Effect of Pulsed Electric Field treatments on permeabilization and extraction of pigments from *Chlorella vulgaris*.

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Abstract.

The effect of PEF treatments of different intensities on the electroporation of the cytoplasmatic membrane of *Chlorella vulgaris* and on the extraction of carotenoids and chlorophylls was investigated**.** Staining the cells with propidium iodide before and after the PEF treatment revealed the existence of reversible and irreversible electroporation. Application of PEF treatments in the range of 20-25 kV/cm caused that most of the population of *C. vulgaris* was irreversible electroporated even at short treatment times (5 pulses of 3 µs). However, at lower electric field strengths (10 kV/cm) cells reversibly electroporated were observed even after 50 pulses of 3 µs.

The electroporation of *C.vulgaris* cells by PEF higher than 15 kV/cm and a duration higher than 15 µs increased significantly the extraction yield of intracellular components of *C. vulgaris.* The application of a 20 kV/cm for 75 µs increased the extraction yield just after the PEF treatment of the carotenoids, chlorophyll a and b 0.5, 0.7 and 0.8 times respectively. However further increments in electric field strength and treatment time did not cause significant increments in extraction yield. The extraction of carotenoids from PEF treated *C.vulgaris* cells after 1 hour of the application of the treatment significantly increased the extraction yield in comparison to the yield obtained from cells extracted just after the PEF treatment. After a PEF treatment at 20 kV/cm for 75 µs, extraction yield for carotenoids, chlorophyll a and b increased 1.2, 1.6 and 2.1 times respectively. A high correlation was observed between irreversible electroporation and percentage of yield increase when the extraction was conducted after 1 hour of the PEF treatment application (R: 0.93), but no when the extraction was conducted just after PEF treatment (R: 0.67).

Introduction.

Microalgae are a diverse group of microorganisms with a great potential for the production of valuable biologically active products such as carotenoids, chlorophylls, phycobilins, fatty acids, vitamins, sterols etc. (Pulz, Gross 2004). The current consumer demands for more natural products with fewer synthetic additives together with their wide range of biological activities of the products produced by these microorganisms have made microalgae bioproducts the focus of interest of the food, cosmetic and pharmaceutical industries (Olaizola, 2003).

In recent years, production of higher yields of microalgae specific bioproducts have been improved by advances based on molecular biology and optimization of cultivation factors (temperature, pH, light, carbon source, salinity, nutrients etc.) (Gassel et al., 2014; Gao et al., 2013; Jeon et al., 2013). These advances together with the possibility of operating large photobioreactors that are able to handle biomass and metabolites at sufficiently high levels are key factors in the economic viability of commercial exploitation of different products from microalgae (Del Campo et al., 2007). However, there are presently still several obstacles to fully taking advantage of bioproducts producing microalgae such as the ability to successfully extract these compounds from the cell biomass. (Cooney et al., 2009)

Bioproducts produced by microalgae are generally localized in the intracellular space or accumulated in organelles (eg. pigments), vesicles or in the cytoplasm. The presence of a cell wall surrounding the cells and especially of an intact cytoplasmic membrane that acts as a semipermeable barrier influences the extraction of these compounds from cells (Vanthoor-Koopmans et al., 2013). Traditionally, extraction of microalgae bioproducts is mainly conducted from dried biomass with organic or aqueous solvents, depending on the polarity of the compound to be extracted (Ceron et

al., 2008). Conventional liquid extraction of compounds from microalgal matrices is time-consuming, and a relatively large amount of solvents has to be used, which, in the case of organic solvents, is expensive and potentially harmful. Generally, in order to reduce time and solvent volumes, cells are mechanically disrupted prior to the extraction process. Mechanical disruption of microalgae can be accomplished in a variety of ways such as bead milling, homogenization and ultrasound (Prabakaran, Ravindran, 2011). However, these mechanical cell disruption methods are characterized by a lack of specificity that causes a range of cell debris or other impurities to be released with the compound of interest. This negatively affects the purification operation downstream (Balasundaram et al., 2009). Moreover, some of these treatments need to be performed in batch mode (bead milling), making it difficult to scale up the technology and they involve high power consumption (ultrasound). The use of supercritical CO_2 extraction has gained acceptance in recent years to extract high-value products from microalgae. The main advantage of this procedure is that the extracts are free of potentially harmful solvent residues (Macias-Sanchez et al., 2010). However, in some cases, extracts with relatively poor selectivity are obtained, and the cost of supercritical fluids and associated equipment make it difficult to compete with classical solvent extraction especially because this technology requires working with dry biomass (Cheng et al., 2011; Mendes et al., 2003). Drying microalgal biomass requires a significant amount of energy and may cause losses of valuable food compounds (Cooney et al., 2009).

