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Soluciones innovadoras basadas en la aplicación de pulsos eléctricos de alto voltaje para mejorar la extracción y liberación de compuestos de interés producidos por microorganismos

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Tesis Doctoral

SOLUCIONES INNOVADORAS BASADAS EN LA APLICACIÓN DE PULSOS ELÉCTRICOS DE ALTO VOLTAJE PARA MEJORAR LA EXTRACCIÓN Y LIBERACIÓN DE COMPUESTOS DE INTERÉS PRODUCIDOS POR MICROORGANISMOS

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Innovative solutions based on the application of pulsed electric fields to improve the extraction of compounds of interest from microorganisms

Soluciones innovadoras basadas en la aplicación de pulsos eléctricos de alto voltaje para mejorar la extracción y liberación de compuestos de interés producidos por microorganismos

Doctoral Thesis

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Certifican:

Que la Tesis Doctoral titulada "Soluciones innovadoras basadas en la aplicación de pulsos eléctricos de alto voltaje para la mejora de la extracción y liberación de compuestos de interés producidos por microorganismos" de la que es autor D. Juan Manuel Martínez Rodríguez, ha sido realizada bajo su dirección, su contenido corresponde con el Proyecto de Tesis aprobado en su momento y cumple las condiciones requeridas para optar al grado de Doctor por la Universidad de Zaragoza.

En Zaragoza, a 22 de Mayo de 2019

Fdo: Dr. Javier Raso Pueyo

Fdo: Dr. Ignacio Álvarez Lanzarote

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ABSTRACT

Microbial cultures such as yeast and microalgae represent a highly promising resource for obtaining high-value products like pigments. Furthermore, some food industry process are based on the release of certain compounds from microorganisms, as is the case of "aging on lees" of wines, in which mannoproteins are released from the cell wall of *Saccharomyces cerevisiae*. Although some of these high valuable compounds are released to the medium during the normal metabolism, the majority remains inside the cells. The recovery of these compounds, which are intracellularly locked should occur through a sustainable "green" biorefinery process in which a crucial role is played by the cell disintegration technique used to improve the efficiency of the extraction step. Pulsed electric fields (PEF) is a non-thermal technology that produces the increment of the permeability of the cytoplasmic membrane to the passage of ions and macromolecules. Thereby, PEF treatment enhances mass transfer phenomena through the cytoplasmic membrane, because the latter loses its selective permeability after the treatment.

The objective of this Doctoral Thesis was to evaluate the potential of PEF technology to improve the extraction of intracellular compounds of interest from yeast and microalgae. It was studied: the release of mannoproteins from *S. cerevisiae* yeast, the extraction of carotenoids from *Rhodotorula glutinis* yeast and the extraction of pigments such as phycocyanin, phycoerythrin and carotenoids from *Artrosphira platensis*, *Porphyridium cruentum* and *Haematococcus pluvialis* microalgae respectively.

The electroporation of cytoplasmic membranes of *S. cerevisiae* triggered autolysis and accelerated the release of mannoproteins from the yeast cell wall during incubation. PEF-induced autolysis and the subsequent release of mannoproteins was faster at higher temperatures and pH, and lower ethanol concentration. The improvement of the mannoprotein release by PEF was associated to the fact that the treatment facilitated the enzyme liberation from vacuoles and the subsequent access of these enzymes to the cell wall. A high concentration of β-glucanase and protease was detected in the extracellular medium containing PEF treated cells of *S. cerevisiae* as compared with the control. Likewise, PEF accelerated the release of mannoproteins during the "aging on lees" of *Chardonnay* wine without negatively affecting its physicochemical properties. The mannoproteins released from PEF treated cells of S. *cerevisiae* featured similar functional properties in the wine than mannoproteins released during the natural autolysis.

On the other hand, the electroporation of fresh biomass of *R. glutinis* yeast was ineffective for extracting carotenoids using ethanol as solvent. However, the incubation of electroporated cells of *R. glutinis* for 24 hours in an aqueous medium permitted the extraction of carotenoids in ethanol. This effect was also observed when the cells were freeze-dried after incubation in an aqueous medium. The necessity of the incubation in an aqueous medium for carotenoid extraction was attributed to the action of esterases that caused the disassociation of carotenoid attached to cell structures. A high concentration of esterases, which activity decreased in presence of ethanol, was detected in extracellular medium containing PEF treated cells of *R. glutinis.*

Finally, PEF treatment permitted selective recovery of C-phycocyanin from fresh biomass of *A. platensis* microalga using water as solvent with greater purity than extracts obtained using bead-beating. The efficacy of PEF treatment on the extraction yield was highly depended on temperature of application. On the other hand, electroporation of *P. cruentum* microalga was influenced by electric field strength and treatment time and allowed the extraction of phycoerythrin. The release of this pigment from PEF-treated cells to the aqueous medium always required a lag time that would indicate the necessity of dissociation of the phycobiliprotein from the cells structures, which was hypothesized to be related to the autolysis PEF triggering effect. Similarly to *R. glutinis*, the electroporation of fresh biomass of *H. pluvialis* microalgae was ineffective for extracting astaxanthin carotenoid using ethanol as solvent. However, PEF-treatment of fresh biomass followed by incubation in its own growth medium permitted the extraction of this compound in ethanol. As mainly astaxanthin in the free form rather than forming esters was identified in the extracts, it was hypothesized that also esterase activity was involved in the observed effect.

Obtained results have demonstrated by first time that the improvement of extraction of intracellular compounds by PEF is not only caused by the enhancement of mass transfer through the cytoplasmic membrane, but also PEF triggering activity of some endogenous enzymes plays a very important role in this effect.

RESUMEN

Los cultivos microbianos como las levaduras y las microalgas representan una fuente muy prometedora para obtener productos de alto valor como pigmentos. Además, algunos procesos de la industria alimentaria se basan en la liberación de ciertos componentes de microorganismos, como es el caso de la "crianza sobre lías" de los vinos, en el cuál las manoproteinas son liberadas de la pared celular de *Saccharomyces cerevisiae.* Aunque algunos de estos componentes de alto valor son liberados al medio durante el metabolismo normal, la mayoría permanecen dentro de las células. La recuperación de estos componentes que están encerrados intracelularmente debería ocurrir a través de un proceso de bio-refinería sostenible y verde en el que la técnica de desintegración celular usada para mejorar la eficiencia de la etapa de extracción juega un papel principal. Los Pulsos Eléctricos de Alto Voltaje (PEAV) es una tecnología no térmica que produce el incremento de la permeabilidad de la membrana citoplasmática al paso de iones y macromoléculas. Por lo tanto, el tratamiento de PEAV mejora el fenómeno de transferencia de masa a través de la membrana citoplasmática porque esta pierde su permeabilidad selectiva después del tratamiento.

El objetivo de esta Tesis Doctoral fue evaluar el potencial de la tecnología de PEAV para mejorar la extracción de compuestos intracelulares de interés de levaduras y microalgas. Se estudió: la liberación de manoproteinas de la levadura *S. cerevisiae,* la extracción de carotenoides de la levadura *Rhodotorula glutinis* y la extracción de pigmentos como ficocianina, ficoeritrina y carotenoides de las microalgas: *Artrosphira platensis, Porphyridium cruentum* y *Haematococcus pluvialis* respectivamente.

La electroporación de la membrana citoplasmática de *S. cerevisiae* desencadenó la autolisis y aceleró la liberación de manoproteinas de la pared celular de la levadura durante la incubación. La autolisis inducida por PEAV y la subsiguiente liberación de manoproteinas fue más rápida a altas temperaturas y pHs, y bajas concentraciones de etanol. La mejora de la liberación de manoproteinas por PEAV se asoció al hecho de que el tratamiento facilitó la liberación de enzimas de las vacuolas y el subsiguiente acceso de dichas enzimas a la pared celular. Se detectó una alta concentración de β-glucanasa y proteasa en el medio extracelular que contenía células de *S. cerevisiae* tratadas por PEAV en comparación con el control.

Así mismo, los PEAV aceleraron la liberación de manoproteinas durante la "crianza sobre lías" del vino *Chardonnay* sin afectar negativamente a sus propiedades fisicoquímicas. Las manoproteinas liberadas de las células de *S. cerevisiae* tratadas por PEAV mostraron propiedades funcionales similares en el vino que las manoproteinas liberadas durante la autolisis natural.

Por otro lado, la electroporación de biomasa fresca de levadura *R. glutinis* fue inefectiva para extraer carotenoides usando etanol como solvente. Sin embargo, la incubación durante 24 horas de las células electroporadas de *R. glutinis* en un medio acuoso permitió la extracción de carotenoides en etanol. Este efecto fue también observado cuando las células fueron liofilizadas después de la incubación en medio acuoso. La necesidad de la incubación en medio acuoso para la extracción de carotenoides fue atribuida a la acción de las esterasas que causarían la disociación de los carotenoides unidos a las estructuras celulares. Una alta concentración de esterasas, cuya actividad disminuyó en presencia de etanol, fue detectada en el medio extracelular que contenía células de *R. glutinis* tratadas por PEAV.

Finalmente, los tratamientos de PEAV permitieron la recuperación de C-ficocianina de biomasa fresca de la microalga *A. platensis* usando agua como solvente con mejor pureza que los extractos obtenidos usando molino de perlas. La eficacia de los tratamientos de PEAV en los rendimientos de extracción fue altamente dependiente de la temperatura de aplicación. Por otro lado, la electroporación de la microalga *P. cruentum* estuvo influenciada por la intensidad del campo eléctrico y el tiempo de tratamiento y permitió la extracción de ficoeritrina. La liberación de este pigmento de las células tratadas por PEAV al medio acuoso requirió en todos los casos un tiempo de demora que indicaría la necesidad de la disociación de la ficobiliproteina de las estructuras celulares, que se asumió que estaba relacionada con el efecto de la autolisis desencadenada por los PEAV. De forma similar a *R. glutinis*, la electroporación de la biomasa fresca de la microalga *H. pluvialis* fue inefectiva para extraer el carotenoide astaxantina usando etanol como solvente. Sin embargo, el tratamiento de PEAV de la biomasa fresca seguido de la incubación en el propio medio de crecimiento permitió la extracción de este compuesto en etanol. Como se detectó principalmente astaxantina en forma libre en los extractos en vez de formando ésteres, se pensó que también la actividad esterasa estaba involucrada en el efecto observado.

Los resultados obtenidos han demostrado por primera vez que la mejora de la extracción de compuestos intracelulares gracias a los PEAV no es solamente causada por la mejora de la transferencia de masa a través de la membrana citoplasmática, sino que también la actividad de algunos enzimas endógenos desencadenada por los PEAV juega un papel muy importante en este efecto.

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1. INTRODUCTION

1.1. Utilising Pulsed Electric Field Processing to Enhance Extraction Processes

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1.1. Utilising Pulsed Electric Field Processing to Enhance Extraction Processes

1.1.1. Challenges of extraction processes in Food Industry

Extraction is an important unit operation that is extensively used in many applications in the food industry. This operation results in a main processing stage for obtaining different food products such as sugar, oils or juices or with the objective of obtaining specific components such as polyphenols, carotenoids, pigments, etc.

When mechanical forces are used to extract and separate liquids, such as oil or juice, from within the cellular structure of plant material, the process is called expression. Different systems (belts, rollers, or rotating screws) provide the force to express intracellular compounds. Extraction by expression is achieved either in a single-stage, or in two stages in which a size reduction of the raw material is followed by separation in a press. The main applications of expression are the extraction of components from plant materials either for direct consumption (olive oil, fruit juices, etc) or for using in subsequent processing (e.g., grape juice for wine or apple juice for cider).

The recovery of valuable soluble components from raw materials by primarily dissolving them in a liquid solvent, so that the components can be separated and recovered later from the liquid is called solid-liquid extraction or leaching. In this process, the solvent is mixed thoroughly with the solid, which usually is a plant based matrix and the contact is kept during the required time. It is considered that extraction of a solute that is located inside a matrix occurs in three steps. Firstly, the solvent penetrates the matrix and the solute is dissolved, then the solute diffuses to the surface of the solid and finally migrates from the superficial part of the matrix to the solvent. After this exchanging period, the mixture is divided in two parts: the liquid one, composed by the dilution of the solute in the solvent and another solid formed by the residue containing the insoluble components and part of the solvent embedded in them. Solid-liquid extraction is used for obtaining sugar from cane or sugar beet or oils from seeds such as soya or sunflower and other seeds. Solid-liquid extraction has also been traditionally used for extracting colorants, flavors, proteins or bioactive compounds from vegetal materials. However, nowadays, microorganisms, such as microalgae, yeasts or bacteria, represent a highly promising resource for obtaining high-value products including polyunsaturated fatty acids, proteins or pigments that are extracted by solid-liquid extraction using organic or aqueous solvents, depending on the polarity of the compound to be extracted. Extraction

of these compounds from microbial cells permits the subsequent concentration and the increment of the bio-availability of those compounds with specific bioactivities.

Components obtained by expression or solid-liquid extractions are generally located within the cells so that they have to cross the cell envelopes to be released. The presence of an intact cytoplasmic membrane which acts as semipermeable barrier and other envelopes such as thick cellular walls influence the extraction velocity and yield of intracellular compounds by expression or leaching greatly. Traditionally, some pre-treatments such as milling, heating or the use of enzymes are applied to disrupt the cell envelopes and facilitate the subsequent extraction. However, the application of these treatments have significant shortcomings such as excessive denaturalization of cell envelopes, producing the release of cell debris which lead to the need of subsequent purification techniques, high energy costs or incompatibility with the extraction of labile substances.

1.1.2. Pulsed Electric Field (PEF) technology

1.1.2.1. Definition and description of the procedure

PEF is a treatment that consists in the intermittent application of direct-current high-voltage pulses (kV) for periods of time ranging from microseconds to milliseconds, through a material placed between two electrodes. This voltage generates an electric field which intensity depends on the gap between the electrodes and the delivered voltage. If this electric field is high enough, a phenomenon called electroporation occurs which consists in the increment of the permeability of the cytoplasmic membrane to the pass of ions and macromolecules.

A typical PEF setup includes a pulse power supply that transforms the sinusoidal alternating current from the power line to pulses with sufficient peak voltage and energy, and a treatment chamber where the high-voltage electric pulses are applied to the product. The PEF treatment chamber is generally composed of two electrodes held in position by insulating material that forms an enclosure through which the products are able to flow.

1.1.2.2. Processing parameters

The main processing parameters that characterize a PEF treatment are the electric field strength and the processing time, which depends on the number of pulses delivered, and the specific energy.

Electric field strength refers to the field strength locally present in the treatment chamber during the treatment. For parallel electrode treatment chambers, apart from some edge effects, the electric field is homogeneous within the inter-electrode space and can be estimated by dividing the voltage measured across the electrodes of the treatment chamber by the distance between the electrodes. In the case of other chamber configurations, such as co-linear electrode configuration, the electric field strength is not uniform and must be estimated by numerical simulation procedures.

Treatment time is defined as total effective time during which the electric field is applied and it is calculated by multiplying the number of pulses applied by the pulse duration.

The specific energy (kJ kg^{-1}) per pulse is the electrical energy required to generate the pulse of high voltage in the treatment chamber. It depends on the pulse characteristics (peak voltage and pulse width) and on the electrical resistance of the treatment chamber. The latter depends on its dimensions and on the electrical conductivity of the product. The total specific energy input required for a given process can be calculated by multiplying the number of pulses applied and the total specific energy per pulse. **Table 1.1.** shows examples of parameters of treatments for electroporating different type of cells with the purpose of improving the extraction of intracellular compounds.

Currently, it is accepted that for manifestation of electroporation an increment in the transmembrane voltage above a given threshold is required. The external electric field strength required to reach this voltage threshold is correlated with the cell size (Heinz et al., 2001). This dependence explains why the critical electric field required to electroporate vegetal cells is lower than that required to electroporate smaller sized microorganisms **(table 1.1.)**. Above the critical electric field strength, permeabilization generally increases with more intense electric field strength and longer treatment durations.

The main advantages of using PEF as a pre-treatment for enhancing the extraction processes as compared to pre-treatments based on heating is due to PEF being a nonthermal process. The low energy requirements for electroporating cells do not cause a significant increase of the temperature of the matrix, preventing negative effects of heating on the quality and purity of the extracts. On the other hand, harsh cell disintegration techniques such as grinding, milling or ultrasound cause complete cell disruption, thus resulting in a nonselective release of the cell components. However, PEF have a specific effect on the cytoplasmic membrane, thus enhancing selective extraction of intracellular compounds without affecting the overall structure of the cell. Therefore, the extracts are usually purer, reducing the requirements for additional purification steps that lead to an increase in costs.

Table 1.1. Electric field strength, treatment time and total specific energy to electroporate different types of cells

Matrix	Extract	Electric field strength (kV cm^{-1})	Treatment time	Total specific Energy (kJ kg^{-1})	Reference
E. coli	Proteins	20	800 µs	129.28	Meglic et al., 2015
S. cerevisiae	Mannoproteins	15	150 µs	56.25	Martínez et al., 2016
C. vulgaris	Carotenoids	20	75 µs	30	Luengo et al., 2015
Red beetroot	Betanin	4	75 µs	6	Luengo et al., 2016
Grape pomace	Polyphenols	1.2	40 ms	18	Brianceau et al., 2015
Grape	Juice	0.4	100 ms	15	Grimi et al., 2009
Grape	Polyphenols	5	150 µs	3.67	Puertolas et al., 2010
Olives	Oil	2	150 µs	5.22	Abenoza et al., 2013
Sugar beet	Sucrose	7	100 µs	3.9	Lopez et al., 2009

Finally, PEF is a process that is low in energy consumption and it can be applied in continuous flow at the processing capacity requirements of the food industry (in the order of tons per hour).

1.1.3. Applications of PEF as pre-treatment for improving extraction

1.1.3.1. Extraction of valuable compounds from microbial cells assisted by PEF

Nowadays, microorganisms, such as microalgae, yeasts or bacteria, represent a highly promising resource for obtaining high-value products, including polyunsaturated fatty acids, proteins or pigments. Some of the advantages in comparison to vegetable sources are that microbial production is not seasonal and does not need lands, they are easily genetically modified to over-produce a specific compound, their biological cycle is shorter and they can use by-products as nutrients that would generally not be used by plants.

Extraction of high-value products from microbial cells has traditionally been conducted from dry biomass. However, drying the biomass prior to the extraction step requires a significant amount of energy and may cause losses of valuable compounds due to oxidation. Recently, several techniques such as microwave, ultrasound pressurized liquid or supercritical fluids have been assessed as a pre-treatment or during the extraction of the dried biomass for improving traditional solvent extraction. Likewise, the ability to successfully extract bio-products from the cell biomass with high extraction yields but without causing significant degradation is one of the main drawbacks of these processes currently available.

The PEF-assisted extraction of relevant molecules from microorganisms has several advantages over the standard techniques, such as the possibility of conducting the extraction with fresh biomass, less release of cell debris and as consequence lower purification requirements, substitution of harmful chemicals by greener solvents and the possibility of selective extraction of molecules.

Bacteria

Genetic engineering has opened the possibility to produce proteins for medicine and industry in recombinant bacteria. The introduction of a gene into a bacteria can lead to the production of recombinant proteins that can also be used in the food industry. As proteins are produced in the cell cytoplasm and remain inside the cell, cell lysis methods are needed in order to obtain them. The main drawbacks of the chemical cell lysis methods (detergents, solvents, enzymes, chelating agents or alkali treatments, etc.) are the cost and the necessity of removing them from the final product. On the other hand,

physical methods such as osmotic shocks, freezing and thawing, wet-milling or high-pressure homogenization have the disadvantage of excessive fragmentation of bacteria and the increase in costs of downstream purification processes.

Besides the application of PEF to assist the DNA transfer between bacteria, or between bacteria and yeast and the enhancement in the plasmid DNA extraction, improving the extraction of proteins or lipids is one of the promising benefits of electroporation. Depending on the size of the pores caused by PEF in membranes, some intracellular proteins can be released through the pores selectively. The different sensitivity to PEF among bacterial strains has been described and the media of incubation after PEF treatments resulted decisively in the amount of protein extracted. The highest amount of proteins was extracted when higher electric field strengths or longer pulse durations were applied (Meglic et al., 2015). The incubation at low temperatures after the treatment seems to increase the concentration of extracted proteins by PEF, since resealing of membrane pores is a slow process (Meglic, 2016).

Yeast

Yeasts are eukaryotic microorganisms belonging to the fungus kingdom, which are widely used for the industrial production of extracts. The possibility of highly-selective and effective extraction of different intracellular components (ions, saccharides, enzymes, proteins or nucleic acids) from yeasts using PEF has recently been demonstrated (Liu et al., 2013). The different thickness of the cell wall of yeast species affects the efficiency of PEF assisted extraction of compounds. This effect could be shown with observations using scanning electron microscopy, demonstrating that PEF also affects wall structure (Ganeva et al., 2014).

In addition to the electric parameters, characteristics of the extraction media are critical in the protein release from yeast. After PEF treatments, an incubation time that depends on each yeast species and each protein is required in order to allow protein efflux from cells. The composition of the media is a parameter that influences the extraction yield and velocity of release. The presence of potassium or sodium chloride in the incubation media accelerated the release of proteins from *Saccharomyces* cells (Meglic, 2016). The incubation in hypertonic media increases the yield of extracted proteins compared to isotonic or hypotonic media, due to the exposure of cells to a hyperosmotic stress after PEF accelerating irreversible membrane damage.

It has recently been demonstrated that PEF treatments trigger the autolysis of yeasts, resulting in the self-degradation of the yeast cell's constituents by their own enzymes after cell death. Among the events that occur during yeast autolysis, it has been observed that electroporation of the cytoplasmic membrane accelerates the degradation of the cell wall by facilitating its contact with hydrolytic enzymes located in intracellular structures (Martínez et al., 2016). Autolysis induced by PEF allows not only the acceleration of the release of compounds that are part of the cell wall of *S. cerevisiae* yeasts, such as mannoproteins, which are of great interest in the wine industry, but also the improvement of extraction of intracellular compounds such as carotenoids after incubating the *Rhodotourla glutinis* yeast cells following the PEF treatment (Martínez et al., 2018). It was hypothesized that this enhancement in the extraction of carotenoids was caused by the disruption of the association of carotenoids with other molecules present in the cytoplasm by enzymes released from the cytoplasmic organelles as consequence of the osmotic imbalance produced in the cytoplasm by electroporation.

Microalgae

Microalgae are a promising source of biocompounds because they are renewable resources that do not need agricultural lands, use inorganic material as nutrients, perform photosynthesis, fix CO_2 and grow in fresh or salty water and even in waste water.

The critical element of microalgae based products to be competitive in the market is the final cost. After growing, extraction of the desired compounds is the highest cost, and the lack of a feasible and economically viable method limits the market for microalgae products. Most of the commercial microalgae compounds are obtained from dried biomass, followed by solvent extraction, resulting in a process high in energy consumption and that negatively affects the product characteristics. As several compounds of interest can be obtained from a given microalga, a recent approach to make the process profitable is the selective extraction of the different compounds without causing their degradation.

The benefits of PEF as a pre-treatment in the extraction of pigments (carotenoids chlorophylls, etc.), proteins, lipids, carbohydrates and other compounds have been demonstrated (Kempkes, 2016). A strong increase in the electrical conductivity of the suspension media or the uptake of propidium iodide after PEF suggest effective microalgae permeabilization. PEF improves the extraction of both water soluble or non-

polar compounds. The reduction of the pulse duration from millisecond to microsecond with a slight increase of the electric field strength allows to maintain or even increase the extraction yield while decreasing the energy requirements. The temperature during PEF processing is another critical parameter affecting microalgae electroporation. The increment of temperature in ranges that do not produce thermal degradation of the compounds (<40 °C) allows reducing the required electric field strength and the treatment time to obtain a given extraction yield and, consequently, decrease the total specific energy delivered by the treatment (Martínez et al., 2016).

Due to the fact that PEF pre-treatment enhances selective release, the purities of the extracts obtained after PEF are higher than those obtained using other techniques based on the complete cell destruction such as milling, ultrasounds or grinding. Besides, the efficiency of PEF pre-treatments is high enough to achieve significant extraction yields. Furthermore, in the case of extraction of non-polar compounds, which are important microalgae-based products, the PEF treatment opens the possibility of introducing green solvents replacing harmful ones.

1.1.3.2. Extraction of compounds of interest from plant based matrices

Extraction of plant pigments

Plant tissues are rich sources of pigments that can be used as natural food colorants but also have health properties, assisting in the prevention of several problems, such as inflammation, cancer, heart disease or diabetes. Commonly, the extraction procedures of these compounds from plants are based on using high temperature and/or polluting solvents, producing the release of undesirable components, degradation of thermolabile compounds and toxicity if the solvents are not completely removed. Post-extraction purification steps represent high costs.

PEF treatment can enhance the diffusivity of components using green solvents at ambient temperatures, preventing thermal degradation of vegetal cell walls and release of debris. Several detailed studies of PEF-assisted plant extraction were conducted by different researchers using different approaches, vegetal tissues and pigments. Much of the attention has been paid to the extractability of betanin, anthocyanin and carotenoids following the PEF-treatment of red beetroot, cabbage, grape, tomato, carrot or paprika

(Brianceau et al., 2015, Grimi et al., 2009, Luengo et al., 2016). Generally, due to the larger size of vegetal cells, the electric field strengths required to electroporate the cells are lower than in the case of microbial cells. Total extraction has been achieved using moderate electric field strengths (0.5-5 kV cm^{-1}) both in the range of microseconds or milliseconds, with low energy consumption. Temperature and pH of the extraction medium have been described as important parameters affecting extraction.

Enhancing expression of juices

In the food industry, mechanical extraction by screw presses, belt presses, hydraulic presses, filter-presses or centrifuges is used to recover the intracellular liquid phase of vegetal tissues, being the base for the production of fruit juices.

The pre-treatment of the material by PEF before pressing enhances energy efficiency and increases the production yield. Traditionally, physical (heating, milling), chemical (alkalis) or enzymatic (maceration) methods are used, but an intense treatment also affect sensory and nutritional properties and extract pollution leading to the need of further clarification. As PEF is a non-thermal and gentle treatment, it can be considered as a valuable alternative to reduce the intensity or even replace these treatments. The benefits of PEF on the extraction of different fruit juices such as carrot, pepper or apple have been demonstrated (Vorobiev and Lebovka, 2008), with positive results using treatments in the range of 1 to 5 kV cm^{-1} prior or during pressing. Generally, PEF can improve extraction efficiency by increasing juice yield, decreasing processing time, reducing energy requirements in comparison to other pretreatments, and decreasing the intensity of subsequent processing steps.

Extraction of edible oils

Extraction is the first step for obtaining edible oils from plants. Oils are extracted from the seeds or fruits using a variety of different methods. In the case of virgin olive oil, the oil is extracted directly from the olives by means of mechanical procedures. For oils obtained from seeds, the process is more complex combining pressing, cooking and solvent extraction.

The pre-processed seeds/beans are treated in a multistage counter current process with solvent until the remaining oil content is reduced to the lowest possible level. The

mixture of oil and solvent is separated by distillation and the solvent is recycled into the extraction process and the crude oil is stored ready for refining.

It was shown that the application of a PEF treatment prior to the extraction process improves the recovery and quality of maize soybean, rapeseed and sesame (Vorobiev and Lebovka, 2016). This enhancement could led to the reduction of the amount of solvents used in the leaching step.

Virgin olive oil is a high-value edible oil which is appreciated for its flavor as well as health properties due to its high oleic acid content and high levels of natural antioxidants (phenols and tocopherols). Olive oil is exclusively extracted from the olives by means of mechanical procedures including crushing, malaxation and centrifugation. These operations affect extraction yield and quality of the final product. Processing plants are interested in optimizing the main operating conditions of the malaxation step to maximize extraction yield without modifying the phenolic content and sensory properties of the olive oil.

The potential of PEF application to assist the extraction of olive oil, both for increasing the yield or enhancing the release of valuable molecules, has been demonstrated. The effect of PEF pre-treatment on olive paste prior to malaxation allows for reducing malaxation time and temperature without negatively affecting its sensorial attributes (Abenoza et al., 2013). Furthermore, paste pretreated by PEF presented higher concentration of desired components such as total polyphenols, phytosterols and tocopherols than the control oil obtained from non-treated olive paste (Puértolas et al. 2016).

Application in winemaking

Winemaking of white and red wines diverges from the first processing step. While white wine is made by fermenting the juice obtained after pressing the grapes, in the case of red wine, fermentation of the must is conducted together with the grape skin. This red wine step is called maceration-fermentation and involves not only the conversion of sugars into ethanol by yeast, but also the extraction of polyphenols located in the grape skin cells. Phenolic compounds strongly affect the quality of red wine as they are responsible for the color, flavor and aging behavior, but are also associated with the health benefits of moderate consumption of these types of wine.

During winemaking, the extraction of these compounds is limited as consequence of the low permeability of the cell walls and cytoplasmic membranes of the hypodermal cells. The most traditional strategy to obtain wines with high phenolic content is extending the maceration time, however, this reduces the production capacity of the wineries because 20 % of the fermentation tanks are occupied by skins. Different methods such as thermovinification, grape freezing, flash-release, or the use of pectolytic enzymes have been used for increasing the permeability of cell envelopes. High energy consumption, costs and in some cases negative effects on wine quality are the main problems of these techniques.

PEF permeabilization of red grape skin cells accelerates and/or increases the release of phenolic compounds and allows for reducing the duration of the maceration step in vinification or to increase the color and concentration of anthocyanins and polyphenolic compounds in the wine without negatively affecting its sensorial attributes (Saldaña et al., 2016). Furthermore, some studies have demonstrated that electroporation improves the extraction of aromatic compounds. This electroporation can be achieved using moderate electric fields (0.5-1 kV cm^{-1}) in the range of milliseconds or higher electric fields (1-10 kV cm^{-1}) in the range of microseconds, which correspond to low energy inputs.

Nowadays, the advantages of PEF, such as the low energy consumption (less than 10 kJ kg^{-1}), the availability of PEF generators with sufficient power to fit with the production requirements of wineries (tons per hour), and the simplicity of implementation of continuous flow treatment chambers into the existing processing lines make this technology viable and very attractive for the wineries.

Sugar production

The traditional process for sugar extraction from beetroot requires prolonged hot water diffusion at 70-75 °C. Such aqueous diffusion at high temperature results in a significant thermal degradation of the cell wall and the release of non-desired components such as pectins, oligo- and polymolecular compounds, decreasing the quality of extracts and forcing the use of complex multistage purification processes. These temperatures also accelerate various chemical reactions such as the Maillard reaction that leads to undesired color in the extracts (Loginova et al., 2011). The reduction of extraction temperatures to

avoid these problems would represent prolonged times to achieve the required yield and would lead to the growth of thermophile microorganisms that consume the sugar.

PEF-assisted aqueous extraction of sugar has significant potential for industrial implementation (Eshtiaghi and Knorr, 2002). The extracts obtained from cold diffusion of PEF-treated sugar beets have lower quantities of pectin and color, as well as higher purities than extracts obtained after thermal diffusion (70 °C). The combination of mild heating at 50 °C and PEF-treatment has been demonstrated to shorten the diffusion time. The use of PEF-assisted diffusion technique in the sugar industry can reduce energy consumption and costs, maintaining the efficiency and avoiding problems related to traditional sugar manufacture (Lopez et al., 2009).

Revalorization of by-products

The food industry is generating massive quantities of by-products and waste annually, which represent a major problem because their disposal is associated with environmental and health related issues. A strategy to valorize these by-products is to extract valuable compounds that can be incorporated into food or cosmetic products. Although conventional extraction methods usually reach high yields, they need enormous quantities of organic solvents, require long times and are high in energy consumption. These high costs are generally not acceptable when re-valuing waste. For that reason, alternative methods, including PEF, are developed and evaluated for by-products valorization (Poojary et al., 2016).

The application of PEF-assisted extraction to recover valuable compounds from fruit and vegetables waste, such as apple pomace, orange, lemon, peach, tomato or mango peels, papaya and borage by-products and blueberries waste, have been explored. Studies show that polyphenols and anthocyanin yield, as well as antioxidant activity improves after application of PEF. Waste recovery assisted by PEF can be a cost-effective approach, thus attracting interest from industry.

The rigid cell structure of lignocellulosic waste and the low moisture content of some vegetal waste, such as seeds, leafs, shoot and branches, hinder the extraction of compounds. However, PEF assisted extraction has been successfully evaluated to recover bio-compounds from these matrices using higher electric field strengths and pulse duration (above 10 kV cm^{-1}). Waste from seeds has been assessed as a source of valuable

compounds such as polyphenols, proteins, carbohydrates, and isothiocyanates from flaxseed, papaya seed, olive seed and sesame seed using PEF treatments to improve extraction efficiency. Valorization of winery waste and by-products for recovery of antioxidants has also been studied (Poojary et al., 2016). The concentration of recovered compounds was dependent on the matrix and the PEF processing conditions, but in any case the improvement was presented.

1.1.4. Conclusions

Electroporation caused by the application of PEF results in improved diffusion between intra- and extracellular parts in microorganisms or vegetal cell material, which can enhance extraction processes in the food industry by leaching or expression **(Figure 1.1.)**. Therefore, this technology can be used for the recovery of valuable compounds from different matrices, allowing the reduction in extraction time and temperature, and therefore leading to extracts with improved nutritional and sensorial properties and better preservation of bioactive compounds. Additionally, the reduction of the use of organic solvents has a double benefit both from the economic and environmental point of view. The existence of commercial PEF equipment able to treat large amounts of products at industrial scale in continuous flow and the simplicity of implementation, as well as the wide range of applications in the food and biotechnological industry position PEF-assisted extraction as a real alternative to traditional techniques.

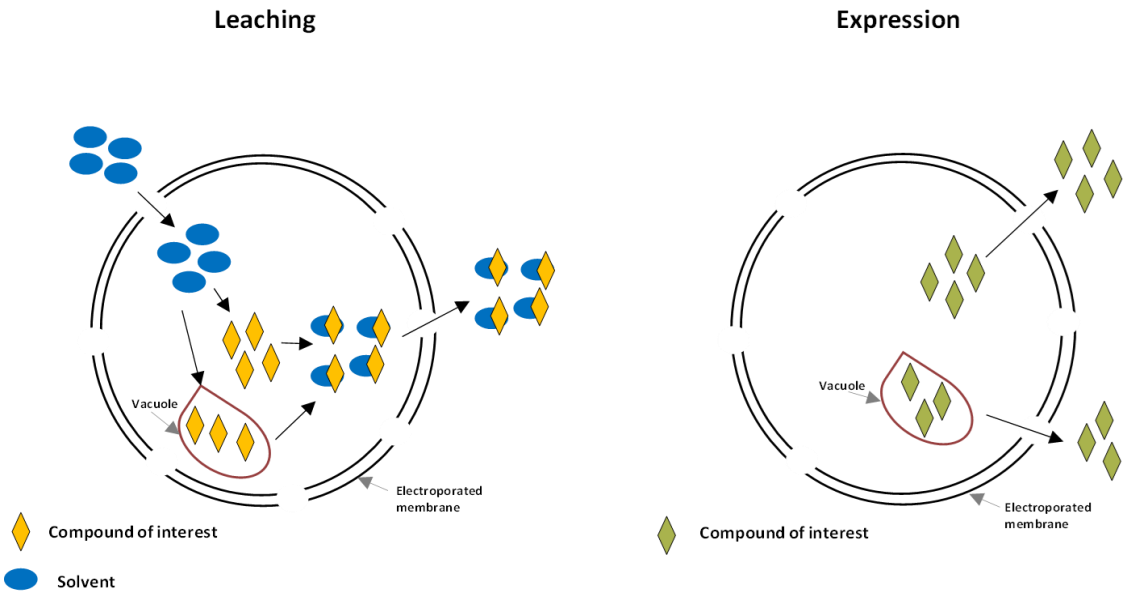

Figure 1.1. Electroporation effect on improving extraction by leaching or expression

1.2. Pulsed Electric Field-assisted extraction of valuable compounds from microorganisms

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1.2. Pulsed Electric Field-assisted extraction of valuable compounds from microorganisms

1.2.1. Challenges and opportunities in the production of compounds from microbial sources

At the present time, when the global human population is increasing exponentially, microbial cultures represent a highly promising resource for obtaining high-value products, including polyunsaturated fatty acids (PUFA), proteins, amino acids, or pigments. Furthermore, the wide variety of methodologies, substrates and microorganisms that can be used for the production of food-related compounds expand the possibilities involved. In general, microorganisms such as microalgae, fungi, yeast, and bacteria, offer several advantages in comparison with vegetables or animals. Microbial cells are able to use inexpensive feedstock and wastes as sources of carbon and energy to produce biomass; they are highly efficient in substrate conversion, feature high productivity derived from the resulting rapid growth rate, and their biological cycle is shorter in comparison with more complex organisms. Besides, as compared to vegetable sources, microbial production is independent of seasonal factors and does not require land. As nutrients, microorganisms can utilize a variety of substrate like agricultural by-products and effluents, industrial wastes, or natural gases such as methane, all of which are generally not used by plants and, moreover, help to decompose pollutants (Huang & Kinsella, 1986; Nasseri et al., 2011; Li et al., 2008). Microalgae, for instance, can grow in fresh or salty water and even in waste water; they perform photosynthesis by fixing CO_2, and use inorganic material as nutrients.

Production of compounds of interest from microbial sources for the food industry can take advantage of the increasing relevance that genetic engineering has acquired in recent years. Microorganisms have a great potential to act as hosts for foreign genes derived from higher organisms such as animals, and can be easily genetically modified to over-produce a specific compound. These characteristics are exploited for recombinant protein production (Olempska-Beer et al., 2006).

