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Pulsed electric field-assisted extraction of carotenoids from fresh biomass of *Rhodotorula glutinis*

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ABSTRACT

The aim of this study was to demonstrate the potential of PEF for inducing autolysis of *R. glutinis*, with the purpose of designing a more efficient and ecofriendly carotenoid extraction process: an extraction from fresh biomass, using cheaper, non-toxic, environmental-friendly solvents.

Propidium iodide uptake and release of intracellular components revealed the irreversible electroporation of *R. glutinis* by PEF. Flow cytometry measurements detected morphological changes in PEF-treated *R. glutinis* cells during incubation caused by the autolysis triggering effect of electroporation.

After submitting the fresh biomass to a PEF treatment (15 kV/cm, 150 μs) that irreversibly electroporated more than the 90% of the cells, ethanol proved ineffective for extracting carotenoids from fresh biomass of *R. glutinis*. However, after incubating the PEF-treated fresh biomass for 24 h at 20 °C in a pH 7 buffer, ca. 240 μg/g d.w. of carotenoids were recovered after 1 h of extraction in ethanol. The highest amount of carotenoids extracted (375 μg/g d.w.) from the PEF-treated cells of *R. glutinis* was obtained after having incubated them at 25 °C for 24 h in a medium of pH 8.0.

The improvement in carotenoid extraction by incubating the *R. glutinis* cells after PEF treatment seems to be caused by PEF-triggered autolysis, which tends to disrupt the association of carotenoids with other molecules present in the cytoplasm, and causes a degradation of the cell wall.

1. Introduction

Carotenoids are natural fat-soluble pigments synthetized by diverse microorganisms and plants that provide an attractive alternative to synthetic food colorants. Additionally, in animals, carotenoids carry out important biological functions due to their provitamin A and antioxidant activities resulting in potential health benefits such as strengthening of the immune system and decreasing the risk of cancer and degenerative diseases (Aksu & Eren, 2007). Since animals cannot synthesize carotenoids, they need to be ingested by dietary intake (Rock, 1997). Currently, carotenoids are in commercial use as feed additives, animal feed supplements, natural food colorants, nutrient supplements, and, more recently, as nutraceuticals for cosmetics and for pharmaceutical purposes (Jaswir and Monsur, 2011).

For commercial use, carotenoids are mainly obtained via chemical procedures or by extracting them from plants. Growing consumer concerns related to synthetic additives, along with the finding that natural carotenoids are more easily absorbed by animals and have improved antioxidant properties, have led to an increased demand for carotenoids obtained from other natural sources such as microorganisms (Frengova & Beshkova, 2009; Stahl & Sies, 2005).

Rhodotorula glutinis yeasts have been taken into consideration as potential sources of natural carotenoids, because they are able to produce high yields while growing in low-cost substrates such as agro-industrial wastes (Buzzini & Martini, 2000). However, as the carotenoids produced by yeast are synthesized within the cell and remain inside it, efficient extraction processes are necessary if they are to be commercially used. The main difficulty in developing an economically viable extraction process is that, owing to the hydrophobic nature of carotenoids, their extraction from yeast is performed after dehydration of biomass using large amounts of organic solvents in multiple extraction steps that release a considerable amount of pollutants.

Several authors have demonstrated that effective yeast cell wall disruption by either chemical or mechanical procedures facilitates the entry of solvents into the cell to solubilize intracellular carotenoids and to improve the carotenoid extraction yield (An et al., 2006; Kaiser, Surmann, Vallentin, & Fuhrmann, 2007; Michelon, de Borba, da Silva Rafael, Burkert, & de Medeiros Burkert, 2012; Middelberg, 1995). However, those techniques have many disadvantages, including a high cost and the release of cellular debris; purification becomes expensive

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and difficult to apply on an industrial scale.

Pulsed electric fields (PEF) is a physical treatment that causes an increase in cytoplasmic membrane permeability (electroporation) by applying intermittent high-intensity pulses of a duration in the order of microseconds. Several studies have demonstrated that the electroporation of bacteria, yeast and microalgae improves the extraction of intracellular compounds of interest such as lipids, proteins, carbohydrates, and pigments, while energy consumption remains low (Ganeva, Galutzov, & Teissié, 2003; Jin et al., 2011; Liu, Lebovka, & Vorobiev, 2013; Luengo, Martínez, Bordetas, Álvarez, & Raso, 2015; Zbinden et al., 2013). However, an eventual improvement in the extraction of carotenoids from yeast via electroporation has not yet been investigated. It has recently been demonstrated that PEF treatments accelerate the subsequent autolysis of yeast that results in the self-degradation of the yeast cell's constituents by its own enzymes after cell death. Among the events that occur during yeast autolysis, it has been observed that electroporation of the cytoplasmic membrane caused by PEF encourages the degradation of the cell wall by facilitating its contact with hydrolytic enzymes located in intracellular structures (Martínez, Cebrián, Álvarez, & Raso, 2016).

The aim of this study was to demonstrate the potential of PEF for inducing autolysis of *R. glutinis*, with the purpose of designing a more efficient and ecofriendly carotenoid extraction process: an extraction from fresh biomass, using cheaper, non-toxic, environmental-friendly solvents.

