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Production of Magnetic Modified Microalgae using Iron Oxide Nanoparticles and Electroporation Technique

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Immobilization of living cells is a useful technique that allows to enhance the biotechnological properties of many organisms. Currently, cell-immobilization is obtained via carrier adsorption, self-aggregation and entrapment. We are currently investigating the possibility to immobilize microalgae cells using magnetic nanoparticles on a magnetic surface. The principle is based on the introduction, inside the cell-body of microalgae, superparamagnetic nanoparticles through electroporation. Once microalgae have absorbed enough magnetic nanoparticles they will be responding to an external magnetic field. By using a flat magnetic surface, it will be possible to trap them in a specific position by means of magnetic force. Importantly, immobilized cells will be covered with a thin layer of water and during cell division only one of the daughter cells will keep the magnetic nanoparticles since they will face permanently the magnetic surface. The first step towards this goal is the introduction of magnetic nanoparticles in the microalgae cells. This work will present preliminary results of magnetic cells preparation. Magnetic nanoparticles covered with lipid, to make them biocompatible, and electroporation method to inset nanoparticles in the cells have been used. The protocol to obtain magnetic cells and viability assay that proved the feasibility of the method will be described.

1. Introduction

The increasing demand of renewable energy from one side, and industry pressure to operate under favourable environmental conditions on the other side, headed to improved chemical biocatalyst processes and to look for new eco-friendly bioprocesses like the use of immobilized enzymes or whole cells. In recent years a noticeable rise of enzyme-catalysed industrial processes has been observed but handling purified enzymes usually implies high costs (Souza et al., 2002). Cell immobilization provides advantages like higher specificity and may boost productions always under lower energy consumption (Souza et al., 2002). Furthermore, whole cells can support higher mechanical strength and increased metabolic activity (Elakkiya et al., 2016), prevent contamination of the final product (Souza et al., 2002) and minimize effluent disposable problems. Immobilization of whole cell biocatalysts can be achieved through absorption, covalent binding, cross-linking, entrapment or by combination of those techniques (Groboillot et al. 1994; Bickerstaff et al. 1997; Souza et al., 2002; Elakkiya et al., 2016). Immobilization enhances stability of cells and also provides resistance to conformational changes under acidic pH, heat and organic solvents (Groboillot et al., 1994).
Microalgae contain numerous bioactive compounds that can be harnessed for commercial use (Molino et al., 2019; Molino et al., 2018a). Their metabolites like lipids, carbohydrates, carotenoids and vitamins and their numerous produced proteins can be used in health products, food and feed additives as well as for cosmetics

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(Di Sanzo et al., 2018; Molino et al., 2018b; Molino et al., 2018c). Algae are also considered as living-cell factories for biofuel production (Priyadarsani et al., 2012). Nowadays, microalgae represent a partially unexplored field even though their physiological and biochemical characteristics provide advantages since they can produce unique fatty acids, sugars and various enzymes (Priyadarsani et al., 2012; Savvidou et al., 2018). Recently, magnetic nanoparticles are used for harvesting algal biomass instead of other well-known procedures as centrifugation, filtration, flocculation, sedimentation, and flotation (Christenson and Sims, 2011; Uduman et al., 2010, Prochazkova et al., 2013) due to their advantages like higher efficiency, lower cost and less energy consumption while the whole procedure is faster (Fraga-Garcia et al., 2018). Polyphenol-coated iron oxides (Fe_3O_4) particles used for oleaginous microalgae harvesting (Wang et al., 2018), functionalized Fe_3O_4-Silica core–shell nanoparticles to enhance biodiesel production (Chiang et al., 2014), magnetic Fe_3O_4−ZnO nanocomposites for algal biomass production (Ge et al., 2015), as well as ferrofluid directed harvesting of marine microalgae *Chlorella sp*. for biodiesel production (Ho et al., 2017) are some of the recent applications combining microalgae cells with nanoparticles. Methods that can be used for delivery of DNA/RNA, proteins and nanoparticles into cells are transformation, conjugation, electroporation, glass beads treatment and carbon whiskers. The most widely used method is electroporation which is a physical method based on electropermeabilization. Specifically, an electric pulse generates temporary micro-pores in the cell membrane enabling the passage of molecules into the cells. The cell wall, shape and size of the cells can inhibit this passage so all electroporation conditions such as electric field strength, electroporation buffer, capacitance, number of pulses or the use of enhancers have to be optimized for each species individually (Munoz et al., 2018). Many species of microalgae cells present over the cell plasma membrane a rigid cell-wall made of, among other molecules, cellulose, hemicellulose and glycoproteins (Popper and Tuohy 2010). In order to manipulate cells and introduce nanoparticles trough the plasma membrane, the cell wall must be removed or at least partially damaged. The procedure to remove the cell wall from plant or microalgae cells is well known and can be executed either enzymatically or mechanically (Gerken et al., 2013; Ometto et al., 2014). Biological and biomedical applications of magnetic particles have been explored in the past, and nowadays several protocols are available mainly for separation/extraction of bioactive molecules used for research purposes such as antibodies, DNA, RNA, widely used proteins and other technological fields (Neuberger et al., 2005; Adamaki et al., 2016; Banis et al., 2017; Kim et al., 2018; Kouli et al., 2018; Rocha-Santos 2014). Magnetofection and magnetophoresis are new biotechnological applications with particles size of nanometers order that imply the direct introduction of magnetic particles inside the cell-body (Plank et al 2003). Even though reliable, these techniques have been used exclusively on mammalian cells.. No use of magnetic nanoparticles has been previously explored as vehicle to immobilize cells with rigid cell-walls and in particular microalgae cells.