1.2.1.1. Macronutrients

The chemical compounds which humans consume in the greatest quantities and which provide us with the bulk of our energy are known as macronutrients. Microorganisms such as algae, fungi, yeast, and bacteria are able to utilize inexpensive feedstock to produce the three primary macronutrients: protein, carbohydrates, and lipids.

Microorganisms produce proteins of great nutritive value, and are thus a successful source of amino acids for humans. Proteins constitute between 50-70 % of the composition of microalgae. In terms of amino acid quality, the nutritional value of proteins from several microalgae compare favorably with egg, soy, and wheat protein while successfully fulfilling WHO/FAO requirements (Chacón-Lee & Gonzalez-Mariño 2010). Yeast extracts, which are rich in peptides, amino acids, nucleotides, and vitamins, are used as supplements in culture media, but also as flavors and taste enhancers (replacing glutamates and nucleotides) in many canned foods. The waste derived from brewer´s yeast is commonly used for the commercial production of food-grade yeast extracts (Chae et al., 2001). However, some microorganisms also synthesize specific proteins with highly applicable functional properties (Yan et al., 2016).

Microalgae store high carbohydrate content in starch grains (pyrenoids), but the cell wall also acts as a reservoir containing sugars such as arabinose, xylose, mannose, galactose, and glucose, which vary according to species and growth phase (Cheng et al., 2011; Ho et al., 2012; Izumo et al., 2011). Similarly, yeast accumulates several kinds of carbohydrates. Some of these microbial polysaccharides can modulate the human immune system, thereby acting as favorable sources of biologically active molecules for food supplements and natural therapeutics (De Oliva-Neto et al., 2016).

Polyunsaturated fatty acids (PUFA) are acknowledged as essential nutritional components that prevent cardiac disorders. Although, animals cannot synthetize PUFAs of more than 18 carbons, oceanic fish consume microalgae and incorporate them; they thus have traditionally served as the most common source. However, the depletion of marine resources and the increment in demand for PUFA have increased efforts in the study of microalgae as potential sources of these health-related compounds (Wang et al., 2015). Linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are microalgae PUFAs of particular interest, with potential

applications in infant formulas and nutritional supplements (Li et al., 2014; Spolaore et al., 2006).

1.2.1.2. Pigments

The color of foods is primarily due to pigments such as carotenoids, anthocyanins, betanin and chlorophylls, either as inherent food constituents or as food or feed additives. These coloring agents that make food attractive have drawn considerable attention in recent years, not only because of their coloring properties, but also due to potential health-promoting effects (Saini & Keum, 2018). Pigments are nowadays mostly chemically produced; however, public scrutiny and the negative assessment of synthetic food dyes on the part of modern consumers have given rise to a strong interest in natural coloring alternatives. Microorganisms have been taken into consideration as potential sources of natural pigments because they are capable of high yields while growing in low-cost substrates such as agro-industrial wastes (Buzzini & Martini, 2000).

Carotenoids are natural fat-soluble pigments synthesized by various microorganisms and plants. They are currently in commercial use as feed additives, natural food colorants, nutrient supplements, precursors of vitamins, and, more recently, as nutraceuticals for cosmetics and pharmaceutical purposes (Jaswir & Monsur, 2011). Although synthetic forms are cheaper, microbial carotenoids have the advantage of supplying natural isomers, which have proven superior in terms of nutritional and therapeutic value (Becker, 2004).

Astaxanthin, a carotenoid, imparts the orange-red color to crustaceans and salmonids, thereby playing an important role in consumer appeal; however, animals cannot synthesize it, thus the compound must be supplemented in diet. *Xanthophyllomyces dendrorhous* yeast and *Haematococcus pluvialis* microalgae accumulate this pigment, and hundreds of scientific papers and patents have dealt with microbial astaxanthin production (Johnson, 2003; Schmidt et al., 2011). The generation of mutants has enabled the development of over-producing strains with high astaxanthin yields (Jacobson et al., 2003). Yeast of the genus *Rhodotorula* accumulates the carotenoids torulene and torularhodin, along with a minute quantity of β-carotene. Torularhodin is an uncommon carotenoid with potential applications ranging from food technology to pharmaceutics. This carotenoid exhibits strong antioxidant activity, acts as a Vitamin A precursor, and has been proposed as a colorant for meat products due to its

characteristic red color (Moliné et al., 2012; Zoz et al., 2015). Recently, the anti-cancer potential of torularhodin has been demonstrated in vivo on mice (Du et al., 2016). Another natural carotenoid, zeaxanthin, plays a critical role in the prevention of age-related eye diseases such as macular degeneration and cataracts. This pigment is increasingly used in the food industry because of its strong antioxidant activity and anti-cancer properties (Zhang et al., 2018). Microorganisms have been regarded as holders of great potential in obtaining highly pure, cost-effective zeaxanthin (Tuli et al., 2015). Many bacterial species, primarily of the genera *Flavobacterium* and *Paracoccus*, have been observed to naturally accumulate zeaxanthin (Bhosale et al., 2004; Manikandan et al., 2016; Hameed et al., 2014), and genetically modified *E. coli* has been reported as a source for zeaxanthin production (Zhang et al., 2018). Other carotenoids synthetized from bacteria are the keto-carotenoid canthaxanthin produced by the photosynthetic bacterium *Bradyrhizobium sp*., as well as the extremely halophilic bacterium *Halobacterium sp.* and the widely used carotenoid astaxanthin is also produced by *Agrobacterium aurantiacum* (Dufossé, 2006).

Microalgae contain basically three types of pigments: carotenoids, chlorophylls, and phycobiliproteins. Among carotenoids apart from α and ß-carotenes, microalgae such as *Chlorella sp* contain lutein, which is approved as a food colorant by the European Union (E-161 b) and has also a potential role in preventing retinal degeneration, some types of cancer, and cardiovascular diseases thanks to its antioxidant capabilities (Carpentier et al., 2009). Similarly to carotenoids, chlorophylls are lipid-soluble compounds with low polarity, and they serve commercially as important natural green pigments (Henriques et al., 2007). Moreover, chlorophyll has been associated with health benefits as a nutraceutical agent with antioxidant, anti-inflammatory, antimutagenic, and antimicrobial properties (da Silva Ferreira & Sant'Anna, 2017). Another type of photosynthetic accessory pigments are phycobiliproteins, which are assembled in the thylakoid membranes of chloroplast. These water-soluble proteins are commercially produced from the cyanobacterium *Arthrospira* and the rhodophyte *Porphyridium* (Viskari & Colyer, 2003; Román et al., 2002). The main potential of these molecules is to serve as natural dyes, but an increasing number of investigations have shown their health-promoting properties along with a broad range of pharmaceutical applications, as well as a potential application as fluorescent biomarkers in immunology (Cheng et al., 2012; Qiu et al., 2004).

The hydrosoluble vitamin riboflavin (Vitamin B2) has a variety of applications as a yellow food colorant, including dressings, sherbet, beverages, instant desserts, ice creams, tablets, and other products. Yeast such as *Candida guilliermundii* or *Debaryomyces subglobosus* produce up to 600 mg/L of riboflavin (Dufossé, 2006).

Anthocyanins, apart from acting as colorants, have potential anti-obesity effects thanks to multiple mechanisms (Xie et al., 2018). Although these flavonoids are currently obtained from plants, the microbial production of anthocyanins using genetically engineered bacteria that express plant anthocyanins is a promising strategy for future therapeutic use, in view of the association between its structure and activity, (Smeriglio et al., 2016; Xie et al. 2018). Recombination techniques, especially in *E.coli*, have been successfully applied to produce anthocyanins from cheap precursors, attaining reasonable yields (Lim et al., 2015; Jones et al., 2016 & 2017).

1.2.1.3. Enzymes

Enzymes are proteins that accelerate chemical reactions having wide-ranging applications in the food industry. Many food processing procedures are based on the enzymatic activity of microorganisms. In winemaking, for example, yeast proteases hydrolyze the peptide linkages between amino acid units of grape proteins, improving the clarification and stabilization of must and wine. This proteolytic activity is also used for protein haze reduction (Van Rensburg & Pretorius, 2000), and plays a major role during the autolysis process in wines aged on yeast lees (Alexandre & Guilloux-Benatier, 2006).

Since the microbial enzymes that participate in food processing are well known, commercial preparations of isolated enzymes are currently used rather than microbial cells. Pure enzymes industrially extracted from yeast and bacteria have been available for several decades. For example, β-glucosidase for the enhancement of wine aroma is commercially isolated from the yeasts *Vitis, Saccharomyces, Oenococcus, Aspergillus* or *Candida* (Longo & Sanromán, 2006). Different species of *Candida*, such as *C. antarctica*, *C. rugosa*, or *C. cylindracea*, are able to produce lipases for the performance of esterification reactions in the production of esters from inexpensive raw materials (i.e, fatty acids and alcohols) with the purpose of enhancing food aroma (Longo & Sanromán, 2006).

Further bacterial enzymes are currently being used in the food industry on a widespread scale: pullulanase for the production of maltose syrup, as well as α-amylase for the refining of sugar, produced by the bacteria *Bacillus licheniformis* or *Pseudomonas fluorescens* (Aiyer, 2005; Olempska-Beer et al., 2006), which as well, together with proteases, catalyze the inflation and maturation of dough, thereby improving the texture and taste of bread (Nadeem et al., 2009). Recently, bacterial enzymes have been added in alcoholic fermentation to supplement endogenous enzymes with the purpose of reducing the viscosity of starch, forming fermentable sugars and limit dextrins which improve the quality of beer (Saranraj et al., 2012). In the dairy industry, galactosidase (lactase) obtained mainly from *Escherichia coli, Kluyveromyces lactis, K. fragilis* and *Candida pseudotropicalis* hydrolyzes the lactose of milk, thus making it digestible for lactose–intolerant consumers (Panesar et al., 2006). Some enzymes also produce the flavors that help to identify cheese and accelerate its maturation, such as *Bacillus spp* and lipases, which, for example, generate the preferred flavors in butter (Sharma et al., 2001). In the production of amino acids to supplement food, certain enzymes such as aminoacylase, or aspartase from *E.coli*, are used to produce aspartic acid (Olempska-Beer et al., 2006). The enzyme tannase produced by yeast *Candida sp, Saccharomyces cerevisiae* and *Mycotorula japonica* catalyzes the hydrolysis of tannins to help avoid their undesirable effects in instant tea (Boadi & Neufeld, 2001). Other yeast enzymes that are useful in the food industry include invertase from *Kluyveromyces fragilis*, *Saccharomyces carlsbergensis* and *S. cerevisiae* for candy and jam manufacturing, and galactosidase from *S. carlsbergensis* for the crystallization of beet sugar.

However, enzymes traditionally isolated from cultivable microorganisms are limited in scope and often not well-adapted to the conditions and methods of modern food production. For this reason, recombinant DNA technology plays an important role in the manufacturing of novel enzymes for food processing. Decades ago, bovine chymosin for the production of cheese was expressed in the bacteria *Escherichia coli K-12* and in yeast *Kluyveromyces marxianus var. lactis*, which thereby became the first recombinant enzymes approved for food use by the FDA. Many further recombinant enzymes are now being used (Flamm, 1991; Olempska-Beer et al., 2006).

1.2.2. Microbial structure and the location of target compounds

Although certain highly valuable compounds are released from bacteria, yeast, or microalgae to the growth medium during the normal metabolism of microbial cells, the majority remain inside the cell. Therefore, it is necessary to know the cell's structure and the compound's location before designing effective strategies for compound extraction and subsequent purification.

1.2.2.1. Bacteria

The cytoplasm of all known microorganisms is encased in a lipid bilayer: the cytoplasmic membrane, composed primarily of phospholipids and embedded proteins. This membrane, which is common in bacteria, yeast, and microalgae, does not have mechanical strength, but maintains concentration gradients among the cell and its surroundings thanks to its selective permeability to ions and organic molecules. The structure of the phospholipid molecule generally consists of two hydrophobic fatty acid "tails" and a hydrophilic "head" made up of a phosphate group. These amphiphilic phospholipids are arranged so that the hydrophobic "tail" regions are isolated from the surrounding water, while the hydrophilic "head" regions interact with the intracellular (cytoplasm) and extracellular faces of the resulting bilayer. The membrane also serves as an attachment surface for several extracellular structures including the cell wall, the configuration of which depends on the specific microorganism.

The envelope of Gram-positive bacteria has a different structure than that of Gram-negative bacteria **(Fig. 1.2.a).** Gram-negative bacteria possess a cell wall consisting in an outer lipid bilayer membrane and a peptidoglycan layer (7-8 nm) that surrounds the cytoplasmic membrane and provides the cell with mechanical strength. The complex outer membrane consists of a lipid bilayer, transmembrane proteins, phospholipids, and lipopolysaccharides. A lipoprotein complex connects the lower portion of the phospholipid bilayer to the crosslinked peptidoglycan layer. Gram-positive bacteria, on the other hand, present a thicker peptidoglycan layer (an outermost layer of 20-80 nm) and a much smaller volume of periplasm than in Gram-negative bacteria. Whereas the cytoplasmic membrane acts as a selective barrier to the free diffusion of solutes and regulates transmembrane ion transport and solute gradients, the cell wall allows the unrestricted diffusion of molecules through itself.

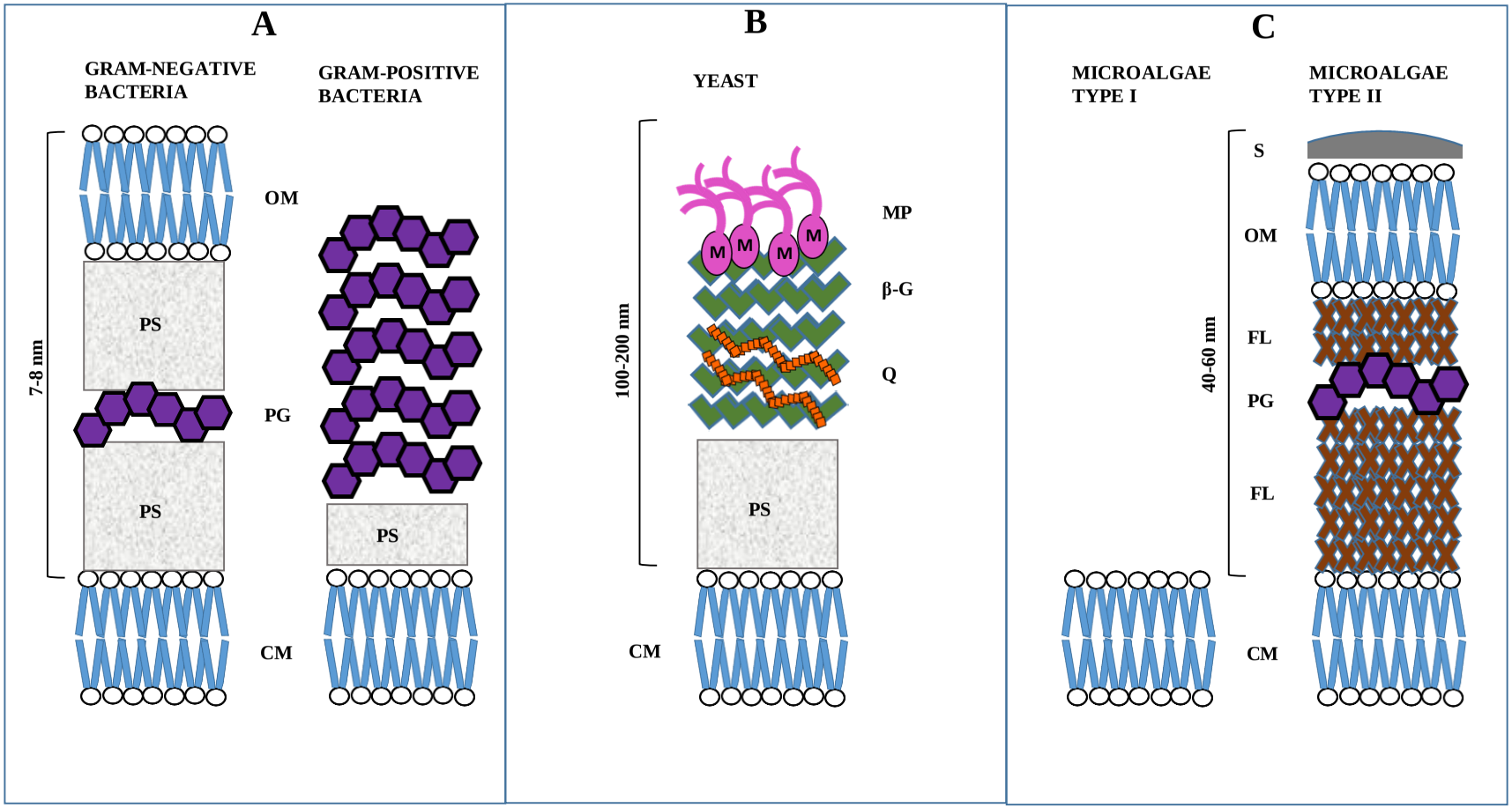


Figure 1.2. Simplified structure of the envelope of Gram-negative and Gram-positive bacteria (A), yeast (B) and microalgae (C). CM: cytoplasmic membrane; PS: periplasmic space; PG: peptidoglycan; OM: outer membrane; MP: mannoproteins; β-G: β-glucans; Q: chitins; FL: fibrillary layer; S: sheath

In comparison with eukaryotes, the intracellular structures of a bacterial cell are extremely simple and, except for the ribosomes (where protein is synthesized), they do not contain organelles. Proteins produced by bacteria are usually located in the cytoplasm and isolated inside the cell by the inner layer (cytoplasmic membrane), while recombinant proteins of *E. coli* are typically located in the periplasmic space between the cytoplasmic membrane and the outer membrane (Meglič, 2016). On the other hand, pigments and lipids in bacteria are located in the cytoplasmic membrane or in outer membrane vesicles (which, in the case of Gram-negative bacteria, are vesicles of lipids released by the outer membranes).

1.2.2.2. Yeast

Although yeast species exhibit great diversity in size, shape and color, and although the composition of cells may even vary within the same species depending on culture conditions, their typical envelopes are the cytoplasmic membrane, the periplasmic space, and the cell wall (Balasundaram et al., 2009) **(Fig 1.2.b).** Using *Saccharomyces cerevisiae* as a model, the outer layer is the rigid cell wall, which is remarkably thick (100 to 200 nm) and serves as a protecting capsule which provides mechanical strength. It is composed of polysaccharides, mainly β-glucans and mannoproteins, which are highly glycosylated proteins, as well as of a minor percentage of chitins and other proteins (Quiros et al., 2012). Between the cell wall and the cytoplasmic membrane, the periplasm constitutes an interrupted space with invaginations of the membrane and irregularities in the inner surface of the cell wall. A number of important active enzymes are located in this space. Yeast cells likewise possess a cytoplasmic membrane composed mainly of a bilayer of phospholipids with functional proteins embedded (Stewart, 2017).

Yeasts have vacuoles, which are organelles containing inorganic and organic molecules including enzymes in solution. Vacuoles are dynamic structures that can rapidly modify their morphology according to the cell's requirements. They are involved in many processes including the homeostasis of cell pH and the concentration of ions, osmoregulation, and the storage of amino acids, as well as polyphosphate and degradative processes. Bio-active compounds (proteins, glycoproteins, polysaccharides, polyphosphates, lipids, nucleic acids, pigments, etc.) are retained at various locations within the cell including cell wall, periplasm, plasma membrane, cytoplasm, and vacuoles (Balasundaram et al., 2009).

1.2.2.3. Microalgae

The architecture and cell wall composition of microalgae and cyanobacteria vary widely, ranging from tiny membranes to multilayered complex structures. Based on the complexity of surface structures, four cell types can be distinguished: (I) a simple phospholipid bilayer cell membrane, (II) cell membrane and additional extracellular material, (III) cell membrane and additional intracellular material in vesicles, and (IV) cell membrane as well as additional intra- and extracellular layers (D´Hondt et al., 2017). The microalgae species most used for biotechnological applications belong to Type I (naked), such as *Dunaliella,* which is vulnerable to disruption, or to type II such as *Spirulina, Chlorella, Haematococcus, Scenedesmus,* or *Nannochloropsis* with a complex cell wall. This cell wall type may include various structures associated with the membrane (cell wall, mucilage and sheaths, scales, frustules, lorica, skeleton), that make it rigid and multilayered. As an example, **Figure 1.2.c** shows the cell wall of *Arthrospira* consisting of five layers. Overlaying the cytoplasmic membrane is the peptidoglycan layer surrounded internally and externally by two fibrillary layers. Microfibrillar framework is embedded in amorphous mucilaginous material composed of polysaccharides, lipids, and proteins. The outer membrane is tightly connected with the peptidoglycan layer, and is covered with a sheath of acidic polysaccharides (Tomaselli, 1997). The constituents of these cell walls include carbohydrates (glucose, rhamnose, mannose, ribose, xylose, fucose, and galactose), proteins, lipids, carotenoids, tannins, and lignins. Polysaccharides in the cell wall include cellulose, chitin-/chitosan-like molecules, hemicelulloses, pectins, fucans, alginates, ulvans, carrageeenans, and lichenins (Zhang et al., 2018).

The patterns of intracellular cell organization in algae can be divided into prokaryotic and eukaryotic groups. Whereas in prokaryotic algae the DNA is uniformly distributed throughout the entire cell, eukaryotic microalgae present a nucleus surrounded by membrane, membrane-bounded plastids, endoplasmic reticulum, mitochondria, Golgi apparatus, pyrenoids, and chloroplasts. The chloroplasts (photosynthetic lamellae, discs, or thylakoids), confined within membranes, may have different structures and contain membrane-bound photosynthetic pigments (chlorophylls, carotenoids and phycobiliproteins). In many eukaryotic microalgae, the pyrenoids are present within the plastids that are the centers for enzymatic condensation of glucose into starch. In addition, cells accumulate lipids in droplets in the cytoplasm.

1.2.3. Obtaining highly valuable compounds from microbial biomass

In order to incorporate highly valuable compounds from microbial biomass to the human diet, two strategies can be applied. The simplest would consist in adding the whole microbial cells to the food. However, to achieve intestinal absorption of the compounds, the microbial cell walls need to be previously removed or enzymatically digested. Furthermore, the ingestion of other compounds present in microbial cells might not be recommendable for the human organism. The other approach involves the extraction and isolation of the compound of interest, thereby avoiding the inclusion of the entire microbial biomass in the food. Extraction of high-value pure compounds from microbial cells permits the subsequent concentration and the increased bio-availability of compounds with specific bioactivities.

Figure 1.3. shows a general process flow diagram for obtaining highly valuable compounds from microorganisms. The process begins with the selection of strains that naturally produce the compound of interest, the selection of mutants that overproduce it, or the introduction of an external gene from a higher organism into a microbial host in order to produce it. The microorganisms are subsequently cultivated under conditions tending to produce high yields of the compound, and are harvested when the concentration of the target compound in the suspension has reached its peak. There is no single best method for harvesting microorganisms; the optimal harvesting technology depends on species, growth medium, production, end product, and the cost-benefit ratio. Low-cost filtration procedures are applicable only to the harvesting of fairly large microbial cells, while small cells should be flocculated into larger aggregates. The decision between applying sedimentation or flotation methods depends on the difference in density between the cell and the growth medium. For oil-laden microalgae with low cell density, flotation technologies should be considered (Barros et al., 2015). Moreover, oxygen release from algae cells and oxygen supersaturation conditions in growth medium support the use of flotation methods. The use of centrifugation is required for smaller microorganisms. After harvesting, the extraction process has traditionally been conducted from dry biomass because this approach permits the storage of raw matter, thereby providing the option of delaying the extraction of the compound of interest. However, drying the biomass prior to the extraction step requires

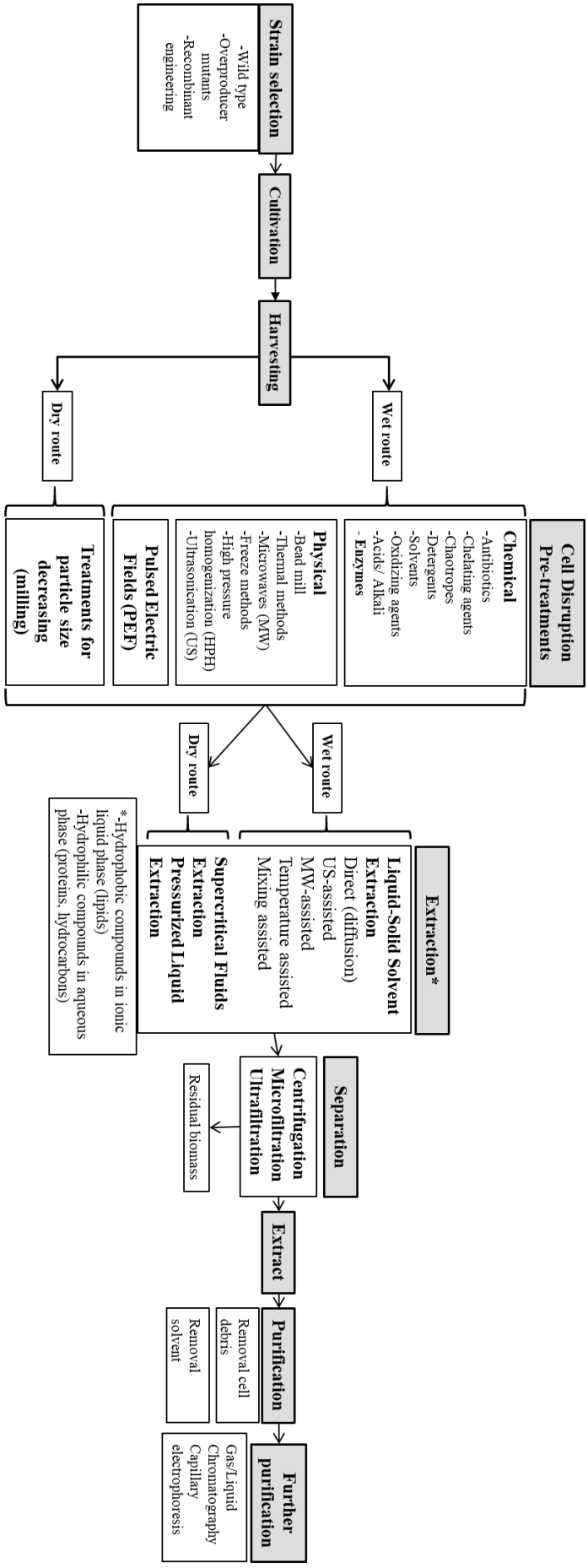


Figure 1.3. Process flow diagram production of high-valuable compounds from microorganisms

a significant amount of energy and may cause losses of valuable compounds due to oxidation: thus, ideally, it should be preferable to use moist biomass. Components are located within the cells, thus either following the dry or wet route; their recovery requires that they cross the cell envelopes to be released. The presence of an intact cytoplasmic membrane which acts as a semipermeable barrier, of membranes of organelles, and of other envelopes such as thick cellular walls, all obstruct the release of valuable compounds. Therefore, cell disruption pre-treatments are applied to break those structures and facilitate subsequent extraction. The treatments are either (bio) chemical, physical, or a combination of both. **Table 1.2.** shows the main advantages and disadvantages inherent in these methods. Chemical treatments permeabilize the envelopes of microorganisms and differ in selectivity and efficiency towards different microbial species (Geciova et al., 2002). Since each chemical substance disrupts the cells in a different way, several mechanisms are involved in the disruption processes. Antibiotics inhibit the production of cell membrane components; chelating agents bind the cations; chaotropes make the surrounding medium less hydrophilic; detergents form micelles; solvents dissolve or perforate the cell membrane/wall; alkalis saponify the membrane lipids, and acids lead to poration of envelopes (Günerken et al., 2015). The main operating parameters are temperature, time, biomass concentration, microorganism species, and the type and concentration of chemical (Lam & Lee, 2015). Although these methods are efficient and upscaling is quite simple, the cost of chemicals and the resulting product quality might reduce the benefits. Furthermore, in the case of food applications, chemical treatments introduce another complicating factor: the "contamination" of the cell suspension by the active chemical, which often is non-food grade, thereby resulting in a higher degree of complexity in terms of downstream process operations. Enzymatic lysis with cellulases, glycosidases, amylases, proteases, xylanases, peptidases, or lipases presents advantages including biological specificity, high selectivity, and prevention of destructive conditions of other chemical methods. However, the drawbacks are high enzyme cost, difficult enzyme recovery, and long incubation times (Lam & Lee, 2015; Middelberg, 1995). The two alternative approaches with potential use in the food industry are the use of external lytic enzymes, or the use of the enzymes pertaining to the cell which is the target of lysis. Autolysis has the advantage of saving the expense of adding external enzymes, but requires specific conditions to achieve an acceptable rate. On the other hand, physical cell disruption methods are usually selected for their simplicity and low selective capacity, allowing the simultaneous extraction of various compounds. The

Table 1.2. Advantages and disadvantages of cell disruption methods in comparison to Pulsed Electric Fields (PEF)

		Advantages	***Disadvantages***
Chemical treatments	*Chemicals*	Efficient Simple upscaling	High cost of chemicals Contamination of product with active chemicals Downstream purification operations
	Enzymes	Biological specificity High selectivity Non-destructive conditions	High enzyme cost Difficult of enzyme recovery Long incubation time
Physical treatments	*Bead mill*	Very effective Simple Fast	High energy consumption Cooling steps Release of cell debris Complicates purification
	Thermal methods	Simple Easily to scale up	Unspecific High energy consumption Degradation of thermo-labile compounds
	Microwaves	Easily to scale up High operational speed	High energy consumption Degradation of thermo-labile compounds
	Freeze methods	Adequate for heat-sensitive compounds	Difficult to scale up Costly in time, energy, and money
	High-pressure homogenization	Effective Easily to scae up	High energetic cost Cooling steps Non-selectivity release Degradation of thermo-labile compounds Purification stage
	Ultrasonication	Efficient Mixing effect	Scale-up not feasible Elevated operational cost Degradation of thermo-labile compounds Non-selective release
Pulsed Electric Field (PEF*)*		Energetically efficient Short process time Non destructive High selectivity No thermal effect Low operation cost Easy to scale up	Dependence on medium composition (conductivity) High cost of the equipment

cells are subjected to high stress, producing breakage; depending on the method, the size of the generated cell fragments varies, and bears a direct influence on the purity of released products (Liu et al., 2016). In comparison with enzymatic methods, mechanical techniques require a much shorter operation time (Nasseri et al., 2011), and do not present the residue problems inherent in chemical methods. However, the application of these mechanical treatments usually produces an increment in operational temperature;

biomass must therefore be cooled down to avoid undesirable heating effects, thereby increasing energy consumption. Some of these methods cannot be applied in continuous flow, and are thus difficult to scale up. Furthermore, the resulting excessive denaturalization of cell envelopes leads to the release of cell debris and therefore requires subsequent purification techniques, while also impeding the extraction of labile compounds that have become degraded (Biller et al., 2016; D'Hondt et al., 2017; McMillan et al., 2013; Skorupskaite et al., 2017; Walther et al., 2017). As an alternative to the disruption methods we have just described, this review provides insights into the potential of electroporation using Pulsed Electric Fields (PEF).

After a cell disruption treatment, biomass can be dried, or the process can continue using the wet biomass. Extraction is usually performed by dissolving target compounds in a solvent, in order to separate them and later recover them from the liquid (liquid-solvent extraction). The solvent (organic or aqueous, depending on the polarity of the compound to be extracted), is mixed thoroughly with the solid, and contact is maintained throughout the required time interval. The extraction of a compound that is located inside a cell requires for the solvent to penetrate the cell and dissolve the compound, then for the compound to diffuse to the surface of the cell, and, finally for it to migrate from the superficial part of the cell to the extracellular solvent. The application of ultrasound (US), microwave (MW), mixing, or heating methods improve the yields from direct solvent diffusion. During extraction, high-frequency microwaves can shatter cells, thereby improving lipid (Lee et al., 2010; Pan et al., 2016) and pigment extraction (Choi et al., 2007; Pasquet el al., 2011). Cavitation due to US during solvent liquid extraction results in micro-jetting, erosion, particle breakdown, macro-turbulences, and micro-mixing, thereby enhancing the hydration of the matrix (Chemat et al., 2017). Likewise, in recent applications of ultrasound, its capability to form fine emulsions of two immiscible phases has been exploited in the simultaneous extraction of polar and nonpolar compounds. US-assisted emulsification-extraction uses two immiscible solvents, which, under US application, form an emulsion that allows close contact of both with the solid sample and a rapid mass transfer of the compounds to be extracted (Delgado-Povedano & de Castro, 2015). Lipids, phenolic compounds, and pigments have been recovered using US-assisted green solvent extraction (Adam et al., 2012; Parniakov et al., 2015).

Supercritical fluid (SCF) extraction has been presented as an alternative method for the extraction of thermolabile compounds while avoiding the use of toxic solvents

(Sahena et al., 2009; Saini & Keum, 2018). SCF temperature and pressure are above the critical point, thereby presenting properties which explain its greater ability to diffuse into the matrix than conventional organic solvents. Many studies deal with SC-CO_2 extraction of carotenoids (Bustamante et al., 2011; Hosseini et al., 2017; Machmudah et al., 2006; Macías-Sanchez et al., 2009; Mussagy et al., 2018) and lipids (Mendes et al., 2006; Sajilata et al., 2008) from microalgae and yeasts (Hasan et al., 2016; Lim et al., 2002; Wang et al., 2012). Co-solvents that enhance the solubilizing power of SC-CO_2 such as ethanol or vegetable oils are occasionally needed to increase the extraction yield (Lim et al., 2002). Similarly, pressurized liquid extraction (PLE) uses conventional solvents at controlled temperatures under high pressure, thereby making it an interesting alternative process to extract antioxidant carotenoids from microalgae (Jaime et al., 2010; Macías-Sanchez et al., 2009; Plaza et al., 2012). When compared to traditional solvent extraction, PLE shows a reduction in extraction time from several hours to minutes, and a decrease in total organic solvent consumption; however, extraction yields are far lower than those reported using other methods (Castro-Puyana et al., 2017; Gilbert-López et al., 2017; Herrero et al., 2006).

After the extraction stage, the mixture is divided into two parts: the liquid portion, composed of the dilution of the compound of interest in the solvent or co-solvent, and the solid phase, formed by the residue containing the insoluble components and part of the solvent embedded therein. Finally, the extract contained in the solvent is purified to different degrees depending on the final application of the extracted compound. For example, the production of phycobiliproteins as biomarkers for immunology assays requires highly purified molecules, while the extracts used as pigments for foods or beverages usually contain a mixture of molecules.

1.2.4. Pulsed Electric Fields technology for improving extraction of valuable compounds from microbial cells

Pulsed electric field (PEF) treatment is an innovative nonthermal technology that has been proposed as an alternative to the cell disruption methods described above. The process consists in the intermittent application of direct-current high-voltage pulses (kV), for time intervals ranging from microseconds to milliseconds, through a material placed between two electrodes. This voltage generates an electric field, the intensity of which depends on the gap between the electrodes and the delivered voltage. If the electric field

is intense enough, a phenomenon called electroporation occurs, which consists in the increment of the permeability of the cytoplasmic membrane to the passage of ions and macromolecules (Kotnik et al., 2012). Thereby, PEF treatment enhances the migration of compounds located in the microbial cytoplasm through the membrane, because the latter loses its selective permeability after the treatment. Depending on intensity of treatment, the cell can still be able to reseal the pores, whereby the permeabilization is transitory (reversible electroporation), or, on the contrary, pores are permanent (irreversible electroporation). Either for purposes of inactivation of microorganisms or for the improvement of mass transfer processes, the objective is to reach irreversible electroporation of cell membranes. Whereas for the inactivation of microorganisms in food processing several $\log_{10}$ cycle reductions of the microbial population are needed, for compound extraction the electropermeabilization of 1-2 $\log_{10}$ cycles, which represent 90-99 % of the population, would be enough to occasion an increment of permeability in the majority of the microbial population in order to extract the compounds of interest.

1.2.4.1. The cell membrane electroporation phenomenon

Cell membrane in an electric field

The cytoplasmic membrane of microorganisms is only two-phospholipid-molecules thick (about 5 nm) with proteins embedded, and behaves partly as a liquid and partly as a gel. The membrane has very low electrical conductivity and can be regarded as a thin insulating sheet, while both extra- and intracellular media surrounding the membrane are aqueous, highly conductive electrolyte solutions **(Fig. 1.4.)**. Thus, the structure formed by the extracellular medium, the lipid bilayer, and the intracellular medium is a conductor-dielectric-conductor that behaves like a capacitor (Ivorra, 2010).

When exposed to a sufficiently strong electric field, the membrane undergoes electrical breakdown, which renders it permeable to molecules that would otherwise be unable to cross it (electroporation). In solid insulators, an electrical breakdown generally causes a permanent structural change. However, as the lipids of the membrane behave has a two-dimensional liquid, the membrane can spontaneously return to its pre-breakdown state after the treatment. If exposure time is sufficiently short and the membrane recovery is sufficiently rapid for the cell to remain viable, electroporation is termed reversible; otherwise, it is termed irreversible (Teissie et al., 2005).

Induced Transmembrane Voltage (ITV)

Electroporation is a dynamic phenomenon that depends on local transmembrane voltage. It is generally accepted that a specific transmembrane voltage threshold is required for the electroporation phenomenon to occur. Under normal conditions, the systems of ions transport through the membrane lead to the irregular distribution of positive and negative ions on both sides of the membrane, thereby generating a difference in potential called resting transmembrane voltage (RTV). Ions involved in RTV represent a low percentage of total ions in cytoplasm or extracellular media. However, a reorganization of charges occurs when a cell is subjected to a high electric field strength and the charges accumulate on both sides of the membrane. This phenomenon supposes an increase in transmembrane voltage, the value of which is designated as induced transmembrane voltage (ITV) (Tsong, 1991). When ITV reaches a determined threshold, electroporation of the cytoplasmic membrane takes place (Zimmerman et al., 1974).

