RECOVERY OF HIGHLY CONCENTRATED MICROALGAE CELLS AFTER ENZYMATIC AND MECHANICAL TREATMENT

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ABSTRACT

Although selection techniques based on spontaneous mutation are conventionally used for strains improvement of autotrophic and heterotrophic organisms, genetic manipulations like DNA transformation and protoplast fusion are supposed to be more effective. The above procedures, when applied to microorganisms that present a rigid cell-wall, require the efficient isolation and regeneration of protoplasts, which facilitate the applications of classical molecular and genetic studies. Protoplast electroporation is a particular method that allows the introduction of foreign DNA or nanoparticles into a great variety of photosynthetic cells by means of electric pulses. Transformed cells can further exploited depending on the effectiveness of the regeneration protocol. *Scenedasmus almeriensis* and *Nannochloropsis oceanica* are two well-known green autotrophic microalgae that are widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics industries. The objective of this study was to develop an efficient protocol for recovery highly concentrated protoplasts from *Scenedasmus almeriensis* and *Nannochloropsis oceanica.* The final goal is to propose a protocol that can find applications when for genetic studies or manipulations of algal species a high volume of biomass is required.

The first step in protoplast preparation is the total or partial removal of the cell-wall through enzymatic digestion. Cell-wall complexity is the major factor that influences the digestion efficiency, so the selection of the appropriate degrading enzymes is the key factor for isolating protoplasts. The algal cell-wall is comprised of algal 'wall protein' and 'wall polysaccharides'. Our results showed that an enzymatic solution containing 2% cellulase was more effective than other kind of enzyme. A second method to reversibly damage the cell-wall is via mechanical stress using e.g. glass beads. Herein, the effects of the mechanical treatment are also presented.

It is worth emphasizing the vital role of different culture medium for the recovery of protoplasts, since, from our experience, it seems that photosynthetic activity is impaired after cell-wall removal. In general, microalgae membranes are constituted by glycoproteins and phospholipids and also single amino acids. 0.1% w/v casamino acids were necessary due to the fact that it can be directly used as the building blocks of the membranes. Also, glucose and fructose are the major components for glycolysis pathway, that can start from glucose degradation or by using fructose and lead to production of glycerol (glyceraldehyde-3-phosphate) and then to triglycerides and phospholipid production. In both strains, 2% w/v of glucose in the culture medium helped to increase the glycosylation as well as the glycoprotein levels and that served for the reconstruction of the cell-wall and membranes. As a constituent, glucose and fructose play a double role for phospholipid and glycoprotein production supporting the membrane synthesis. In conclusion, this work provides for the first time a very efficient protocol for the preparation of protoplasts from *Scenedasmus*

almeriensis and *Nannochloropsis oceanica* and the recovery of highly concentrated microalgae cells, which can find useful applications in both classical molecular biology and genetic studies.

