulgaris* was proven experimentally (Rausch, 1991). Of course there might be a reduction in ATP synthesis due to enhanced permeability of the other membrane enclosed organelles of the cell. A portion of the permeated ions will diffuse to the surrounding medium after surmounting the diffusive resistance, attributing to the observed increase in EC of the medium. This change in energy currency of the cell would hypothetically be a possible reason for the observed hormetic response of the cells as well.

Figure 4

Once the electric field is turned off, the voltage drops to zero within microseconds, but the pores persist in the membrane for several months. The number of pores decreases exponentially with time by a constant of $\tau = 8.45\times10^5$ h (in equation 21). Accordingly, it takes about several months for microalgae to retrieve their initial condition; however, this long resealing time cannot be explained by suggested mechanisms for growth stimulation such as EF activation of some cellular proteins and enzymes (Hunt, 2009) because these only have a short-term effect on cells. Shirakashi et al. (Shirakashi, 2004) observed a similar response in mammalian cells

exposed to a moderate pulsed electric field, 2-3 kV cm^{-1}. The resealing time of the cell membranes exposed to a 20 µs pulsed electric field was experimentally determined to be 200 to 300 seconds, an order of 10^5 in resealing time compared to the exposure time.

4.1.2 Algal growth retardation through enhanced peroxide accumulation

The stimulatory effect of EF on growth was masked by necrosis at high treatment times, Fig. 2D. The electric field could alter the energy levels of free electrons and thereby affect the relative probabilities of radical pair recombination. The concentration of free radicals in the algal media has revealed an enhancement with continuous EF treatment, Fig. 5. At low treatment times, the potential damage by the hydroxyl radicals not only can be masked by the stimulatory effect of permeabilization but also by a stimulatory effect attributed to low concentrations of free radicals (Small, 2012). But at high treatment times, there are possibilities of irreversible electrical breakdown and free radical damage to the cells. The damage by the hydroxyl radicals can be neutralized by microalgae to an extent depending on the algal concentration (Wang, 2008). That is a possible explanation for a shift in the optimal treatment time from 30 min to 70 min with an increase in algal concentration from 0.25 g L^{-1} to 1.0 g L^{-1}. Wang et al. (Wang, 2008) observed a similar decreasing trend in the growth rate of *C. vulgaris* at high magnetic field intensities and treatment times due to an increase in free radicals of the media.

Figure 5

Treatment of algae with EF might influence cell metabolism and it is possible this has a consequence on the growth rate of the cells. An analysis of the changes in the biochemical composition of the algae after EF treatment was performed to better understand which molecular sites were influenced by the EF treatment. This is described in the next section.

4.2 Effect of EF treatment on algal biochemical composition

Electric fields have been proposed to influence biological systems at different levels from the individual cell to the whole culture (Hunt, 2009). These fields were observed to affect different biochemical processes in biological systems including photochemical charge separations in photosystems, modulating the interaction of signaling ions with ligands, regulating macromolecule activities through EF-induced conformational changes, and so on (Hunt, 2009). To find out the general effect of these possible changes in biochemical reactions, the quantities of cellular pigments including carotenoids and total chlorophylls were determined, as well as the contents of cellular lipid, soluble carbohydrate, and proteins were measured.

4.2.1 Variations in the contents of cellular pigments

Total chlorophyll and carotenoids were measured by the use of UV-vis spectrophotometer and presented in Table 4. The contents of total cellular chlorophyll show an increase up to a maximum followed by a decline after exposure to EF at different time intervals of 10, 30, 50, and 70 min. The treatment time leading to the highest chlorophyll content was found to be consistent with that of the growth rate, Fig. 2D. For an algal suspension of 0.25 g L^{-1}, the chlorophyll content increased about 26% compared to that of the control cells after 30 min exposure to EF. For the case of 0.4 g $L^{-1}$and 1.0 g L^{-1}, this increase was about 59% and 89% and observed after the

optimal treatment times of 50 min and 70 min, respectively. This consistency is attributed to the dependence of algal growth on photosynthesis and on chlorophyll as the essential component of photosynthesis in green algal species (Lv, 2010). The higher percentage of increase at high algal suspensions is because of the corresponding lower content of total chlorophyll, Table 4. The decline in total chlorophyll contents after reaching the maximum should not be related to nitrogen exhaustion from the culture media. To eliminate any such possibility, nitrate concentration in the medium was continuously measured and no significant decrease (from 10 mM to about 9.4 mM) leading to chlorophyll consumption was observed. There were no significant changes in total carotenoids of the cells exposed to EF at different time intervals. This can be explained by the high carotenoids content of *C. vulgaris* which would not be influenced by EF stress. In other studies concerning the antioxidant defense system of *C. vulgaris* with magnetic field treatment (Small, 2012; Wang, 2008), no visible change was observed in total carotenoids as well.