The ITV that occurs prior to the electroporation phenomenon depends on the electric field strength applied, as well as the size and shape of the cell. For a single spherical cell with a nonconductive plasma membrane, the Laplace equation is solved in the spherical coordinate system, yielding the expression often referred to as the steady-state Schwan equation (Schwan, 1957) (equation):

$$\Delta Vi = \frac{3}{2} |E| r(cos\theta)$$

where ΔVi is the induced transmembrane voltage, E is the electric field strength applied, r is the cell radius and θ is the angle measured from the center of the cell with respect to the direction of the field **(Fig 1.4.).**

Therefore, from this equation one can deduce that the external electric field strength required to reach the transmembrane voltage threshold is inversely correlated to cell size. Consequently, the external electric field strength required to induce electroporation in microbial cells (1-10 µm) is higher (> 10 kV/cm) than that required for eukaryotic plant cells (40-200 µm; < 5 kV/cm) (Donsi et al., 2010). While the size of bacteria is typically 0.5–5.0 µm, yeast have a diameter ranging from 5 to 8 µm, and microalgae range from 3 µm (e.g. *Chlorella* species) to several hundred µm (e.g. *Haematococcus pluvialis*). **Table 1.3.** shows the electric field strength and total specific energy required to electroporate a certain percentage of different types of cells showing

this correlation. Heinz et al., (2001) provided a graph that also takes size and orientation into account.

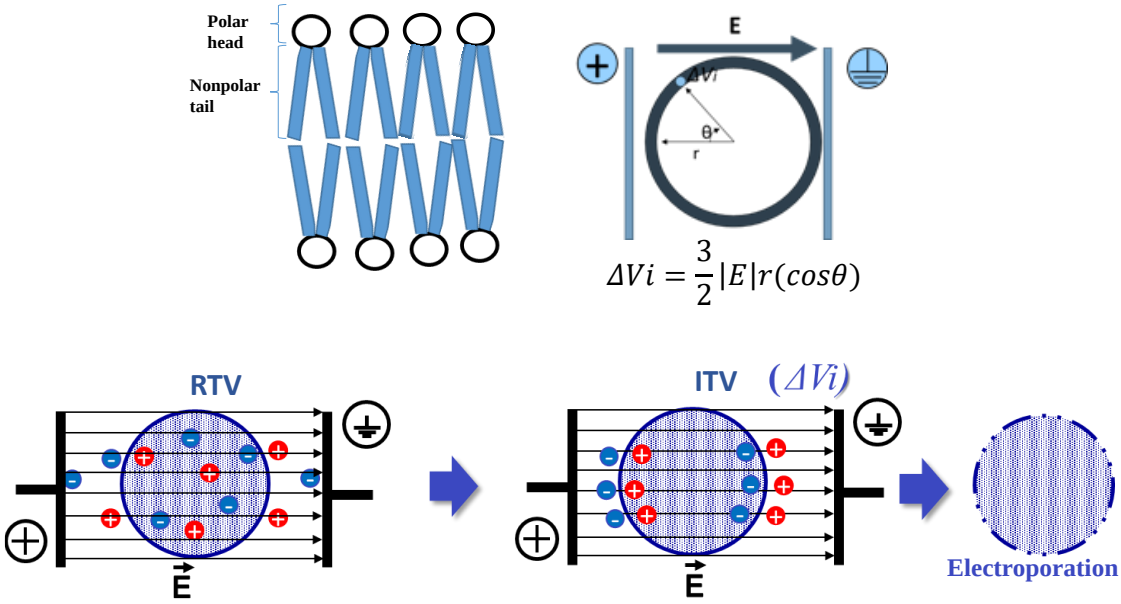


Figure 1.4. Above: Conformation of phospholipid bilayer of cytoplasmic membrane (left side) and terms of Laplace equation for a spherical cell subjected to an electric field (right side). Below: exposure to electric fields of a cell suspended in an aqueous electrolyte medium

According to the above equation, electroporation is not uniform across the entire cytoplasmic membrane. Zones that are perpendicular to field strength direction ($\theta \approx 0$) will be easily electroporated, since they are subjected to a higher ITV. Application of an electric field between two electrodes provokes a current running from the negative to the positive pole. Therefore, charge accumulation on both sides of membranes is mainly produced in cell areas located in parallel position with respect to the electrodes; thus, transmembrane voltage increases at a greater rate in those parts of the cell. This phenomenon has been observed in various studies performed with fluorescent dyes, in which it has likewise been observed that those areas in which transmembrane potential is at a maximum are electroporated earlier (Gross et al., 1986; Hibino et al., 1993; Kotnik et al., 2010; Kotnik, 2016). Finally, from the above equation it can be also concluded that the cell area subjected to electroporation depends on the electric field strength applied: a greater electric field strength will allow the attainment of an ITV required to produce electroporation in areas where lower electric fields did not reach the ITV threshold.

Table 1.3. Electric field strength and total specific energy to electroporate different types of cells at pH 7

Microorganism	Shape and size	Irreversibly permeabilized percentage (P.I. intake)	Electric field strength (kV cm^{-1})	Total specific energy (kJ kg^{-1})	Reference
		Bacteria			
E. coli NCTC5934	Rod-shaped, 2 µm long and 0.25–1.0 µm in diameter	99 %	25 kV/cm	500 kJ/kg	García, Gómez, Mañas, Raso, & Pagán,2007
S. senftenberg 775W	Rod-shaped, 2 to 5 µm long and 0.7-1.5 µm in diameter	75 %	19 kV/cm	144.4 kJ/kg	García et al., 2007
L. monocytogenes	Rod-shaped, 2 µm long and 0.5 µm in diameter	50 %	19 kV/cm	288.8 kJ/kg	García et al., 2007
		Yeast			
S. cerevisiae	Round to ovoid, 5–8 µm in diameter	99 %	10 kV/cm	34.08 kJ/kg	Aronsson & Rönner, 2001
S. cerevisiae	Round to ovoid, 5-8 µm in diameter	90 %	20 kV/cm	30 kJ/kg	Martínez et al., 2016
R. glutinis	Round oval elongate, 3-5 µm in diameter	90 %	20 kV/cm	72 kJ/kg	Martínez et al., 2018b
		Microalgae			
C. vulgaris	Spherical, 3-8 µm in diameter	90 %	15 kV/cm	16.8 kJ/kg	Luengo et al., 2015a
P. cruentum	Spherical to ovoid, 10-16 µm diameter	90 %	10 kV/cm	9 kJ/kg	Martínez et al., 2019b
Chlamydomonas reinhardtii	Spherical to ovoid, 8-10 µm diameter	85 %	3.5 kV/cm	13 kJ/kg	Bodénes et al., 2019

Data regarding total specific energy depends on the conductivity of the media in which treatments where applied, a parameter which is crucial

When application of the electric field has concluded, the pores formed during treatment can reseal, or they can persist in the absence of an electric field. As the pores formed during treatment can be reversible or irreversible, two ITV threshold values can be distinguished. Surpassing the first one would produce electroporation, initiating the formation of the first pores in the membrane. By augmenting treatment intensity, either via an increase in electric field strength or in treatment time, the surpassing of the second ITV threshold leads to the defects' irreversibility: the pores thus remain permanent, even after treatment (Ivorra, 2010). Irreversible electroporation has been associated with pore size, since the larger the size of the pore created, the longer it will take it to close once the electric field strength has ceased (Saulis et al., 1991). In this sense, if the radius of a pore surpasses a critical value, it remains stable after treatment. Otherwise, if the size of a pore does not reach the critical radius, the cell will be able to reseal it and the membrane will return to its previous state (Tomov, 1995; Joshi et al., 2003; Joshi & Hu, 2012). In order to improve mass transfer process, and thus the extraction of intracellular compounds, an irreversible permeabilization of the membrane is required. Electric field amplitude and pulse duration have a tremendous impact on the pore size obtained, but also affect the amount of energy spent and the temperature of the medium during the process (Bodenés et al., 2016; Saulis et al., 2013; Smith et al., 2014). On the other hand, the extraction of certain intracellular target molecules is highly dependent on the pore size of membranes and their solubility in water and/or organic solvents (Bodenés et al., 2019).

In the case of bacteria, the shape of different cells ranges from spherical (coccus) to rod-shaped (bacillus), but it can also be filamentous, spiral, or pleomorphic. Similarly, yeast cells can be spherical, globose, ellipsoidal, elongate, rectangular, pear-shaped, apiculate, ogival, or even tetrahedral. The shape of microalgae varies enormously from species to species as well. Equations similar to the one described above can be derived for nonspherical cells, provided that the latter resemble a regular geometrical body such as a cylinder, an oblate spheroid, or a prolate spheroid (e.g. bacilli) (Gimsa & Wachner, 2001; Kotnik & Miklavic, 2000).

Hydrophilic pore formation theory

As described above, a specific transmembrane voltage threshold is required for the electroporation phenomenon to occur. However, the ultimate reason that explains how

an increment in transmembrane voltage can lead to the formation of pores in the cytoplasmic membrane has not been fully elucidated. The most accepted model used to explain electroporation phenomenon is currently the hydrophilic pore formation theory (Chen Smye et al., 2006). Phospholipids that constitute the bilayer of the cytoplasmic membrane are held together only by weak (noncovalent) interactions: they can easily move laterally within a specific layer, and can even occasionally flip into the other layer, but practically never leave the bilayer. Despite the relative weakness of pairwise interactions between lipids, the cooperative nature of such interactions makes the lipid bilayer a very stable structure and an almost impenetrable barrier. However, it is supposed that under normal conditions hydrophilic pores appear spontaneously in the cytoplasmic membrane, thereby allowing ions, water, and hydrophilic molecules to cross rapidly (passive form) through the cytoplasmic membrane. Such pores with radii below a nanometer and lifetimes below a nanosecond would form and reseal due to thermal and mechanical fluctuations (Kotnik et al., 2010).

According to this theory, when an electric field is applied, a voltage is induced across the bilayer, thereby reducing the energy required for spontaneous hydrophilic pore formation. This facilitates the formation of a greater number of pores which are more stable than they would have been in the absence of the electric field (Kotnik et al., 2012). Therefore the observed increase in membrane permeability to compounds that would be unable to cross the membrane under normal conditions is a consequence of the formation of a number of pores of a large enough size and with a sufficiently long lifetime.

However, aqueous pores in the bilayer are too small to be observed under optical microscopy, and under electron microscopy they cannot be clearly distinguished from artifacts derived from the required sample preparation (Spugnini et al., 2007).

The main evidence supporting the theory of aqueous pore formation is that simulations of molecular dynamics permit the modelling of a sequence of molecular-scale events that includes the formation of pores when a lipid bilayer membrane is exposed to the direct action of an external field of sufficient intensity (Leontiadou et al., 2004).

Quantification of electropermeabilization in cell suspension

The term “electropermeabilization” refers to the percentage of cells with a cytoplasmic membrane permeable to a certain substance after PEF, and it is used to

evaluate efficacy of treatment (Saulis et al., 2013). Several techniques can be used to assay electropermeabilization of cells; however, pores of different sizes can be considered, depending on the size of the molecule that is used to evaluate the capability of crossing through the membrane. If the detection of permeablization is performed by measuring ion transport through membrane, the formation of small-size pores is enough. However, the use of molecules of larger size such as propidium iodide (PI) or bleomycin can only detect the formation of larger pores. The fluorescent dye PI is a small (660 Da) hydrophilic molecule that is unable to penetrate intact cytoplasmic membrane. However, the addition of an adequate solution of this dye to a suspension that has been subjected to a PEF treatment is used to evaluate irreversible permeabilization, because the dye bonds with the cell's DNA; thus, the fluorescent cell count corresponds with the number of permeabilized cells. An evaluation of pore sizes can be obtained by dyeing the cell suspension after treatment with a range of dyes of different atomic mass, such as fluorescent Dextrane FITC (3000 Da) and Sytox Green (600 Da). While Dextrane FITC requires pores of minimum diameter of 0.8-0.9 nm, Syxtox Green is able to cross through pores of 0.5-0.7 nm (Saulis & Saulé, 2012).

The release of nucleic acids and proteins to the medium after PEF treatment of cells is another indicator of the degree of permeabilization. It can be evidenced by the increment of optical density of the media at 260 and 280-nm, respectively. Likewise, plasmolysis of cells due to the entrance of water into the cytoplasm denotes electroporation and can be observed microscopically.

1.2.4.2. PEF-assisted extraction from bacteria

The effect of PEF on bacteria has been widely investigated as a nonthermal procedure of microbial inactivation rather than as a procedure for enhanced extraction of compounds of interest from microbial cells. One of the consequences of the loss of the integrity and functionality of the cytoplasmic membrane caused by irreversible electroporation is cell death. Therefore, PEF has significant potential as an alternative to thermal food preservation treatments, since the PEF method avoids the undesirable changes induced by heat in foods (Saldaña et al., 2014).

Reversible electroporation is currently used as a standard to assist DNA transfer between bacteria, or between bacteria and yeast, and for the enhancement of plasmid DNA extraction. On the other hand, irreversible electroporation of bacteria is one of the

promising applications of electroporation to help improve the extraction of proteins or lipids (Meglič et al., 2015). **Table 1.4.** shows the benefits of PEF on the recovery of different compounds from bacteria and the conditions of treatments applied. It has been observed that some intracellular proteins can be released through the pores selectively, depending on the size of the pores induced by PEF in the cytoplasmic membrane (Ohshima et al., 2000). A varying sensitivity to PEF among bacterial strains has been observed, and the media of incubation after PEF treatments has a decisive influence on the amount of protein extracted. Ohshima et al. (2000) studied the effect of different parameters of PEF treatments on the extraction of three recombinant proteins from *E. coli*. After PEF treatment and the addition of 5% glycine that increases cell membrane permeability, *E. coli/pNC1* released the highest amount of β-glucosidase, corresponding to 26 % of the amount obtained after ultrasonic treatment. On the other hand, α-amylase, which is accumulated in the periplasmic space, was easily released after PEF treatments when *E. coli/pHI301A* bacteria were suspended in 0.9 % NaCl and 10 % polyethylene glycol (PEG) solution. An amount of 89 % of α-amylase was extracted, and the enzyme had nine times more specific activity compared with the effect of ultrasonic treatment. These results indicated that PEF treatments can also affect the outer membrane of this Gram-negative bacterium, thereby proving useful for the selective release of periplasmic protein. Shiina et al., (2007) studied the extracellular release of recombinant α-amylase from *E. coli HB101/pHI301A* by PEF during fed-batch cultivation. When PEF was applied intermittently from the beginning of stationary phase, the amount of active α-amylase released was about 30% of the total amount of α-amylase produced in the cells. Therefore, suitable PEF treatment was shown to be useful for easy and effective release of periplasmic proteins by fed-batch cultivation from recombinant *E. coli*.

The influence of PEF parameters on protein extraction from *E. coli* has also been studied. The highest amount of protein was extracted when greater electric field strengths or longer pulse durations were applied (Meglič et al., 2015). Incubation at low temperatures after treatment seems to increase the concentration of proteins extracted with the assistance of PEF. This effect was associated with a slower resealing of bacteria membrane pores at lower temperatures (Meglič, 2016). Bacterial growth phase is supposed to affect bacterial metabolism, as well as cell wall structure and porosity. However, while Coustets et al. (2015) obtained the highest amount of extracted proteins from cells in middle exponential phase and no effect of PEF in stationary phase, Meglič

Table 1.4. Recovery of intracelullar components from microorganisms by Pulsed Electric Fields (PEF)

Microorganism	*Extract*	*Improvement*	*Electric field strength (kV cm^{-1})*	*Total specific energy (kJ kg^{-1})*	*Reference*
		Bacteria			
E. coli/pNC1	β-glucosidase	Recovery: 26 % of total	10 kV/cm	280 kJ/kg	Ohshima et al., 2000
E. coli/pHI301A	α-amilase	Recovery: 89% of total	10 kV/cm	200 kJ/kg	Ohshima et al., 2000
E. coli BL21(DE3)	Enzyme GADPH	Yield 2 times greater than from untreated	7 kV/cm	735 kJ/kg	Coustets et al., 2015
E. coli K12 TOP10/pEGFP-N1	Total protein	15 µg/mL more protein than from untreated cells	20 kV/cm	533.76 kJ/Kg	Meglič et al., 2015
		Yeast			
S. cerevisiae	Trehalose	Efficiency greater than microwave and ultrasound	19.97 kV/cm		Jin et al., 2011
S. cerevisiae	β-galactosidase	Recovery: 45 % of total content	5.2 kV/cm		Ganeva et al., 2015
S. cerevisiae	Mannoproteins	After 18 days, 4.2- fold better release than from untreated	15 kV/cm	10.12 kJ/kg	Martínez et al., 2016
R. glutinis	Carotenoids	3 times greater extraction than from untreated	15 kV/cm	67.5 kJ/kg	Martínez et al., 2018b
		Microalgae			
C. vulgaris	Lutein	3.5-4.2 fold in comparison to untreated	25 kV/cm	61.38 kJ/kg	Luengo et al., 2015b
Chlorella	Carbohydrates and small ionic solutes	Recovery of 39 and 75 % respectively	17.1 kV/cm	99.9 kJ/kg	Postma et al., 2016b
A. platensis	C-phycocyanin	Total content extraction after 6 h	25 kV/cm	93 kJ/kg	Martínez et al., 2017
Nannochloropsis	Water-soluble proteins	Recovery: 10 % of total proteins	20 kV/cm	53,1 kJ/kg	Grimi et al., 2014
Nannochloropsis	Proteins, pigments	Efficient extraction of proteins in water at the first step and improved extraction in DMSO/ethanol of pigments (second step)	20 kV/cm	96 kJ/kg	Parniakov et al., 2015c
P. cruentum	β-pycoerythrin	Total content extraction after 24 h	8 kV/cm	15 kJ/kg	Martínez et al., 2019b
C. reinhardtii	Proteins	Recovery 70 %	15 kV/cm	12.5 kJ/kg	‘t Lam et al., 2017
H. pluvialis	Proteins	Release 8 times greater than from untreated	3 kV/cm	8.1 kJ/kg	Coustets et al., 2015
A. falcatus	Lipids	130 % extraction with respect to untreated	45 kV/cm	42 kJ/kg	Zbinden et al., 2013
Scenedesmus spp	Lipids	3.1-fold more crude lipid and fatty acid methyl ester	30 kV/cm	216 KJ/kg	Lai et al., 2014
A. protothecoides	Lipids	6-fold yield with respect to untreated samples	16 kV/cm	150 kJ/kg	Eing et al., 2013
A. protothecoides	Lipids	Recovery of 90-97 % from PEF-treated, in comparison to 10 % from untreated	10 kV/cm	150 kJ/kg	Silve et al., 2018

Data regarding total specific energy depends on the conductivity of the media in which treatments where applied, a parameter which is crucial.

et al. (2015), did not find differences between growth phases in terms of the amount of proteins extracted.

Genetic engineering offers the possibility of producing proteins such as enzymes in recombinant bacteria; pigments such as carotenoids or anthocyanins produced by recombinant bacteria can likewise represent a continuous, feasible, and reliable source for the medical field and the food industry. Research into PEF-assisted extraction of these molecules from bacterial cells has been scarce until now. However, for the extraction of proteins from *E. coli*, which is one of the most preferred and popular host systems for producing recombinant proteins, pre-treatment by PEF shows great promise as a specific cell lysis method. Similarly, the extraction of lipophilic molecules could be improved, and the ratio of harmful solvents decreased, by treating bacteria previously with PEF. However, in order to optimize the protocol, several parameters need to be considered and optimized for each bacteria species separately: electric field strength, treatment time, pulse waveform and width, temperature, electroporation media, extraction media after PEF, etc.

1.2.4.3. PEF-assisted extraction from yeast

The possibility of highly selective and efficient extraction of various intracellular components (ions, saccharides, enzymes, proteins, or nucleic acids) from yeasts using PEF has been widely demonstrated **(Table 1.4).** Observations using scanning electron microscopy show that PEF also affects cell wall structure (Ganeva et al., 2014), thereby perhaps explaining why varying thicknesses of the cell wall in yeast species affect the efficiency of PEF-assisted compound extraction. The PEF-assisted extraction of different proteins that act as enzymes (dehydrogenases, kinases, β-D-galactosidase, etc.) from various yeast species (*Saccharomyces cerevisiae*, *Kluveromyces lactis* and *Schizosaccharomyces pombe*) has been described, showing that their specific activities were higher than those obtained by mechanical disintegration or enzymatic lysis (Meglič, 2016). Moreover, carbohydrate trehalose (Jin et al., 2011) and intracellular proteins recovered from yeast via PEF treatment have been reported (Ganeva et al., 2003; Marx et al., 2011; Ohshima et al., 1995). Yeast membrane permeabilization and the associated extraction by PEF are greatly dependent on electric field strength and pulse duration.

Some authors noted that a selective release of intracellular proteins can be achieved by adjusting electric field strength: at lower electric field strengths they

extracted mostly invertase, which is located around the cell membrane, while at higher electric field strengths mainly alcohol dehydrogenase (located in the cytoplasm near the center of the cell) was extracted (Meglič, 2016). Optimized electric field strengths made it easy to exclusively release periplasmic space material (recombinant protein) through the pores while minimizing the release of contaminants or cell debris (Liu et al., 2013; Liu et al., 2016). Ganeva et al. (2015) achieved the selective and efficient recovery of large intracellular proteins from yeast by combining PEF with lytic enzymes. PEF treatment enabled the extraction of a portion of all proteins, after which the addition of lyticase improved the recovery of larger proteins.

Various authors have observed that yeast cells need to be incubated after PEF for several hours in order to allow protein efflux from cells; the time interval depends on the yeast species and the type of protein (Ganeva et al., 2014; Suga & Hatakeyama, 2009). In addition to electric parameters, characteristics of the extraction media are critical in the release of protein from yeast. The composition of the media is a parameter that bears an influence on extraction yield and velocity of release. The presence of potassium or sodium chloride in the incubation media accelerated the release of proteins from *Saccharomyces* cells (Suga & Hatakeyama, 2009). Incubation in hypertonic media increases the yield of extracted proteins compared with isotonic or hypotonic media, due to the exposure of cells to a post-PEF hyperosmotic stress that accelerates irreversible membrane damage. Martínez et al. (2018) reported that the amount of released intracellular compounds depended on incubation conditions (pH, temperature, and alcohol concentration), which probably affect the disorganization of the electroporated membrane or the evolution of pore size. On the other hand, independently of the effect of incubation temperature on the evolution of pore size, it is well known that temperature also affects mass transfer, and as a consequence, the rate of a compound's release from the cytoplasm.

Yeast autolysis induced by PEF

The permeabilization of the cytoplasmic membrane has been widely described as the cause of improved release of certain compounds thanks to PEF; additionally, it has been recently demonstrated that PEF treatments also trigger the autolysis of yeasts, thereby resulting in the self-degradation of the constituents of yeast cells by their own enzymes after cell death (Martínez et al., 2016). After PEF treatments that caused permeabilization, the release of intracellular proteins and nucleic acids from *S. cerevisiae*

was observed mainly during the initial hours. The amount of leaked molecules was correlated with the proportion of permeabilized and dead cells in the suspension. However, the release of mannoproteins, which form part of the cell wall, progressed gradually and did not reach its maximum until after 25 days of incubation. At this time, the amount of mannoproteins released from PEF-treated cells was ten times higher than that of those released from untreated cells (Martínez et al., 2016). Mannoprotein release is a consequence of the degradation of the cell wall by cytoplasmic enzymes such as glucanases and proteases. The disorganization of the membranous systems of yeasts during autolysis permits endogenous enzymes to come in contact with the cell wall; as a consequence, mannoproteins, along with other cell wall constituents, are released into the surrounding medium (Alexandre & Guilloux-Benatier, 2006). Whereas natural autolysis is a very slow process, the electroporation of yeast by PEF induced cell autolysis and a significant release of mannoproteins to the extracellular medium after only 24 h of incubation (Martínez et al., 2016). Several mechanisms related to electroporation could be involved in the induction of autolysis by PEF. On the one hand, electroporation leads to the formation of a water inlet from the surrounding media to the cytoplasm, an effect that is indicated by the decrease in the treated suspension's absorbance at 600 nm. The decrease of osmotic pressure within the cytoplasm as a consequence of the water inlet could lead to plasmolysis of the organelles and the release of the enzymes they contain. On the other hand, the electroporation of the cytoplasmic membrane by PEF could facilitate the contact of those released enzymes with the outermost layer of the yeast cell wall, where the mannoproteins are located **(Fig. 1.5.).**

It has been demonstrated that the rate of mannoprotein release from PEF-treated *S. cerevisiae* is affected by factors that influence on enzymatic activity (Martínez et al., 2018). The release of mannoproteins from PEF-treated yeast cells was influenced by pH, temperature, and alcohol concentration, all of which likewise affect natural autolysis.

The potential of PEF for triggering autolysis and accelerating the release of mannoproteins was also evaluated during the aging on lees of Chardonnay white wine. These glycoproteins are associated with positive effects such as haze formation reduction, the prevention of tartaric salt precipitation, and the diminution of astringency, along with the improvement of mouthfeel, aroma intensity, and color stability, thereby considerably improving wine quality. The amount of released mannoproteins increased drastically in wines containing PEF-treated yeast and reached its peak after 30 days, while untreated

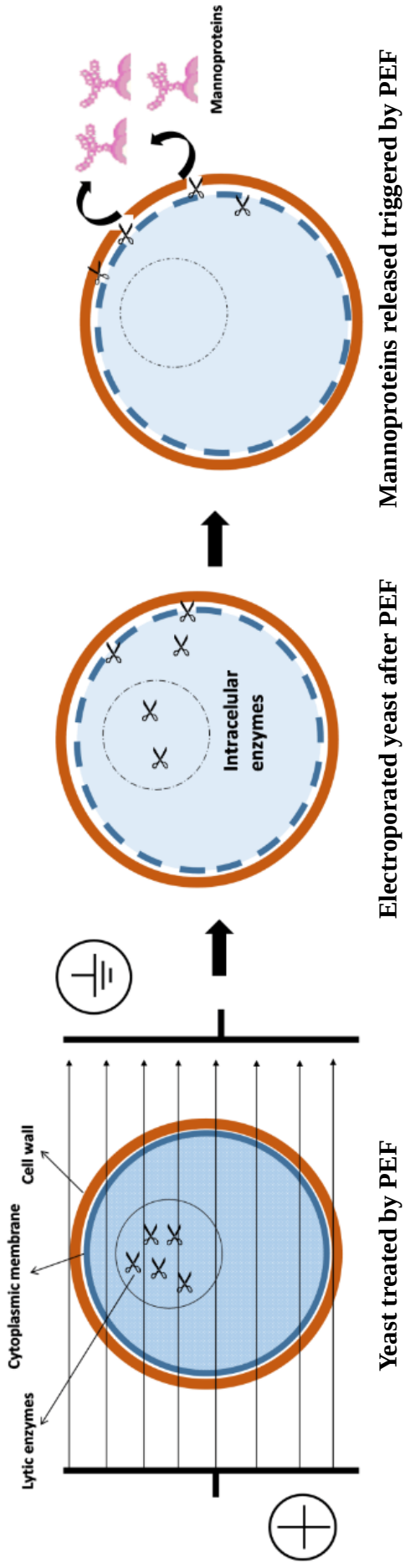


Figure 1.5. Mechanism involved in the triggering of mannoprotein release by Pulsed Electric Fields.

cells required six months. The mannoproteins released in a shorter time from PEF-treated cells featured functional properties similar to those of mannoproteins released during natural autolysis from untreated yeast (Martínez et al., 2019).

Similarly, the potential of PEF for inducing autolysis has been studied in the red yeast *Rhodotourla glutinis* with the purpose of designing a more efficient and ecofriendly process of carotenoid extraction from fresh biomass. While an extended incubation of *R. glutinis* biomass in ethanol after PEF treatment resulted in negligible extraction, only 24 h of previous incubation of the treated cells in aqueous medium were necessary to achieve subsequent carotenoid extraction in ethanol (95 %). This fact was associated with the trigger effect of PEF on enzymatic reactions. Flow cytometry measurements detected morphological changes in PEF-treated *R. glutinis* cells during aqueous incubation caused by the autolysis triggering effect of electroporation. It was hypothesized that this enhancement in the extraction of carotenoids was caused by the disruption of the association of carotenoids with other molecules present in the cytoplasm by enzymes released from the cytoplasmic organelles, as a consequence of the osmotic imbalance produced in the cytoplasm by electroporation (Martínez et al., 2018).

1.2.4.4. PEF-assisted extraction from microalgae

The benefits of PEF as a pre-treatment in the extraction of pigments (carotenoids chlorophylls, etc.), proteins, lipids, carbohydrates, and other compounds from microalgae have been widely demonstrated (Kempkes, 2016). In general, PEF pre-treatment improves the subsequent extraction of water-soluble as well as non-polar compounds utilizing an appropriate solvent (**Table 1.4.**). Electric field strength, treatment time and specific energy all influence on the effectivity of PEF treatment. After subjecting a microalgae suspension to a PEF treatment of sufficient intensity, a strong increase in the electrical conductivity of the suspension media and the uptake of propidium iodide can be observed, thereby suggesting that an effective permeabilization of the microalgae has taken place (Postma et al., 2016). The critical electric field required to electroporate microalgae cells is lower than that required to electroporate smaller-sized microorganisms such as bacteria. Beyond the critical electric field strength, permeabilization generally increases along with more intense electric field strength and longer treatment durations. However, the reduction of pulse duration from milliseconds to microseconds, combined with a slight increase of electric field strength, allows to maintain or even increase the

extraction yield while reducing the energy requirements (Luengo et al., 2015). Another critical parameter affecting the electroporation of microalgae is temperature during PEF processing. The increment of temperature within ranges that do not cause thermal degradation of the compounds (<40 °C) allows a reduction of the required electric field strength and treatment time to obtain a given extraction yield and, consequently, a decrease in the total specific energy delivered by the treatment (Luengo et al., 2015; Martínez et al., 2017).

The extraction of small intracellular products from *Auxenochlorella protothecoides* after electroporation by PEF was described by Goettel et al., (2013). Electroporation permitted the release of carbohydrates and amino acids from the cells, but high molecular compounds were not able to pass through the cytoplasmic membrane. Similarly, PEF-assisted extraction from *Nannochloropsis* carried out by Grimi et al. (2014) allowed for the recovery of ionic solutes, amino acids and small water soluble proteins. Focusing on protein extraction, Coustets et al., (2013) developed a method for the release of water-soluble protein from cytoplasm of *C. vulgaris* and *N. salina*, consisting in a 24-h incubation of the PEF-treated cells in a salty buffer. After that, they attained efficient protein release in phosphate buffer from both microalgae genera after a single passage through the pulsation chamber (3 or 6 kV/cm, 15 bipolar pulses of 2 ms). Similar results were obtained when the impact of PEF on the extraction of cytoplasmic proteins from *Haematococcus pluvialis* was evaluated (Coustets et al., 2015). Parniakov et al. (2015) investigated the effect of combining PEF with subsequent alkaline protein extraction at different pH levels (8.5, 11 or 12) achieving the highest yield of 10 % at the highest pH (12). Postma et al., (2016) studied the effect of temperature on the extraction of proteins and carbohydrates from *Chlorella vulgaris.* While PEF allowed for selective release of small water-soluble components, over 95 % of proteins were still retained inside the microalgal cell after PEF. In addition, the cell wall of microalgae has been mentioned by some authors as an obstacle against the effect of PEF and a barrier that impedes the extraction of large molecules. ‘t Lam et al. (2017) investigated this assumption, reporting the complete release of hydrophilic proteins from the cell-wall-free mutants, whereas PEF treatment of the species containing cell wall resulted in substantially lower protein yields.

Similarly, PEF has been successfully applied to recover lipids from microalgae at low energy intensities. The extraction of hydrophobic lipids requires the use of organic

solvents. In some studies the extraction yield was increased after PEF and, in others, a PEF pre-treatment allowed to reduce the proportion of organic solvents or to substitute them with a more eco-friendly solvent such as ethanol. The effect of PEF treatment on lipid recovery from the microalga *Auxenochlorella protothecoides* was studied by Eing et al., (2013). After extraction of water-soluble components from the PEF-treated microalgae suspension, they achieved improved lipid extraction from residual biomass using 70 % ethanol as solvent. Lai et al., (2014) applied PEF treatments to the microalga *Scenedesmus* prior to extraction and yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME) after recovery in different solvent mixtures. In another study, PEF was used as a pretreatment prior to extraction from cyanobacterium *Synechocystis PCC 6803* as feedstock of nonpetroleum-based diesel fuel. Treatment by PEF enhanced the potential of the low-toxicity solvent isopropanol to access lipid molecules during subsequent solvent extraction, leading to lower usage of isopropanol for the same extraction efficiency. Thus, PEF showed promise in lowering the costs and environmental effects of the lipid-extraction step (Sheng et al., 2011). Other authors studied the effect of PEF followed by the extraction of lipids from *Ankistrodesmus falcatus*, reporting a 130 % increase with respect to control (Zbinden et al., 2013). And, more recently, Gonçalves et al., (2016) applied PEF for the extraction of lipids in an economically viable microalgal production process associated with wastewater treatment. Silve et al. (2018) incubated PEF-treated cells of *Auxenochlorella protothecoides* in aqueous media under inert conditions, thereby enhancing the efficiency of the subsequent extraction of lipids in solvents while reducing specific treatment energy. Almost total extraction was achieved after a 20-hour incubation period at 25 °C, while incubation on ice was still beneficial but less efficient than at 25 °C. They suggested that the spontaneous release of ions and carbohydrates due to electroporation facilitated subsequent successful lipid extraction, although a direct causality between the two phenomena was not demonstrated.

In the area of pigment extraction, Luengo et al., (2014) and Luengo et al., (2015) studied and optimized the extraction of chlorophylls, carotenoids, and, specifically, lutein from *Chlorella vulgaris* regarding influence of electrical parameters and temperature. A PEF treatment of 20 kV/cm for 75 μs increased extraction yields for carotenoids, and chlorophylls a and b 1.2, 1.6, and 2.1 times, respectively (Luengo et al., 2014). A high correlation was observed between irreversible electroporation and the percentage of yield increase when the extraction was conducted 1 h after the application of PEF treatment,

but not when the extraction was conducted just after PEF treatment. Authors compared the effect of microsecond versus millisecond pulses in combination with electric field strength (Luengo et al., 2015). To achieve a maximum extraction yield, the energy of treatments required in the millisecond order was 150 kJ/L, while for treatments in the microsecond order, while slightly increasing the electric field strength, the required energy was nevertheless only 30 kJ/L. Regarding the influence of treatment medium temperature, a treatment of 25 kV/cm-100 μs at 25-30 °C increased the lutein extraction yield 3.5-4.2-fold in comparison with control, resulting in the most suitable treatment conditions for maximizing lutein extraction from *Chlorella vulgaris* at the lowest energy cost (Luengo et al., 2015).

Martínez et al. (2017) applied PEF to fresh biomass of *Artrosphira platensis* to enhance the selective extraction of the water-soluble protein phycocyanin in aqueous media. Electric field strength (15-25 kV/cm), treatment time (60-150 μs), and temperature of application (10-40°C) were found to influence extraction yields, but a delay of 150 min at the onset of extraction was observed for all conditions. This delay was attributed to the fact that low molecular weight compounds can cross the cytoplasmic membrane immediately after electroporation, whereas the release of molecules of larger molecular weight might require that the pores created by PEF treatment enlarge over the course of time. Similarly, Jaeschke et al. (2019) achieved high yields of proteins and phycocyanin from *A. platensis* after PEF treatments of 40 kV/cm using 1-μs pulses (112 kJ/kg). The yield increased with incubation time after PEF treatment.

More recently, Martínez et al., (2019) studied the extraction of another water-soluble phycobiliprotein, phycoerythrin (BPE), into aqueous media by the application of PEF to fresh *Porphyridium cruentum*. While the release of this water-soluble protein was undetectable in the untreated cells even after long incubation times, the entire content was released from PEF-treated cells after 24 hours of extraction. The protein was not released immediately, however; a lag time of over 6 hours was necessary until the compound could be detected in the extraction medium. This behavior indicates that BPE extraction requires not only the diffusion of the compound across the cell membrane, but also the dissociation of the compound from the cell structures. In this sense, it was hypothesized that PEF could trigger the release of hydrolytic enzymes from the *P. cruentum* organelles that would disassemble the associations between BPE and other structures of the cell; in this way, the water-BPE complex could diffuse across the

cell membrane, driven by a concentration gradient. An improved grasp of the kinetics and mechanism of the enzymes participating in microalgae autolysis, and of the autolysis trigger by PEF, will allow for this process to be developed on an industrial scale.

Overall, PEF treatments show to be a very promising technique useful for microalgal cell perforation on a large scale. The efficiency of PEF pre-treatments is high enough to achieve significant extraction yields. Due to the fact that PEF pre-treatment enhances selective release, the purities of extracts obtained after PEF are higher than those obtained using other techniques based on complete cell destruction such as milling, ultrasound, or grinding. The energy consumption of PEF is much lower than in those conventional techniques (de Boer et al., 2012). Furthermore, in the case of extraction of non-polar compounds, which are important microalgae-based products, PEF treatment opens the possibility to introduce green solvents that replace harmful ones.

1.2.5. Conclusions

This review has provided insight into the PEF-assisted extraction of compounds from bacteria, yeast, and microalgae. The main advantages of using PEF as a pre-treatment for enhancing extraction processes compared with other pre-treatments based on heating is due to the fact that PEF is a non-thermal process. The low energy requirements for electroporating cells do not cause a significant increase in the temperature of the matrix, thereby preventing the negative effects of heat on the quality and purity of the extracts. On the other hand, harsh cell disintegration techniques such as grinding, milling, or ultrasound cause complete cell disruption, thus resulting in a nonselective release of cell components. PEF nevertheless does have a specific effect on the cytoplasmic membrane and can thus enhance the selective extraction of intracellular compounds without affecting the cell's overall structure. The obtained extracts are therefore usually purer, reducing the requirements for additional purification steps (which would lead to an increase in costs).