Treatment of fresh microalgal biomass by pulsed electric fields (PEF) could replace conventional techniques that aim improving bioproduct extraction from microalgae. PEF is a technology that causes electroporation of the cell membranes by application of intermittent electric field strength of high intensity for periods of time in the order of

microseconds. Electroporation causes the increment of the cell membrane permeability to ions and macromolecules due to the formation of local defects or pores in the cell membranes. Depending on the intensity of the treatment and cell characteristics, reversible or irreversible pores can be formed (Weaver, Chizmadzhev, 1996). This technology has been proved as an effective method for irreversible permeabilization of cell membranes of both eukaryotes and prokaryotes (Bousseta et al., 2013; Donsi et al., 2010; Monfort et al., 2012). It has been demonstrated that PEF increase the extraction rates and yield of different intracellular compounds of interest from plant cells such as sugar, polyphenols, anthocyanins, chlorophylls, carotenoids and betalains (Puertolas et al., 2012).

The application of PEF for improving microalgal lipid extraction has been previously observed (Goettel et al., 2013; Grimi et al., 2014; Sheng et al., 2011; Zbinden et al., 2013). However, a better understanding of the process conditions required for microalgae electroporation and the mechanisms involved in this effect is required to define the processing conditions necessary for obtaining the maximum extraction yield of metabolites of microalgae with lower energetic consumption.

Chlorella vulgaris is a unicellular *Chlorophyta* alga that is able to accumulate high levels of the carotenoid lutein and other pigments such as chlorophyll a and b (Gouveia et al., 1996). The objective of this study was to investigate the relationship between reversible or irreversible electroporation of *C. vulgaris* cells, loss of viability and enhanced extraction of carotenoids and chlorophyll a and b.

Material and Methods

Cell culture.

C. vulgaris (BNA 10-007, National Bank of Algae, Canary Islands, Spain), were grown in BG-11 medium contaning the following components: 15 g L^{-1} $NaNO_3$; 4.0 g L^{-1} K_2HPO_4; 7.5 g L^{-1} $MgSO_4.7H_2O$; 3.6 g L^{-1} $CaCl_2.2H2O$; 0.6 g L^{-1} citric acid; 6 g L^{-1} ammonium ferric citrate green; 0.1 g L^{-1} $EDTA.Na_2$; 2.0 g L^{-1} Na_2CO_3 and trace metal solution (H_3BO_3 2.86 g L^{-1}; $MnCl_2.4H_2O$ 1.81 g L^{-1} ; $ZnSO_4.7H_2O$ 0.22 g L^{-1}; $Na_2MoO_4.2H_2O$ 0.39 g L^{-1}; $CuSO_4.5H_2O$ 0.08 g L^{-1}; $Co(NO_3)2.6H_2O$ 0.05 g L^{-1}). For solid medium, 1.5 g of technical agar was added to 100 mL of medium. Medium BG 11 (liquid and solid) was autoclaved at 121 ºC for 20 min.

Cells were cultured photoautotrophically in 1 l Roux flask bubbled with air (6 mL/s), at 30 ºC, in light:dark cycles (12:12 h) with white fluorescent lamps (15 mmol m^{-2} s^{-1}). The cultivation medium was initially inoculated at a concentration of 1 × 10^6 cells/mL using a pre-culture obtained from a single colony. Cell density was determined by microscope (microscope L-Kc, Nikkon, Tokyo, Japan) in a Thoma cell chamber (ServiQuimia, Constantí, Spain). Experiments were performed with cells at the stationary phase of growth after an incubation time between the 10 and 20 days. Dry weight of microalgae was determined by vacuum drying (GeneVac Ltd, UK) at 60ºC using 1 mL of the microalgal suspension.

PEF treatments.

The PEF equipment and treatment chamber used in this investigation was previously described by Saldaña et al., 2010. Microalgae were treated in a tempered batch parallel-electrode treatment chamber (25.0 ± 0.1 °C) with a distance between electrodes of 0.25 cm and an area of 1.76 cm^2. The temperature of the treatment medium was measured

with a thermocouple before and after PEF treatment and the temperature variation was always lower than 2 °C. The energy per pulse (W) was calculated using the following equation:

$$W = \int_0^i k \cdot E(t)^2 dt \quad (1)$$

where k (S/m) is the electrical conductivity of the treatment medium, E (V/m) is the electric field strength and t(s) is the duration of the pulse. The total energy (kJ) applied was calculated by multiplying the energy per pulse by the number of pulses. The total specific energy (kJ/kg) applied was determined by dividing the total energy by the mass of treated medium.