In this work we propose a pioneering approach based on the usage of iron oxide nanoparticles to confer magnetic properties to microalgae cells which subsequently can be trapped by means of a gradient magnetic field in order to exploit and enhance both enzymatic activities and biomass production. To achieve that, many preliminary steps had to be optimized. These steps include: i) cells pre-treatment to enhance electroporation efficiency; ii) optimization of electroporation parameters; iii) recovery of electroporated cells, that consist in obtaining the highest possible number of viable cells with magnetic nanoparticles inside.

2. Materials and Methods

2.1 Organism

Scenedesmus almeriensis culture was purchase from AlgaRes Srl, an Italian company that is working in collaboration with the University of Almeria (prof. Molina Grima). Cells from a preculture, in the exponential phase and at a light intensity of 60-80 μE/m^2/sec and at a temperature of 20°C, were inoculated into 1000-ml Erlenmeyer flask containing 600 ml Mann and Myers medium, with a maximum concentration of microalgae of about 1 g/l.

2.2 Nanoparticles

Experimental investigations were carried out by using ferrofluid nanoparticles (fluidMAG-lipid from Chemicell, Germany) consisting of an aqueous dispersion of magnetic iron oxides with diameter of 80-100 nm and covered with phosphatidylcholine. Phosphatidylcholine forms, over the iron oxides core, a coating layer similar to cell-membrane, this characteristic gives an excellent biocompatibility since coated nanoparticles do not cross react with molecules and organelles of the cytosol.

2.3 Electroporation protocol

S. almeriensis was grown to a maximum concentration of about 1 g/l. A total of 100 ml cultivation were harvested by centrifugation at 2000 g for 10 min at 4 °C, and washed three times with 1 ml 0.6 M

sorbitol/mannitol buffer, then resuspended in 10 ml 0.6 M sorbitol/mannitol buffer. Under standard conditions, an aliquot of 300 µl cells was mixed with 2 µL (50 µg) of fluidMAG-lipid, then transferred into a 2-mm electroporation cuvettes. Electroporation was performed with a pulse generator (MicroPulser) from BioRad (USA). The electroporation system was adjusted to 2 kV field strength.

2.4 Prussian Blue staining

Prussian Blue reagent pack has been purchased from BioPAL (USA). The reagent pack consisted of Reagent A (4% Prussian blue $K_4Fe(CN)_6$ in water), Reagent B (4% HCL) and Phosphate Buffered Saline (PBS). Briefly, cells were fixed in methanol/acetone for 10 minutes at -20 °C, a sufficient amount of Prussian Blue cell staining reagent was prepared mixing equal amounts of Reagent A with Reagent B. Fixed cells and cell staining reagents were mixed and left for 60 minutes at room temperature. During this time, if iron is present, a blue color will develop that can be appreciate whit microscope observation.