Specifically in the case of microorganisms, the PEF-assisted extraction of relevant molecules presents an advantage over standard techniques: the possibility of conducting the extraction with fresh biomass instead of after dehydration, leading to the substitution of harmful chemicals with greener solvents and the possibility of extracting molecules selectively. Finally, PEF is a process that is low in energy consumption and can be applied in continuous flow, allowing for a feasible scale-up to processing capacities in the order

of tons per hour. Furthermore, the simplicity, speed, and viability of adaptation of PEF to industrial equipment harbor the possibility of combining it with other methods.

In general, PEF pre-treatment allows the selective extraction of various compounds achieving high yields. However, PEF parameters should be tailored to each species, considering their structure, size, and other factors affecting efficiency. Furthermore, the recent discovery of the triggering effect of enzymatic activity of cells after electroporation and incubation open up the possibility of new applications of PEF for the facilitation of extraction of compounds that are bounded or assembled in structures. As in all other cases, PEF parameters, along with suspension storage conditions, must be optimized to reach the desired effect. Further research will lead to a more exact understanding of the mechanism implied, and of how the process can be applied on an industrial scale.

2. JUSTIFICATION AND OBJECTIVES

At the present time, when the global human population is increasing exponentially, microbial cultures such as yeast and microalgae represent a highly promising resource for obtaining high-value products, including nutrients, pigments, and enzymes. They are able to metabolize inexpensive feedstock and wastes to produce biomass, have fast growth rate, high efficiency in substrate conversion and high productivity. Besides, their biological cycle is shorter in comparison to more complex organisms like plants, and microbial production is independent of seasonal factors and does not need lands. Likewise, the extensive variety of methodologies, substrates and species that can be used multiplies the possibilities of this source of high-value products. In addition, microorganisms are able to be genetically modified to produce new compounds own of higher organisms or to over-produce a specific compound. Furthermore, some techniques applied in food industry are based on the release of certain compounds from microorganisms, as is the case of "aging on lees" of wines. During this procedure, mannoproteins are released from the cell wall of *Saccharomyces cerevisiae* when the yeasts are deliberately in contact with the wine after the fermentation.

Although some of the high valuable compounds from yeast and microalgae are released to the growth medium during the normal metabolism, the majority remains inside the cells. In order to incorporate these compounds to the diet, the whole microbial cell could be added to the food but the ingestion of other compounds present in microbial cells could not be adequate for the human organism. Furthermore, to achieve intestinal absorption of the compounds, the microbial cell walls must be previously removed or enzymatically digested. Therefore, it seems preferable to extract and isolate the compound of interest from the microorganism, allowing its subsequent concentration to be added in the food and incrementing its bio-availability.

The structure of the cells and the location of the high valuable compounds are specific for each microorganism. Therefore, the design of effective strategies for their extraction and subsequent purification requires an exhaustive research of these features. In any case, compounds have to cross the cell envelopes to be recovered. The presence of an intact cytoplasmic membrane which acts as semipermeable barrier, membranes of organelles and other envelopes such as thick cellular walls difficult the release of valuable compounds. The recovery of these compounds, which are intracellularly locked should occur through a sustainable "green" biorefinery process in which a

crucial role is played by the cell disintegration technique used to improve the efficiency of the extraction step. The selected technique should allow a mild cell disruption of biomass to selectively improve the extraction efficiency, reducing processing times, temperature and amount of organic solvents but maintaining the integrity of the extracted molecules with no losses of functionally. Cell disruption pre-treatments such as chemicals, enzymes or physical methods are applied to break these structures and facilitate the subsequent extraction.

Chemical treatments are effective, but for food applications, the contamination of the suspensions by the active chemical, which often is non-food grade, results in the complication of downstream purification. Enzymes require long incubation times, are expensive and are limited to optimal conditions of use. Regarding the application of mechanical treatments, they usually produce the increment of operational temperature and biomass must be cooled down to avoid undesirable heating effects, increasing energy consumption. Furthermore, these techniques generally produce excessive denaturalization of cell envelopes, leading to the release of cell debris requiring subsequent purification techniques, but also impeding the extraction of labile compounds.

Pulsed electric field (PEF) treatment is an innovative non-thermal technology that consists in the intermittent application of direct-current high-voltage pulses (kV), for time intervals ranging from microseconds to milliseconds, through a material placed between two electrodes. This voltage generates an electric field, which, if it is intense enough produces the increment of the permeability of the cytoplasmic membrane to the passage of ions and macromolecules (Kotnik et al., 2012). Thereby, PEF treatment enhances the migration of compounds located in the microbial cytoplasm through the membrane, because the latter loses its selective permeability after the treatment.

This doctoral thesis provides insights of the potential of electroporation by PEF as a pre-treatment to improve the subsequent extraction of compounds from microbial cells such as yeast and microalgae.

Several studies have demonstrated the potential of PEF to electroporate the cell membranes and to improve extraction processes. However, the implementation of PEF technology at industrial scale for electroproation of microbial cells and the consequent enhancement of the extraction of interest compounds requires further understandings.

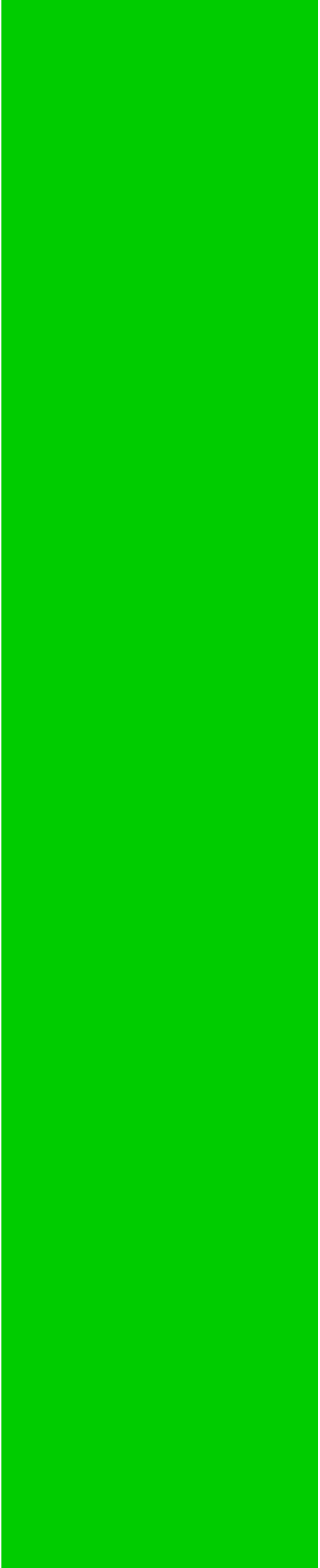
For each one of the biotechnological processes of production of compounds of interest by microorganisms, the main factors affecting electroporation of membranes, the most adequate treatment conditions and the effects of treatment on the extraction of the compound and its quality must be identified.

In general, food and biotechnology industries requires fast extraction processes, because this is reflected in decrease of total processing time and increment of productivity, which lead to a reduction of costs. Furthermore, the reduction of processing time usually implies an improved conservation of the properties of the compound of interest. Therefore, PEF application could be an interesting strategy for making profitable processes.

The general objective of this doctoral thesis was to evaluate the potential of PEF technology to improve the extraction of intracellular compounds of interest from yeast and microalgae. It was studied: the release of mannoproteins from *Sacharomyces cerevisiae* yeast, the extraction of carotenoids from *Rhodotorula glutinis* yeast and the extraction of pigments such as phycocyanin, phycoerythrin and carotenoids from *Artrosphira platensis*, *Porphyridium cruentum* and *Haematococcus pluvialis* microalgae respectively. To achieve this general objective, the accomplishment of the following partial objectives was necessary:

- Development of the methodology for the application of PEF treatments to microbial suspensions and further extraction of the compounds of interest
- Quantification of the effects of PEF treatments on the different microbial cells
- Optimization of extraction yields and analysis of the specific compounds obtained
- Identification of the advantages of the electroporation of microbial cells by PEF in terms of process yields, processing time, extract quality and purity and energetic consumption
- Understanding in the basic mechanisms beyond the electroporation phenomenon of the improvement of mass transfer assisted by PEF

3. MATERIAL AND METHODS



3.1. Release of mannoproteins during *Saccharomyces cerevisiae* autolysis induced by pulsed electric fields

3.1.1. *Saccharomyces cerevisiae*

3.1.1.1. Strain, medium and culture conditions

A strain of *S. cerevisiae* from an industrial preparation for winery applications was used (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland).

Potato-Dextrose agar (PDA) and Sabouraud-Dextrose (SD) broth were supplied by Oxoid (Basingtok, Hampshire, UK) and were prepared following supplier instructions. All the media were sterilized during 20 min at 121ºC in an autoclave (Darlab K-400, Terrassa, Barcelona).

In order to obtain the *S. cerevisiae* suspensions, 1 g of the commercial freeze-dried yeast was hydrated in 20 mL SD broth. After 24h of incubation at 25 ºC of the suspension, a small volume with an inoculating loop was spread on the surface of PDA Petri plates and plates were then incubated during 48h at 25ºC. From an isolated colony selected growth on PDA Petri plates, a tube containing 5mL SD broth was inoculated and incubated during 48h at 25ºC to obtain the pre-culture. After determining the cellular concentration of these pre-culture by microscopical counting (Y-FL, Nikkon, Tokio, Japan) using a Thoma chamber (ServiQuimia, Constantí, Spain), it was inoculated the required volume in 1000 mL flasks containing 600 mL of SD broth to obtain an initial concentration of 10^4 CFU/mL. Incubation of the cultures was carried out in an orbital shaker (Unimax 1010, Heidolph, Schwabach, Germany) at shaking velocity of 185 rpm during 48h at 25°C. To stablish the incubation time to obtain the microbial suspension, the corresponding growing curve was built.

3.1.1.2. Growth curve

In order to elaborate the *S. cerevisiae* growth curves at 25°C, samples were collected along time, aliquots of 1 mL were pour-plated in PDA plates, the optical densities (OD) at 600-nm were measured (directly related to the cellular density) and the dry weight was calculated. Figure 3.1 shows the growing curve along the incubation time which illustrates the evolution of cultures in number of cells and biomass amount.

Plate counting

Aliquots of 1 mL of the culture suspensions were collected at established time intervals. After carrying out the corresponding dilution in sterile Peptone water, 0.1 mL aliquots were pour-plated in PDA Petri plates in triplicate and were incubated at 25ºC during 48h. After this time, colonies were counted with an automatic colony counter (mod. Protos, Analytical Measuring System, Cambridge, UK) adapted by our research group for the counting of densely populated plates. The counting of plates containing below 100 colonies was performed with the naked eye.

Optical density

Optical density of the culture was measured at room temperature using a spectrophotometer (Libra S12, Biochrom, UK) at 600-nm wavelength in plastic cuvettes. Absorbance at 600-nm (OD600-nm) is correlated with the number of cells (CFU/mL).

Dry weight

To determine the dry weight per volume of culture, aliquots of 10 mL of the culture were collected at determined time intervals and centrifuged at 3000 rpm during 2 minutes at room temperature in a centrifuge Heraeus Megafuge 1.0r (Heraeus Instruments gmbH, Hanau, Germany). The supernatant was discarded and the pellet was dehydrated until constant weight in a vacuum dryer (GeneVac Ltd, UK). Dried weight was determined in triplicate for each one of the samples.

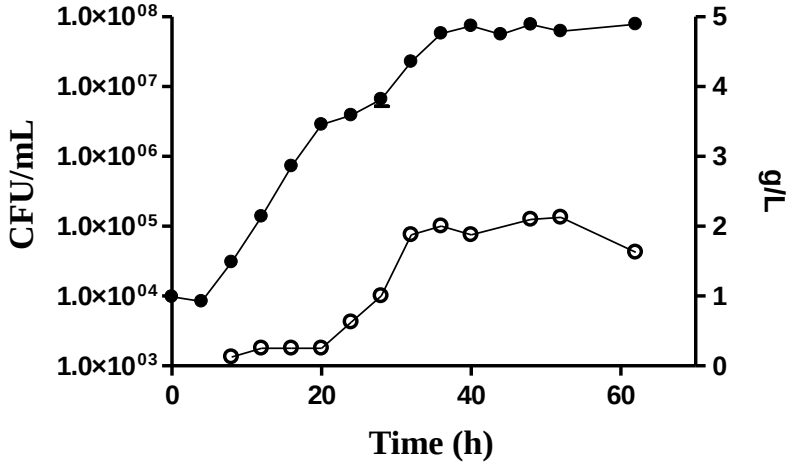


Figure 3.1. Growth curve of *S. cerevisiae* (Levuline Sélection C.I.V.C.) at 25°C expressed in CFU/mL (●) and evolution of dry weight in g/L (○) along incubation time.

3.1.1.3. Chardonnay winemaking, production of yeast in wine and "aging on lees"

Chardonnay grapes (200 kg) were received in our laboratory, destemmed and crushed, and then pressed to extract the must. After the clear must was inoculated with the strain of *S. cerevisiae* (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland) as recommended by manufacturer. Alcoholic fermentation of must was conducted at 18°C during 10 days. Density of wine and residual sugar were monitored during fermentation process. Once fermentation concluded (residual sugars below 1.8 g/L), yeast were left to sediment and it was collected on the one hand the wine and on the other hand the deposited lees (mainly a concentrated suspension of yeasts in wine). This concentrated suspension of yeast in *Chardonnay* wine (1.5×10^9 CFU/mL; conductivity: 1 mS/cm) was PEF treated and other untreated. After that, the yeasts (PEF or non-PEF treated) were mixed with the wine to carry out the aging on lees of wines using PEF and non-PEF treated yeasts.

3.1.2 PEF treatments

Depending of the investigation carried out, two different PEF configurations (static or dynamic) were used. To evaluate the PEF resistance and the autolysis of yeasts, PEF were applied in static mode. In the case of the studies of the PEF effect when "aging on lees" of *Chardonnay* wine, PEF treatments were applied in continuous conditions.

3.1.2.1. PEF treatments in static mode

PEF Generator

For the application of treatments, a PEF generator supplied by the Company ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden) was used. Its electric squeme is shown in Figure 3.2. It is composed mainly of a transformer (DCPS D10-400, ScandiNova) that converts the three-phase current (380 V, 16 A) into a direct current of 1 kV, which is transferred to 6 IGBT switches connected in series (Switch rack SR-6, ScandiNova). An external electric signal (TTL, 5 V) controls the opening and closing of the IGBT modules, provoking the discharge of the 1 kV current in a first pulsating signal of square wave. Finally, a pulses transformer (Pulse transformer, ScandiNova) converts this first pulsating signal into the desired high voltage signal. Thanks to this circuit, the

equipment is able to generate square wave pulses of 3 μs duration (Figure 3.3) up to 30 kV of voltage and 200 A of current intensity at a frequency up to 300 Hz. The equipment is designed to work with a treatment chamber with an optimal electric resistance between 100 and 170 Ω. In these conditions, it is achieved a totally square pulse, in which the voltage increases until the set value at a velocity of 47 kV/μs and, once finished the pulse, the velocity of descent is 56 kV/μs. Along the duration of the pulse, the voltage reached oscillates below 2%.

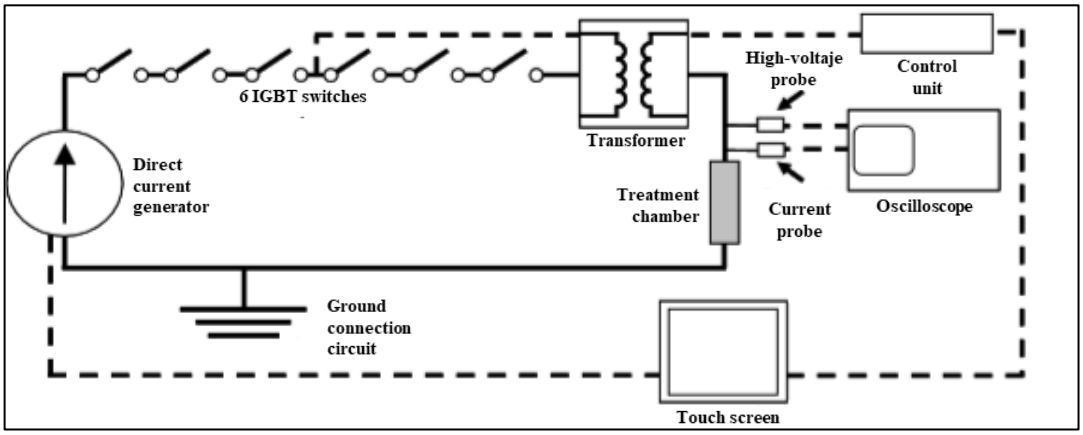


Figure 3.2. ScandiNova PEF equipment electrical configuration.

During the application of the treatments, some of the electrical energy generated is dissipated in form of heating (until 1 kW). In order to avoid system over-heating, the equipment possess a cooling system by means of low conductivity dielectric oil. This oil is cooled down by a heat exchanger in which the refrigerating fluid is water. The water must circulate at a minimum flow rate of 9 L/min, a pressure between 3 and 8 bar, and its temperature must be in the range of 10-40°C. For stable working conditions, the water temperature during PEF application must be equal to its initial temperature ±2.5 °C. The specific software designed by the supplier company (K1-15m, ScandiNova) controls the equipment through a touch screen of liquid glass (Simatic panel, Siemens, Munich, Germany). Due to the fact that the equipment applies high voltages and current intensities, a security system that allows manual disconnection of the electric circuit in the case of an eventual emergency was integrated. This security system consist of an easy-to-operate external push button (RS Amidata, Pozuelo de Alarcón, Spain) connected to the equipment by a plug type RS-232 (RS Amidata). As soon as the push button is actuated, circuit is closed preventing the current pass through it, allowing safe manipulation of its external components.

In order to determine the actual voltage and amperage applied and thereby to know the effective treatment conditions, the system is completed with a high-voltage probe (P6015A, Tektronix, Wilsonville, Oregon, EE.UU.) and a probe to measure the electric current intensity (Stangenes Industries, Palo Alto, California, EE.UU.) both connected to the equipment output, whose measurements are registered in a two channels digital oscilloscope (TDS 220, Tektronix).

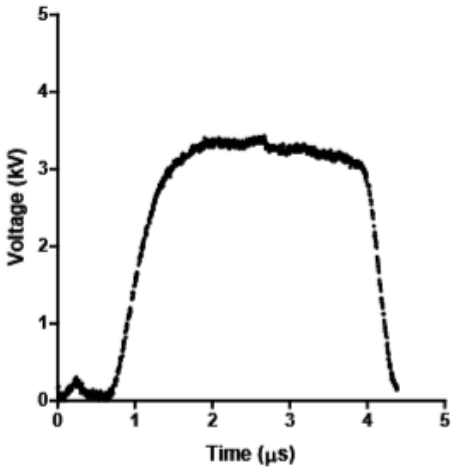


Figure 3.3. Example of the pulses generated by the PEF generator and registered by the oscilloscope.

Static treatment chamber

For the experiments performed using *S. cerevisiae* suspended in buffer to apply the PEF treatments, a tempered parallel electrode static chamber was used. This chamber consists of a polyethylene tube closed in the sides by two stainless steel cylinders of 16 mm diameter and 4 cm length, separated by a distance of 2.5 mm. The inside of electrodes is hollow, which permits circulation of dielectric oil (1.4 µS/cm) tempered at selected temperatures (Figure 3.4). The oil is pumped by means of a peristaltic pump (CC1, Huber, Madrid, Spain). Previously to the pass through the electrodes, the oil is tempered forcing it to circulate inside of a coil stainless-steel exchanger (Ø inner 2 mm, Ø external 3mm, 200 cm length) which is submerged in a thermostatic bath. This system permits to set the treatment medium to different temperatures and avoids any considerable increment of treatment temperature during the application of PEF, allowing to keep constant temperature during treatment application (Saldaña, 2010). In order to fill and empty the chamber, it is disposed a hole in the polyethylene tube of 1.0 mm diameter, which is closed during the treatment by Scotch tape.

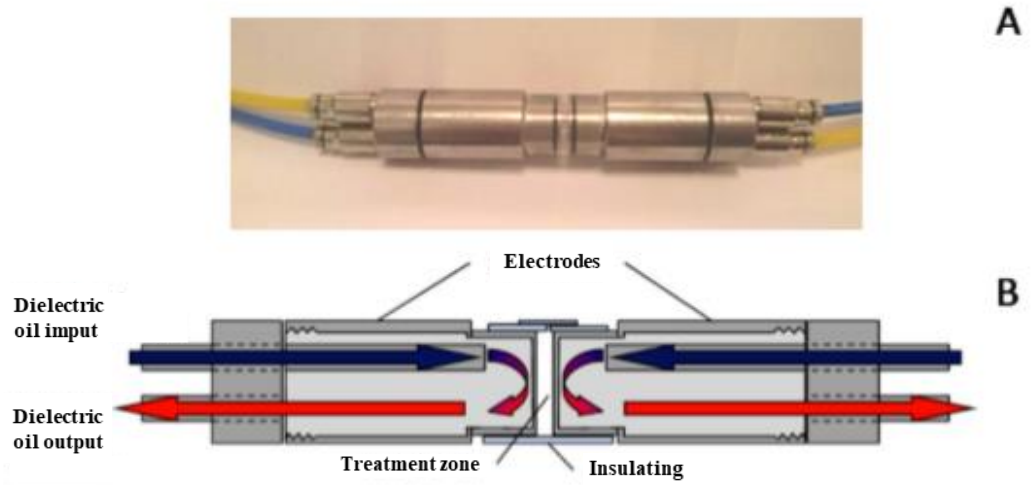


Figure 3.4. Photograph (A) and scheme (B) of the static treatment chamber with tempered parallel electrodes used for the treatments. The arrows indicate the direction of dielectric oil inside the electrodes. Distance between the electrodes: 2.5 mm. Electrode length: 40.0 mm. Electrode diameter: 16.00 mm.

PEF treatment conditions

In a first study in which was evaluated the resistance to PEF treatments of this strain of *S. cerevisiae*, suspensions were subjected to treatments between 5 and 80 monopolar square-wave pulses of 3 µs at electric field strengths of 5, 10, 15, 20 and 25 kV/cm. These treatments corresponded to specific energies ranging from 0.38 to 150 KJ/kg. The frequency of application of pulses was 0.5 Hz and the temperature 25 °C.

For a second study regarding factors affecting *S. cerevisiae* autolysis, a treatment of 15 monopolar square-wave pulses of 3 µs at 25 kV/cm of electric field strength was selected. This treatment corresponded to a specific energy input of 56.25 KJ/kg.

Experiments were performed using *S. cerevisiae* cells at stationary growth phase, which was achieved after 48 h of incubation. Before the treatment, fresh biomass was centrifuged at 3000 × g during 10 minutes at a temperature of 25°C ± 1°C (Heraeus Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany) and was re-suspended in McIlvaine citrate-phosphate buffer (pH 7.0; 1 mS/cm). The suspension containing *S. cerevisiae* (0.44 mL) re-suspended in a concentration of 10^9 CFU/mL was placed into the treatment chamber by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium). After each treatment, the sample was extracted with a new sterile syringe.

3.1.2.2 PEF treatment in continuous mode

The PEF-treatments of *S. cerevisiae* cells that had participated in fermentation of *Chardonnay* grapes were performed in continuous mode.

PEF Generator

The PEF generator used in the continuous mode treatments was ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden) described in section 3.1.2.1.

Continuous treatment chamber

An eight-roll peristaltic pump (Ismatec, Glattbrugg, Suiza) was used to pump the lees (mainly composed by yeast) suspended in *Chardonnay* wine from the reservoir through silicone tubes to the treatment chamber, which consisted of two parallel stainless steel electrodes with a gap of 5.5mm and an electrode area of 2.2cm^2 (Figure 3.5.). The flow rate was set at 3.5 L/h, and the calculated mean residence time in the treatment chamber was 1.24 s. Frequency was calculated by dividing the number of pulses by the residence time.

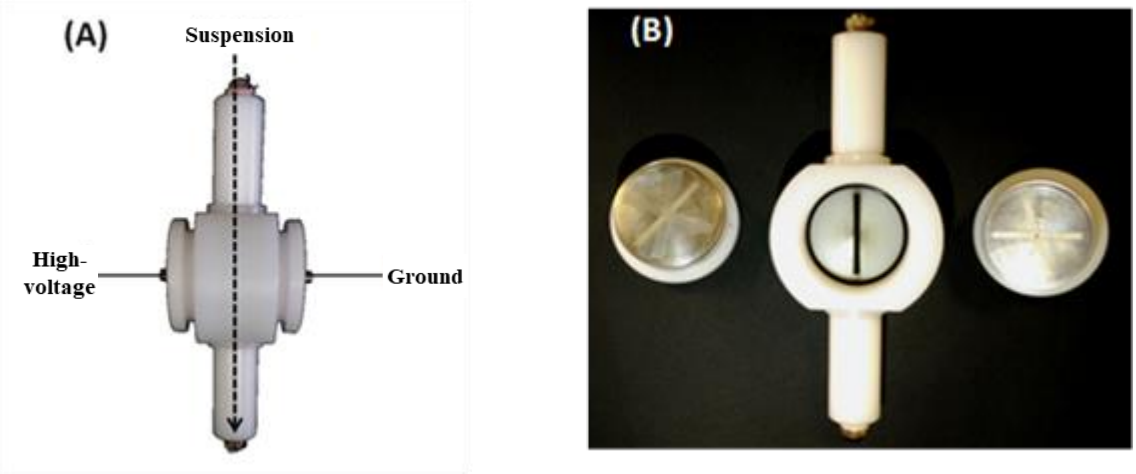


Figure 3.5. Parallel electrodes continuous mode PEF-treatment chamber. (A) Scheme of electrodes location and flow of suspension through the chamber. (B) Open chamber.

A heat exchanger consisting in a stainless steel coil submerged in a thermostatic batch was used to set the initial temperature of the suspension before the treatment.

Temperature of the lees suspension was measured with thermocouples located before and after the heat exchanger, and just after the PEF treatment chamber.

The specific energy input (W) per pulse expressed in kJ/kg pulse was calculated by the following equation:

$$W = m \times V \times I \times t$$

where m (kg) is the mass of the lees suspension contained in the volume of the treatment chamber; V is the applied voltage (kV); I is the current intensity (A); and t is the pulse width (μs), in all cases measured with the previously described probes. The total specific energy was calculated by multiplying the specific energy input per pulse by the number of pulses.

PEF treatment conditions

S. cerevisiae cells suspended in the *Chardonnay* white wine were PEF-treated at electric field strengths between 5 and 25 kV/cm along treatment times between 30 and 105 μs, which represent frequencies between 8 and 28 Hz and total specific energies ranging from 0.85 to 72.71 kJ/kg. The initial temperature before the chamber was set to 20°C, and the final temperatures after the treatments ranged between 20.2 and 37.98°C. PEF treatments were performed in triplicate. These treatments were carried out to evaluate the resistance of *S. cerevisiae* cells suspended in white wine.

However, to evaluate the effect of PEF treatments to accelerate autolysis during "aging-on-lees" two treatments were selected. One that did not cause inactivation (5 kV/cm, 75 μs) and another that inactivated 1 $\log_{10}$ cycle the *S. cerevisiae* population (10 kV/cm, 75 μs). These treatments correspond to a total specific energy of 2.14 kJ/kg and 9.17 kJ/kg, respectively. Aging on lees was performed in triplicate for each one of the conditions. A concentrated yeast suspension (1.5×10^9 CFU/mL) was PEF-treated in continuous mode at the two different intensities. After that, aliquots of the three distinct lots containing lees (10 kV/cm-75 μs; 5 kV/cm-75 μs and untreated) were dispensed in 20L tanks and filled with the same *Chardonnay* wine that had been fermented in our pilot plant. Yeast concentration in wine was 1.5×10^8 CFU/mL. The tanks were stored at 18°C±1ºC for 6 months. To avoid rotting, the sediment of lees at the bottom of the deposits was periodically re-suspended by smoothly shaking the wine.

3.1.3. Enumeration of viable cells after PEF-treatments

After the static PEF-treatments, samples were properly diluted in sterile peptone water and aliquots of 0.1 mL were pour-plated in PDA Petri plates. After that, plates were incubated during 48 h at 25 ºC. After incubation, plate counting was performed using an automatic colony counter (mod. Protos, Analytical Measuring System, Cambridge, UK) adapted by our research group for the counting of densely populated plates. The counting of plates containing below 100 colonies was performed with the naked eye. Inactivation data was expressed as the ratio between the initial number of survivors (N_0) and the number of survivors after different treatment times (N_t).

Likewise, it was evaluated the resistance of *S. cerevisiae* suspended in *Chardonnay* wine to the continuous mode PEF-treatments.

3.1.4. Evaluation of cell permeabilization by PEF treatments: Staining cells with propidium iodide

Quantification of the number of *S. cerevisiae* electroporated cells was performed by measuring the entry of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Barcelona, Spain). PI is a small (660 Da), hydrophilic molecule that is unable to cross through intact cytoplasmic membranes. However, when membranes are damaged, PI goes through them, bonds to the DNA and emits fluorescence. By this way, it can be estimated the number of permeabilized cells from the fluorescent emitted by the cell population (Figure 3.6.).

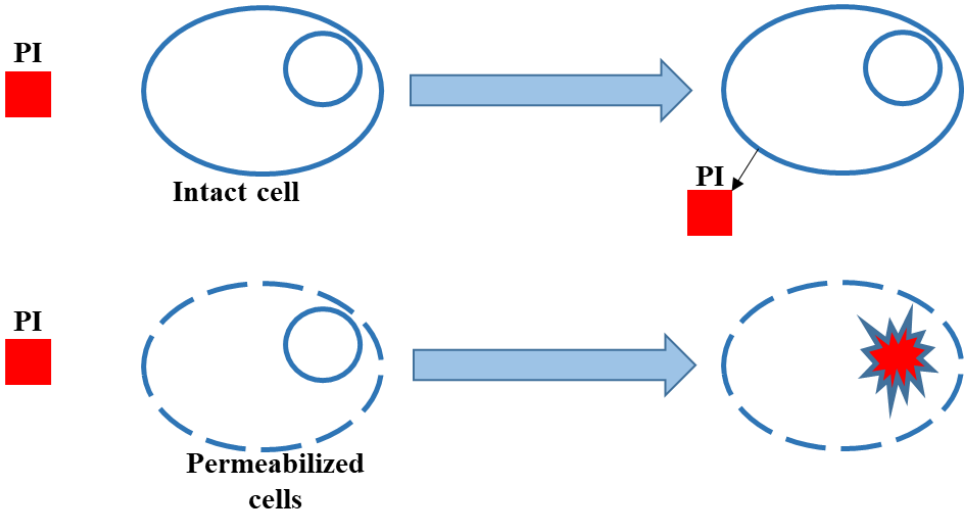


Figure 3.6. Schematic representation of the principles of PI staining methodology.

Two alternative dying protocols were follow under the same experimental conditions in order to detect the reversible and irreversible electroporation. The fluorescent dye PI was added previously to the PEF-treatment in one case (i) or after the PEF-treatment in the other (ii).

i) When PI is added to the suspension previously to the treatment, the fluorescent dye is in contact with the yeasts during the application of PEF. The cells emitting fluorescence (dyed cells) obtained after this procedure correspond to the sum of both irreversibly and reversibly permeabilized cells. For that, the cells were centrifuged (3000 × g) during 10 min at 25°C and re-suspended in McIlvaine citrate-phosphate buffer (pH 7.0; 1 mS/cm) to a concentration of 10^9 CFU/mL. Then, 50 µL of PI (0.1 mg/mL) were added to 450 µL of *S. cerevisiae* suspension, resulting in a final PI concentration of 0.015 mM and the suspension was subjected to the PEF-treatments. Previous experiments showed that the presence of PI during the PEF-treatment did not modify the characteristics of the treatment medium neither the resistance of studied microorganisms to the PEF treatment. After the PEF treatments, suspensions were incubated for 10 min in darkness. Previous experiments showed that longer incubation times did not influence the number of cells permeabilized to the fluorescence dye. After incubation, the cell suspension was centrifuged and washed twice until no extracellular PI remained in the buffer.

ii) When the PI is added to the suspension after the treatment, PI is only able to cross the membrane through the pores that remain open. Therefore, the permeabilization degree when cells are dyed after the PEF treatment corresponds to the irreversible permeabilized cells. For that, PI was added 10 min after the PEF treatment to a final concentration of 0,015 mM and was incubated similarly to the former case (i) during 10 min in darkness. Afterwards, suspensions were washed twice.

PI trapped inside the cells was quantified by spectrofluorophotometry (mod. Genios, Tecan, Austria) using 535-nm excitation filter (523–547 nm) and a 625-nm emission filter (608–642 nm). Results were expressed as the percentage of permeabilized cells based on the fluorescence value obtained for cells permeabilized by the most intense PEF treatment used in this investigation (240 µs at 25 kV/cm) that inactivated at least

99% of microbial population. Under these conditions, the permeabilization of individual cells was also checked using an epi-fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan).

The presence of cells with irreversible pores was determined from the fluorescence measurements by applying post-treatment dying (ii). Reversible permeabilization was quantified by calculating the difference between the fluorescent measurements obtained by pre-treatment dying (i) and the fluorescence measurements obtained by post-treatment dying (ii).

3.1.5. Conditions of storage of cellular suspensions

In the first study, suspensions containing untreated and PEF-treated cells in pH 7 buffer were stored at 25°C in a refrigerated incubator (Trade Raypa, Barcelona, Spain). Samples were periodically collected along storage time that lasted 25 days.

In a second study designed to evaluate factors affecting PEF-induced autolysis, control and PEF-treated cells were re-suspended in citrate-phosphate McIlvaine buffer of different pH (3.5, 5.0 and 7.0), and stored at 25ºC. Cells re-suspended in McIlvaine buffer of pH 7.0 were also stored at different temperatures (7°, 25° and 43°C) or with different ethanol concentrations (6, 12 and 25%, vol/vol; 25°C). Control and PEF-treated cells were also incubated in a medium that simulated the composition of white wine (10 % ethanol vol/vol; Tartaric Acid 4 g/L; Malic Acid 3 g/L; Acetic 0.1 g/L; pH 3.5) at 25ºC, with the purpose of evaluating the interaction of the presence of ethanol and low pH in PEF induced autolysis. Three samples were prepared for each storage condition.

In the third study, the yeasts that fermented *Chardonnay* must were used. The yeasts were PEF-treated suspended in the *Chardonnay* wine. The tanks containing the yeast suspended in *Chardonnay* wine were stored at 18°C (to mimic the conditions of real winemaking) during 6 months and shacked periodically to evaluate the PEF-accelerated aging on lees.

3.1.6. Determination of yeast viability

Similarly to the evaluation of the PEF resistance, the viability of cells along the storage period was determined by collecting samples periodically and pour plating serial dilutions in PDA. After 48 h of incubation at 25°C, colony-formed units were counted.

3.1.7. Monitoring of cell lysis caused by PEF

In order to monitor the release of components during storage of cellular suspensions, different measurements were performed in untreated and PEF-treated samples.

3.1.7.1. Measurement of turbidity of suspensions

Turbidity of the suspension during the storage was measured by the absorbance at 600-nm (Abs600) to monitor leakage of cellular content and cellular density.

3.1.7.2. Measurement of release of intracellular components

Absorbance at 260-nm (Abs260) and 280-nm (Abs280) of the supernatant was measured in order to monitor the presence of intracellular material outside the cell (Aronsson et al. 2005). These wavelengths correspond with the maximum absorbance of the nucleic acids and proteins, respectively.

3.1.7.3. Measurement of the mannose concentration in the medium

When indicated in the text, the concentration of mannoproteins released to the extracellular medium was determined after hydrolyzing the supernatant with sulfuric acid (final concentration 1.5 M) at 100°C for 90 min. Cooled samples were neutralized with NaOH 3 M. Quantitative analysis of mannose was conducted by an enzymatic method (D-Mannose, D-Fructose and D-Glucose assay procedure, Megazyme International, Wicklow, Ireland) (Dupin et al., 2000).

3.1.8. Analysis of *Chardonnay* wines subjected to "aging on lees" periods of varying length

After 1, 2, 3, or 6 months of "aging on lees", samples were collected from the deposits containing untreated or PEF-treated yeasts. After centrifugation, lees were eliminated, and the supernatants were bottled. Different analyses were performed on the wines obtained.

Volatile acidity, pH, and ethanol concentration analysis were performed according to the specifications established by the "Organisation Internationale de la Vigne et du

Vin" (2005). Total polyphenol index and total tannin content were determined using a Libra S12 spectrophotometer (Biochrom, UK). The total polyphenol index (TPI) was measured by directly reading the absorbance of diluted wine 1/20 (v/v) at 280 nm (Ribéreau-Gayon et al., 2006). Quantification of condensed tannins was measured by precipitation with methyl cellulose according to Sarneckis et al. (2006), and results were expressed as epicatechin equivalents.

The chromatic characteristics of the wines were determined by directly measuring their absorbance at 420, 520, and 620 nm using a spectrophotometer (Libra S12) with a 10-mm path-length quartz cuvette. Color intensity (CI) was calculated as the sum of absorbance at 420, 520, and 620 nm. Tint was determined as the proportion of absorbance measured at 420 and 520 nm (Glories, 1984; Sudrau, 1958).

CIELAB parameters (L*,a*,b*,C*, H*) were determined using the original MSCV software (Pérez-Caballero et al., 2003) according to regulations established by the "Commission Internationale de l'Eclairage" (Commission Internationale de l'Eclairage (CIE) 1986), which include L* (lightness), a* (red-green coordinate), b* (yellow-blue coordinate), C* (chroma), and H* (hue).