Before the treatments, microalgae were centrifuged at 3000g for 10 min at 25 °C and re-suspended in a citrate–phosphate McIlvaine buffer (1mS/cm; pH 7). With this conductivity (1mS/cm), the resistance of the treatment chamber (140 Ω) was in the range of resistances that permits to obtain square wave pulses with the PEF equipment used in this investigation. The microalgal suspension (0.5 mL) at a concentration of 10^9 CFU/mL was placed into the treatment chamber by means of a 1 ml sterile syringe (TERUMO, Leuven, Belgium). *C. vulgaris* cells were subjected to up 50 square waveform pulses of 3µs at 10, 15, 20 and 25 kV/cm corresponding with specific energies per pulse of 0.30, 0.66, 1.2, 1.86 kJ/l of culture (0.009, 0.021, 0.038, 0.059 kJ/kg dry weight). Frequency of pulse delivery was 0,5 Hz.

Enumeration of viable cells

PEF treated and control cell suspensions were serially diluted in McIlvaine buffer (1mS/cm; pH 7) sterile solution. From selected dilutions, 20 µL were streak plated into solid media. Plates were incubated at 30 °C for 7 days with the same light regime used

for the liquid culture. Longer incubation times did not increase the microalgal counts. After incubation colonies were counted to determine the number of survivors.

Staining cells with propidium iodide.

Detection of electroporation of *C. vulgaris* cells was performed with the uptake of the fluorescend dye propidium iodine (PI) (Sigma-Aldrich, Barcelona, Spain). PI is a small (660 Da) hydrophilic molecule that is unable to cross through an intact cytoplasmatic membrane. Staining cells by PI were observed using an epifluorescent microscope ((Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan) and the fluorescent of the whole population was measured with a spectrofluorophotometer (mod. Genios, Tecan, Austria) using a 535 nm excitation filter (523–547 nm) and a 625 nm emission filter (608–642 nm). Two alternative staining protocols were followed under the same experimental conditions to detect reversible and irreverversible electroporation.

Staining cells before PEF treatments. Before PEF treatments microalgae were centrifuged at 3000g for 10 min at 25 °C and re-suspended in a citrate–phosphate McIlvaine buffer (1mS/cm; pH 7) to a final concentration of approximately 10^9 cells/mLl. After that PI was added to cell suspensions to a final concentration of 0.8 mM and the suspension was treated by PEF. After PEF treatment, microalgae in contact with PI were incubated for 10 min. Previous experiments showed that longer incubation times did not influence fluorescence measurements. After incubation, cell suspensions were centrifuged and washed two times until no extracellular PI remained in the buffer. PI trapped inside the cells was quantified by spectrofluorophotometry. Results were expressed as the percentage of permeabilized cells based on the fluorescence value obtained for cells permeabilized by the most intense PEF treatment (150 μs at 25

kV/cm) used in this investigation Under these conditions, the permeabilization of individual cells was also checked using an epifluorescent microscope.

The degree of permeabilization evaluated following this protocol corresponds to the sum of the irreversible and reversible permeabilizad cells.

Staining cells after PEF treatment. PI was added to a final concentration of 0.8 mM after application of the PEF treatment to the microalgae suspension. After the addition of the PI, suspension was incubated for 10 min, centrifuged and washed two times until no extracellular PI remained in the buffer, and then fluorescence was measured. The degree of permeabilization when cells were stained after the PEF treatment corresponded to irreversible permeabilized cells. Reversible permeabilization was determined by comparing the fluorescent measured following the two staining protocols.

Fluorescence measures were based on mean values obtained from at least two independent experiments.

Pigment extraction.

For pigment extraction, 100 µL of non-treated or PEF treated suspension just after the PEF treatment or after 1 hour of incubation in the treatment medium at 20ºC were added to 1 ml of 96% ethanol and vortex. The mixture was incubated in the dark at room temperature for 20 minutes and centrifugated at 6000g for 90s. The absorbance of the supernatant was measured at 470, 649 and 664 nm against a 96% ethanol blank. The concentration of total carotenoids and chlorophyll a and b and were calculated according to the following equations (Lichtenthaler, 1987):

Chlorophyll a (Ca): (13.36xA664)-(5.19xA649) (2)

Chlorophyll b (Cb): (27.43xA649)-(8.12xA664) (3)

Total carotenoids: (1000xA470-2.13xCa-97.64xCb)/209 (4)

Statistical analysis

Results correspond to the average of two independent experiments conducted with two different microalgae suspensions. The presented results are means ± standard deviation. One-way analysis of variance (ANOVA) using the Tukey test was performed to evaluate the significance of differences between means values. Differences were considered significant at $p < 0.05$. GraphPad PRISM (GraphPad Software, San Diego, California USA) was used to perform the statistical analysis.