2.5 Viability

After electroporation, protoplast viability was assessed by placing the inoculum to sterile culture vessels containing 10 mL Mann and Myers medium (1968) without agitation and with low light intensity ~60 µE/m2/sec for about 4 days. The protoplasts were examined using an optical microscope and measuring the optical density at 680_{nm} every day for 8 days.

3. Results and discussion

3.1 Cells pre-treatment: protoplast preparation

An enzymatic reaction was used to hydrolyze cellulose by cellulase. After enzymatic treatment and a partial or total cell-wall removal, the microalgae cells were designated as protoplast. The enzymatic reaction was tested at different incubation temperature, enzyme concentration (1, 2, and 4 % by weight) and a time course of 2, 4, 8, 12 and 16 hours. The best results were obtained with 2% of enzyme at 30°C and overnight incubation (12 hours) regarding the electorporation efficiency and the viability of the electroporated cells. Furthermore, since the rigid cell wall plays a critical role during osmotic fluctuation, an important parameter that it must be considered during protoplast preparation is the medium composition. To prevent osmotic shocks, during cell pre-treatment and in all downstream applications, sorbitol and mannitol were added in the medium. These two sugar-based molecules increase the osmotic pressure of media by equilibrating the pressure between the internal cell-compartment and the external environment. The optimized concentration of mannitol and sorbitol was 0.6 M, and was used in all media during digestion, electroporation and recovery along with Mann and Myers medium. Zheng et al. (2011), demonstrate that cellulose and lysozyme in *C. vulgaris* resulted in lipid concentration of 22% and 24% respectively having the highest efficiency comparing to other enzymes. Alkaline pretreatment following by enzymatic or mechanically treatment in *Nannochloropsis sp*. increased the lipid extraction efficiency (Aarthy et al., 2018). Protoplasts from *Chlorella vulgaris* K-73122 were obtained by enzymatic digestion with a mixture of acromopeptidase, cellulase onozura R-10, chitosanase KI, gluczyme, and uskizyme (Ken-ichi et al., 2003). Azencott et al. (2007) compared electroporation and sonication in *Chlamydomonas reinhardtii* resulted to the fact that electroporation can reduce viability of the cells and that cell without the cell wall are more susceptible to lysis. In our study even with only one enzyme and not a combination of those we demonstrate a successful cell wall digestion.

3.2 Electroporation of *Scenedesmus almeriensis* pre-treated cells.

Electroporation is a biomedical technique that, by means of high voltage electrostatic field, allows to temporary destabilize the plasma membrane of living cells permitting at the same time to introduce in the cytosol small molecules and/or nucleic acids (Weaver 2018). This method has been chosen as the main way to insert magnetic nanoparticles inside the microalgae cells. The quantity of nanoparticles used in electroporation experiments have been optimized in 200 ng/ml, for a cell contrition of $20x10^6$/ml. In other words, we used roughly 10 pg of nanoparticles with a radius of 80-100 nm, covered with lipid, per single cell.
Presence of magnetic nanoparticles inside cells was analysed by Prussian blue staining which is a simple, but reliable method. (Figure1). Briefly, cells with nanoparticles were fixed with a mix of methanol/acetone for 10 minutes at -20 °C, then washed with PBS and stained with a solution containing ferrocyanide salt that upon oxidation in presence of iron gives a blue pigment unsoluble in water. In our case, as this reagent was penetrating inside cells, the intracellular blue colour is index of presence of iron particles. By using permanent neodymium magnets, we verified that magnetic microalgae were attracted by magnetic fields (Figure 2). This proves that such kind of cells can be immobilized on a magnetic surface and by calibrating the water speed and flow they could even withstand the water flow.

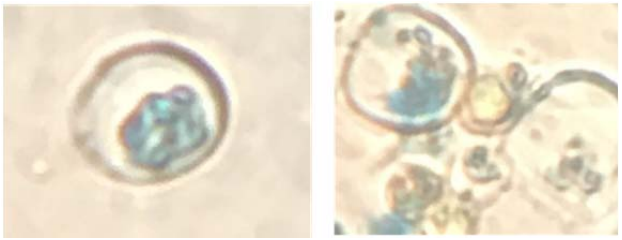

Figure 1: Representative transmission light microscope pictures of Scenedesmus almeriensis cells stained with Prussian Blue. The blue colour of the cytoplasm indicates presence of iron. Due to the fixative used all subcellular organelles formed a solid precipitated.