Turbidity measurements of the wines after centrifugation (3000×g; 5min) and removal of lees were performed using a turbidimeter (HI 83749, Hanna Instruments, Woonsocket, USA). The forming foam capacity of the different wines was also measured. Aliquots of 40 mL were dispensed in 50-mL volumetric flasks until the pear-shaped part was filled. After filling, the flask was flipped 5 fold, immediately after which the foam height in the neck of the volumetric flask was measured.

3.1.9. Statistical data analysis

The results represent the mean ± standard error of three replicates of treatments analyzed in triplicate. A one-way ANOVA test was conducted to assess significant differences between treatments. The differences were considered significant at $p < 0.05$.

3.2. Pulsed electric field-assisted extraction of carotenoids from *Rhodotorula glutinis* yeast

3.2.1. *Rhodotorula glutinis*

3.2.1.1. Strain, medium and culture conditions

The commercial strain of *Rhodotorula glutinis var. glutinis (ATCC 2527)* was provided by the *Colección Española de Cultivos Tipo (CECT).*

In order to obtain the suspensions of *R. glutinis,* the freeze-dried yeasts were re-hydrated in tubes containing 5 mL of Potato-Dextrose broth (PDB, Oxoid, Basingstoke, UK) and incubated during 48 h at 25ºC. After that, inocula were spread on Potato-Dextrose agar (PDA, Oxoid, Basingstoke, UK) Petri plates and incubated at 25ºC during 48 h. Using one isolated colony from the PDA plates some tubes containing PDB were inoculated and after that, tubes were incubated at 25ºC during 48 h to obtain the pre-culture. After determine the cellular concentration of these pre-cultures by counting under microscope (Y-FL, Nikkon, Tokio, Japan) in Thoma counting chamber (ServiQuimia, Constantí, Spain), it was inoculated the required volume in 500 mL flasks containing 250 mL of PDB to obtain an initial concentration of 10^4 CFU/mL. Incubation of cultures was performed in an orbital shaker Unimax 1010 (Heidolph, Schwabach, Germany) at a speed of 185 rpm and temperature of 25 °C during 72 h. To stablish the incubation time to obtain the microbial suspension, the corresponding growth curve was elaborated.

3.2.1.2. Growth curve

In order to elaborate the growth curve of the yeast *Rhodotorula glutinis* at 25°C samples were collected each two hours along four days.

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 the plate counting method in PDA. Dry weight (dw) of yeasts was determined by vacuum drying (GeneVac, Ltd, UK) at 60 °C until constant weight. Stationary growth phase was achieved after 48 h of incubation (Figure 3.7). However, experiments were performed with cells after 72 h of culture, which corresponded with the highest absorbance at 474 nm and thus maximum

carotenoid accumulation (Figure 3.8). Biomass concentration at this time was 10 g_{dw}/mL and 10^8 CFU/mL.

Plate counting

Samples of 1 mL of culture suspension were collected each two hours. After performing the corresponding dilutions in sterile peptone water, aliquots of 0.1 mL were pour-plated in triplicate in PDA Petri plates. Plates were incubated at 25ºC during 48 h.

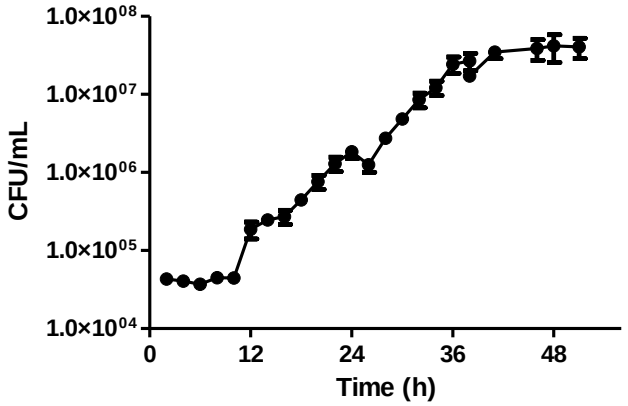


Figure 3.7. Growth curve of *R. glutinis var. glutinis (CBS 20)* in PDB at 25°C

Optical density

Culture optical density was measured at room temperature using an spectrophotometer (Libra S12, Biochrom, UK) at wavelengths of 600-nm and 474-nm periodically to evaluate turbidity (cells in suspension) and carotenoids concentration, respectively (Figure 3.8). While after 48 hours, maxima Abs600 values were achieved, similarly to the growth curve (Figure 3.7), Abs474 required at least 72 h (Figure 3.8).

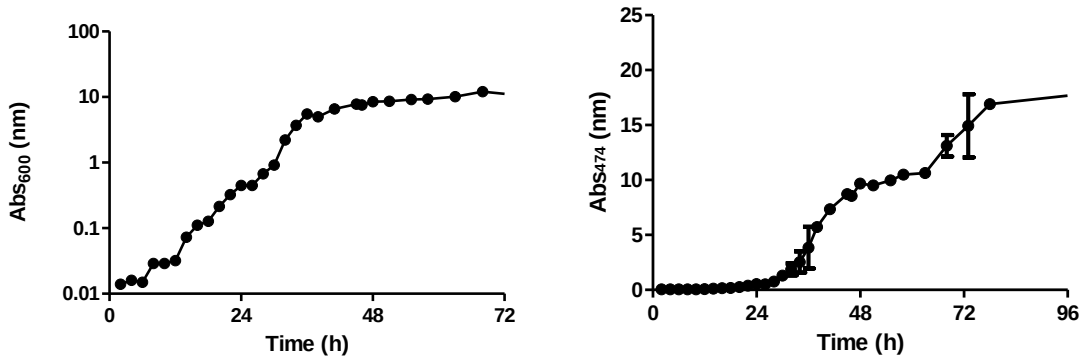


Figure 3.8. Evolution of Absorbance at 600 and 474 nm during the growing of cells of *R. glutinis var. glutinis (CBS 20)* in PDB at 25°C.

Dry weight

To determine the dry weight of a known volume of culture, 10 mL of culture at different times were collected and centrifuged (4000 x g) during 5 min at room temperature in a centrifuge Heraeus Megafuge 1.0r (Heraeus Instruments gmbH, Hanau, Germany). Supernatant was discarded and the pellet was dehydrated until constant weight in a vacuum dryer (GeneVac Ltd, UK). Dry weight was determined in triplicate.

3.2.2. PEF treatments

To evaluate the effect of PEF treatments on the extraction of carotenoids from fresh biomass of *R. glutinis* a square waveform pulse PEF generator was used. However, the combined effect of PEF and the subsequent drying of biomass on the CO_2 supercritical fluid extraction and solvent extraction was evaluated during a research stay at Institute of Food Technology of BOKU (Wien, Austria). In that occasion, an exponential decay pulse generator was utilized.

3.2.2.1. Square waveform PEF treatment

PEF Generator

Treatments were applied using the PEF equipment 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. It is a compact equipment, of 800×600×400 mm dimensions and 80 kg weight. The equipment is controlled by means of a touch screen in which it is possible to modify the applied voltage (until 10 kV), the pulse width (from 5 to 200 μs) and the frequency of the pulses (from 1 to 200 Hz) (Figure 3.9). The pulse generator is based on the discharge of capacitors from a Marx type generator, whose simplified circuit is shown in Figure 3.9.

Treatment chamber

The chamber used to PEF-treat *R. glutinis* was the parallel electrode continuous chamber described in section 3.1.2.2. Dimensions of the chamber were 4 cm length and

0.55 cm width and the gap between electrodes was 0.50 cm resulting in a total treatment volume of 1.2 mL. The flow rate was set at 4 L/h and the residence time was 1.09 seconds.

PEF-treatment conditions

Prior to each treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 x g for 5 minutes 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 CFU/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) achieving an initial temperature of the suspension of 15 ºC. The suspension of fresh biomass of *R. glutinis* was PEF-treated at 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. Outlet temperature was monitored during all treatments: even after the most intense treatments, it never surpassed 40 °C.

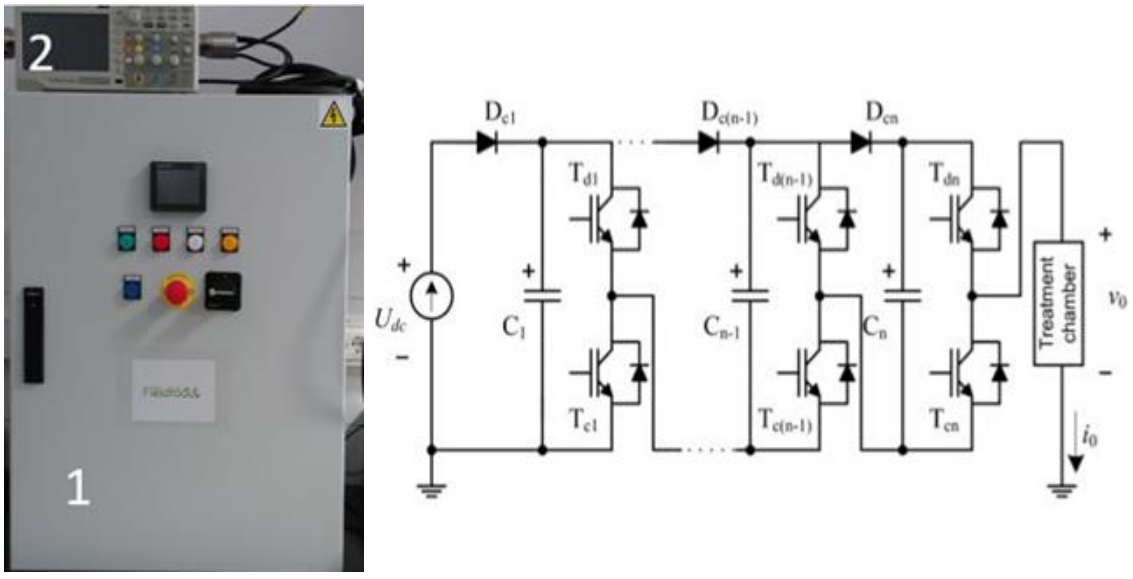


Figure 3.9. Left side: Pulsed Electric Field (PEF) equipment of square waveform pulses. (1) PEF generator and (2) Oscilloscope. Right side: Simplified circuit of the PEF generator based on Marx technology.

3.2.2.2. Exponential decay PEF treatment

PEF Generator

The PEF equipment used in this study was manufactured by the German Institute for Food Technologies (DIL, Quakenbrück, Germany). The apparatus generates exponential waveform pulses at frequencies of 1 to 4 Hz. Discharge of the capacitor is performed by a spark gap, and provides voltages in the range of 1.5 – 30 kV.

Treatment chamber

The treatment chamber used in these experiments was a parallel plate batch chamber consisting of two parallel stainless steel electrodes of 77 mm x 61 mm and 1 mm of thickness, separated by a polyoxymethylene (POM) insulator. The volume of the chamber was 40 mL. Distance between the electrodes was 1 cm.

PEF-treatment conditions

Actual voltage applied in the treatment chamber was measured using a high voltage probe (Tektronix P6015A, Beaverton, USA) connected to an oscilloscope (Tektronix TBS1102B-EDU, Beaverton, USA). Voltage data registered by the oscilloscope were plotted and voltage decay was fitted using an exponential equation, to calculate the pulse width at 37 % of the maximum voltage. The following equation was used to calculate the energy delivered per pulse by the discharge of the capacitor.

$$W = 0.5\, U^2\, C$$

With C being the capacity of the capacitor (0.5 µF), and U being the maximum voltage measured across the electrodes of the treatment chamber. The total specific energy input [kJ/kg] was calculated by multiplying the pulse energy (W) by the number of pulses divided by the mass of sample in the treatment chamber.

Prior to the treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 x g for 5 min at room temperature, and re-suspended in citrate-phosphate McIlvaine buffer (pH 7.0; 5.0 mS/cm) to a final concentration of approximately 10^8 CFU/mL.

A previous screening of PEF treatments at electric field strengths of 10–20 kV/cm and 100–400 pulses was performed in order to evaluate the parameters to achieve

different levels of inactivation. Afterwards, suspensions were subjected to the selected treatment consisting of the application of 400 exponential decay pulses of a pulse width of 6.8 microseconds at an electric field strength of 15 kV/cm. This treatment caused the permeabilization of at least 99% of population.

3.2.3. Evaluation of cell permeabilization after PEF treatments: Staining cells with propidium iodide

Quantification of the number of electroporated *R. glutinis* cells after square waveform PEF-treatments was performed by measuring the uptake of the fluorescent dye propidium iodide (PI) according to the principles described in section 3.1.4.

Aliquots of 50 µL of PI (0.1 mg/mL) were added to 450 µL of *R. glutinis* suspension (10^8 CFU/mL), resulting in a final PI 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 similarly to the ones explained for *S. cerevisiae* (section 3.1.4.). 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 by the two different staining protocols.

Similarly, after the exponential decay PEF-treatments, PEF treated and untreated cells were stained with PI using the same protocol. Samples were analyzed by flow cytometry (BD Accuri C6, New Jersey, USA) in order to check the effectivity of PEF-treatments by means of the measurement of percentage of permeabilized cells.

3.2.4. Evaluation of *R. glutinis* inactivation caused by PEF

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 plates.

3.2.5. Monitoring release of intracellular compounds after PEF treatment

Leakage of intracellular components was monitored by measuring absorbance at 260-nm (Abs260) and 280-nm (Abs280) of the supernatant. These wavelengths correspond with the absorbance maxima of nucleic acids and proteins, respectively (Aronsson et al. 2005).

3.2.6. 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, 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 (Figure 3.10). A total of 5,000 events were measured in each replicate at a flow rate of approximately 83 events.

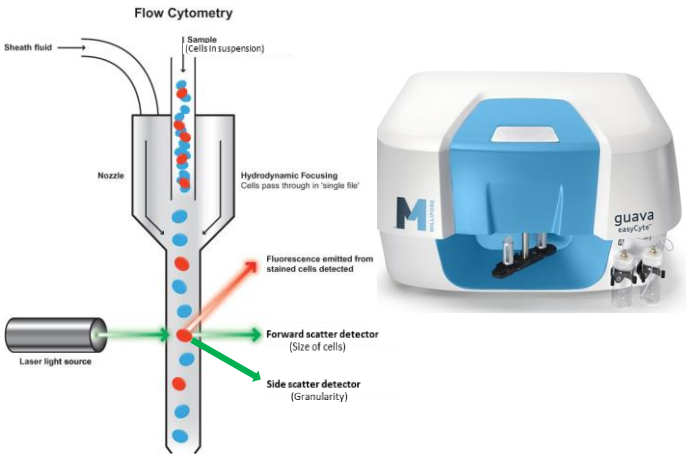


Figure 3.10. Scheme of flow cytometry technique and picture of the cytomerter (Millipore/Guava Easycyte, Germany).

3.2.7. Carotenoid extraction

3.2.7.1. Carotenoid extraction from *fresh* biomass

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 ºC, were centrifuged at 3000 x g for 5 minutes at room temperature and re-suspended in 1 mL of 96% ethanol. Then after different incubation times in presence of ethanol, suspensions were centrifuged at 14,000 x g during 2 minutes (MiniSpin Plus, Eppendorf Ibérica, Madrid, Spain) and the carotenoid concentration of supernatant of untreated and PEF treated cells was measured.

In order to investigate the influence of the pH of the incubation media and of incubation temperature of the storage conditions on the extraction of carotenoids, untreated and PEF-treated cells of *R. glutinis* were incubated in McIlvaine buffer of different pH (5.0, 6.5, 8.0) at distinct temperatures (15º, 25º, and 37º C) for 24 hours. Similarly to described above, suspensions were centrifuged after different incubation times and carotenoids were quantified in the supernatants.

3.2.7.2. Carotenoid extraction from *dry* biomass

Intermediate incubation and freeze-drying

After the exponential decay PEF-treatment, untreated and PEF-treated suspensions were freeze-dried immediately or incubated in the treatment medium (citrate-phosphate McIlvaine buffer, pH 7.0) for 24 hours at 25 °C in darkness and then freeze-dried. The freezing was performed at -40 °C, and samples were subsequently lyophilized (FreeZone6, Labconco, Kansas City, USA) until total dehydration, which was achieved after 48 hours (Figure 3.11). Freeze-dried samples were stored in nitrogen atmosphere at 4ºC until use.

Supercritical CO_2 extraction

Supercritical Fluid Extraction (SFE) is the process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent. Carbon dioxide (CO_2) is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31 °C and critical pressure of 74 bar (Figure 3.12.). Addition of modifiers may slightly alter this.

Figure 3.11. Aspect of *R. glutinis* biomass just after freeze-drying

The supercritical fluid extraction (SFE) was carried out in the Institute of Chemical Process Fundamentals of the CAS (Prague, Czech Republic). It was used a 24 mL extraction column (I.D. 14 mm) filled with freeze-dried biomass of *R. glutinis* (4 g of control or 1.5 g of PEF treated sample) placed between layers of glass beads serving as solvent flow distributors. The extraction column was heated in an air-conditioned oven to the desired temperature and then pressurized by a high pressure pump for liquid CO_2 (Applied Separations, USA). A pressure of 50 MPa and a temperature of 80 °C was set as optimal conditions for SFE of carotenoids from yeast as described by Lim et al. (2002). After 15 minutes of static extraction, a heated micrometer valve downstream of the extractor was opened and the extraction in the dynamic mode was started. The CO_2 flow rate was adjusted using the micrometer valve to a low value of 0.8 g/min, ensuring the solvent saturation by a solute. The extract was collected at ambient temperature in pre-

weighed glass vials serving as a separator. By gradual weighing of the extract during the experiment, the amount of SC-CO_2 required to obtain the total extract from the control and PEF treated *R. glutinis* was determined as 10.3 and 61.3 g/g_{dw}, respectively. After the SFE with pure SC-CO_2 terminated and the first fraction of extract was obtained, the ethanol was added into the SC-CO_2 as co-solvent. Ethanol was supplied at a constant flow rate by a high-pressure pump LCP 4020.3 (ECOM s.r.o.) and mixed with SC-CO_2 before entering the column to reach a concentration of 20 % (w/w) in the SC-CO_2. The second fraction was obtained at a SC-CO_2-to-feed ratio of 12 g/g_{dw}. Ethanol was evaporated at ambient pressure under a nitrogen stream.

Vials with dry extracts from both extraction steps were weighed, tightly closed, and stored in a refrigerator until the analysis.

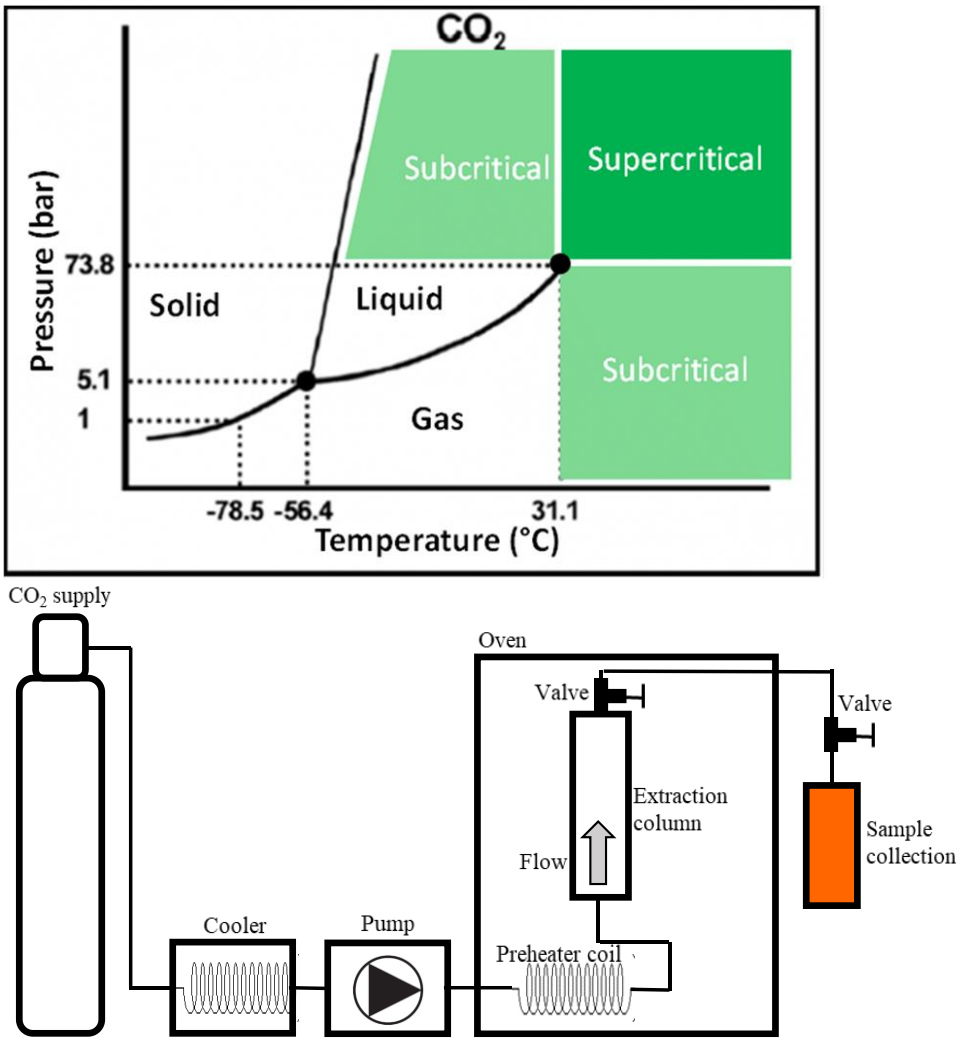


Figure 3.12. Above: CO_2 phase diagram showing the supercritical region. Below: Schematic representation of the Supercritical CO_2 fluid extraction system.

Solvent extraction

Control and PEF treated samples of 0.05 g of freeze-dried biomass were placed in tubes and 10 mL of the different solvents were added (ethanol, hexane or acetone). The tubes were placed in a shaking device (PTR-60, Grant Bio Multirotator, Grant Instruments, Cambridge, UK) at 75 rpm, for 12 hours. Samples were collected periodically and the carotenoid content of the supernatants after centrifugation (3000 x g, 5 min) was measured.

3.2.7.3 Total extraction

Total carotenoids were extracted by suspending 0.05 grams of freeze-dried biomass in 5 mL dimethyl sulfoxide (DMSO), vortexing for 1 min and incubating while shaking at room temperature for 1 h. This was followed by the addition of 5 mL hexane, after that the mixture was vortexed for 1 min and further shaken for 15 minutes. Then, tubes were centrifuged (4000 x g, 10 min) resulting in a yellow colored hexane upper phase. This hexane phase was collected and the procedure was repeated until the collected hexane phase appeared transparent. This way, the non-polar carotenoids could be collected. The remaining DMSO phase was still pink colored, i.e. it still contained more polar carotenoids. To extract these, 1 mL diethylether and 1 mL of saturated NaCl solution were added to the DMSO. The tubes were vortexed for one minute and shacked for 15 minutes. After that the tube was centrifuged, as previously described. The pink pigmented upper phase was collected and the procedure was repeated until the collected diethylether phase appeared transparent. The diethylether extracts were evaporated under continuous nitrogen flux. This extract was dissolved in hexane and pooled with the hexane previously collected for spectrophotometric quantification of total carotenoids.

3.2.8 Carotenoid quantification

3.2.8.1. Photometric carotenoid analysis

The carotenoid content of the extracts was determined using an ultraviolet light (UV) spectrophotometer (UV-1800, Shimazdu, Kioto, Japan) and the UV absorption spectra were recorded. The carotenoid extraction yield was calculated from the measured absorption, using the molar extinction coefficient of β-carotene in ethanol (ε=2620),

acetone (ε=2500) or hexane (ε=2592) and was expressed as mg of carotenoids/mL of suspension, using the following equation:

$$c_{carotenoids} = \frac{Absorption_{454\,nm} \cdot 10}{\varepsilon}$$

3.2.8.2. Carotenoid analysis by HPLC

High performance liquid chromatography (HPLC) was accomplished using an Alliance Waters 2695 Separations Module (Waters, Milford, MA, USA) with integrated autosampler and a photodiode array detector. A 4.6 x 250 mm C-30 carotenoid column of 5 µm of particle size (YMC, Wilmington, USA) was used.

The solvents were HPLC grade methanol (VWR, Paris, France) and methyl-tert-butyl-ether (MTBE; Fisher Scientific, Pittsburgh, PA, USA). A gradient system was used involving two separately mixed mobile phases. Mobile phase A was methanol/MTBE/water (81:15:4) and mobile phase B was methanol/MTBE (9:91). The initial values were 100 % of A and 0 % of B, to 50 % A and 50 % B in 45 min, followed by 100 % B within 25 min. The flow rate was 1.0 mL/min throughout the entire run. All samples were injected via a 20 µL loop using a 100-µL syringe.

On the basis of the maxima absorbance for the carotenoids of *R. glutinis,* detection was done at 450 and 485 nm by a Waters 2998 Photodiode Array Detector. The elution profile of β-carotene standard with the C30 column was obtained and standard curves were constructed by plotting HPLC peak absorbance area versus concentration of the β-carotene in the injected sample.

After the HPLC analyses of the samples, a saponification protocol was performed with the samples, according to Granado et al. (2001), in order to break ester bonds, and chromatograms were repeated.

3.2.9. Statistical data analysis

Experiments were performed in triplicate, and the presented results are mean ± 95% confidence interval. t-Test and One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences between the means values. The differences were considered significant at $p < 0.05$.

3.3. C-phycocyanin extraction assisted by pulsed electric fields from *Artrosphira platensis*

3.3.1. Strain, medium and culture conditions

Arthrospira platensis (BNA 0007B, National Bank of Algae, Canary Islands, Spain) were grown in a modified *Spirulina* medium (Aiba & Ogawa, 1977) (Table 3.1).

Table 3.1. Composition of modified *Spirulina* medium

$NaHCO_2$	13.61 g/L
Na_2CO_3	4.03 g/L
K_2HPO_4	0.50 g/L
$NaNO_3$	2.50 g/L
K_2SO_4	1 g/L
NaCl	0.2 g/L
$MgSO_4\ 7H_2O$	0.04 g/L
$CaCl_2\ 2H_2O$	0.01 g/L
$FeSO_4\ 7H_2O$	0.08 g/L
$Na_2EDTA\ 2H_2O$	0.8 g/L
$FeSO_4\ 7H_2O$	0.7 g/L
$ZnSO_4\ 7H_2O$	1 mg/L
$MnSO_4\ 7H_2O$	2 mg/L
H_3BO_3	10 mg/L
$Co\ (NO_3)_2\ 6H_2O$	1 mg/L
$Na_2MoO_4\ 2H_2O$	1 mg/L
$CuSO_4\ 5H_2O$	0.005 mg/L
Vitamin B_{12}	5 mg/L

Cells were cultured photoautrophically in 2-L tubes of 8 cm diameter and 53 cm height, bubbled with air (6 mL /s) at 30°C, in light:dark cycles (12:12 h) using white fluorescent lamps (15 mmol /m s) (Figure 3.12). The culture medium was initially inoculated at an optical density of 0.1 at 560 nm using a pre-culture. To determine biomass concentration, samples were taken every 24 hours. Experiments were performed using cells at the stationary phase of growth after an incubation time between 7 and 9 days. Biomass concentration at the stationary phase was around 1 g_{dw}/L. Dry weight (dw) of microalgae was determined by vacuum drying (GeneVac Ltd, UK) at 60ºC using 1 mL of the cell suspension until reaching constant weight (around 1 hour).

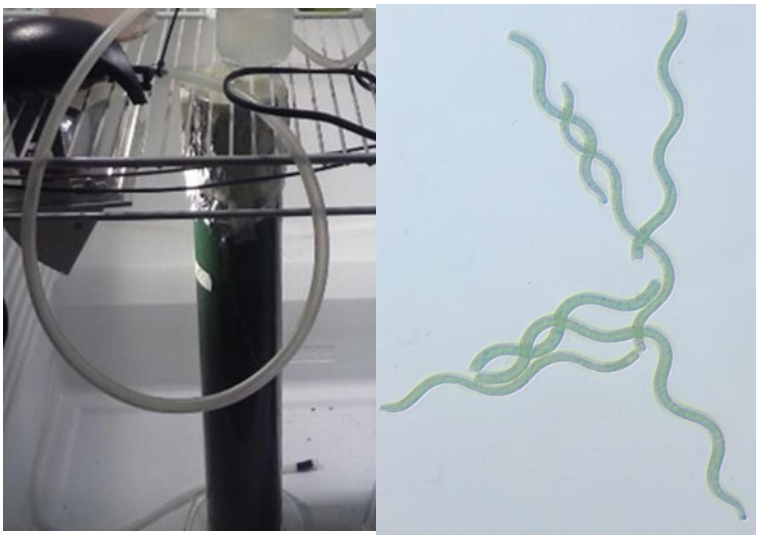


Figure 3.12. *A. platensis* culture in bubbled 2-L tubes and microscopic picture (400x) of the cells.

3.3.2. Cell disruption

In order to determine the total amount of C-phycocyanin, an aliquot of 150 µL of wet cell biomass was blended with 1350 µL of distilled water, and the mixture was disrupted by bead-beating using a bead beater (bead diameter 0.1 mm, BioSpec Products INC, USA) at a speed of 4800 rpm (10 cycles of 10 s). Following each cycle, the sample was cooled down in water at 0º C to avoid overheating of the sample.

3.3.3. PEF treatments

PEF Generator

The PEF equipment used in this investigation (ScandiNova PEF equipment) was previously described in section 3.1.2.1.

PEF treatment chamber

Fresh biomass of *A. platensis* was treated at different temperatures using the tempered batch parallel-electrode treatment chamber described in section 3.1.2.1.

PEF treatment conditions

The treatment medium was tempered at 10.0, 25.0 or 40.0 °C during the PEF treatments thanks as indicated in section 3.1.2.1. The temperature of the treatment

medium was measured with a thermocouple before and after the PEF treatment. Temperature variations were always lower than 2 °C.

Before treatments, fresh biomass of *A. platensis* was centrifuged at 3000 ×g for 10 min at 25 °C and suspended in distilled water. Three cycles of centrifugation and resuspension in distilled water were conducted to obtain a final electrical conductivity of 1.0 mS/cm at 25°C. This conductivity did not change at 10º C and increased to 1.1 mS/cm at 40ºC. The *A. platensis* suspension (0.44 mL) was placed in the treatment chamber by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium). The suspension was subjected from 15 to 50 monopolar square 3 µs waveform pulses of 3.75, 5 and 6.25 kV. These voltages resulted in electric field strengths of 15, 20, 25 kV/cm respectively that corresponded with total specific energies ranging from 13.5 to 110.1 kJ/kg of suspension. The energy per pulse (*W*) was calculated using the following equation:

$$W = \int_0^i \sigma \cdot E(t)^2 dt$$

in which σ (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 (*W*) 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. Frequency of application of the treatments was 0.5 Hz.

3.3.4. C-Phycocynin extraction

For C-phycocyanin extraction, 1 mL of the untreated or PEF-treated *A. platensis* suspension was added to 19 mL of distilled water. The extraction was conducted in a rotary shaker at 20ºC in the dark. In order to obtain extraction curves, samples were gradually collected until 420 min. After centrifugation (6000 x g for 90 s), the supernatant's optical density was measured at 615 and 652 nm. C-phycocyanin concentration was calculated according to the following equation (Bennett & Bogorad, 1973):

$$PC = \frac{OD_{615} - 0.474 \times OD_{652}}{5.34}$$

in which PC is the C-phycocyanin concentration (mg/ mL), OD_{615} is the optical density of the sample at 615 nm, and OD_{652} is the optical density of the sample at 652nm.

The purity of C-phycocyanin extract was monitored spectrophotometrically by the following equation (Abelde et al., 1998):

$$EP = \frac{OD_{615}}{OD_{280}}$$

in which EP is the protein extract purity and OD_{615} indicates the phycocyanin concentration; OD_{280} is the optical density of the sample at 280 nm, indicating the total concentration of proteins in the solution.

The C-phycocyanin extraction yield (PEY) was calculated as:

$$PEY = PC \times V/d_w$$

in which PC is the C-phycocyanin concentration (mg/mL), V is the volume of solvent (mL), and d_w is the dried biomass (g).

3.3.5. Experimental design

Response surface methodology (RSM) was used to evaluate the effect of the electric field strength (15-25 kV/cm), treatment time (60-150 µs), and temperature (10-40ºC) on the C-phycocyanin extraction yield (PEY) from *A. platensis* after 360 min of extraction.

The data obtained after having treated the cells under the conditions described in section 2.2 were modeled with the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i>j}^{k} \beta_{ij} X_i X_j$$

in which Y is the response variable to be modeled, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear coefficients, β_{ii} is the quadratic coefficients, β_{ij} is the cross-product coefficients, and k is the total number of independent factors. A backward regression procedure was used to determine the models' parameters. This procedure systematically removed the effects that were not significantly associated ($p > 0.05$) with the response until a model with a significant effect was obtained.

3.3.6. Statistical data analysis

Experiments were performed in triplicate, and the presented results are means ± 95% confidence interval. One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences between the mean values. The differences were considered significant at $p < 0.05$. Multiple regression analysis was conducted for fitting the equation 5 to the experimental data and significant terms of the model was determined by ANOVA.

The central composite design and the corresponding data analysis were carried out by using the software package Design-Expert 6.0.6 (Stat-Ease Inc., Minneapolis, MN, USA).

3.4. Pulsed electric field permeabilization and extraction of phycoerythrin from *Porphyridium cruentum*

3.4.1. Strain, medium and culture conditions

Porphyridium cruentum (UTEX 161) was obtained from the Culture Collection of Algae at the University of Texas, Austin (USA). The cells were grown in batch culture in 0.5-liter columns of 5.3 cm in diameter at 25±1 °C in artificial seawater (ASW) medium (Jones et al., 1963). For a solid medium, 1.5 g of technical agar were added to 100 mL of the medium. Medium ASW (liquid and solid) was autoclaved at 121° C for 20 min.

Table 3.2. Composition of artificial seawater medium.

NaCl	18 g/L
Mg SO_4 7 H2O	2.6 g/L
KCl	0.6 g/L
$NaNO_3$	1 g/L
$CaCl_2$ 2H_2O	0.3 g/L
KH_2PO_4	0.05 g/L
Tricine	4.48 g/L
Na_2EDTA 2H_2O	30 mg/L
H_3BO_3	11.4 mg/L
$FeCl_3$ 6H_2O	0.49 mg/L
$MnSO_4$ H_2O	1.64 mg/L
$ZnSO_4$ 7H_2O	0.22 mg/L
$CoCl_2$ 6H_2O	0.048 mg/L
HCl	0.22 mM
$FeCl_3$ 6H_2O	1.62 mg/L
Vitamin B12	0.135 mg/L

The cultures were illuminated continuously by fluorescent cool-white lamps (15 mmol/m^2s). The medium was bubbled with air (6 mL/s). The cultures were initially inoculated with 1 x 10^4 cells/mL. Algae growth was monitored by measuring the number of cells with a Thoma counting chamber (ServiQuimia, Constantí, Spain) and an optic microscope (microscope L-Kc, Nikon, Tokyo, Japan). Biomass concentration was determined every 24 h. Experiments were performed using cells at stationary growth phase, achieved after an incubation time of 15 to 18 days. Biomass concentration at the stationary phase was around 2 g_{dw}/L. Dry weight of microalgae was determined by vacuum drying (GeneVac Ltd., UK) similarly to *Arthrospira platensis*.

3.4.2 PEF treatments

PEF Generator

The PEF equipment (ScandiNova PEF equipment) used to treat *P. cruentum* cells has been previously described in section 3.1.2.1.

PEF treatment chamber

Fresh biomass of *P. cruentum* was treated using the tempered batch parallel-electrode treatment chamber as previously described in section 3.1.2.1.

PEF treatment conditions

Prior to treatment, fresh biomass of *P. cruentum* was centrifuged at 1000×g for 1 min at 25° C and re-suspended in a citrate-phosphate Mcllvaine buffer (pH 7.0; 1 mS/cm) to a final concentration of approximately 10^7 cells/mL. The *P. cruentum* suspension (0.44 mL) was placed in the static parallel electrode treatment chamber (gap: 0.25 cm; diameter: 1.6 cm) by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium).

The suspension was subjected to 10 to 50 monopolar square waveform pulses of 3 µs at electric field strengths ranging from 2 to 10 kV/cm, and a frequency of 0.5 Hz. These treatments correspond to specific energy inputs ranging from 0.12 to 15 kJ/kg. The temperature of the treatment medium was measured by a thermocouple before and after the PEF treatment. Initial temperature was 22±1 °C, and after treatments the sample temperature was always lower than 30º C.

3.4.3. Cell disruption treatments

In order to determine the total amount of pigments, an aliquot of 150 µL of wet cell biomass was blended with 1350 µL of distilled water, and the mixture was disrupted with a bead beater (bead diameter 0.1 mm, BioSpec Products INC, USA) at a speed of 4800 rpm (10 cycles of 10 s). Following each cycle, the sample was cooled down in water at 0º C to avoid overheating of the sample.

3.4.4 Enumeration of viable cells

PEF-treated and untreated (control) cell suspensions were serially diluted in a sterile solution of peptone water. From the selected dilutions, 20 µL were plated into ASW solid media. The plates were incubated at 25° C for 8 days with the same light regime used for the liquid culture, and the number of CFU/mL were counted to determine the inactivation rate after treatment. Longer incubation times did not increase the colony counts.