Results and Discussion

Figure 1 shows the influence of treatment time at different electric field strengths on the electroporation of the cytoplasmatic membrane of *C. vulgaris* when PI was added before (1A) and after (1B) the PEF treatment. Independently of the staining protocol, the uptake of PI increased with the treatment time and intensity of the electric field strength. However, at 10 kV/cm and after treatment times equal or lower than 75 µs at 15, 20 and 25 kV/cm, PI uptake was higher when the dye was added before the PEF treatment. For example, after 10 kV/cm for 75 µs, the PI uptake was near 80% when PI was added before the PEF treatment but it was only12 % when it was added after the treatment. The difference between the PI uptakes under the same PEF treatment conditions reveals the existence of reversible electroporation. It means that in a proportion of microalgal cells, that correspond to the reversibly electroproated population, the permeabilization caused by PEF disappeared after the treatment.

Consequently, in these cells, PI could enter into the cytoplasm during the PEF treatment but it was not able to cross the cytoplasmic membrane if PI was added after the treatment. It is generally accepted that a specific transmembrane voltage threshold exists for the manifestation of the electroproation phenomenon. This threshold depends on the intensity of the external electric field applied but also on the size and dimension of the cell. When the external voltage applied generates a cell transmembrane voltage around the critical value, reversible electroporation occurs while if the transmembrane voltage generated is higher than the critical value the electroporation is irreversible (Ivorra, 2010). In this study, it has been observed that PEF treatments of an electric field strength ≥ 20 kV/cm even with short treatment times (2 pulses of 3 µs) caused the irreversible electroporation of most of population of *C. vulgaris* (small differences were observed in the percentage of PI uptake when the PI was added before or after the treatment). However, at lower electric field strengths the PEF treatment caused both reversible and irreversible electroporation in the population of cells of *C. vulgaris*. Similar results have been obtained by other authors investigating the electroporation of different bacteria by PEF (Garcia et al., 2007; Wouters et al., 2001). The existence of both types of electroporated microalgal cells could be explained because the induced transmembrane voltage at lower electric field strength was not high enough for causing irreversible electroporation in the smaller size cells of the microalgal population.

The relationships between the percentage of PI uptake when the PI was added before (figure 2A) or after (figure 2B) the PEF treatment and the percentage of dead cells estimated by plate counting after the treatment are shown in figure 2. A theoretical straight line with slope 1 and intercept 0 that represents a perfect agreement between percentage of PI uptake and cell death has been included in figure 2. According to the results shown in figure 2A, cell death was not correlated with percentage of PI uptake.

While the percentage of PI uptake ranged from 60 to 100% the percentage of dead cells ranged from 0 to 100%. For example, a treatment that permeabilized the 70% of the cells when PI was added before the treatment did not cause significant death in the population of *C. vulgaris* (figure 2A). According to figure 2A, when the % of dead cells was lower than 80%, the number permeabilized microalgal cells was higher than the number of dead cells. Therefore, a percentage of electroporated closed the pores after the treatment and as consequence they survived. Gram-positive bacteria capable of resealing their pores after the PEF treatment and surviving have been also observed by other authors (Garcia et al., 2007; Wouters et al., 2001). On the other hand, when the cells were stained after the PEF treatment (figure 2B) no correlation was observed between percentage of PI uptake and dead cells when the percentage of dead microalgal cells was lower than 80 %. However, in this case the percentage of irreversible permeabilized cells was lower than the percentage of death cells. Hence, according to these results, a percentage of the cells that death during the treatment was able to recover the integrity of the membrane becoming the cytoplasmatic membrane not permeable to PI when the dye was added after the treatment. Other authors have also observed within a population of Gram-negative bacteria treated by PEF the presence of dead cells with unpermeabilized cytoplamatic membranes when they were treated in a medium of pH 7 (Aronsson et al., 2005; Garcia et al., 2007). According to these authors death of these cells could be caused by secondary damages to other structures or functions.

In summary, the application of mild PEF treatment to a population of *C. vulgaris* may result in non electroporated cells and electroporated cells. Between the electroporated cells can be find live reversibly electroporated cells, dead cells with their cytoplasmatic membranes not permeabilized and death cells with their cytoplasmatic membranes

permeabilized. No relationship between the occurrence of membrane permeabilization by PEF and cell death would indicate that the quantification of the number of inactivated cells is not a good index for the estimation of the efficacy of electroporation for improving extraction of intracellular compounds from *C. vulgaris*.

Effect of PEF on the extraction of carotenoids, chlorophyll a and b from *Chlorella vulgaris* cells.