INTRODUCTION

Enhancing fatty acids, proteins and pigments production are the main reasons why microalgae are extensively explored those days as a major source. The above mentioned products demonstrate anti-microbial, anti-inflammatory and anti-cancer activity [1]. New efforts had been raised targeting genetic manipulations of microalgae through DNA-mediated transformations increasing in that way the expression of specific enzymes which regulates the amounts of desired products.
One factor that affects the transformation efficiency of microalgae is their rigid cell-wall which is constituted by polysaccharides like cellulose, pectin and alganean, and also from glycoproteins and peptides [2,3]. Cell-wall disruption can be achieved by enzymatic hydrolysis [4], mechanical methods [5] chemical and heating methods [6,7] even by microwave treatment [8]. Enzymatic and mechanical processes are preferable. Usually cellulase or pectinase are used for enzymatic treatment, while mechanical process can be achieved by ultrasonication, high pressure homogenization and bead milling [5,9,4] resulting to protoplast formation. Subsequently the algae protoplasts need to be recovered from the above procedures since they go through a strong biological stress, to be grown and finally to be transformed in the desired products. Specific compounds like glucose, fructose, glycerol and casamino acids that have been used in various microalgae culture media, increase the growth capability of microalgae.
In the current study, *Scenedasmus almeriensis* and *Nannochloropsis oceanica*, two well-known microalgae strains due to their health food and feed supplement usage, as well as the several applications they have in pharmaceutical and cosmetics industries, are examined regarding the efficiency of protoplast production, recovery and final viability. An enzymatic solution containing 2% cellulase was more effective than other enzymes tested for protoplasts formation on *Scenedasmus almeriensis* and *Nannochloropsis oceanica* microalgae. Scenedasmus almeriensis protoplast formation was achieved by 2% cellulase treatment for 4h, and furthermore 2% w/v glucose in the culture medium increased their viability. In *Nannochloropsis oceanica* the addition of 2% w/v fructose was more effective regarding the protoplasts survival after pre-treatment with 2% cellulase for 8h. Mechanical process for protoplast formation with glass beads resulted to higher growth on *Scenedasmus almeriensis* in the presence of 2% w/v glucose and 0.1% w/v casamino acids in the case of *Nannochloropsis oceanica.*

MATERIALS & METHODS

Strains and culture conditions

Scenedesmus almeriensis was obtained from AlgaRes Srl, an Italian company in collaboration with the University of Almeria. Cells from a preculture, in the exponential phase, a light intensity of 60-80 μE/m^2/sec and a temperature of 20°C, were inoculated into 1000-ml Erlenmeyer flask containing 600 ml Mann and Myers medium. *Nannochloropsis oceanica* was purchased from Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The cultivations were carried out in flasks at 20^{o}C with continuous illumination at 100 μE/m^2/sec for 10-12 days.

Induction of protoplasts

Scenedasmus and *Nannochloropsis* cells in log-phase were harvested by centrifugation at 1000 g for 10 min, then the cell pellet was suspended in buffer containing 0.6 M D-mannitol and different cell-wall-degrading enzymes were tested in order to get the optimum digestion conditions.
Regarding the mechanical treatment, both strains were harvested as before, mixed briefly with 200 mg glass beads and then agitated on a Vortex mixer for 30 sec in 1.5ml conical disposable

polypropylene centrifuge tubes.

Regeneration of the protoplasts

Mann & Myers medium and F/2 medium for *Scenedasmus* and *Nannochloropsis* cells containing different concentrations of glucose, fructose and casamino acids, were used as the regeneration medium. Mann & Myers medium and F/2 mediums without carbon and nitrogen sources were used as control. Protoplasts were incubated at 20°C for about a week and regenerated protoplasts were counted by spectrophotometric measurements.

RESULTS & DISCUSSION

This paper provides for the first time an effective protocol for genetic manipulation of *Scenedasmus almeriensis* and *Nannochloropsis oceanica* microalgae by enhancing their transformation efficiency through protoplasts generation. The cell-wall was treated with various enzymes resulting in 2% cellulase as the best candidate for protoplast formation in both microalgae species, while *Nannochloropsis oceanica* demands more enzymatic incubation time (8h) comparing to *Scenedasmus almeriensis* (4h). Growth capability of microalgae is directly regulated by their culture medium nutrients and specifically by glucose, fructose, glycerol and casamino acids. Those nutrients were examined in their recovery efficiency of protoplasts in order to increase the viability and growth. From the above mentioned medium components, 2% w/v fructose increased *Nannochloropsis oceanica* viability more efficiently, while 2% w/v glucose was the best component regarding *Scenedasmus almeriensis* growth.