3.4.5 Staining cells with propidium iodide

Quantification of the number of *P. cruentum* electroporated cells was performed by measuring the entry of the fluorescent dye propidium iodide (PI) as described in section 3.1.4. After the PEF treatments, 50 µL of PI (0.1 mg/mL) were added to 450 µL of *P. cruentum* suspension (10^7 cells/mL), resulting in a final PI concentration of 0.015 mM. Suspensions were incubated for 10 min. Previous experiments showed that longer incubation times did not influence the fluorescence measurements of *P. cruentum*. After incubation, the cell suspension was centrifuged and washed twice until no extracellular PI remained in the buffer. PI trapped inside the cells (permeabilization of individual cells) was checked using an epi-fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan). The percentage of fluorescent cells was calculated after observing a population of at least 150 cells.

3.4.6 Pigment (β-phycoerythrin) extraction

For β-phycoerythrin extraction, untreated or PEF-treated cells of *P. cruentum* suspended in citrate-phosphate McIlvaine buffer of pH 7 were kept in a dark location. In order to obtain extraction curves, samples were periodically collected over a period of 48 hours. After centrifugation (6000 x g for 2min), the optical density (OD) of the supernatant was measured at 280, 565, 620 and 650 nm. The β-phycoerythrin concentration was calculated according to the following equations (Bermejo et al., 2001):

$$B-PE\ (\frac{mg}{mL}) = \frac{(OD_{565} - 2.8\ (R-PC) - 1.34(APC))}{12.7}$$

$$R-PC\ (\frac{mg}{mL}) = \frac{(OD_{620} - 0.7OD_{650})}{7.38}$$

$$APC(\frac{mg}{mL}) = \frac{(OD_{650} - 0.19OD_{620})}{5.65}$$

in which B-PE is the β-phycoerythrin concentration, and R-PC and APC correspond with R-phycocyanin and allophycocyanin, respectively.

The purity of B-PE extract was monitored spectrophotometrically, and the following equation was applied:

$$Extract\ purity = \frac{OD_{565}}{OD_{280}}$$

in which OD_{565} indicates the optical density at 565 nm (absorption maxima of β-phycoerythrin), and OD_{280} is the optical density of the sample at 280 nm which is correlated with the total concentration of proteins in the solution.

3.4.7 Statistical data analysis

Experiments were performed in triplicate, and the presented results are means ± 95% confidence interval. Replicates correspond to three biological replicates of different experiments, both for treatments and controls. T-tests and one-way analysis of variance (ANOVA) using Tukey's test were performed to evaluate the significance of differences between the mean values. The differences were considered significant at $p < 0.05$.

3.5. Pulsed electric field permeabilization to extract astaxanthin from the nordic microalgal strain *Haematococcus pluvialis*

3.5.1. Alga strain identification and cultivation

The microalgal strain used in this work was isolated from freshwater in Umeå, Northern Sweden, and initially identified as *Haematococcus pluvialis* (also known as *Haematococcus lacustris*) based on cell morphology and culture physiology. The taxonomy of the isolate was confirmed by genomic DNA extraction (NucleoSpin Soil DNA extraction kit, MACHEREY-NAGEL, Germany) and PCR amplification of the ITS2 (Internal Transcribed Spacer 2) sequence in the algal rRNA gene, as described in Ferro et al. (2018). The amplicon was sequenced and a phylogenetic tree (supplementary material of manuscript VIII) was inferred including ITS2 sequences of 27 *Haematococcus* isolates using Mega7 software (http://www.megasoftware.net/) and a neighbor-joining method validated at 1000 bootstrap replications.

Table 3.3. Bold´s Basal Medium composition of different N concentration

	BBM 2.94 mM N	**BBM 30 mM N**	**BBM N free**
$NaNO_3$	250 mg/L	2522 mg/L	-
KH_2PO_4	175 mg/L	1750 mg/L	1750 mg/L
K_2HPO_4	75 mg/L	750 mg/L	750 mg/L
NaCl	25 mg/L	25 mg/L	25 mg/L
Mg SO_4 $7H_2O$	75 mg/L	75 mg/L	75 mg/L
$CaCl_2$ $2H_2O$	25 mg/L	25 mg/L	25 mg/L
Na_2EDTA	50 mg/L	50 mg/L	50 mg/L
KOH	31 mg/L	31 mg/L	31 mg/L
$FeSO_4$ $7H_2O$	4.98 mg/L	4.98 mg/L	4.98 mg/L
H_2SO_4	1 mg/L	1 mg/L	1 mg/L
$MnCl_2$ $4H_2O$	0.232 mg/L	0.232 mg/L	0.232 mg/L
ZnSO4 $7H_2O$	1.412 mg/L	1.412 mg/L	1.412 mg/L
Na_2MoO_4 $2H_2O$	0.192 mg/L	0.192 mg/L	0.192 mg/L
$CuSO_4$ $5H_2O$	0.252 mg/L	0.252 mg/L	0.252 mg/L
$Co(NO_3)_2$ $6H_2O$	0.080 mg/L	0.080 mg/L	0.080 mg/L
H_3BO_3	1.142 mg/L	45.68 mg/L	182.72 mg/L

Microalgae *Haematococcus pluvialis* isolated from Northern Sweden was cultivated in two steps. In the first stage, the required volume of microalgae inoculum

was added in 250 mL flasks (Figure 3.13) filled up to 70% volume with Bold's Basal Medium of 30 mM N (BBM, Bischoff and Bold, 1963) to an initial optical density OD_{750} of 0.1 (Table 3.3). These cultures were maintained at 20°C and bubbled with 1 L/L of air with 3% CO_2 for seven days.

After the first stage of biomass production, cultures were harvested and centrifuged under sterile conditions. The pellet was re-suspended and washed twice in the culture media tested for the second step (stress induction). Cells grown in a multicultivator photobioreactor (Figure 3.13) were exposed to different stress conditions: high light intensities (200-1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$), addition of 5-10 g/L NaCl, sudden nitrogen starvation (BBM N free shown in Table 3.3) and N starvation combined with mixotrophic growth in the presence of xylose or glucose to induce astaxanthin accumulation.

The multicultivator MC1000-OD (Photon System Instruments, Czech Republic) is a bench scale photobioreactor for cultivation of multiple samples that consists of 8 cultivation vessels, of 85 mL culture volume that can be cultivated under controlled temperature, light intensity and aeration conditions (Figure 3.13). Each vessel is independently illuminated by an array of white LEDs. Cultures were bubbled with 1 L/L of air with 3% CO_2.

In parallel, locally isolated strain *Chlorella vulgaris* 13-1 (Ferro et al., 2018) was cultivated in BBM and subjected to the same pre-treatments than *H. pluvialis* for comparison of disruption efficiency.

3.5.2. Biomass concentration and optical density

The parameters pH, maximal photosynthetic efficiency of photosystem II (Qy), and optical density (measured at 530, 680 and 750 nm) were monitored daily for each algal culture. The biomass concentration of the cultures was determined after filtration of a known culture volume over pre-dried and pre-weighted glass fiber filters (Whatman GC) by measuring the weight increase of the dried filters, as described in Gojkovic et al. (2014). The biomass concentration was calculated and expressed in g/L of culture.

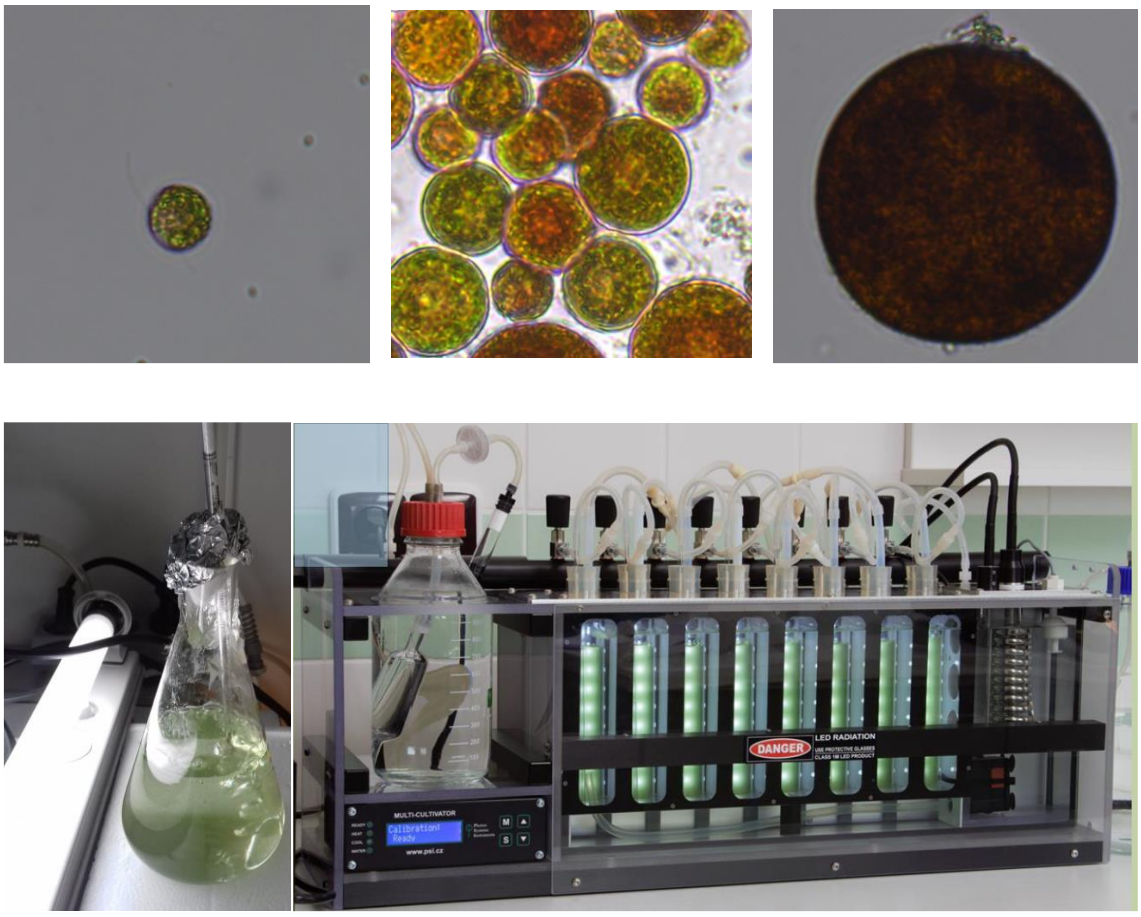


Figure **3.13.** Microscopic observation (400 x) of the different growth phase of *H. pluvialis (biflagellate green vegetative cell, immature cyst, red mature cyst)*. Cultures of *H. pluvialis* in Erlenmeyer flask (first stage) and cultures in multicultivator bioreactor (second stage).

3.5.3. Chlorophyll and carotenoids determination spectrophotometrically

Pigment content (chlorophylls and total carotenoids) was determined spectrophotometrically using a multi-step methanol extraction method as explained in Gojkovic et al. (2014). The chlorophylls and carotenoids content of the extracts were determined using an UV-vis spectrophotometer (T90+Uv/vis spectrometer, PG instruments, Ltd, UK) at 470, 647 and 663 nm. Results were expressed as mg of carotenoids/chlorophylls per mL of suspension and calculated using the modified Arnon's equations (Lichtenthaler, 1987).

3.5.4. Cell disruption treatments

The obtained algal biomass was harvested and subjected to different disruption treatments. Each biomass sample after each treatment was observed under the light

microscope (Leica DMi1, 40x magnification) to determine the degree of intact cells and access the efficiency of each disruption procedure: PEF, beat-beating, US, thermal, freezing-thawing and dimethyl sulfoxide treatment.

PEF treatments

The PEF equipment used in this investigation (Figure 3.14) was a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). The equipment consists of a set of capacitors, with a maximum capacitance of 3275 F, which generates square waveform pulses ranging in duration from 0.05 to 5 ms with a maximum output voltage of 3000 V. A parallel electrode treatment chamber composed of a cylindrical methacrylate tube closed with two polished stainless steel cylinders was used to apply the PEF-treatments. The electrode diameter was 10 mm and the gap between the electrodes was 50 mm.

Microalgae biomass was directly PEF-treated suspended in the own cultivation medium that had a conductivity of 1 mS/cm. In a first screening, the sensitivity of this strain of *H. pluvialis* to PEF treatments was tested. The PEF treatments ranged from 10 to 80 pulses of 5 ms (50-400 ms) with a frequency of 1 Hz at electric field strengths ranging from 0.2 to 1.0 kV/cm. The specific energy of these treatments ranged from 2 to 400 kJ/kg. Specific energy input per treatment expressed in kJ/kg was calculated as described in section 3.1.2.

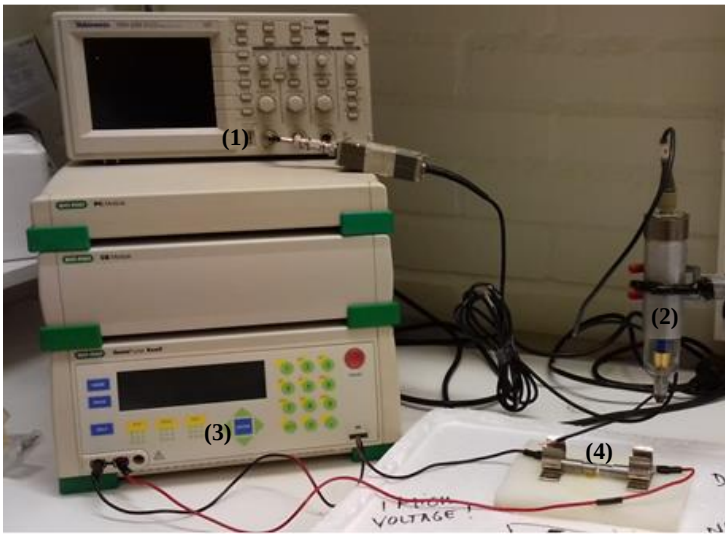


Figure 3.14. PEF equipment. (1) Oscilloscope, (2) High-voltage probe, (3) PEF-generator, (4) Treatment chamber

For the extraction experiments, treatment of 10 pulses of 5 ms at 1 kV/cm (50 kJ/kg) was selected. PEF-treated cells were incubated in their own aqueous treatment media from 1 to 12 h at room temperature in absence of light. Suspensions were then centrifuged and biomass was re-suspended in the extraction solvents.

Bead-beating

An aliquot of 150 µL of wet cell biomass was blended with 1350 µL of distilled water, and the mixture was disrupted by bead-beating using a bead beater (Bullet Blender Storm 24, Next advance, Troy, USA) and 12 mg of 0.1 mm diameter glass beads at a speed of 4800 rpm (between 5 and 10 cycles of 60 s). Following each cycle, the sample was cooled down on ice for 180 s to prevent overheating.

Ultrasounds treatment

An aliquot of 5 mL of microalgae biomass was diluted with 45 mL of ethanol. Suspension was sonicated 10 times during 10 seconds at 80% of amplitude in an 20 kHz Ultrasound apparatus 450 W Sonifier SFX550 Cell Disruptors (Emerson, San Luis, USA) equipped with a Branson model 102 c (CE) (Emerson). Between cycles, sample was cooled down on ice.

Thermal treatment

Aliquots of 1 mL were placed in 5 mL glass tubes. Tubes were submerged in a Thermostatic Bath (Grant TC120, Grant Instruments, Cambridge, UK) at 70ºC for 1 h.

Freezing-Thawing

Aliquots of 1 mL of microalgae biomass were placed in Eppendorf tubes and centrifuged. Pellet was subjected to fast freezing under liquid nitrogen and after that it was left to melt on ice (slow melting). Cycles of freezing-thawing were repeated 5 times.

Dimethyl Sulfoxide treatment

Aliquots of 0.1 mL of culture were mixed with 0.9 mL of dimethyl sulfoxide (DMSO) in a 2 mL tube. Approximately 12 mg of 0.1 mm glass beads were added and

tubes were then subjected to repeated bead-beating cycles until the pellet became colorless. This method was used as reference of total content of carotenoids.

3.5.5. Evaluation of viability of *Haematococcus pluvialis* after PEF treatments

Photosystem II (PSII) maximum quantum yield (Qy) was determined by measuring of the chlorophyll fluorescence in a portable pulse-amplitude-modulation (PAM) fluorimeter (AquaPen AP-100, Photon Systems Instruments, Czech Republic) according to the user manual. Cultures were adapted to dark for 15 minutes prior to measurement.

Cells after the PEF treatments were left for 1 h at room temperature and their photosynthetic efficiency was subsequently measured. It was considered that Qy values bellow 0.3 indicated that the cells were considerably affected by PEF.

3.5.6. Solvent extraction of pre-treated biomass

After the disruption treatments, samples were suspended in the different solvents (acetone, methanol and ethanol), mixed and incubated during 1 h in order to permit the extraction of carotenoids from the cell to the solvent.

3.5.7 HPLC analysis of the extracts

The HPLC method used in this study was described by Yuan and Chen (1997). Briefly, HPLC analysis was performed using a Varian ProStar high performance liquid chromatograph (Varian Inc., Walnut Creek, CA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler and a ProStar 335 photodiode array detector. The system was controlled with the Star chromatography workstation v.6.41 (Varian). Separation was achieved on a reverse-phase column (LC Luna® 100 Å C18 250 x 4.6 mm; 5 μm particle size, Phenomenex) with a precolumn (LC Luna 50 x 4.6 mm; 5 μm particle size, Phenomenex) of the same material. Chromatographic peaks were identified by comparing retention times and spectra against known standards.

3.5.8. Statistical data analysis

Results represent the mean ± standard error of three biological replicates of treatments. One-way ANOVA tests were conducted to assess significant differences between treatments. The differences were considered significant at $p < 0.05$.

3.6. Evaluation of the enzymatic activity in the extracellular media during incubation of yeast

In addition to the studies presented in the manuscripts attached in the results section, the presence of enzymes in the extracellular media during incubation and the activities of these enzymes under different conditions were evaluated. The results of these experiments have been included in the discussion of this thesis and the methodology used is described below.

3.6.1. Determination of protease activity in yeast supernatants during incubation

Peptidase/protease enzymatic activity in the supernatants of the untreated (control) and PEF-treated cells was determined by the EnzChek® Peptidase/Protease Assay Kit (E33758, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The analyses were performed periodically in *R. glutinis and S. cerevisiae* suspensions during incubation time after treatments.

It is a FRET (fluorescence resonance energy transfer)-based method for the accurate quantitation of a wide range of protease activities. The substrate comprises a fluorophore and a quencher moiety separated by an amino acid sequence. Upon sequence cleavage by proteases, the fluorophore separates from the quencher and is free to emit a detectable fluorescent signal (excitation and emission maxima of 502 and 528 nm, respectively). The magnitude of the resultant signal is proportional to the degree of substrate cleavage, and can therefore be used to quantitate the enzyme activity present.

During all steps, the peptidase/protease substrate (both concentrate and working solution) was protected from light as much as was possible. The kit components were allowed to equilibrate to room temperature prior to use. Different dilutions of the standard enzyme and samples were prepared in the digestion buffer (10 mM Tris-HCl, pH 7.8), as well as one negative control composed only by buffer. In order to load microplate wells, 50 µL of protease dilutions were added (samples or standard) into separate wells of a microplate, then 50 µL of peptidase/protease substrate working solution was added. Analysis were performed in triplicate. After that, plate was mixed well. Plates were incubated for 60 minutes at 25°C, protected from light. The fluorescence was measured after incubation using the microplate reader (mod. Genios, Tecan, Austria) at excitation

and emission maxima of 502 and 528 nm, respectively. Standard curve using trypsin, in which protease amount was plotted vs. fluorescence and fitted a line to the data points, was used to determine protease activity.

3.6.2. Evaluation of the protease activity under different conditions

The influence of temperature and ethanol concentration of medium on the activity of the peptidase/protease enzymes released from the yeast species was evaluated. The supernatants containing proteases were collected and the assays of substrate conversion were performed as described in section 3.6.1 but the environmental conditions were varied. The cleavage by proteases was performed at 7, 25 and 43 °C in the normal buffer or at 25°C in buffers containing 6, 12 and 25 % (vol/vol) ethanol. Protease activity obtained under the different conditions was expressed as percentage of the activity measured at 25°C and 0 % ethanol.

3.6.3. Determination of esterase activity in yeast supernatants during incubation

Esterase enzymatic activity in the supernatants of the untreated (control) and PEF-treated cells were determined using a chromogenic assay described in Gilham and Lehner (2005) adapted to cuvettes scale. The analyses were performed periodically in *R. glutinis* suspensions during incubation time after treatments.

The method makes use of *p*-nitrophenyl ester (*p*-nitrophenyl acetate). When this compound is mixed with a solution of esterase enzymes, it is produced the release of *p*-nitrophenol, which is measured spectrophotometrically at 410-nm.

A stock solution of 250 mM *p*-nitrophenyl acetate was prepared using CH_2Cl_2 (dichloromethane). Inmediately prior to initiation of the assay, 20 µL of the stock is diluted into 10 mL of citrate-phosphate pH 8 McIlvaine buffer. Supernatant samples of 100 µs were incubated with 1 mL substrate solution in 1.5 mL disposable cuvettes. A negative control was also prepared by mixing 100 µs of buffer with 1 mL substrate solution. During an incubation period at 37°C of 30 min, the liberation of *p*-nitrophenol is measured as the increase in absorbance at 410 nm in an ultraviolet-visible

spectrophotometer against a blank without enzyme. The absorbance of *p*-nitrophenol is dramatically affected at acidic pH, therefore assays were performed at neutral pH.

3.6.4. Evaluation of the esterase activity under different ethanol concentration

The influence of the medium ethanol concentration on the activity of esterase enzymes obtained from *R. glutins* was evaluated. The assays were performed as described in section 3.6.3. but the concentrations of ethanol in the substrate conversion media were varied to 6, 12 and 25 % ethanol (vol/vol). Esterase activity measured under the different ethanol concentrations was expressed as percentage of the activity measured in 0 % ethanol.

3.6.5. Determination of β-glucanase activity in yeast supernatants during incubation

Similarly, β-glucanase enzymatic activity in the supernatants of the untreated (control) and PEF-treated cells was determined using the azo-barley glucan method (malt and bacterial β-glucanase assay procedure, Megazyme International, Wicklow, Ireland). The analyses were performed periodically in *R. glutinis and S. cerevisiae* suspensions during the incubation time after treatments.

The supernatants of the microbial suspensions are incubated with Azo-Barley glucan substrate under defined conditions. The dyed substrate is depolymerized by β-glucanase to fragments which are soluble in the presence of precipitant solution. After centrifugation of the precipitant-treated reaction mixture, the absorbance at 590 nm of the supernatant solution is directly related to the level of β-glucanase/Celullase in the supernatants of microbial suspensions.

Aliquots of 0.05 mL of Azo-Barley glucan substrate solution (pre-warmed at 30° C) were dispensed into Eppendorf tubes which were pre-incubate at 30°C for 5 minutes. To each tube of Azo-Barley substrate, 1 mL aliquot of supernatant of each microbial suspensions was added. After that, tubes were incubated at 30°C for exactly 10 min (from time to addition). After that, 3 mL of the precipitant solution (40 g/L sodium acetate, 4 g/L Zinc acetate and HCl to adjust to pH 5 in distilled water) were added and tubes were vortexed vigorously. Tubes were allowed to stand at room temperature for 5 minutes,

stirred again and centrifuged (1000 $_x$ g, 10 min). Absorbance at 590 nm of supernatant of each sample was read and the reaction blank against distilled water.

Malt β-glucanase standard curve on Azo-Barley Glucan was used to obtain the equation that correlates absorbances and enzyme concentration.

3.6.6. Evaluation of the β-glucanase activity under different ethanol concentration and temperature

The solution of β-glucanase obtained from the supernatants of PEF-treated *S. cerevisiae* suspension was collected and its enzymatic activity was measured under different conditions. The assays described in section 3.6.5 were performed at incubation temperatures: 7, 25 and 43 °C in the normal buffer or at 25°C in buffers containing 6, 12 and 25 % (vol/vol) ethanol. B-glucanase activity obtained under the different conditions was expressed as percentage of the activity measured at 25°C and 0 % ethanol.

4. RESULTS

4.1. Mannoproteins extraction during *Saccharomyces cerevisiae* yeast autolysis induced by Pulsed Electric Field

4.1.1. Release of mannoproteins during *Saccharomyces cerevisiae* autolysis induced by Pulsed Electric Field *(Manuscript I)*

4.1.2. Factors influencing autolysis of *Saccharomyces cerevisiae* cells induced by Pulsed Electric Fields *(Manuscript II)*

4.1.3. Pulsed Electric Fields accelerate release of mannoproteins from *Saccharomyces cerevisiae* during aging on lees of *Chardonnay* wine. *(Manuscript III)*

4.1.1. Release of mannoproteins during *Saccharomyces cerevisiae* autolysis induced by Pulsed Electric Field *(Manuscript I)*

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Release of Mannoproteins during *Saccharomyces cerevisiae* Autolysis Induced by Pulsed Electric Field

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The potential of the application of pulsed electric fields (PEF) to induce accelerate autolysis of a commercial strain of *Saccharomyces cerevisiae* for winemaking use was evaluated. The influence of PEF treatments of different intensity (5–25 kV/cm for 30–240 μs) on cell viability, cytoplasmic membrane permeabilization and release of mannoproteins and compounds absorbing at 260 and 280 nm has been investigated. After 8 days of incubation at 25°C the Abs_{600} of the suspension containing the control cells was kept constant while the Abs_{600} of the suspension containing the cells treated by PEF decreased. The measurement of the absorbance at 260 and 280 nm revealed no release of UV absorbing material from untreated cells after 8 days of incubation but the amount of UV absorbing material released drastically increased in the samples that contained cells treated by PEF after the same storage period. After 18 days of storage the amount of mannoproteins released from the untreated cell was negligible. Conversely, mannoprotein concentration increased linearly for the samples containing cells of *S. cerevisiae* treated by PEF. After 18 days of incubation the concentration of mannoproteins in the supernatant increased 4.2 times for the samples containing cells treated by PEF at 15 and 25 kV/cm for 45 and 150 μs. Results obtained in this study indicates that PEF could be used in winemaking to accelerate the *sur lie* aging or to obtain mannoproteins from yeast cultures.

Keywords: pulsed electric fields, mannoproteins, autholysis, *Saccharomyces cerevisiae*, winemaking

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INTRODUCTION

Yeast cell wall, which represents up to 20% of yeast cell dry weight, is mainly composed of β-glucans and mannoproteins. These mannoproteins are highly glycosylated (∼90% sugars, mainly mannose) and are located in the outermost layer of the yeast cells acting as structural components (Quiros et al., 2012). Mannoproteins have been associated with positive quality and technological traits of wines. It has been shown that mannoproteins reduce haze formation, prevent the precipitation of tartaric salt, contribute to the mouthfeel, influence the intensity of the aroma of wine and can interact with phenolic compounds, thus improving color stability and reducing the astringency of wine (Pérez-Serradilla and De Castro, 2008). Furthermore, different studies have demonstrated important emulsifying and stabilizing properties of mannoproteins due to the amphipathic structure of their molecule (da Silva Araújo et al., 2014).

Mannoproteins are released from the yeast cell wall during yeast autolysis. Autolysis is a phenomenon that begins with the disorganization of membranous systems (cytoplasmic membrane and other organelle membranes) caused by cell's death. During autolysis enzymes glucanase and proteinase degrade the cell wall and, as result, the cell wall becomes porous and different compounds such as mannoproteins are released into the surrounding medium (Alexandre and Guilloux-Benatier, 2006).

Releasing of mannoproteins from yeast autholysis occurs during the alcoholic fermentation but mainly during the aging on the lees of certain types of wines such as white, red, or sparkling. Wine lees are a residue that is formed at the bottom of the recipes containing wine after fermentation and that is mainly composed by yeast. The autolysis of yeast in wine is a very slow process lasting from a few months to years. Therefore accelerating this process is highly desirable to reduce the risk of microbial spoilage of wine and decrease production costs (Alexandre and Guilloux-Benatier, 2006; Comuzzo et al., 2015).

Different strategies have been suggested for accelerating yeast autolysis. Enzymes able to hydrolize B-glucans from yeast cell walls and thermolysis are the most widely proposed tools (Martínez-Rodríguez et al., 2001; Comuzzo et al., 2012; Bzducha-Wróbel et al., 2014). Recently the potential of non-thermal processing technologies such as high pressure homogenization (Comuzzo et al., 2015) and ultrasound (Martín et al., 2013) have been also investigated to induce autolysis of wine yeasts.

High pressure homogenization is one of the most commonly employed mechanical methods for large scale disruption of microbial cells. This method results in effective breakage of cells and high recovery of bio-products. However, HPH causes non-selective release of the products and its final products contain large quantity of cell debris which complicates the downstream process of purification (Comuzzo et al., 2015).

Pulsed electric fields (PEF) is a technology that causes loss of the barrier function of the cell membranes by application of intermittent electric fields of high intensity for short periods of time (μs–ms) (Barba et al., 2015; Puértolas and Barba, 2016). The phenomenon, that is called electroporation, is mainly associated to the formation of local defects or pores in the cytoplasmic membrane of the cells increasing its permeability and causing uncontrolled molecular transport across microbial membranes. Recently, it has been reported that PEF provokes not only cytoplasmic membrane permeabilization but also changes in the cell wall structure (Ganeva et al., 2014; Pillet et al., 2016). This technology has been successfully applied to recover different intracellular components such as proteins, nucleic acids, and ionic substances from different yeast species (Ganeva et al., 2003; Liu et al., 2013).

The aim of this study was to evaluate the potential application of PEF to induce accelerate autolysis of a commercial strain of *Saccharomyces cerevisiae* for winemaking use. The effect of PEF treatments of different intensity on cell viability, cytoplasmic membrane permeabilization, and release of mannoproteins and compounds absorbing at 260 and 280 nm has been investigated.

MATERIALS AND METHODS

Strains, Medium, and Propagation Conditions

A strain of *S. cerevisiae* from an industrial preparation for winery applications was used (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland). Yeasts were grown in in 1000 mL glass flasks containing 600 mL of Sabouraud-Dextrose broth (Oxoid, Basingstoke, UK) under agitation at 25°C. Yeast's growth was monitored by measuring the absorbance at 600 nm and the number of cells using a Thoma counting chamber and the plate counting method in Potato-Dextrose-Agar (PDA, Oxoid, Basingstoke, UK). The experiments were performed with cells at stationary growth phase, which was achieved after 48 h of incubation.

PEF Treatment

The PEF equipment used in this investigation was previously described by Saldaña et al. (2010). Before treatment, fresh biomass of *S. cerevisiae* was centrifuged at 3000 × *g* for 10 min at 25°C and re-suspended in a citrate-phosphate Mcllvaine buffer (pH 7.0; 1 mS/cm) to a final concentration of approximately 10^9 cells mL^{-1}. The *S. cerevisiae* suspension (0.44 mL) was placed in the treatment chamber by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium). Cells were subjected to 5–80 monopolar square waveform pulses of 3 μs of electric field strengths between 5 and 25 kV/cm at room temperature and applied at a frequency of 0.5 Hz.

PEF Inactivation

After the PEF treatments, cells were plated in PDA in order to monitor inactivation after different treatment conditions. Serial dilutions 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. Inactivation data was expressed as the ratio between the initial number of survivors (N_o) and the number of survivors after different treatment times (N_t).

Staining Cells with Propidium Iodide

Quantification of the number of *S. cerevisiae* electroporated cells was performed by measuring the entry of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Barcelona, Spain). PI is a small (660 Da) hydrophilic molecule that is unable to cross through intact cytoplasmatic membranes. 50 μL of PI (0.1 mg mL^{-1}) were added to 450 μL of *S. cerevisiae* 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 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 (240 μs at 25 kV cm^{-1}) used in

this investigation. Under these conditions, the permeabilization of individual cells was also checked using an epi-fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan). Fluorescence was measured with a spectrofluorophotometer (mod. Genios, Tecan, Austria) using 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 irreversible electroporation.

Staining Cells before PEF Treatments

When PI was added before PEF treatments stain cells corresponded to the sum of both the irreversibly and reversibly permeabilized cells.

Staining Cells after PEF Treatments

The degree of permeabilization when cells were stained after the PEF treatment corresponded to irreversibly permeabilized cells. Reversible permeabilization was calculated by comparing the fluorescent measurements obtained following the two staining protocols.

Storage of Cellular Suspensions and Determination of Yeast Viability

Control and PEF treated cells were re-suspended in buffer of pH 7.0 and stored at 25°C. Samples were collected at different time points along the period of storage which lasted 25 days.

The viability of cells during the storage was determined by pour plating of serial dilutions and counting the colony-formed after 48 h of incubation at 25°C.

Monitoring Cell Lysis Caused by PEF

In order to monitor the release of components during storage of cellular suspensions, different measurements were performed in untreated and PEF treated samples.

Turbidity of the suspension during the storage was measured by the absorbance at 600-nm (Abs_{600}) to monitor leakage of cellular content. Absorbance at 260-nm (Abs_{260}) and 280-nm (Abs_{280}) of the supernatant was measured in order to monitor the presence of intracellular material outside the cell (Aronsson et al., 2005).

The concentration of mannoproteins in the extracellular medium was determined after hydrolyzing the supernatant with sulfuric acid (final concentration 1.5 M) at 100°C for 90 min. Cooled samples were neutralized with NaOH 3 M. Quantitative analysis of mannose was conducted by an enzymatic method (D-Mannose, D-Fructose, and D-Glucose assay procedure, Megazyme International, Wicklow, Ireland) (Dupin et al., 2000).

Statistical Data Treatment

The results represent the mean ± standard error of the mean of three replicates. One-way ANOVA test was conducted to assess significant differences between treatments. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

PEF Inactivation of *S. cerevisiae* as Function of the Electric Field Strength and Treatment Time

The inactivation curves of *S. cerevisiae* after exposure to PEF treatments of different electric field strengths and duration is shown in **Figure 1**. It can be observed that treatments below 10 kV/cm were ineffective to inactivate *S. cerevisiae*. These results confirms data obtained by other authors showing that electric field higher than 10 kV/cm were required to inactivate different types of yeast when pulses of a duration of microseconds were applied (Cserhalmi et al., 2002; Aronsson et al., 2005). Currently, it is accepted that the main mechanism involved in microbial inactivation by PEF is electroporation that is is a consequence of an increment in the transmembrane voltage (Heinz et al., 2001). The external electric field strength required to reach the transmembrane voltage threshold to induce electroporation is correlated with the cell size (Heinz et al., 2001). This dependence explains why the critical electric field required to electroporate yeast is lower than that required to electroporate bacteria -which size is lower- and higher than that required to electroporate eukaryotic cells of plants or animal tissues -which size is higher-.

As it has been reported by other authors, above the critical electric field strength *S. cerevisiae* inactivation increased with more intense electric field strength and longer treatment durations (Saldaña et al., 2014). However, the inactivation kinetics of *S. cerevisiae* was non-linear. Thus, at any electric field

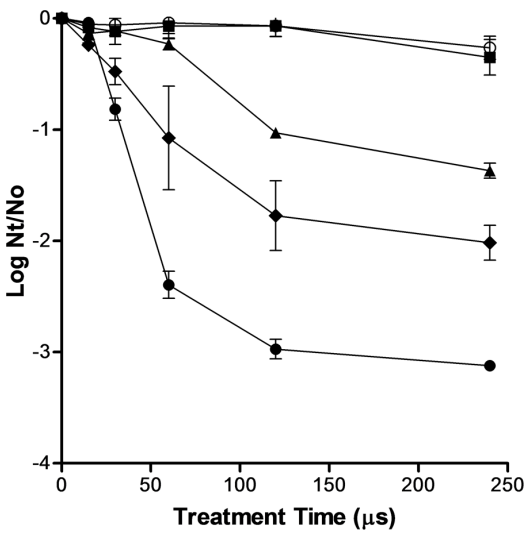

FIGURE 1 | Inactivation of *Saccharomyces cerevisiae* by pulsed electric fields (PEF) treatments of different electric field strengths. 5 kV/cm (○), 10 kV/cm (■), 15 kV/cm (▲), 20 kV/cm (♦), 25 kV/cm (●). Inactivation data was expressed as the ratio between the initial number of survivors (N_O) and the number of survivors after different treatment times (N_t).

strength assayed, the inactivation was faster in the first moments of the treatment and then the number of survivors decreased more slowly as the treatment time increased. A treatment of 120 μs (40 pulses of 3 μs) inactivated around 1.0, 1.7, and 2.7 log cycles the population of *S. cerevisiae* when applied at 15, 20, and 25 kV/cm, respectively (**Figure 1**). Nevertheless, a further increment of treatment duration from 120 to 240 μs scarcely increased the lethality of PEF.

PI Entry into *S. cerevisiae* Cells as Function of the Electric Field Strength and Treatment Time

Figure 2 shows the percentage of cells permeabilized to PI after PEF treatments of different electric field strength and duration when PI was added before or after the treatment. For comparison purposes, the percentage of *S. cerevisiae* cells inactived by the same PEF treatments are also shown in **Figure 2**. As it can be observed in the figure, the entry of PI increased with the treatment time and intensity of the electric field strength, regardless of the staining protocol. In order to detect significant permeabilization to PI an electric field strength equal or higher than 15 kV/cm was required. At electric field strengths of 15 and 20 kV/cm, the difference between the PI entry observed by the different staining protocols under the same PEF treatment conditions reveals the existence of reversible electroporation. It means that in a proportion of cells, the permeabilization caused by PEF disappeared after the treatment. However, at the highest electric field strength assayed (25 kV/cm) all the population was irreversibly electroporated. This dependence between the intensity of the electric field strength and the proportion of cells reversibly electroporated has been previously observed by other authors in other microorganisms such as bacteria (García et al., 2007; Cebrián et al., 2015), microalgae (Luengo et al., 2014) and other species of yeasts (Aronsson et al., 2005).