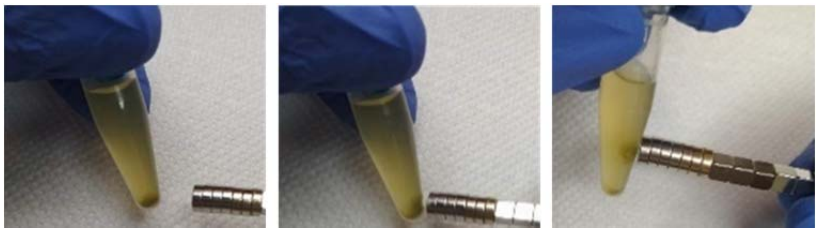

Figure 2: Scenedesmus almeriensis cells transformed with magnetic nanoparticles are susceptible to magnetic field generated by permanent neodymium magnets

3.3 Recovery of electroporated *Scenedesmus almeriensis* pre-treated cells and viability test

An important step was the protoplast recover that consisted on the optimization of culture parameter allowing microalgae cells to properly reconstruct the cell wall and subsequently to grow. After electroporation and nanoparticle internalization, cells deprived of their natural shield cannot survive if they are directly immobilized on top of a magnetic surface because of osmotic issues and of the fragility of their body. Indeed, a recovery period is necessary both for the synthesis/repair of the cell-wall and to overcome the stress induced by electric shock. Initially, electroporated cells in specific flasks were incubated in 21 °C with shaking and normal light fluence, roughly 60-80μE/m^2/sec. Unfortunately, after many attempts it wasn't possible to re-grow protoplast cells. The cells couldn't stay viable in the next few days. Next, reduction of the light fluence by covering flasks with aluminum resulted in a slight improvement. Cell culture lasted for a few days more, but still was not possible to have vital cells for long. The above cell survival difficulty led us to examine this issue in two parts since it was not easy to understand if cells were dying because of the deprivation from cell-wall, or due to electric shock, or a combination of both. Therefore, first the protocol to recover protoplast cells without electroporation was optimized and subsequently such procedure was applied to microalgae protoplasts that undergo electroporation. To do so, we cultured protoplast cells in different ways by changing on different parameters each time. Specifically, cultures were kept in: dark and agitation; dark and no agitation; light and agitation; light and no agitation. Different temperatures (18-25 °C) were also tested for each of the above conditions. Surprisingly, it was verified that the best way to recover majority of the culture was to incubate them without agitation and with low light fluence ~60 μE/m2/sec for about 4 days (figure 3). The results of this study agree with an analogous effort of protoplast formation with different protocol using cellulase reported by Raimundo et al. (2018), where it was also found that after 4 days the *Penium Margaritaceum* cells start to acquire their proper formation.

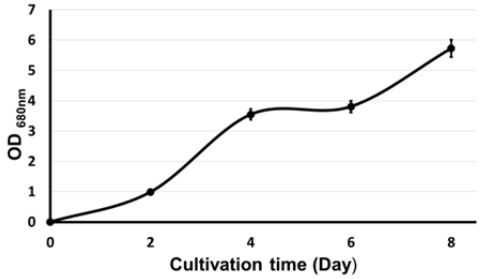

Figure 3. Growth curve of Scenedasmus almeriensis in Mann and Myers medium. Data correspond to the mean ± SD of three independent experiments.

4. Conclusion

Production of magnetically modified cells is one of the challenges towards magnetic cell immobilization. It's the first time, till now in our best knowledge, referring to magnetically modified viable microalgae cells. Results presented in this work demonstrated that, even though with not exceptional efficiency, it is possible to introduce iron oxide nanoparticles in cells without compromise significantly their viability. To achieve this goal, several research and preliminary optimization work was done about pre-treatment, transformation via electroporation and finally recovery of transformed cells, however, further investigations are required in order to carry out the fine tuning of the protocol for magnetic modified microalgae production using iron oxide nanoparticles and to improve efficiency growth.

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