The effects of the electric field strength and treatment time on the extraction of carotenoids and chlorophyll a and b from *C. vulgaris* cells treated by PEF is shown in figure 3. As the extracted compounds are lipophilic, ethanol was used as a solvent. The extraction was conducted just after the PEF treatment (Fig 3 A,B,C) and after pre-incubating the cells for 1 h after applying the PEF treatment (Fig 3 C,D,E). Solid black bars correspond to the extraction from untreated *C.vulgaris* cells (control). Extraction yield increased by increasing electric field strength and treatment time independently of the extraction protocol followed but the extraction protocol did not affect the extraction yield of the three compounds investigated for the control sample. A pre-incubation for 1 hour before extraction did not increase extraction yield for control cells ($p > 0.05$). However, for the samples treated by PEF, the extraction yield of investigated compounds was higher. For example, after a PEF treatment at 20 kV/cm for 75 µs the extraction yields for carotenoids and chlorophyll a and b were 42%, 54% 195% higher, respectively, when extraction was conducted after 1 hour of pre-incubation. Statistically significant increments of the extraction yields were not observed for increasing treatment time form 75 to 150 µs ($p > 0.05$) at any electric field strength applied. However, the influence of the electric field depended on the extraction protocol followed. When the extraction was conducted just after PEF treatment, the application of a PEF treatment of 15 kV/cm or lower did not increase significantly the extraction

yield of the three compounds in comparison to the control ($p > 0.05$). However, the application of a PEF treatment of 15 kV/cm for 75 µs improved a 104%, 142% and 176% the extraction yield of the carotenoids, chlorophyll a and b, respectively when samples were pre-incubated for 1 hour. The application of a PEF treatment at 20 kV/cm for 75 µs increased significantly the extraction yield of the carotenoids and chlorophyll a and b for 124%, 164% and 218%, respectively, but further increments of the electric field did not cause a significant increment in the extraction yields of the three compounds. The electric field strength applied to obtain the higher pigment extraction yield was intermediate between the 45 kV/cm used for enhancing lipid extraction from the microalgae *Ankistrodesmus falcatus* (Zbinden et al., 2013) and the 3-4.5 kV/cm used for extraction of proteins from *C. vulgaris* (Coustets et al., 2013). This difference in the electric field strengths required for microalgae electroporation could be related to the pulse duration used in the different studies. While in this research pulses with durations of microseconds were applied, pulses of nanoseconds and milliseconds in duration were used for the extraction of lipids and proteins respectively. The relationship between the pulse duration and electric field strength required to cause cell electroporation needs further investigation for a better understanding of the influence of this parameter. On the other hand, the smaller size of *C. vulgaris* cells compared with eukaryote cells of plant tissues could explain the reason why higher electric fields were required for improving extraction from microalgae. Generally, electric field strengths lower than 7 kV are used to improve the extraction of different compounds from eukaryote cells of plant tissues when pulses in the microsecond range are used (Puertolas et al., 2012).

The higher extraction yield of the three pigments after one hour of incubation in the samples treated by PEF was not caused by an increment of the degree of permeabilization in the cells treated by PEF. No statistical significant ($p > 0.05$)

differences between PI uptake just after application of PEF treatment and after 1 hour of incubation were observed (data not shown). The increment observed could be caused by by the plasmolisis of the chloroplast during the incubation time. As pigments such as carotenoids and chlorophyll a and b are located in the chloroplast, their extraction requires that these compounds first cross the chloroplast membrane and then, the cytoplasmatic membrane. The chloroplast plasmolisis could be due to an osmolitic disequilibrium in the periplasmatic space as a consequence of the loss of selective permeability of the cytoplasmatic membrane in the electroporated cells. When the extraction was conducted just after the PEF treatment the cytoplasmatic membrane was permeabilized but not the chloroplast membrane. Nevertheless, after 1 hour of incubation, both membranes could become permeabilized and, consequently, the extraction of the three pigments should be facilitated.

Figure 4 shows a high positive correlation R= 0.93 between the percentage of PI uptake when PI was added after the treatment (irreversible electroporation) and the percentage of yield increase when the extraction was conducted after 1 hour of the application of the PEF treatment (Fig 4 B). However, no good correlation (R= 0.67) was observed when the extraction was conducted just after 1 h (Fig 4 A). Similar results were obtained with chlorophyll a and b (data not shown). This behavior could also be related with the fact that pigments need to cross chloroplast and cytoplasmatic membranes for extraction. As the chloroplast membrane is intact after treatment, no correlation was observed between extraction and irreversible electroporation. However, as the integrity of the chloroplast membrane was reduced after 1 hour, a high correlation was observed between percentage of yield increase and irreversible electroporation.

As conclusion, results obtained in this investigation demonstrated the potential of PEF for improving extraction of compounds of interest from the microalgae C. *vulgaris*. The

efficacy of PEF on extraction enhancement depended not only of the processing parameters (electric field strength and treatment time) but also of the elapsed time from the application of the treatment and the extraction process. Due to the differences in compounds of interest that may be extracted from microalgae and in cell size, cell shape and cell envelopes between different microalgae species definition of processing conditions for optimization extraction will required specific studies for each application.

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Figure captions.

Figure 1. Influence of treatment time at different electric field strengths on the PI uptake when PI was added before (A) and after (B) the PEF treatment. 10kV/cm (■),15kV/cm (○),20kV/cm (▲) and 25 kV/cm (x).