According to **Figure 2**, the number of irreversibly permeabilized yeast cells was, in general, lower than the number of dead cells but the difference decreased for the PEF treatments applied at higher intensity. These results indicate that a percentage of yeasts cell that are inactivated 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- or that death of these cells could be caused by secondary damages to other structures or functions (Aronsson et al., 2005; García et al., 2007). Other authors have also observed the presence of dead microbial cells of bacteria and microalgal with non-permeabilized cytoplasmatic membranes after the application of PEF treatments at moderate intensity (García et al., 2007; Luengo et al., 2014; Cebrián et al., 2015). As it was also reported by these authors our results confirm that after applying intense PEF treatments (25 kV/cm for 75 μs) no difference between the percentage of *S. cerevisiae* cells inactivated and irreversively electroporated was observed.

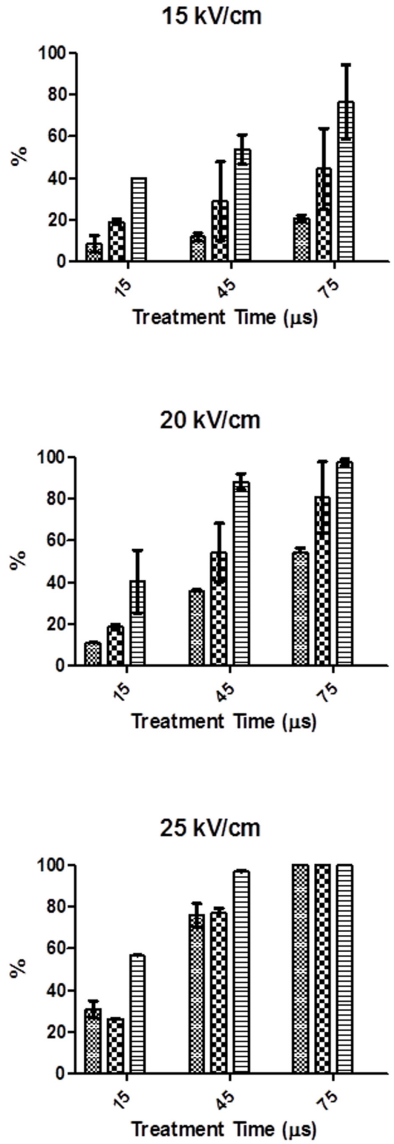

FIGURE 2 | Percentage of *S. cerevisiae* cells inactivated and stained when propidium iodine (PI) was add before or after PEF treatments of different electric field strength and treatment time. 512 % of stained cells when PI added after PEF treatment, % of stained cells when PI added before PEF treatment, % of inactivated cells.

Decrease of the Optical Density and Leakage of Intracellular Material after Application of PEF Treatments

Decrease in the absorbance at 600 nm and presence of UV absorbing material in the suspension medium were used as indicators of the degree of cell lysis caused by PEF. Thus,

when the permeability of the cytoplasmatic membrane of the microorganism is altered water diffuses from the external medium to the cytoplasm causing a decrease in the optical density of the cell suspension. On the other hand, the presence of intracellular material outside the cell can be detected by measuring the absorbance of the suspending medium at 260 and 280 nm, which corresponds with the absorbance maxima of nucleic acids and proteins, respectively.

In order to evaluate the potential of PEF for inducing lysis of *S. cerevisiae*, four treatments of different intensity were selected: a treatment that inactivated around 50% of S. *cerevisiae* (15 kV/cm, 45 μs), two treatments that inactivated around a 90% the population of *S. cerevisiae* at low (15 kV/cm, 150 μs) and high (25 kV/cm, 45 μs) electric fields and a treatment that inactivated around 99.9% the population of *S. cerevisiae* (25 kV/cm, 150 μs). **Figure 3** illustrates the inactivation obtained just after the PEF treatment (time 0) and the viability of the PEF treated *S. cerevisiae* cells along the incubation time. Statistically significant differences ($p < 0.05$) in the survivor number were not observed in the control cells and in cells treated with the most intense PEF treatments (15 kV/cm, 150 μs and 25 kV/cm for 45 and 150 μs) after 25 days of incubation. Conversely, the population of the *S. cerevisiae* cell treated at 15 kV/cm for 45 μs decreased progressively from day 3 to day 8 of incubation. Thus, after 8 days of incubation the number of viable cells in this suspension was similar to the number of viable cell in the suspensions containing cells treated at 15 kV/cm for 45 μs and 25 kV/cm for 150 μs. These results indicate that when PEF treatments are applied at low intensities a proportion of the population is injured rather than inactivated. Since incubation of the microorganisms in a buffer of pH 7.0 is not an optimal recovery condition, subletally injured cells of *S. cerevisiae* would not be able to repair this damage and they would dead during incubation. Inactivation by a subsequent incubation under non-favorable conditions of yeast and bacteria treated by PEF treatments of moderate intensity that did not cause a significant inactivation has been previously observed by other authors (Somolinos et al., 2007, 2008).

Figures 4A–C shows the evolution along the time of the absorbance at 600 nm of the yeast suspension and of UV absorbing material of the suspension medium at 260 and 280 nm, respectively, after the application of the PEF treatments.

Figure 4A shows that the decrease of abs_{600} of the yeast suspension was a function of the intensity of the PEF treatment applied. After 24 h of incubation the absorbance of the suspension containing untreated yeast was maintained constant: By contrast, the absorbance of the suspensions containing PEF treated yeast decreased around 25% for the cells exposed to treatment of 15 kV/cm for 45 μs and 62.5% for the cells treated at 15 kV/cm for 150 μs. Differences statistically not significant ($p < 0.05$) were observed in the abs_{600} decrease between this last treatment and both treatments applied 25 kV/cm. On the other hand, it should be noted that while further incubation of the suspensions containing cells treated at 15 kV/cm for 150 μs or at 25 kV/cm resulted in almost no changes in their abs_{600}, the abs_{600} of the suspension containing cells treated at 15 kV/cm for 45 μs progressively decreased until reaching the same value as the OD of the rest of suspensions after 6 days of incubation. After 25 days of incubation the decrease in abs_{600} of the suspension containing control cells was still 50% lower than the abs_{600} of the suspension containing cells treated by PEF.

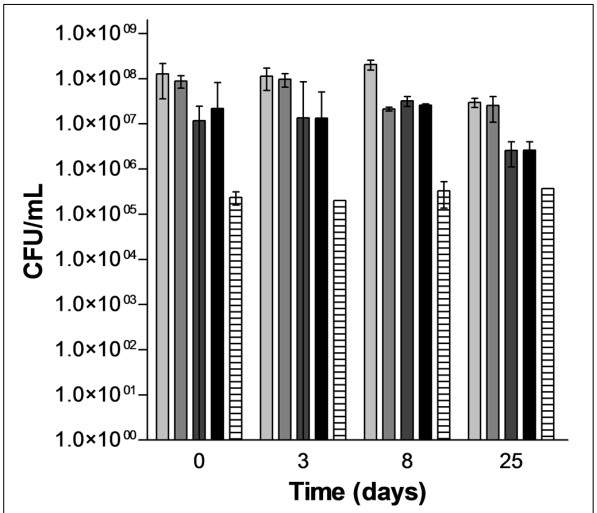

FIGURE 3 | Evolution of the population of untreated and PEF treated cells of *S. cerevisiae* along the incubation time. Untreated, 15 kV/cm 45 μs, 15 kV/cm 150 μs, 25 kV/cm 45 μs, 25 kV/cm 150 μs.

Measurement of UV absorbing substances at 260 and 280 nm was used as an index of the amount of intracellular components (mainly nucleic acids and proteins) leaking from cells after exposure to PEF. In the first moments after the treatment, the leakage of UV absorbing components as measured at both wavelengths was higher after the treatments carried out at higher electric field strength. The less severe treatment (15 kV/cm, 45 ms) resulted in an increase in 0.5 and 0.2 absorption units at 260 and 280 nm, respectively, after 24 h. By contrast, the rest of the treatments yielded increases of 0.95 and 0.43 units at 260 and 280 nm, respectively, and the absorbance values reached were maintained almost constant during all the incubation time.

Regarding the evolution of the absorption values at 260 and 280 nm of the medium containing the yeast cells treated at the lowest PEF treatment intensity, the values progressively increased until days 8–10 of incubation. The time required to reach the maximum absorbance values also corresponded with the time of incubation required for the death of cells treated at 15 kV/cm for 45 μs. Therefore, inactivation of the sublethal injured cells was accompanied by the release of nucleic acids and proteins to the extracellular environment. These observations suggest that PEF treatment applied in this investigation caused the formation of pores large enough to permit the leakage of molecules such as proteins that are much bigger that PI. On the other hand, results obtained indicate that the amount of molecules leaked was

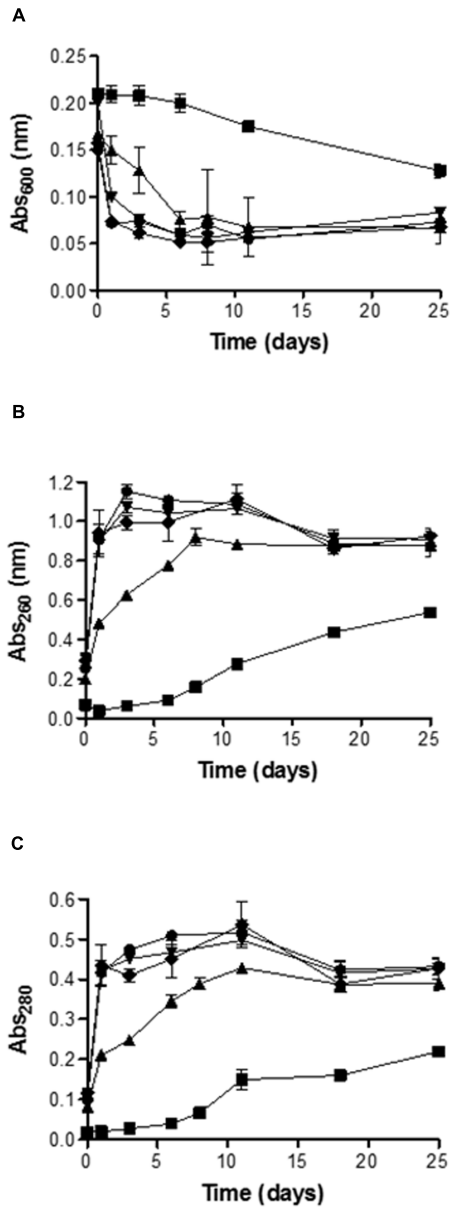

FIGURE 4 | Evolution along the time of the absorbance at 600-nm (A), 260-nm (B) and 280-nm (C) of the medium containing untreated and PEF treated cells of *S. cerevisiae*. Untreated (■), 15 kV/cm 45 μs (▲), 15 kV/cm 150 μs (▼), 25 kV/cm 45 μs (♦), 25 kV/cm 150 μs (●).

correlated with the proportion of dead cells in the suspension. However, no significant differences were observed in abs_{600} decrease or leakage of nucleic acids and proteins when the proportion of dead cells in the suspension was higher than the 90%.

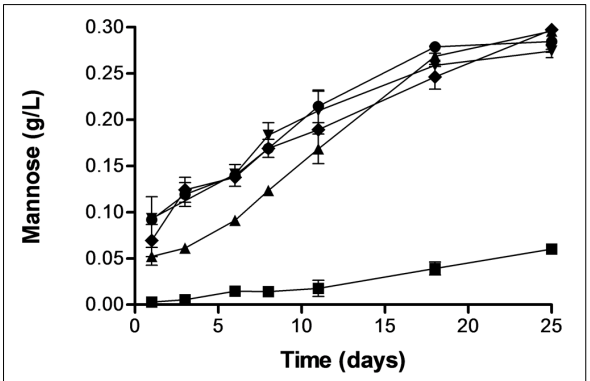

FIGURE 5 | Release of mannose from *S. cerevisiae* cells untreated and treated by PEF treatments of different intensity. Untreated (■), 15 kV/cm 45 μs (▲), 15 kV/cm 150 μs (▼), 25 kV/cm 45 μs (♦), 25 kV/cm 150 μs (●).

Release of Mannoproteins to the Extracellular Environment as Function of the Intensity of the Electric Field Strength Treatment

Release of mannoproteins to the extracellular media from suspensions containing untreated cells and PEF treated cells at the same treatment intensities described above was monitored by determining the mannose concentration of the supernatant after hydrolyzing the polymeric forms into monomeric sugar by addition of sulfuric acid. **Figure 5** shows that the concentration of polymeric mannose into the extracellular environment increased drastically along the time for the samples containing cells treated by PEF but mannose was hardly detected in the suspension containing untreated cells. After 25 days of incubation the concentration of mannose in the samples containing PEF treated cells of *S. cerevisiae* was 10 times higher than in the control. On the other hand, release of mannose in the sample containing cells of *S. cerevisiae* treated at 15 kV/cm for 45 μs was lower than in the rest of the samples containing cells treated by more intense PEF treatments. However, after 18 days of incubation no statistically significant differences ($p < 0.05$) in the polymeric mannose concentration were observed in all samples containing cells treated by PEF. Polymeric mannose release was a process slower than the decrease of the OD or the release of UV absorbing substances. In the samples treated by PEF the concentration of polymeric mannose in the extracellular environment clearly increased until the 18 days of incubation and them concentration remained almost constant.

The process of natural yeast autolysis begins as consequence of the disorganization of membranous systems of the cell, such as the cytoplasmic membrane and other organelles when that occurs with the death of the cell. This permits the endogenous enzymes to come in contact with cellular constituents which are degraded and render soluble. The enzymes glucanase and protease play a significant role in the degradation of cell wall constituents of the yeast and as consequence the cell wall becomes porous and

mannoproteins, among other cell wall constituents, are released into the surrounding medium (Alexandre and Guilloux-Benatier, 2006). Results obtained in this investigation confirm that natural autolysis is a slow process (Pérez-Serradilla and De Castro, 2008). Conversely, the electroporation of the yeast by PEF induced autolysis of the cells and a significant amount of mannoproteins were detected in the extracellular medium after only 24 h of incubation. Several mechanism related to electroporation could be involved in the induced autolysis by PEF. On the one hand, electroporation causes a water inlet in the cytoplasm, what has been demonstrated by the increment of the absorbance at 600 nm of the suspension. The decrease of the osmotic pressure in the cytoplasm as consequence of the water inlet could cause the plasmolysis of the organelles and the release of the enzymes. On the other hand, the electroporation of the cytoplasmic membrane by PEF could facilitate the contact of these enzymes with the outermost layer of the yeast cell wall were the mannoproteins are located.

According to observation in this investigation, PEF could be used in winemaking to accelerate the *sur lie* aging reducing the risk of microbial spoilage by yeast such as *Brettanomyces* and biogenic amine contamination or to obtain mannoproteins from yeast cultures to be used in winemaking. Furthermore, mannoproteins obtained by PEF induced yeast autholysis could be used for other applications in the food industry because these molecules have interesting emulsifying and stabilizing properties due to the amphipathic structure of the mannoprotein molecule (da Silva Araújo et al., 2014).

CONCLUSION

Results obtained in this study show the potential of PEF to induce autolysis in *S. cerevisiae* cells and to accelerate the release of mannoproteins to the extracellular medium. The major advantage of PEF, as compared to other process such as thermolysis, is that the lytic process occurs without thermal damage, thus avoiding the formation of odorant compounds reported by other authors when high temperatures are applied during the processing of yeast-derivated products (Münch and Schieberle, 1998; Pozo-Bayón et al., 2009).

AUTHOR CONTRIBUTIONS

JM: substantial contributions to the acquisition and analysis, data for the work; drafting the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GC: substantial contributions to the acquisition, analysis, data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IA: substantial contributions to the conception or design of the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JR: substantial contributions to the conception or design of the work; analysis, and interpretation; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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4.1.2. Factors influencing autolysis of *Saccharomyces cerevisiae* cells induced by Pulsed Electric Fields *(Manuscript II)*

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Factors influencing autolysis of *Saccharomyces cerevisiae* cells induced by pulsed electric fields

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ABSTRACT

The influence of temperature (7-43 °C), pH (3.5–7.0) and ethanol concentration (6–25%) on PEFinduced autolysis and the release of mannose from *Saccharomyces cerevisiae* was investigated. Changes in the release of intracellular compounds absorbing at 260 nm and 280 nm depended on storage conditions and differed among untreated and PEF-treated cells. For untreated cells, the increase of the Abs_{260} and Abs_{280} values during 3 weeks of storage was very low when incubated in media of different pH, different ethanol concentrations, or at 7° and 25 °C. Conversely, Abs_{260} and Abs_{280} values progressively increased for PEF-treated cells stored under the same conditions. Although the PEF treatment intensity was the same in all cases, the amount of intracellular material released depended on incubation conditions. Except for cells stored at 43 °C, for which the concentration of mannose in the media after 21 days was around 90 mg L^{-1}, the amount of mannose released from untreated cells after 21 days of storage was lower than 60 mg L^{-1} under all other conditions assayed. After the same incubation time, the amount of mannose released from PEF treated cells ranged from 80 mg L^{-1}, when they were stored in media with 25% ethanol, to 190 mg L^{-1} when they were stored at 43 °C. Interaction among assayed factors affecting mannose release was investigated in a medium containing 10% ethanol (v/v) and pH 3.5 for 21 days. Although the interaction of both factors delayed mannose release, the medium containing PEF-treated yeasts had approximately twice the amount of mannoproteins as those containing untreated yeasts.

1. Introduction

Yeast autolysis is an irreversible process that occurs following the death of the cell. This process consists in the self-degradation of the cellular constituents by the action of its own enzymes. As a consequence, the medium in which the cells are suspended is enriched by the compounds released from the degradation of intracellular constituents (Tao et al., 2014).

The autolysis of yeast is a process that is exploited in the food industry with the purpose of obtaining various ingredients, such as yeast extracts and autolysates. Yeast extract is used in soups, gravies, spreads, dressings, and meat products as a flavor contributor and flavor enhancer. Autolysates are similar to yeast extracts, but cell walls are not removed in the production process: texture and viscosity are thus enhanced, in addition to flavor. In the wine industry, on the other hand, the autolysis of yeast occurs during the production of wines with prolonged yeast contact ("ageing on lees"). This process leads to the release of mannoproteins from the cell wall. Mannoproteins are highly glycosylated proteins that have been associated with positive qualities and technological traits of wine such as haze reduction, the prevention of tartaric precipitation, mouthfeel contribution, the reduction of astringency, and the enhancement of the aroma and color of red wine (Charlier et al., 2007; Pérez-Serradilla and De Castro, 2008). Moreover, several studies have demonstrated that mannoproteins possess important emulsifying and stabilizing properties, owing to their amphipathic structure (Da Silva Araújo et al., 2014).

In industrial applications, yeast autolysis is induced by physical (heating, osmotic pressure modification, or alternate freezing and thawing) or chemical treatments (pH, detergents and antibiotics) with the purpose of reducing duration of the process to a few days. However, in the ageing-on-lees process, the autolysis of yeast takes

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place very slowly: the process lasts from a few months to several years (Alexandre and Guilloux-Benatier, 2006; Comuzzo et al., 2017).

Pulsed electric fields (PEF) is a technology that causes the loss of barrier function of cell membranes (electroporation) by applying intermittent electric fields of high intensity and extremely short duration (from μs to ms) (Barbosa-Canovas et al., 2001; Puertolas and Barba, 2016). Recently it has been demonstrated that PEF could serve as an alternative physical treatment to accelerate the release of mannoproteins (Martínez et al., 2016). The influence of the principal factors that may affect PEF-induced autolysis is nevertheless unknown.

The aim of this study was to investigate the influence of temperature, pH and ethanol concentration on PEF-induced autolysis and mannoprotein release from *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Culture conditions

A strain of *S. cerevisiae* for winery applications was used (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland). Yeasts were grown in 1000-mL glass flasks containing 600 mL of Sabouraud-Dextrose broth (Oxoid, Basingstoke, UK) under agitation at 25 °C. Yeast growth was monitored by measuring optical density at 600 nm, by microscopy counting using a Thoma counting chamber, and by plate counting in Potato-Dextrose-Agar (PDA) (Oxoid, Basingstoke, UK). The experiments were performed with cells in stationary growth phase, which was achieved after 48 h of incubation.

2.2. PEF treatment

The PEF equipment used in this investigation has been previously described by Saldaña et al. (2010). Prior to treatment, fresh biomass of *S. cerevisiae* was centrifuged at 3000 × *g* for 10 min at 25 °C and re-suspended in a citrate-phosphate McIlvaine buffer (pH 7.0; 1 mS/cm) to a final concentration of approximately 10^9 cells mL^{-1}. The *S. cerevisiae* suspension (0.44 mL) was placed in a static parallel treatment chamber (gap: 0.25 cm; diameter: 1.6 cm) by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium). Cells were subjected to 15 monopolar square waveform pulses of 3 μs of 25 kV/cm electric field strength, at room temperature and a frequency of 0.5 Hz. This treatment corresponds to a specific energy input of 56.25 kJ kg^{-1}. Sample temperature after treatment was lower than 30 °C.

2.3. PEF inactivation

After the PEF treatments, serial decimal dilutions in peptone water of the treated suspension were pour plated in PDA in order to monitor the degree of inactivation and the number of viable cells expressed in colony-forming units (CFU), corresponding to the number of colonies counted after 48 h of incubation at 25 °C.

2.4. Storage conditions of yeast after PEF treatments

Control and PEF-treated cells were re-suspended in citrate-phosphate McIlvaine buffer of different pH (3.5, 5.0 and 7.0), and stored at 25 °C. Cells re-suspended in McIlvaine buffer of pH 7.0 were also stored at different temperatures (7°, 25° and 43 °C) or with different ethanol concentrations (6, 12 and 25%, vol/vol; 25 °C). Control and PEF-treated cells were also incubated in a medium that simulated the composition of white wine (10% ethanol vol/vol; Tartaric Acid 4 g L^{-1}; Malic Acid 3 g L^{-1}; Acetic 0.1 g L^{-1}; pH 3.5; 25 °C), with the purpose of investigating the interaction of the presence of ethanol and low pH in PEF induced autolysis. Three samples were prepared for each storage condition.

2.5. Determination of yeast viability

The viability of cells along the storage period was determined by pour plating serial dilutions in PDA and counting the colony-formed units after 48 h of incubation at 25 °C.

2.6. Monitoring of cell autolysis and release of components

Control and treated samples were collected at different points in time during the storage period, which lasted three weeks. A series of measurements were performed in order to monitor the release of components and evidence cell lysis.

Absorbance at 260-nm (Abs_{260}) and 280-nm (Abs_{280}) of the supernatant was measured in order to monitor the release of intracellular components (Aronsson et al., 2005; Liu et al., 2013). These wavelengths correspond with the absorbance maxima of nucleic acids and proteins, respectively.

In addition, the concentration of mannoproteins in the extracellular medium was determined after hydrolyzing the supernatant with sulfuric acid (final concentration 1.5 M) at 100 °C for 90 min. Cooled samples were neutralized with NaOH 3 M. Quantitative analysis of mannose was conducted by an enzymatic method (D-Mannose, D-Fructose and D-Glucose assay procedure, Megazyme International, Wicklow, Ireland) (Dupin et al., 2000).

2.7. Statistical data treatment

The results represent the mean and standard errors of the mean of three independent experiments.

3. Results

In order to evaluate the effect of different factors on PEF-induced autolysis and mannoprotein release, a suspension of 10^8 CFU mL^{-1} of *S. cerevisiae* was subjected to PEF treatment that caused the inactivation of around 90% of the population (1-Log_{10} cycle). After the treatment, untreated and PEF treated cells of *S. cerevisiae* were suspended for three weeks in media of different pH, in media containing different ethanol concentrations, and in a media of pH 7 at different temperatures. During incubation, yeast cell viability, changes in Abs_{260} and Abs_{280} and mannose release were monitored.

3.1. Yeast cell viability over time at different incubation conditions

The evolution of the number of survivors over time for untreated and PEF treated *S. cerevisiae* cells incubated under different conditions is shown in Fig. 1. Yeast growth was not observed under any conditions whatsoever: the population of *S. cerevisiae* either remained stable or decreased over time in the case of both untreated and PEF treated cells, depending on the incubation condition assayed. Changes in the number of survivors across incubation time were not observed for the untreated or PEF-treated cells when incubated at pH 5.0 and 7.0 (Fig. 1A) or at 7 °C and 25 °C (Fig. 1C). By contrast, a loss of viability across incubation time was observed for all the other incubation conditions. This decrease in the number of viable cells was generally greater for those populations previously treated with PEF. For example, the number of viable cells did not decrease for the untreated population in the medium of pH 3.5, but an additional 1.5 Log cycle of inactivation was observed in the population treated by PEF after 21 days of incubation. Incubation in

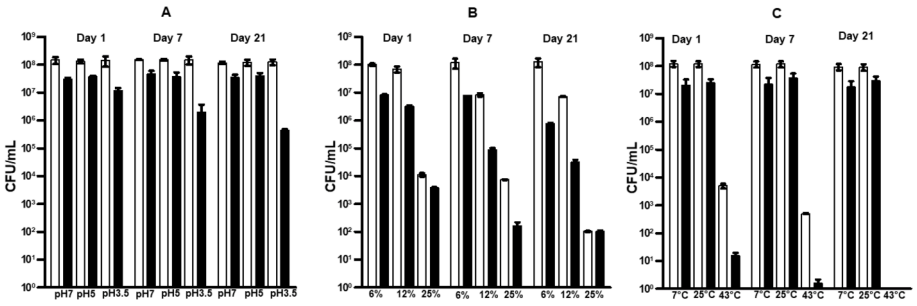

Fig. 1. Evolution over time of the population of untreated (white bars) and PEF-treated (black bars) cells of *S. cerevisiae* stored at different pH (A), different ethanol concentrations (B) and at different temperatures (C). Different letters correspond to statistically significant differences ($p < .05$).

media (pH 7.0) containing 6, 12 and 25% of ethanol or at 43 °C affected yeast viability: the population reduction was greater in the case of PEF treated cells. These data support results obtained by other authors, who indicate that PEF treatment may cause sublethal injury in a proportion of the microbial population (Cebrián et al., 2015; Garcia et al., 2005; Somolinos et al., 2007). It is well known that sublethally injured microorganisms are not able to recover damages when incubated under non-optimal conditions, and they die in the course of time.

3.2. Release of intracellular compounds to the media containing cells of S. cerevisiae under different incubation conditions

Among other effects, yeast autolysis leads to a disorganization of the cytoplasmic membrane that allows hydrolytic enzymes (glucanases and proteinases) located in the cytoplasm to interact with cell wall polymers, leading to the release of compounds such as mannoproteins to the extracellular environment. The presence in the extracellular environment of compounds absorbing at 260 nm (Fig. 2) and 280 nm (Fig. 3) was used to monitor the permeability increment of the cytoplasmic membrane of untreated and PEF-treated cells of *S. cerevisiae* under different incubation conditions over time.

As is shown in Figs. 2 and 3, the release of specific intracellular compounds absorbing at 260 nm (nucleic acids) and at 280 nm (proteins/peptides) was also a function of the PEF treatment and of storage conditions. For control cells, the increase in Abs_{260} and Abs_{280} values after 3 weeks was very low when incubated in media of different pH, different ethanol concentrations, or at 7 °C and 25 °C. Such low release of these compounds was observed even when incubation conditions caused a significant decrease in cell viability. For example, after 7 days of incubation, the population of control cells decreased by 1 and 4-Log_{10} cycles when stored in media containing 12% and 25% of ethanol respectively, whereas Abs_{260} and Abs_{280} barely increased when compared with storage conditions in which loss of viability was not observed (6% of ethanol). For untreated cells stored at 43 °C, a significant absorbance increase at 260 and 280 nm was observed, whereby the release of UV-absorbing material measured after 3 weeks of storage was similar to that observed for PEF treated cells stored under the same conditions. These results seem to indicate that when untreated cells are stored at 43 °C, inactivation would be accompanied by a certain amount of increase in permeability of the cytoplasmic membrane, thereby allowing the release of UV-absorbing compounds. However, it should be noted that the significant increment of UV-absorbing material in the extracellular media took place after the loss of cell viability. During the first 24 h of incubation at 43 °C, 99.99% of the population was inactivated; however, 3 days of incubation were required to detect substantial release of UV-absorbing compounds to the extracellular media.

The increase in Abs_{260} and Abs_{280} values was much more rapid and attained higher values for PEF treated cells than for untreated ones. The maximum absorbance values attained depended on storage conditions. The greatest release of compounds absorbing at 260 and 280 nm was obtained at pH 7.0, and when cells were incubated at 43 °C. In the presence of ethanol, the maximum release of those compounds from PEF-treated cells was lower than in the other media, and the influence of ethanol concentration on this event was very low. These results indicate that, although the same PEF treatment was applied to cells before storing them under

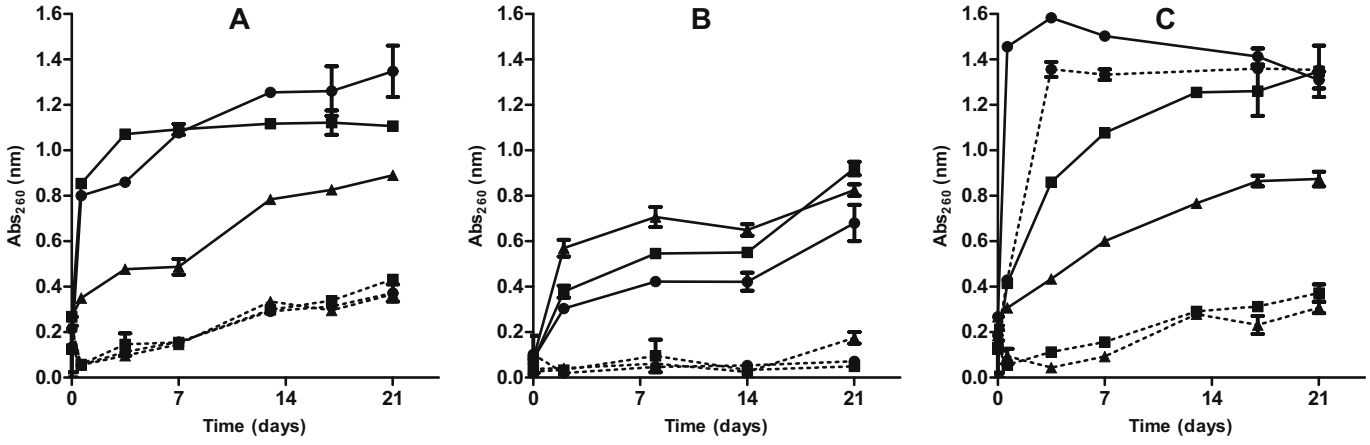

Fig. 2. Evolution over time of the absorbance at 260 nm of the media containing untreated (dotted lines) and PEF (continuous lines) treated cells of *S. cerevisiae* stored at different pH (A): 3.5(▲), 5.0(■) and 7.0(●); ethanol concentrations (B): 6%(▲), 12%(■) and 25%(●) and different temperatures (C): 7 °C (▲), 25 °C (■) and 43 °C (●).

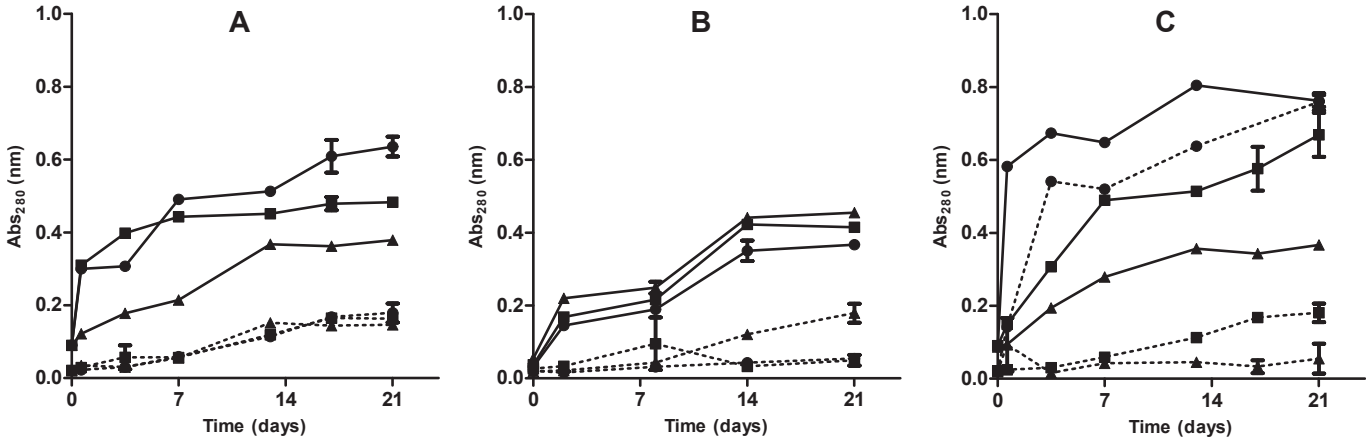

Fig. 3. Evolution over time of the absorbance at 280 nm of the media containing untreated (dotted lines) and PEF (continuous lines) treated cells of *S. cerevisiae* stored at different pH (A): 3.5(▲), 5.0(■) and 7.0(●); ethanol concentrations (B): 6%(▲), 12%(■) and 25%(●) and different temperatures (C): 7 °C (▲), 25 °C (■) and 43 °C (●).

different conditions, the diffusion of cytoplasmic compounds through the pores, or the presence of local defects caused by PEF both depend on incubation conditions.

3.3. Release of mannoproteins from untreated and PEF treated cells of S. cerevisiae under different incubation conditions

Mannoproteins that form the outer cell wall layer are highly glycosylated with a carbohydrate fraction of mannose (Bowman et al., 2006; Klis et al., 2002). In order to obtain an indicative value of mannoprotein release during yeast autolysis, several different procedures are employed to determine the concentration of mannose in the extracellular media after acid hydrolysis (Dalliest et al., 1998; Quiros et al., 2012).

The release of mannose to the extracellular environment from untreated and PEF-treated *S. cerevisiae* cells as a function of incubation conditions during three weeks is shown in Fig. 4. Under all investigated storage conditions, the concentration of mannose in the media in which yeast cells were suspended was higher when they had been previously treated by PEF, as observed in the release of intracellular compounds absorbing at 260 nm and at 280 nm. The concentration of mannose in the media containing untreated cells stored at 43 °C was around 90 mg L^{-1} after 21 days of storage, but the amount of mannose released from untreated cells stored under all other conditions was lower than 60 mg L^{-1}. After the same incubation time, the amount of mannose released from PEF-treated cells ranged from 80 mg L^{-1} (in the case of cells stored in media with 25% ethanol) to 190 mg L^{-1} (when cells stored at 43 °C).

In order to compare morphological changes occurring in untreated and PEF-treated yeast cells during storage under different conditions, the cells were observed under optical microscopy. Fig. 5 compares the morphology of stationary-phase yeast cells prior to storage in pH 7.0 buffer (control cells) (A) with the morphology of untreated (B) and PEF-treated (C) yeast after 21 and 7 days of storage in pH 7.0 buffer, respectively. Fig. 5A shows the typical yeast morphology, which consists in elongated, ovoid cells with a large vacuole whiting the cytoplasm. After incubation, the volume of

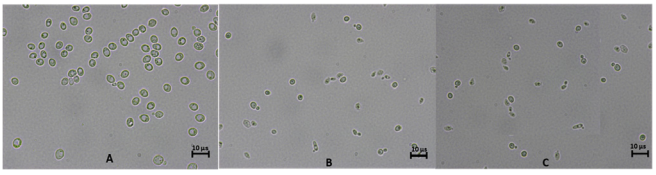

Fig. 5. Optical microscopy observation (600x) of control cells at stationary growth phase (A), untreated cells stored 21 days in pH 7.0 buffer (B) and PEF treated cells stored 7 days in pH 7.0 buffer (C) of *S. cerevisiae*.

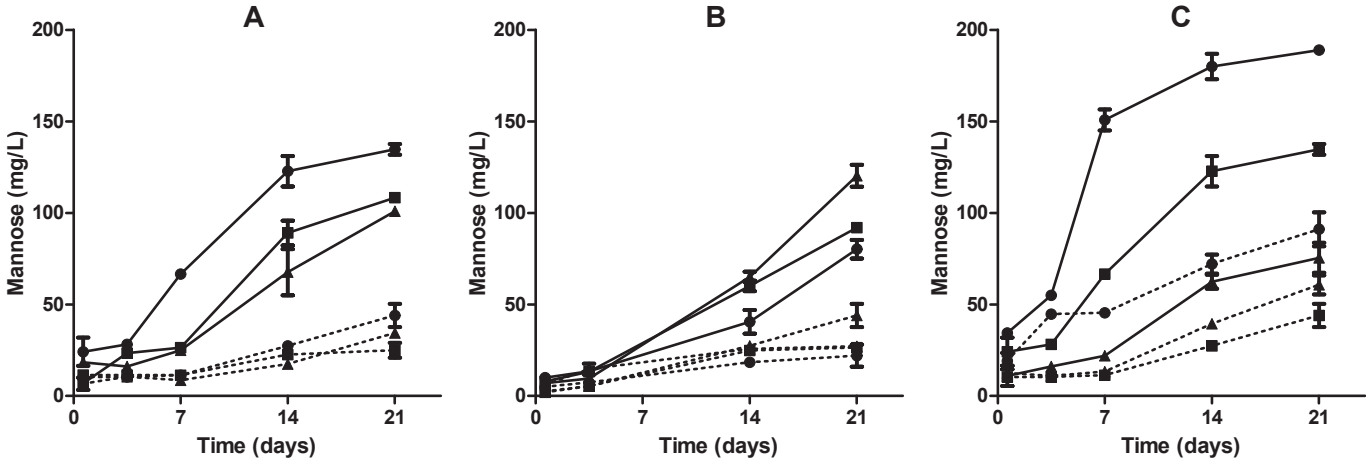

Fig. 4. Release of mannose from *S. cerevisiae* cells, both untreated (discontinuous lines) and PEF treated (continuous lines), stored at different pH (A): 3.5(▲), 5.0(■) and 7.0(●); ethanol concentrations (B): 6%(▲), 12%(■) and 25%(●) and different temperatures (C): 7 °C (▲), 25 °C (■) 43 °C (●).

untreated (5B) and PEF treated (5C) yeast cells was much smaller due to the release of cytoplasm content that takes place during autolysis. Although PEF treatment accelerated the release of intracellular compounds and manoproteins from the cell wall, no significant morphological changes different from those occurring in the untreated yeast were observed.

