Bioelectrochemistry 103 (2015) 74–81

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Optimization of protein electroextraction from microalgae by a flow process

Mathilde Coustets [a,b,1], Vanessa Joubert-Durigneux [c,1], Josiane Hérault [d], Benoît Schoefs [e], Vincent Blanckaert [d], Jean-Pierre Garnier [c], Justin Teissié [a,b,*]

[a] CNRS UMR5089–IPBS (Institut de Pharmacologie et de Biologie Structurale), 205 Route de Narbonne, BP 64182, F-31077 Toulouse, France
[b] Université de Toulouse, UPS, IPBS, F-31077 Toulouse, France
[c] CERPEM, Maison de la Technopole, 6 rue Léonard de Vinci, F-53000 Laval, France
[d] Sea, Molecules, Health (EA2160), IUML — FR 3473 CNRS, Université du Maine, IUT de Laval, Rue des Drs Calmette et Guerin, F-53020 Laval Cedex 9, France
[e] MicroMar, Mer, Molécules, Santé, IUML — FR 3473 CNRS, University of Le Mans, Avenue Olivier Messiaen, F-72085 Le Mans cedex 9, France

ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 22 July 2014
Accepted 12 August 2014
Available online 29 August 2014

Keywords:
Pulsed electric field
Electroextraction
Microalgae
Flow process

ABSTRACT

Classical methods, used for large scale treatments such as mechanical or chemical extractions, affect the integrity of extracted cytosolic protein by releasing proteases contained in vacuoles. Our previous experiments on flow processes electroextraction on yeasts proved that pulsed electric field technology allows preserving the integrity of released cytosolic proteins, by not affecting vacuole membranes. Furthermore, large cell culture volumes are easily treated by the flow technology. Based on this previous knowledge, we developed a new protocol in order to electro-extract total cytoplasmic proteins from microalgae (*Nannochloropsis salina*, *Chlorella vulgaris* and *Haematococcus pluvialis*). Given that induction of electropermeabilization is under the control of target cell size, as the mean diameter for *N. salina* is only 2.5 μm, we used repetitive 2 ms long pulses of alternating polarities with stronger field strengths than previously described for yeasts. The electric treatment was followed by a 24 h incubation period in a salty buffer. The amount of total protein release was observed by a classical Bradford assay. A more accurate evaluation of protein release was obtained by SDS-PAGE. Similar results were obtained with *C. vulgaris* and *H. pluvialis* under milder electrical conditions as expected from their larger size.

1. Introduction

The simple growth requirements of microalgae make these microorganisms attractive bioreactors systems for the production of high-value heterologous proteins [1]. It is possible to produce recombinant protein either in cytosol or chloroplast of microalgae [2]. One of the most recent examples was given by the production of unique immunotoxin cancer therapeutics in algal chloroplasts [3].

Algae cultivation can be achieved by fermentation or in photobioreactors (PBRs) which provide an accurate control of culture environment. Extraction of proteins is diminished by the cell wall barrier. Microalgae with fragile cell wall did not show significant differences with their protein extract, which was the complete opposite for the microalgae having a rigid cell wall. Therefore, disrupting the rigid cell wall of *Chlorella vulgaris* was previously required to obtain a complete protein release after extraction [4]. Optimization of algal cell disruption methods is fundamental from an economic point of view [5].

Pulsed electric fields are now recognized as an efficient tool for Biotechnological processing [6]. Cytoplasmic proteins can be extracted from yeasts by electropulsation (pulsed electric field technology, PEF) [7]. Flow process treatment of yeasts (*Saccharomyces cerevisiae*), with high intensity electric field pulses, allows the release of the intracellular protein content on large culture volumes. The proof of concept of the flow process protocol was previously validated [7]. Flow process electroextraction is indeed patented to the CNRS (FR # 0013415; Euro/PCT # 1982525.6). A pre-industrial pilot was developed in our group during the FP7 "Electroextraction" project ([FP7-SME-2007-1], Grant agreement n° 222220). Standard methods, classically used for large scale treatments, such as mechanical disintegration (glass beads grinding), chemical extraction (solvents) and enzymatic digestion (lyticase enzyme), affect protein stability [8,9]. This is due to the disintegration of vacuoles, releasing proteases. This negative step does not occur with PEF, where the specific activity of extracted proteins is always higher than with classical methods (either glass bead disruption or enzymatic treatment) [7]. Electroextraction is a promising approach for Biotechnology. A general consensus supports PEF as the most developed approach to affect the algae envelope [10]. On cyanobacteria (*Synechocystis*), PEF is used to induce cell disruption prior to a solvent extraction, using propanol, to extract lipid molecules [11]. It is proposed

* Corresponding author at: 205, Route de Narbonne, BP 64182, F-31077 Toulouse, France. Tel.: +33 5 61 17 59 96; fax: +33 5 61 17 59 94.
E-mail address: justin.teissie@ipbs.fr (J. Teissié).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.bioelechem.2014.08.022
1567-5394/

to improve cellulose hydrolysis to sugar from wood chips [12]. Electropermeabilization for small molecules can be achieved under reversible conditions preserving the microalgae viability [13].

In the present study, by using the pre-industrial scale pilot system, a new protocol was designed in order to electro-extract total proteins from a large volume of microalgae, taking into account that they were indeed photosynthetic yeasts [14]. Batch process for RNA extraction was recently described for detection of harmful algal blooms [15].

Our experiments were performed on microalgae with rigid cell walls. We further selected two fresh water species *C. vulgaris* and *Haematococcus pluvialis* and a marine one *Nannochloropsis salina*.

Chlorella (Trebouxiophyceae) is a genus of unicellular green microalgae that is ubiquitous in fresh water environments. *C. vulgaris* is bigger than *N. salina* (diameter = 3–6 μm) and it contains highly nutritious substances such as proteins, vitamins, minerals, fatty acids and nucleic acids.