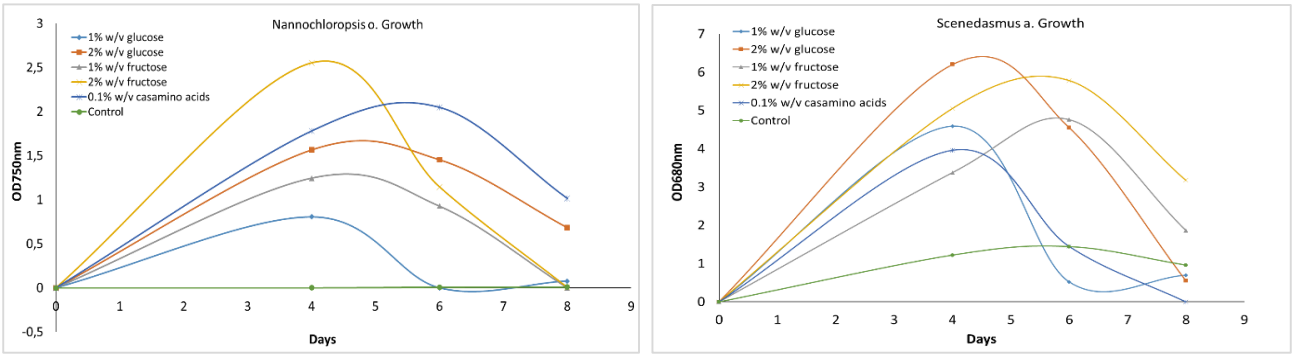

Figure 1. *Relationship between incubation (Days) time and viability of Nannochloropsis oceanica and Scenedasmus almeriensis after enzymatic treatment with 2% cellulase in different enriched culture media*

Besides enzymatic digestion, protoplast preparation was performed by a mechanical process with glass beads and protoplast recovery and viability were examined as well. Enhanced growth on *Scenedasmus almeriensis* was achieved in the presence of 2% w/v glucose in the culture medium whereas 0.1% w/v casamino acids in the case of Nannochloropsis oceanica.

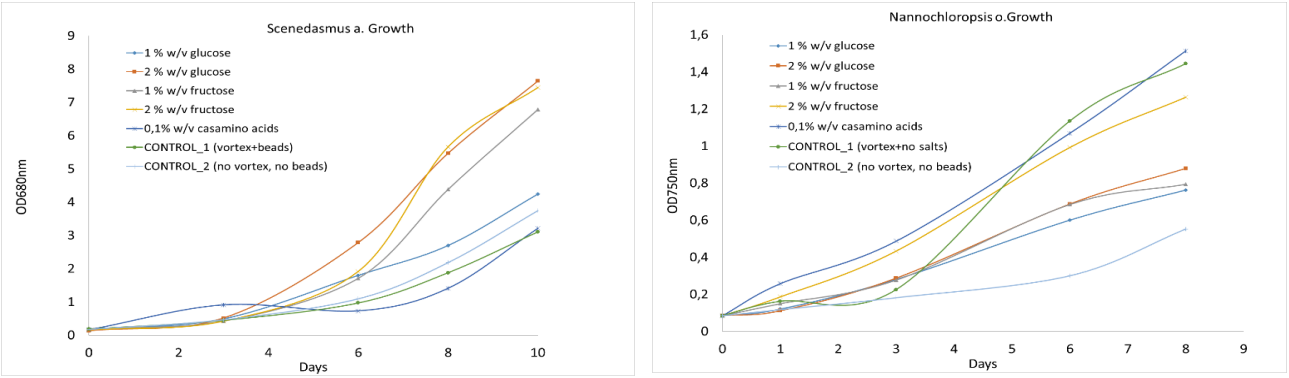

Figure 2. *Relationship between incubation (Days) time and viability of Nannochloropsis oceanica and Scenedasmus almeriensis after mechanical treatment with glass beads in different enriched culture media*

All of the abovementioned nutrients can support growth since glucose and fructose through glycolysis pathway lead to glycerol formation which supports lipid production and finally the energy needs for microalgae cells growth. Casamino acids are the building blocks of proteins, and by enhancing the protein formation may increase also the synthesis of cell-wall conjugated proteins (phospholipids, glycoproteins) leading to recovery and final growth increment.

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