Figure 2.Relationship between the percentages of cell permeabilization assessed by PI staining before (A) and after PEF (B) against the percentage of death cells. To show the degree to which each treatment causes membrane permeabilization, a theorical straight line with slope =1 and intercept =0, is included.

Figure 3. Influence of treatment time at different electric field strengths on the extraction yield of carotenoids (A) chlorophyll a (B) and chlorophyll b (C) from *C. vulgaris* just after the PEF treatment, and extraction yield of carotenoids (D) chlorophyll a (E) and chlorophyll b (F) from *C. vulgaris* after 1 hour of incubation after the PEF-treatment. Control (■); 10 kV/cm (▤); 15 kV/cm (▩); 20 kV/cm (□); 25 kV/cm (▨).

Figure 4. Relationship between the percentages of cell permeabilization assessed by PI and after PEF treatment against the percentage of carotenoids extraction yield increase in comparison to the control when extractions was performed just after the PEF treatment (A) and 1 hour after the PEF treatment (B).

Figure 1.

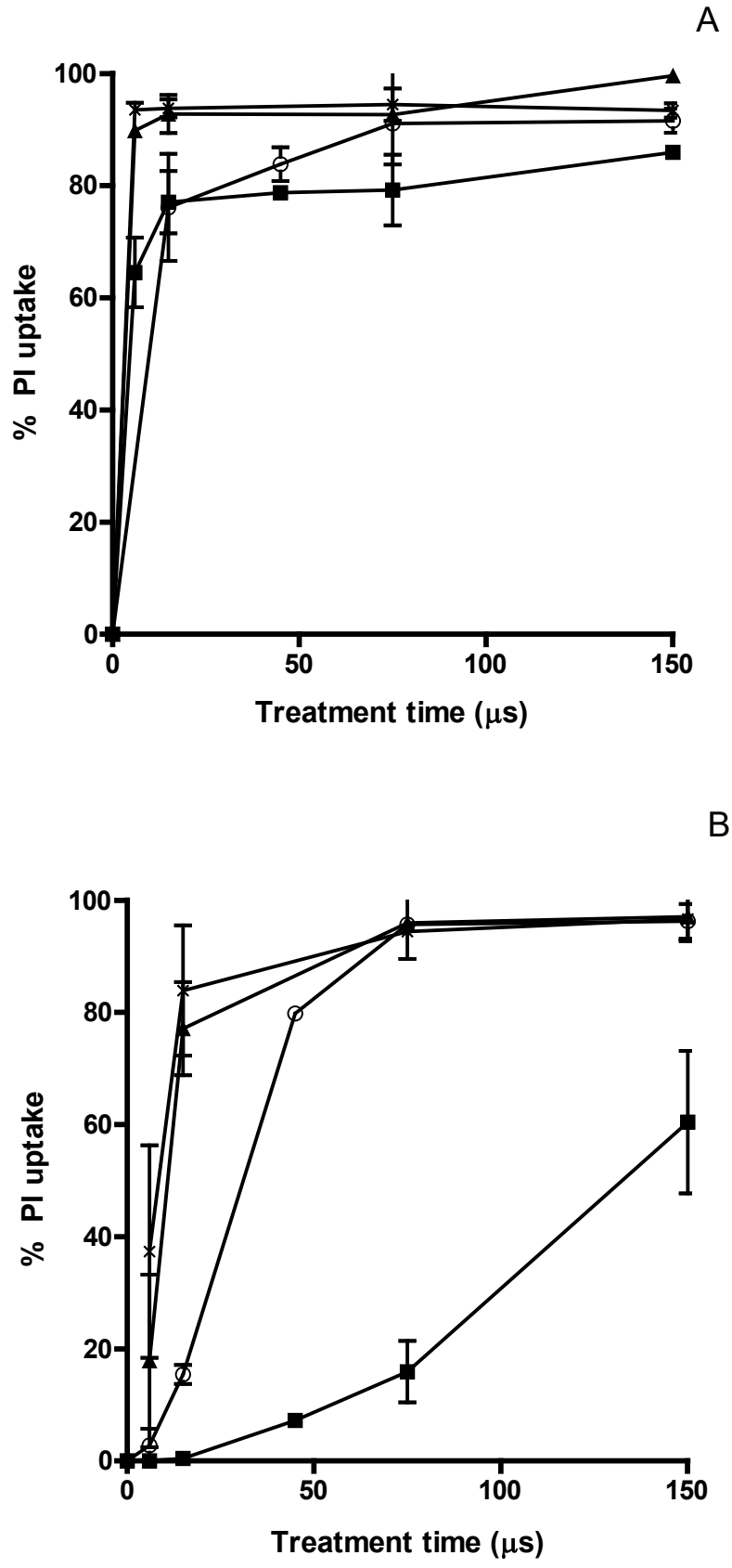

A
100
80
60
40
20
0
% PI uptake
0
50
100
150
Treatment time (μs)
B
100
80
60
40
20
0
% PI uptake
0
50
100
150
Treatment time (μs)

Figure 2.