A continuous decrease of up to 55% and 50% in total chlorophylls and carotenoids respectively was noticed with an increase in the concentration of algal suspension from 0.25 g L^{-1} to 1.0 g L^{-1}. This decrease can be attributed to an increase in algal concentration leading to self-shading of light. The strong dependence of total chlorophyll content on light intensity was reported by Cheirsilp and Torpee (Cheirsilp, 2012) and a similar total chlorophyll content (of about 25 mg g^{-1}) for *Chlorella sp.* was reported under nearly the same experimental conditions.

Table 4 Effect of EF treatment on total chlorophyll and carotenoids of *C. vulgaris* ISC33 at different algal concentrations

4.2.2 Variations in soluble carbohydrate, lipid, and protein contents

The variation of the main biochemical components including soluble carbohydrate, lipid, and protein with EF treatment at different algal concentrations of 0.25, 0.4, and 1.0 g L^{-1} were measured after 4 days of cultivation. Fig. 6 (A), (B), and (C) show these changes for algal concentrations of 0.25, 0.4, and 1.0 g L^{-1}, respectively. There was no significant variation of these biochemical components with EF treatment. The high standard deviations from the mean value could be related to the complexity of the process and the methods used for determination of the components. The latter can be improved to an extent by the use of other measurement protocols based on Fourier Transform Infrared spectroscopy (Pistorius, 2009).

Figure 6

Although the EF treatment imposed no significant effect on the biochemical composition, a noticeable change can be determined with inoculum size. The inoculum size of 42.1 mg L^{-1} prepared from the moderate algal suspension of 0.4 g L^{-1} showed an increase of up to 59% and 78% compared to those of the highest inoculum size (105.3 mg L^{-1}) and the lowest inoculum size (26.3 mg L^{-1}), respectively. A consistent increase of up to 65% and 111% in soluble carbohydrate of this algal suspension was observed compared to those of the highest and the lowest algal concentrations, respectively. Concomitant with these biochemical enhancements, the protein content in this moderate algal suspension was up to 45% and 43% less than those of the highest and the lowest algal concentrations, respectively. A similar observation was reported by Stephenson et al.(Stephenson, 2010) regarding the effect

of inoculum size on the lipid content of *C. vulgaris*. They found an increase in lipid content at lower algal concentrations due to more light available per cell. These researchers also reported a decline in protein content concomitant with an increase in total lipid. Lu et al. (Lu, 2012) investigated the effect of inoculum size from 1×10^7 cells mL^{-1} to 10^4 cells mL^{-1} on growth and lipid content of *C. sorokiniana*. They found no significant alteration in lipid content with a change in inoculum size.

Several researchers investigated the effect of initial biomass density on the biochemical composition of different algal species. Differences in the experimental conditions of these works and the present study make the quantitative comparison impracticable. The relationship between physiological responses and inoculum size can surely be attributed to the changes in the local environment around the cells. The lower content of soluble carbohydrate and lipid at high cell density compared to that of the moderate one might be a result of less available nutrients to individual cells and less light permeation into the culture with a consequent reduction in the required energy currency for metabolite and lipid formation (Lu, 2012). The decline in lipid content at low algal suspension (inoculum size of 26.3 mg L^{-1}) might be because of high cellular reproduction, Fig. 2A and 2D, with a subsequent reduction in the lipid accumulation.

The results of the present study demonstrated the importance of EF treatment for the achievement of high biomass productivity. Depending on the purpose of microalgae cultivation (either single cell protein or lipid production), microalgae with a cell concentration of 1.3×10^7 cells mL^{-1} (0.4 g L^{-1}) can be exposed to EF with an intensity of 2.7 kV $cm^{-1}$for about 50 minutes and then the concentration be adjusted to an appropriate level prior to cultivation. To successfully apply this strategy to commercial algae cultivation systems, further research considering the effect of biofouling on electrode surfaces and the mode of algal treatment (continuous or intermittent) needs to be conducted.

5. Conclusions

Moderate static electric fields have a stimulatory effect on the growth of *C. vulgaris* ISC33. This effect was attributed to the enhancement of membrane permeability which is influenced by the concentration of the algal suspension. Long-term exposure of microalgae to EF also enhanced peroxide accumulation in the medium masking the stimulatory effect at long-term treatments. There was a consistent increase in chlorophyll content with growth rate but an insignificant change was observed in total carotenoids. The cell metabolism seems not to be influenced by the EF treatment while both algal growth and metabolism were significantly affected by the inoculum size.

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