2. Material and methods

2.1. Strain, medium, and culture conditions

A commercial strain of *Rhodotorula glutinis var. glutinis* (ATCC 2527), provided by *Colección Española de Cultivos Tipo* (CECT) was used. The yeast cells were grown at 25 °C in 500 mL glass flasks containing 250 mL of Potato-Dextrose broth (PDB, Oxoid, Basingstoke, UK) under orbital shaking at 185 rpm (Heidolph, Schwabach, Germany). Yeast growth was monitored by measuring absorbance at 474 and 600 nm (correlated with carotenoid production and cellular density, respectively) and the number of cells was monitored using a Thoma counting chamber and plate-counting method in Potato-Dextrose-Agar (PDA, Oxoid, Basingstoke, UK). Dry weight (dw) of yeast was determined by vacuum drying (GeneVac, Ltd., UK) at 60 °C until constant weight. Stationary growth phase was achieved after 48 h of incubation; however, experiments were performed with cells after 72 h of culture, which corresponded with the highest absorbance at 474 nm. Biomass concentration at this time was 10 g dw/mL and 10^8 cells/mL.

2.2. PEF treatment: equipment, chamber, and conditions

The PEF equipment used in this investigation was the commercial model EPULSUS®-PM1–10 (Energy Pulse System, Lisbon, Portugal). It consists of a Marx generator of square waveform pulses with 10 kV of maximum voltage, 180 A of maximum current, and 3.5 kW of power. Fresh biomass of *R. glutinis* was PEF-treated in a parallel electrode continuous chamber of 4 cm length and 0.55 cm width. The gap between electrodes was 0.50 cm, resulting in a total treatment volume of 1.2 mL. The flow rate was 4 L/h and the residence time was 1.09 s.

PEF treatment was performed using three different electric fields: 10, 15 and 20 kV/cm. Different amounts of monopolar square waveform pulses of 3 μs were applied to achieve treatment times between 15 and 300 μs. The total specific energy of the treatments ranged from 1.65 kJ/kg to 132 kJ/kg.

Prior to treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 × *g* for 5 min at room temperature, and re-suspended in a citrate-phosphate McIlvaine buffer (pH 7.0; 2 ms/cm) to a final concentration of approximately 10^8 cells/mL. The microbial suspension was pumped across a heat exchanger submerged in a tempered batch at 10 °C by a peristaltic pump (BVP, Ismatec, Wertheim, Germany). Outlet temperature was monitored during all treatments: even after the most intense treatments, it never surpassed 40 °C.

2.3. Staining cells with propidium iodide

Quantification of the number of electroporated *R. glutinis* cells was performed by measuring the uptake of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Barcelona, Spain). PI is a small (660 Da) hydrophilic molecule that is unable to penetrate intact cytoplasmatic membranes. 50 μL of PI (0.1 mg/mL) were added to 450 μL of *R. glutinis* suspension, resulting in a final concentration of 0.015 mM. After the PEF treatments, suspensions were incubated for 10 min. Previous experiments showed that longer incubation times did not influence the fluorescence measurements. After incubation, the cell suspension was centrifuged and washed twice until no extracellular PI remained in the buffer. The permeabilization of individual cells was determined using an epi-fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan). Results were expressed as the percentage of permeabilized cells after counting ca. 200 cells in each sample.

In order to detect reversible and irreversible electroporation, two alternative staining protocols were followed under the same experimental conditions. When PI was added prior to PEF treatments, stained cells corresponded to the sum of both the irreversibly and reversibly permeabilized cells. On the other hand, when cells were stained after the PEF treatment, the count of fluorescent cells corresponded to that of irreversibly permeabilized cells. Reversible permeabilization was calculated by comparing the number of fluorescent cells obtained from following the two different staining protocols.

2.4. PEF inactivation

After PEF treatments, serial dilutions of the suspensions were pour plated and the number of viable cells, expressed in colony forming units (CFU), corresponded to the number of colonies counted after 48 h of incubation at 25 °C in PDA.

2.5. Monitoring release of intracellular compounds after PEF treatment

Leakage of intracellular components was monitored by measuring absorbance at 260-nm (Abs_{260}) and 280-nm (Abs_{280}) of the supernatant. These wavelengths correspond with the absorbance maxima of nucleic acids and proteins, respectively (Aronsson, Rönner, & Borch, 2005).

2.6. Monitoring morphological changes by flow cytometry

The size and granularity of the *R. glutinis* cells during storage in buffer was assessed by flow cytometry (Millipore/Guava Easycyte, Germany). When a suspension is run through the cytometer, the cells are focused through a small nozzle in a tiny stream that only lets one cell pass at a time. Light scattered by the cells is detected as they pass through the laser beam. A detector in front of the light beam measures forward scatter (FS), which is correlated with cell size, and several lateral detectors measure side scatter (SS), which is correlated with cell complexity. A total of 5000 events were measured in each replicate at a flow rate of approximately 83 events/s.

2.7. Carotenoid extraction

For carotenoid extraction, 1 mL of the non-treated or PEF-treated suspension, either immediately after PEF treatment or after 1, 2 or 5 days of incubation in buffer of pH 7 at 20 °C, was centrifuged at 3000 × *g* for 5 min at room temperature and re-suspended in 1 mL of 96% ethanol. After different incubation times, suspensions were centrifuged at 14,000 × *g* during 2 min (MiniSpin Plus, Eppendorf Ibérica,