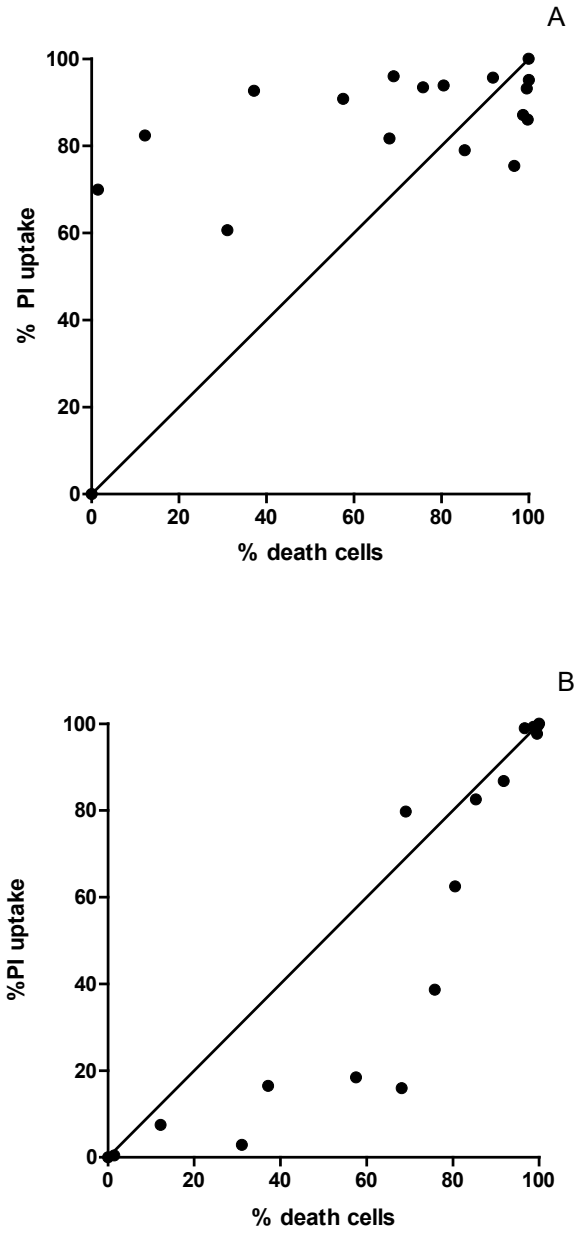

A
100
80
60
40
20
0
% PI uptake
0
20
40
60
80
100
% death cells
B
100
80
60
40
20
0
%PI uptake
0
20
40
60
80
100
% death cells

Figure 3.