H. pluvialis is a freshwater green species of Chlorophyta from the family *Haematococcaceae*. This species is bigger than *N. salina* and *C. vulgaris* (diameter = 5–25 μm) and its morphology evolves during its life cycle. Naturally green, it turns red under conditions of stress. The red color is due to a pigment: astaxanthin, which is important in cosmetics and aquaculture [16]. *H. pluvialis* contains carotenoids, fatty acid, protein carbohydrates and minerals.

Field effects on a cell are linked to target's size [17,18]. High fields are required to permeabilize small cells and their organelles. Given that the electroextraction system has already been optimized for yeasts, where the advantage conferred by this new method was to leave vacuole membranes intact (vacuoles contain proteases). With the small sized *N. salina* (2.5 μm), we were also interested to electro-extract proteins, which were located in the chloroplasts of microalgae (diameter ~1 μm). Due to the size-dependence of the PEF effect and the screening effect of the plasma membrane, the smaller organelles required larger field strength than the plasma membrane to induce electropermeabilization [19]. Previous experiments, at 6 kV/cm, were conducted on bacteria (*Escherichia coli*), which had a mean diameter similar to the chloroplast, to trigger protein electroextraction (unpublished data). Therefore, to permeabilize the saline microalgae, the bacterial parameters would be applied. The results on protein extraction from yeasts were conclusive of the need of repetitive ms long pulses application. Similar results were obtained with *C. vulgaris* and *H. pluvialis* under milder electrical conditions as expected from their larger size.

The simple electroextraction method allows a high yield extraction of total cytosolic proteins, as it was shown on yeasts [7] and reported on microalgae [20].

2. Materials and methods

2.1. Strain and culture conditions

C. vulgaris and *Dunaliella salina* cultures and algae fertilizer were purchased from Teramer (Nimes, France). They grew in a photoreactor built in the lab [20]. The algae starter culture was added and left for two to three weeks to allow the algae to proliferate. Growth was checked by measuring the density of algae under an inverted microscope with a Malassez slide.

N. salina was grown in closed photobioreactor system (Phytolutions GmbH, Bremen, Germany).

H. pluvialis, cultured in MLA medium, was kindly provided by Soliance (Ile Grande, France).

2.2. Sample preparation

A 100 mL volume of *C. vulgaris*, at a concentration of 10^7 cells/mL, was centrifuged at 2500 ×*g* during 5 min at room temperature. Supernatant was removed and cells were resuspended in 100 mL of distilled water (final conductivity *C. vulgaris* = 200 μS/cm) (HI8820N conductimeter, Hanna, Italy). This final conductivity was carefully checked before electric treatment.

A 100 mL volume of *N. salina* suspension in water was centrifuged at 1400 ×*g* during 10 min at room temperature. Supernatant was removed and the cells resuspended in 100 mL of distilled water (final conductivity = 50 μS/cm).

Concerning *H. pluvialis*, a 200 mL volume of microalgae suspension was centrifuged at 2500 ×*g* for 10 min at room temperature. Supernatant was removed, and cells resuspended at a concentration of 10^6 cells/mL (except in a set of experiments, 10^5 cells/mL) in distilled water with a final conductivity around 30 μS/cm.

2.3. Controls

A sample of 5 mL of microalgae washed total suspension was centrifuged at 2500 ×*g* during 5 min at room temperature. The pellet was resuspended in 10 mL of distilled water or phosphate buffer (PB 105 mM, 0.3 M Glycerol, 1 mM DTT, pH = 7) and incubated at room temperature overnight. The suspension was then centrifuged at 2500 ×*g* during 5 min at room temperature. The final analyses were made on the supernatants.

2.4. PEF delivery

The PEF delivery system was previously described [20]. Briefly, pulse generators (DEEX-Bio, Betatech, France) and flow through applicators (CNRS) were designed to apply trains of pulses on the flow. In the pulsing chamber, the distance between electrodes was 6 mm. Another chamber with a smaller gap between the electrodes (3 mm) was used when stronger fields were delivered.

Washed suspensions of microalgae were flow processed through the pulsing chamber, according to the parameters (E = 3 kV/cm or 6 kV/cm, duration of pulses = 2 ms, bipolar impulsions).

In a set of experiments, a microalgal suspension was exposed to 9 pulses (duration = 2 ms) with an electric field of 3 kV/cm (protocol named P1) followed by 9 pulses (duration = 2 ms) at 6 kV/cm (protocol named P2).

After the electric treatment, the pulsed suspension was collected and diluted 5 times in water or phosphate buffer (PB 105 mM, 0.3 M Glycerol, 1 mM DTT, pH = 7). Samples were incubated 30 min or overnight at room temperature or at 4 °C. The supernatants were collected after centrifuging for 5 min at 2500 g.

Electric field strength was estimated as the voltage applied divided by the distance between the plate electrodes, which was 6 mm or 3 mm.

2.5. Total proteins

Total protein concentration was determined using a commercial kit according to the method of Bradford (Bio-Rad, Marnes-la-Coquette, France) with bovine serum albumin (Sigma, St Louis, USA) as the standard [21].

2.6. Electrophoresis

Protein samples were analyzed by SDS-PAGE on a Mini Protean II (Bio-Rad) using a 4.5 and 12% polyacrylamide stacking and running gels, respectively with Tris/glycine/SDS buffer. Prior to the SDS-PAGE analysis, protein samples were denatured with loading buffer at 98 °C for 8 min. PiNK Plus Prestained Protein Ladder (GeneDirex®, Interchim, France) was used as a protein molecular weight marker and protein bands were stained using Coomassie blue G250 dye (Bio-Rad).