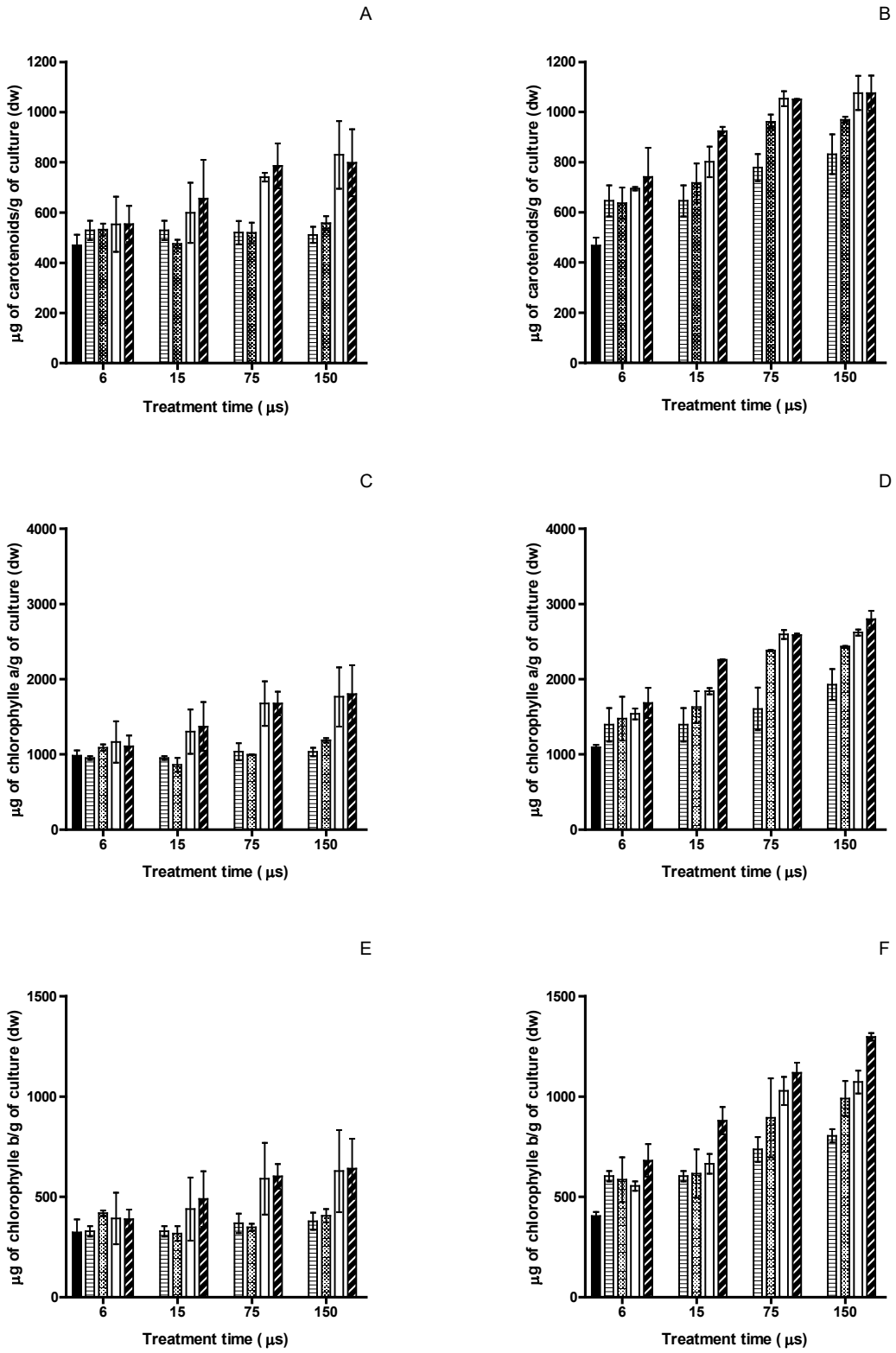
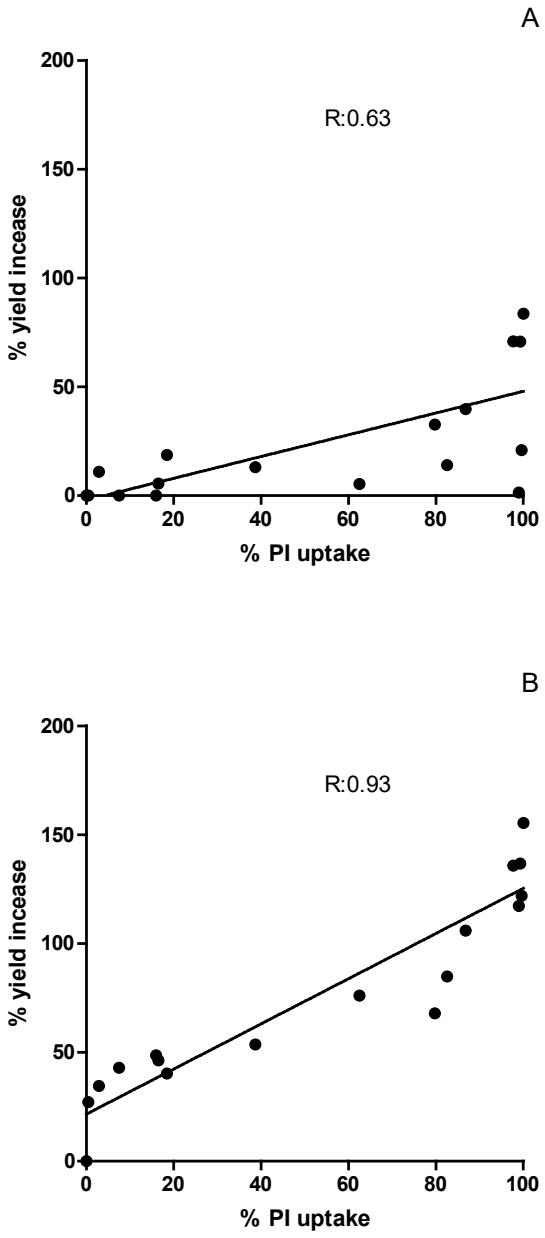

A
B
C
D
E
F
µg of carotenoids/g of culture (dw)
µg of chlorophylle a/g of culture (dw)
µg of chlorophylle b/g of culture (dw)
Treatment time (µs)
6
15
75
150

Figure 4.


A
R:0.63
% yield incease
% PI uptake
0
50
100
150
200
0
20
40
60
80
100
B
R:0.93
% yield incease
% PI uptake
0
50
100
150
200
0
20
40
60
80
100