One of the major applications of yeast autolysis in the food industry is the enrichment of wine in mannoproteins during a process called “aging on the lees”. In the course of this operation, the wine is maintained in contact with the yeast that participated in its fermentation process. In order to ascertain whether PEF could be an effective treatment to accelerate the aging-on-lees stage, the release of mannose in a medium containing several organic acids and in which pH and ethanol content was similar to white wine was investigated. As shown in Fig. 6, the amount of mannose released was higher in the medium containing PEF-treated yeast. The total amount of mannose released after 21 days of incubation was lower than the amount detected in media of pH 3.5 or in media with 12% of ethanol: this indicates that the combination of both factors could act synergistically to hinder mannoprotein release. In any case, the yeast treated with PEF released approximately twice the amount of mannose than untreated yeast, thereby confirming the potential of PEF to accelerate the aging-on-lees stage in winemaking.

4. Discussion

The process of autolysis is associated with cell death, which is required to initiate the process of degradation of the cell’s constituents by the action of its own enzymes. Since the natural autolysis of yeast caused by aging is a slow process, several physical procedures that cause microbial inactivation have been assayed as triggers for the process (Comuzzo et al., 2017; Liu et al., 2015; Martín et al., 2013). It has been recently demonstrated that PEF accelerates a series of events that occur during yeast autolysis such as the release to the extracellular environment of compounds absorbing at 260 and 280 nm, as well as mannoproteins (Martínez et al., 2016). This effect has been attributed to the electrical breakdown of the cytoplasmic membrane when exposed to a sufficiently strong electric field that renders it permeable to molecules that would otherwise be unable to cross it. One can therefore assume that the electroporation of the cytoplasmic membrane caused by PEF encourages the release of cell wall components by facilitating the contact of hydrolytic enzymes located in intracellular structures with the cell wall. As compared with other physical microbial cell disruption methods such as bead milling, sonication, or high-pressure homogenization, PEF does not provoke a mechanical destruction of the cells (Fig. 5C). Therefore a release of selective products and cell debris which might impinge upon the purity of products derived from yeast autolysis should not take place (Comuzzo et al., 2012).

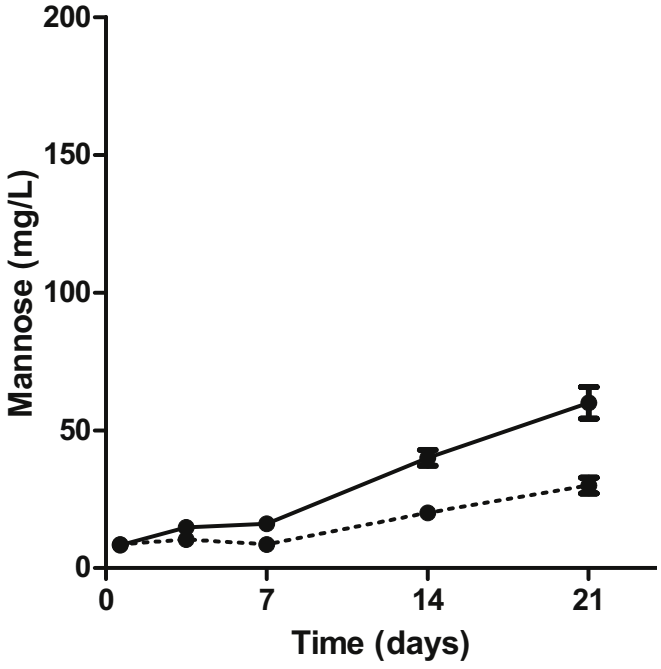

Fig. 6. Release of mannose from untreated (discontinuous lines) and PEF-treated (continuous lines) *S. cerevisiae* cells stored in a medium of pH 3.5 containing ethanol (10%).

Similarly to what has been observed by other authors studying natural autolysis, this investigation shows that induced autolysis triggered by PEF is also influenced by several factors that affect cell viability and enzymatic activity. Although it has been reported that natural yeast autolysis is strongly influenced by pH, the presence of ethanol, or incubation temperature, the effect of those factors on the release of manoproteins and compounds absorbing at 260 and 280 nm was only observed in this study at the highest incubation temperature (43 °C) for the control cells. This was probably due to the fact that 21 days is too short a period to induce natural autolysis. After 3 weeks of incubation, loss of viability was not detected after most of the incubation conditions assayed. Excluding incubation at 43 °C, in all other incubation conditions the population decreased between 1 and 6 log cycles at pH 7. Nevertheless, that loss of viability did not lead to substantial release of intracellular compounds, which indicates that cell death was not associated with an increment of cytoplasmic membrane permeabilization. It has been previously reported that microbial inactivation is not always associated with an immediate increase in membrane permeabilization (Virto et al., 2005). The rapid release of intracellular compounds from the control cells incubated at 43 °C indicates that an increment of cytoplasmic permeability is indeed involved in yeast inactivation at that higher temperature.

The characteristic mechanism of electroporation explains the rapid increment observed in the concentration of intracellular compounds in the extracellular media. Although the yeast cells were submitted to the same PEF treatment in all cases, the amount of release of intracellular compounds depended on incubation conditions. Those conditions could have an effect on the disorganization of the electroporated membrane of the yeast cell by enzymatic lysis, or on the progress of the size of the pores caused by electroporation over time which, as has been reported by other authors, may depend on the media in which cells are suspended (Luengo et al., 2015; Saulis, 2010; Vorobiev, 2006). Regarding the temperature factor, its effect on mass transfer could also exert an influence on the release rate of intracellular compounds.

Mannoprotein release requires that enzymes, mainly located in vacuoles of the cytoplasm, achieve access to the cell wall in order to degrade its constituents. Similarly to that which occurred with the release of intracellular compounds, substantial mannoprotein release was not observed for control cells. This was probably due to the fact that longer incubation times are required for the disorganization of the organelle and cytoplasmic membranes (Alexandre y Guilloux-Benatier, 2006; Fornairon-Bonnefond, 2002). On the other hand, the electroporation of the cytoplasmic membrane caused by PEF would decrease the time that hydrolytic enzymes require to achieve contact with the cell wall and, as a consequence, accelerate the release of mannoproteins to the extracellular environment. It is important to remark that the increment in permeability of the cytoplasmic membrane that permits the contact of hydrolitic enzymes with the cell wall is not the only precondition

for mannoprotein release. It is well known that enzymatic activity is highly dependent on environmental conditions. Therefore, the differences observed in the amount of mannoproteins released from the PEF-treated cells suspended in different conditions seem to be a consequence of both the evolution in quantity and size of the pores caused by the PEF treatment along storage – which may influence the time required until the hydrolytic enzymes contact the cell wall – and the effect of storage conditions on enzymatic activity.

In conclusion, this study has demonstrated that the autolysis induced by PEF and the subsequent release of manoproteins from the yeast cell wall are influenced by different well-known factors that likewise affect natural autolysis. However, independently of the conditions in which autolysis occurred, the release of mannose was more rapid when the yeast cells were previously treated by PEF. Therefore, PEF treatment may turn out to be an effective procedure to reduce the time for obtaining mannoproteins from yeast, or for shortening the ageing-on-lees process that occurs during the elaboration of certain wines. The possibility of submitting large volumes in continuous flow to PEF treatment, and the low energy consumption required to electroporate yeast, are two key advantages that could enable PEF technology to become a commercially viable method capable of accelerating yeast autolysis.

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4.1.3. Pulsed Electric Fields accelerate release of mannoproteins from *Saccharomyces cerevisiae* during aging on lees of *Chardonnay* wine *(Manuscript III)*

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Pulsed electric fields accelerate release of mannoproteins from *Saccharomyces cerevisiae* during aging on the lees of Chardonnay wine

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ABSTRACT

The potential of PEF for triggering autolysis of *Saccharomyces cerevisiae* and accelerating the release of mannoproteins during aging on the lees of Chardonnay wine was evaluated.

Release of mannoproteins in *Chardonnay* wine increased drastically in samples containing PEF-treated (5 and 10 kV/cm, 75 μs) yeasts. No mannoprotein release was observed in the first seven days of aging on the lees in wine containing untreated yeast; however, after the same time interval, the concentration of those compounds increased by 40 and 60% in wines containing yeast treated by PEF at 5 and 10 kV/cm, respectively. After 30 days of incubation, the mannoprotein concentration in wines containing yeast treated under the most intense PEF conditions reached the maximum value. Control cells, on the other hand, required six months to reach that maximum level.

Chromatic characteristics, total polyphenol index, total volatile acidity, pH, ethanol, and CIELAB parameters of the wine were not affected during aging on the lees with untreated and PEF-treated yeast. On the other hand, the capability of the mannoproteins released from yeast treated by PEF for decreasing wine turbidity, foaming, and interacting with tannins was similar to that of those released from untreated yeast; the differences observed were a consequence of the varying concentration of mannoproteins.

The result obtained demonstrates that PEF permits the acceleration of the aging-on-lees step while avoiding or reducing the problems associated with it. To achieve this effect, intense treatment is not required. Therefore, wineries could process lees by using the most economical PEF devices on the market.

1. Introduction

Mannoproteins are highly glycosylated proteins which constitute the major component of the cell wall in yeast. It is well known that their presence in wine produces positive effects such as haze formation reduction, the prevention of tartaric salt precipitation, the diminution of astringency, and the improvement of mouthfeel, aroma intensity, and color stability (Pérez-Serradilla & De Castro 2008).

Traditionally, the mannoprotein enrichment of certain types of wines occurs during yeast autolysis in the "aging on lees" step. In this practice, the wine is deliberately left in contact with the lees sediment (mainly composed of yeast). Autolysis causes disorganization of membranous systems and thus permits the release of enzymes such as glucanase and proteinase, thereby leading to the degradation of the cell wall and the subsequent release of mannoproteins into the wine. This process, associated with yeast death, is very slow – lasting from a few months to years (Alexandre & Guilloux-Benatier 2006).

Aging over lees is traditional practice in the manufacture of white wines fermented in barrels (Bourgogne wines), natural sparkling wines (champagne, cava), and in French "vin jaune" flor sherry wine, a white wine that spends six years in an oak barrel under a velum of *S. cerevisiae* (Palomero, Morata, Benito, González, & Suárez-Lepe 2007). The released mannoproteins, together with glucans and cytoplasmic compounds such as proteins, peptides, amino acids, fatty acids and nucleotides during autolysis, provide the peculiar properties that have made these wines so renowned (Charpentier, Santos, & Feuillat 2004).

Aging on the lees is a technique that requires considerable investment on the part of wineries in equipment (tanks, barrels), entails elevated labor costs (periodic stirring – *bâtonnage* – and sensorial analyses), and implies immobilization of winery stocks. Furthermore, this practice may negatively affect wine quality (Palomero, Morata, Benito, Calderón, & Suárez-Lepe 2009). Periodic stirring required during natural autolysis to maintain the lees sediment in suspension increases the risk of wine oxidation. The storage of wines in contact with dead yeast

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during long periods has been associated with increased bacterial contamination and *Brettanomyces* colonization. Although manufacturers have a great interest in accelerating this process, enological procedures aiming to increase the mannoprotein content of wine are not yet fully established; they can be time–consuming, or they can exceedingly augment production costs (Pérez-Serradilla & De Castro 2008).

Different strategies have been suggested for the acceleration of yeast autolysis, including enzymes capable of hydrolyzing β-glucans from yeast cell walls, thermolysis, or mechanical methods for large-scale disruption of microbial cells (such as ultrasound, microwaves, and high pressure homogenization) (Martínez, Cebrián, Álvarez, & Raso 2016). Another interesting alternative can be found in the use of selected yeasts that over-produce mannoproteins, but this selection criterion is difficult to accomplish; moreover, the use of genetically modified organisms in food applications, particularly in wine, would limit the usefulness of such approaches (Pérez-Través, Querol, & Pérez-Torrado 2016).

Pulsed Electric Fields (PEF) is a technology that provokes the increment of the permeability of cytoplasmic membrane of cells (electroporation) via the application of pulses of high electric field strength (kV/cm) and short duration (μs-ms). It has been recently proven that PEF trigger the autolysis of *S. cerevisiae* and the accelerated release of mannoproteins in buffer (Martínez et al. 2016). The extension of that effect depends on different factors, such as temperature, pH, or presence of ethanol (Martínez et al. 2017).

The aim of the present study was to investigate the potential of PEF for triggering autolysis of *S. cerevisiae* and accelerating the release of mannoproteins during aging on the lees of *Chardonnay* wine.

2. Material and methods

2.1. Winemaking

Chardonnay grapes (200 kg) were received in our laboratory, destemmed and crushed, then pressed to extract the must. After the clear must was racked, it was inoculated with a strain of *Saccharomyces cerevisiae.* Alcoholic fermentation of must was conducted at 18 °C during 10 days.

2.2. Yeast strain

The yeast used in this investigation derived from a strain of *S. cerevisiae* from an industrial preparation (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland). For the experiments involving inactivation and aging on lees, the *S. cerevisiae* cells derived from the same yeast which participated in the fermentation of Chardonnay grapes performed in our laboratory. Once fermentation had taken place, the yeasts were collected by removing the wine after sedimentation of the lees. A concentrated yeast suspension (1.5×10^9 cells mL^{-1}, conductivity: 1 mS cm^{-1}) was directly used for the PEF treatments or maintained untreated, and then mixed with the wine for aging-on-lees.

2.3. PEF equipment

The PEF equipment used in this investigation (Modulator PG, ScandiNova, Uppsala, Sweden) generates square waveform pulses of a width of 3 μs and a frequency of up to 300 Hz. The maximum output voltage and current were 30 kV and 200 A, respectively. The actual voltage and current intensity applied were measured using a high-voltage probe (Tektronix, P6015A, Wilsonville, OR, USA) and a current probe (Stangenes Industries Inc. Palo Alto, CA, USA), respectively, connected to an oscilloscope (Tektronix, TDS 220, Wilsonville, OR, USA).

Treatments were performed in continuous mode. An eight-roll peristaltic pump (Ismatec, Glattbrugg, Switzerland) was used to pump the lees from the reservoir through silicone tubes to the treatment chamber, which consisted of two parallel stainless steel electrodes with a gap of 5.5 mm and an electrode area of 2.2 cm^2. The flow rate was set at 3.5 L h^{-1}, and the calculated mean residence time in the treatment chamber was 1.24 s. Frequency was calculated by dividing the number of pulses by the residence time. A heat exchanger consisting in a stainless steel coil submerged in a thermostatic batch was used to set the initial temperature of the suspension before the treatment. Temperature of the lees suspension was measured with thermocouples located before and after the heat exchanger, and just after the PEF treatment chamber.

The specific energy input (W) per pulse expressed in kJ kg^{-1} pulse $^{-1}$ was calculated by the following equation (Eq. 1):

$$W = m \cdot V \cdot I \cdot t \quad (1)$$

where m (kg) is the mass of the lees suspension contained in the volume of the treatment chamber; V is the input voltage (kV); I is the current intensity (A); and t is the pulse width (μs). The total specific energy was calculated by multiplying the specific energy input per pulse by the number of pulses.

2.4. PEF treatments

2.4.1. Evaluation of the resistance to PEF of S. cerevisiae in Chardonnay wine

S. cerevisiae cells suspended in the wine were PEF-treated at electric field strengths between 5 and 25 kV/cm along treatment times between 30 and 105 μs, which represent frequencies between 8 and 28 Hz and total specific energies between 0.85 and 72.71 kJ kg^{-1}. The initial temperature before the chamber was set to 20 °C, and the final temperatures after the treatments ranged between 20.2 and 37.98 °C. PEF-treatments were performed in triplicate.

After the treatments, serial decimal dilutions were pour-plated in Potato-Dextrose-Agar to monitor inactivation. 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. Inactivation data was expressed as the ratio between the initial number of survivors (No) and the number of survivors after different treatment times (Nt).

2.4.2. Evaluation of PEF treatments designed to accelerate autolysis during aging-on-lees

Two different PEF treatments were selected for the aging-on-lees experiments: one that did not cause inactivation (5 kV/cm, 75 μs) and another that inactivated 1 log cycle (10 kV/cm, 75 μs). These treatments correspond to a total specific energy of 2.14 kJ kg^{-1} and 9.17 kJ kg^{-1}, respectively. Aging on lees was performed in triplicate for each one of the conditions. A concentrated yeast suspension (1.5×10^9 cells mL^{-1}) was PEF-treated in continuous mode at the two different intensities. After that, aliquots of the three different lots containing lees (10 kV/cm-75 μs; 5 kV/cm-75 μs and untreated) were dispensed in 20 L tanks and filled with the same Chardonnay wine that had been fermented in our pilot plant. Yeast concentration in wine was 1.5×10^8 cells mL^{-1}. The tanks were stored at 18 °C for 6 months. To avoid rotting, the sediment of lees at the bottom of the deposits was periodically re-suspended by smoothly shaking the wine.

2.5. Monitoring of mannoproteins release

Release of mannoproteins from untreated (control) and PEF-treated (5 and 10 kV/cm, 75 μs) yeast of *S. cerevisiae* was monitored along the aging-on-lees storage period. Samples were periodically collected and centrifuged (3000 × *g* 5 min), and the mannoprotein concentration in the supernatant of the wine was determined after hydrolyzing it with sulfuric acid (final concentration 1.5 M) at 100 °C for 90 min. Cooled samples were neutralized with NaOH 3 M. Quantitative analysis of mannose was conducted by an enzymatic method (D-Mannose, D-

Fructose, and D-Glucose assay procedure, (Megazyme International, Wicklow, Ireland) (Dupin et al. 2000).

2.6. Analysis of wines subjected to aging-on-lees periods of varying length

After 1, 2, 3, or 6 months of aging on lees, samples were collected from the deposits containing untreated or PEF-treated yeasts. After centrifugation, lees were eliminated, and the supernatants were bottled. Different analyses were performed on the wines obtained.

Volatile acidity, pH, and ethanol concentration analysis were performed according to the specifications established by the Organisation Internationale de la Vigne et du Vin (2005). **Total polyphenol index** and total tannin content were determined using a Libra S12 spectrophotometer (Biochrom, UK). The total polyphenol index (TPI) was measured by directly reading the absorbance of diluted wine 1/20 (*v*/v) at 280 nm (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu 2006).

The chromatic characteristics of the wines were determined by directly measuring their absorbance at 420, 520, and 620 nm using a spectrophotometer (Libra S12) with a 10-mm path-length quartz cuvette. Color intensity (CI) was calculated as the sum of absorbance at 420, 520, and 620 nm. Tint was determined as the proportion of absorbance measured at 420 and 520 nm (Glories 1984; Sudrau 1958).

CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, H^*) were determined using the original MSCV software (Pérez-Caballero, Ayala, Echávarri, & Negueruela 2003) according to regulations established by the Commission Internationale de l'Eclairage (Commission Internationale de l'Eclairage (CIE) 1986), which include L^* (lightness), a^* (red-green coordinate), b^* (yellow-blue coordinate), C^* (chroma), and H^* (hue).

Turbidity measurements of the wines after centrifugation (3000 × *g*; 5 min) and removal of lees were performed using a turbidimeter (HI 83749, Hanna Instruments, Woonsocket, USA).

The **forming foam** capacity of the different wines was also measured. Aliquots of 40 mL were dispensed in 50-mL volumetric flasks until the pear-shaped part was filled. After filling, the flask was flipped 5 times, immediately after which the foam height in the neck of the volumetric flask was measured.

Quantification of **condensed tannins** was measured by precipitation with methyl cellulose according to Sarneckis et al. (2006), and results were expressed as epicatechin equivalents.

2.7. Statistical data treatment

The results represent the mean ± standard error of the mean of three replicates of treatments analyzed in triplicate. A one-way ANOVA test was conducted to assess significant differences between treatments. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. PEF inactivation of S. cerevisiae in Chardonnay wine as a function of electric field strength and treatment time

The inactivation along time of *S. cerevisiae* suspended in white wine after exposure to PEF treatments of varying electric field strengths (5–25 kV/cm) is shown in Fig. 1. As reported above, inactivation increased with electric field strength and treatment duration. Under electric fields of 10 kV/cm or higher, a very rapid inactivation was observed after the first 30 μs (10 pulses of 3 μs), after which the number of survivors decreased more gradually as treatment time increased. This treatment duration inactivated around 0.7, 1.7 and 2.5 log cycles of the population of *S. cerevisiae* when applied at 10, 15 and 20 kV/cm respectively, but a further 75-μs increment in treatment time only brought about an increased inactivation of 0.3 to 0.7 $\log_{10}$ cycles.

Yeast growth in the must during wine fermentation and subsequent treatment in the same wine significantly affected PEF resistance as compared with results reported by other authors when yeast were grown in cultivation media and treated in buffer. As shown in Fig. 1, although a treatment of 5 kV/cm did not inactivate the strain of *S. cerevisiae* used in this investigation, an inactivation of around 1 $\log_{10}$ cycle was observed after a treatment at 10 kV/cm that lasted 75 μs. To obtain that level of inactivation when the same yeast strain was cultivated in a Sabouraud-Dextrose broth and treated in McIlvaine buffer of pH 7, it was necessary to increase the electric field to 15 kV/cm, and to extend the treatment time to 120 μs (Martínez et al. 2016). Aronsson, Rönner, and Borch (2005) also reported treatment conditions of the same order to achieve 1-$\log_{10}$ cycle of inactivation of another strain of *S. cerevisiae* in phosphate buffer (pH 7.0). In addition to the fact that the strain used in this investigation was grown for 7 days in the fermenting must, the low pH and the presence of ethanol in the wine in which the cells were treated could explain the lower PEF resistance displayed by our strain. It has been previously reported that both factors affect microbial inactivation by PEF (Aronsson & Rönner 2001; Puértolas, López, Condón, Raso, & Álvarez 2009).

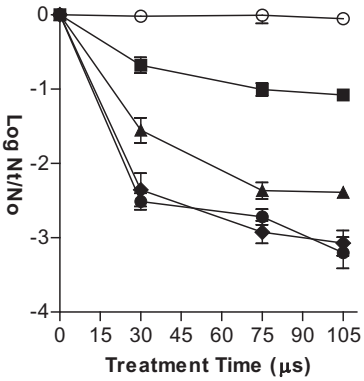


Fig. 1. Inactivation of *Saccharomyces cerevisiae* by Pulsed Electric Felds (PEF) treatment of different electric field strengths. 5 kV/cm (○), 10 kV/cm (■), 15 kV/cm (▲), 20 kV/cm (◆), 25 kV/cm (●). Inactivation data was expressed as the ratio between the initial number of survivors (No) and the number of survivors after different treatment times (Nt).

Previous studies in which the potential of PEF for triggering *S. cerevisiae* autolysis and accelerating mannoprotein release has been demonstrated show that an inactivation of > 90% of the population (1-$\log_{10}$ cycle) was not required in order to achieve the highest rate of mannoprotein release; even treatments that did not affect yeast viability (determined by colony count in PDA) were nonetheless capable of triggering autolysis (Martínez et al. 2016). In accordance with those results, two treatments were selected to evaluate the release of mannoproteins from PEF treated *S. cerevisiae* cells in Chardonnay wine: one which did not cause inactivation of the yeast population (5 kV/cm, 75 μs, 2.14 kJ kg^{-1}) and another which brought about a 90% reduction thereof (10 kV/cm, 75 μs, 9.17 kJ kg^{-1}). Although the specify energy of this last treatment was slightly greater that the specific energy of treatments at higher electric field with similar or higher inactivation, the treatment with the lowest electric field to obtain the target inactivation (90% of the population) was selected. Lower requirement in electric field strength for microbial inactivation by PEF is an issue of interest for practical applications on an industrial scale. This procedure implies the possibility of using lower energetic inputs, less powerful PEF modulators and as consequence a considerable reduction in costs.

3.2. Release of mannoproteins from lees in Chardonnay wine

Release of mannoproteins during yeast autolysis is generally monitored by determining the concentration of mannose in the extracellular media after acid hydrolysis (Dallies, Francois, & Paquet 1998; Quirós, Gonzalez, & Morales 2012). Release of mannose to the wine from untreated cells and PEF-treated cells at 5 kV/cm and 10 kV/cm for 75 μs

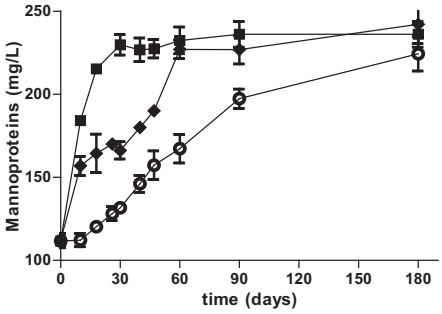


Fig. 2. Release of mannoproteins in Chardonnay wine from *S. cerevisiae* cells untreated and treated by PEF treatments of different intensity: 5 KV/cm 75 μs (♦); 10 kV/cm 75 μs (■) or control cells (○).

during the aging on lees of Chardonnay wine is shown in Fig. 2.

Mannose concentration in the wine previous to aging on lees was 110 mg/L. It is well known that mannoproteins are released in the course of alcoholic fermentation by the fermenting yeast, thereby increasing their concentration in the wines which have just fermented (Domizio, Liu, Bisson, & Barile 2017; Escot, Feuillat, Dulau, & Charpentier 2001). Release of mannose in Chardonnay wine increased drastically in the samples containing PEF-treated yeast cells as compared with those containing untreated yeast. No mannose release was observed in the first 7 days of aging on the lees in the wine containing untreated yeast; however, after the same period of time, mannose concentration increased by 40 and 60% of the total in those wines which contained the yeast treated by PEF at 5 kV/cm and 10 kV/cm, respectively.

Mannoprotein release is a process subsequent to yeast death that permits endogenous enzymes to come in contact with the cell wall as a consequence of the disorganization of the cell's membranous systems (cytoplasmic and organelle membranes). Natural autolysis is a slow process; thus, more than one week of incubation was necessary to detect an increment in the concentration of mannoproteins in the wine. On the other hand, the triggering of yeast autolysis by PEF permitted a significant amount of mannoproteins to be detected in the wine after only 7 days of incubation.

After 30 days of aging on the lees, the mannose concentration in wines containing yeast treated at the most intense PEF conditions attained the maximum value (230 mg/L), whereas the concentration in wines containing yeast treated at 5 kV/cm 75 μs was still only 165 mg/L. Later on, after 60 days of incubation, the mannose concentration in wines containing PEF-treated yeast under both conditions equalized at around 230 mg/L. On the other hand, after 60 days, mannose release from control cells was still half the amount than that released from PEF-treated cells. Wines containing untreated cells required six months of incubation to reach the maximum release of mannose, thereby confirming that natural autolysis in wine is indeed a slow process.

The release of mannoproteins not only depends on the time required for cytoplasmic hydrolytic enzymes to contact the cell wall, but also on the influence of environmental factors on the activity of the enzymes that degrade the cell wall. In a previous study, a release of 100 mg/L of mannose was detected from the same *S. cerevisiae* yeast previously treated by a PEF treatment that had inactivated the 90% of the population after 7 days of incubation in a buffer of pH 7 (Martínez et al. 2017). However, as it shown in Fig. 2, mannose release in the wine after the same incubation time was 65 mg/L. As the PEF treatments applied in both populations caused the same level of inactivation, the more gradual release of mannose in Chardonnay wine seems to be associated with the influence of environmental factors on autolysis. The usual conditions of winemaking are not the most suitable for this event to occur (Fornairon-Bonnefond, Camarasa, Moutounet, & Salmon 2002). The low pH of wine, along with the presence of ethanol, make the autolytic process more likely to occur at a much slower rate. It has already been proven that low pH and ethanol prolonged the time required for mannoprotein release during PEF-induced autolysis in buffer (Martínez et al. 2017) but further investigation in wine is now required.

It is also important to remark that even a PEF treatment which did not affect yeast viability (provided that post-treatment recovery is measured by colony count in PDA) proved nevertheless capable of accelerating the release of mannoproteins in Chardonnay wine. After 2 months of incubation, the concentration of mannose in the wine that contained yeast subjected to the lowest-intensity treatment was comparable to the concentration in the wine containing yeast treated by the most intense PEF treatment (inactivation of 90% of the population). As has been described elsewhere, when PEF treatments are applied at low intensities, a proportion of the population is injured rather than inactivated (Somolinos, García, Condón, Mañas, & Pagán 2007; Somolinos, García, Mañas, Condón, & Pagán 2008). Injured microorganisms are able to recover from damage and grow under optimal conditions such as nutritive plating media, but not under less-than-optimal conditions. Low pH and the presence of ethanol in the wine should prevent the recovery of the PEF-injured yeast: as a consequence, the yeast's death, autolysis and subsequent release of mannoproteins speeded up.

3.3. Analysis of wines after aging on untreated and PEF-treated lees

Previous studies have reported that the methods used to accelerate yeast autolysis may exert an influence on the characteristics of the mannoprotein fraction obtained, and thus, on its ability to improve the characteristics of wines (Núñez, Carrascosa, Gonzalez, Polo, & Martínez-Rodríguez 2006). For example, it has been observed that enzymatically obtained mannoproteins are more effective in avoiding protein haze in white wines than mannoproteins obtained from yeast treated by heat (Dupin et al. 2000; Moine-Ledoux & Dubourdieu 1999). In order to ascertain the effect on wine of mannoproteins released from yeast treated by PEF, physico-chemical characteristics of the wines obtained via untreated and PEF-treated yeast were analyzed in the course of the aging-on-lees process. Furthermore, we analyzed turbidity, foaming capability, and tannin concentration of wines containing untreated and PEF-treated yeast in order to evaluate the functional properties of mannoproteins released from yeast whose autolysis was PEF-induced.

3.3.1. Physicochemical characteristics of the wines

Chromatic characteristics, total polyphenol index, total volatile acidity, pH, ethanol, and CIELAB parameters of the wine before aging on the lees and after 1 viz. 3 months of aging on the lees with untreated and PEF-treated yeast are shown in Table 1. Significant statistical differences regarding these characteristics were not found between the treatments, neither did they occur between aging-on-lees time intervals. It would therefore seem that such physicochemical characteristics of wines are not a reflection of different levels of mannoprotein release.

3.3.2. Turbidity measurements

Turbidity of wines after centrifugation conducted with the purpose of precipitating yeast propagation after different aging-on-lees periods are shown in Fig. 3. One can observe that, after 30 days of storage, the turbidity of wine containing PEF-treated cells at 5 kV/cm and 10 kV/cm was 11 and 1 NTU, respectively, whereas the turbidity of wine containing untreated cells was much more pronounced (23 NTU). The photograph adjacent to Fig. 3 offers a comparison of the aspect of those wines after centrifugation. This effect seems to be a consequence of mannoprotein interaction with wine proteins and other compounds responsible for wine turbidity, thereby facilitating their precipitation during centrifugation. After 60 days of storage, the turbidity of the two wines which contained untreated and PEF-treated yeast was below 5 NTU; no statistically significant differences were observed between the

Table 1
Physico-chemical characteristics of the initial wine, and of the wine containing untreated or PEF-treated cells at 5 and 10 kV/cm during 1 or 3 months of aging on lees.

	Initial wine	1 month aging on lees			3 months aging on lees		
		Control	5 kV/cm	10 kV/cm	Control	5 kV/cm	10 kV/cm
Color intensity	1.51 ± 0.2 (a)	1.50 ± 0.2 (a)	1.48 ± 0.3 (a)	1.52 ± 0.2 (a)	1.48 ± 0.3 (a)	1.51 ± 0.3 (a)	1.52 ± 0.2 (a)
Tint	3.89 ± 0.3 (a)	3.91 ± 0.3 (a)	4.01 ± 0.2 (a)	4.11 ± 0.3 (a)	4.02 ± 0.4 (a)	3.91 ± 0.3 (a)	3.89 ± 0.3 (a)
Total phenols (OD 280 nm)	10.1 ± 0.2 (a)	10.2 ± 0.2 (a)	9.8 ± 0.3 (a)	10.2 ± 0.3 (a)	10.3 ± 0.3 (a)	10.4 ± 0.2 (a)	9.9 ± 0.3 (a)
pH	3.17 ± 0.2 (a)	3.20 ± 0.3 (a)	3.19 ± 0.3 (a)	3.17 ± 0.3 (a)	3.18 ± 0.4 (a)	3.20 ± 0.3 (a)	3.21 ± 0.3 (a)
Alcohol	14.50 ± 0.20	14.51 ± 0.30	14.50 ± 0.20	14.52 ± 0.20	14.50 ± 0.30	14.51 ± 0.40	14.51 ± 0.20
Volatile acidity (g/L)	0.32 ± 0.2 (a)	0.32 ± 0.1 (a)	0.31 ± 0.1 (a)	0.32 ± 0.1 (a)	0.31 ± 0.2 (a)	0.33 ± 0.2 (a)	0.30 ± 0.2 (a)
L*	97.9 ± 1.5 (a)	96.1 ± 2.0 (a)	96 ± 1.6 (a)	97.05 ± 2.3 (a)	96.8 ± 2.5 (a)	97.1 ± 1.9 (a)	97.0 ± 2.4 (a)
C*	6.8 ± 1.8 (a)	6.86 ± 1.3 (a)	6.5 ± 1.3 (a)	6.4 ± 1.5 (a)	7.45 ± 1.3 (a)	7.85 ± 1.4 (a)	7.50 ± 1.2 (a)
h*	95.17 ± 3.6 (a)	93.64 ± 3.0 (a)	91.39 ± 3.2 (a)	92.45 ± 3.5 (a)	92.5 ± 2.7 (a)	95.5 ± 4.0 (a)	93.20 ± 2.2 (a)
a*	−0.87 ± 0.5 (a)	−0.32 ± 0.4 (a)	0.23 ± 0.3 (a)	−0.20 ± 0.5 (a)	−0.31 ± 0.5 (a)	−0.70 ± 0.6 (a)	−0.41 ± 0.4 (a)
b*	6.75 ± 2.1 (a)	7.85 ± 1.9 (a)	6.57 ± 2.4 (a)	6.39 ± 2.3 (a)	7.43 ± 1.9 (a)	7.85 ± 2.4 (a)	7.51 ± 2.6 (a)

turbidity of the three wines. It therefore seems that the concentration of mannoproteins in the control wine after two months of aging on the lees is sufficient to facilitate the precipitation of those compounds responsible for wine turbidity. Further studies would be required to demonstrate whether the effect of the mannoproteins obtained from yeast treated by PEF may help to prevent visible wine protein haze formation once the wine has been bottled. At the present time, winemakers are using bentonite fining to prevent haze formation. Bentonite, however, is a laborious option which can affect wine quality, since it can eliminate certain aroma compounds (Moriwaki, Matioli, Arévalo-Villena, Barbosa, & Briones 2015; Puig-Deu, Lopez-Tamames, Buxaderas, & Torre-Boronat 1996).

3.3.3. Foam formation

Glycoproteins are the most prominent macromolecules responsible for the foam of sparkling wines; it is well known that the mannoproteins of yeast released during aging on lees play an essential role in the improvement of wine foam properties (Núñez et al. 2006Núñez, Carrascosa, González, Polo, & Martínez-Rodríguez 2005Senée, Robillard, & Vignes-Adler 1999). The effect of mannoprotein released from untreated or PEF-treated yeast on foam formation in the course of varying aging-on-the-lees time intervals is shown in Fig. 4. The foam formation capacity of the different wines aged on lees was expressed as the percentage of foam height in relation to the wine with the greatest foam height. After thirty days of aging on lees, the height of the foam thereby formed was 2.36 and 5.65 times higher for the wines containing yeast treated at 5 and 10 kV/cm, respectively, in comparison with those containing untreated yeast. On the other hand, the foam formation capacity of the wine containing the highest concentration of mannoproteins was 80% of the maximum foam capacity, equivalent to the foam capacity of the same wine after 90 days of aging on the lees. After sixty days of aging, the concentration of mannoproteins in the control wine increased, thereby significantly decreasing the differences in the resulting wines' foam formation capability, which were 1.8 and

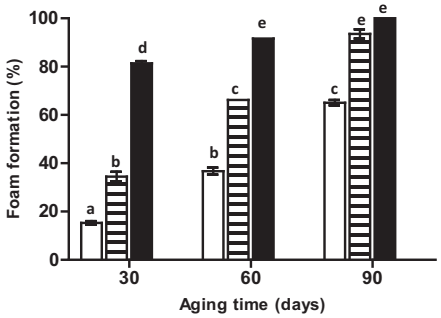
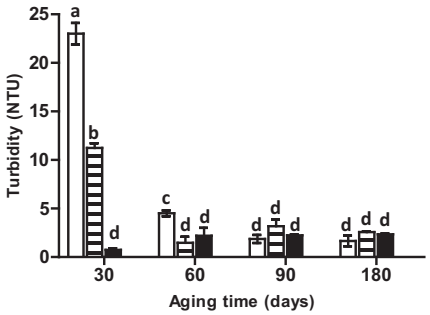


Fig. 4. Foam formation measurements of Chardonnay wine containing *S. cerevisiae* cells either untreated (white bars) or treated by PEF treatments of different intensity: 5 kV/cm 75 µs (striped bars) or 10 kV/cm 75 µs (black bars) and stored in different aging-on-lees periods.

2.5 times higher for 5 and 10 kV/cm, respectively. Finally, after ninety days of aging, although the forming capacity for wines containing untreated yeast was still 65% of the maximum, it became equal for both wines containing PEF-treated yeast, thereby reaching the maximum value obtained. When aging on the lees was extended from 1 month to 3 months, we observed an increment of the foaming capacity of the wine containing the yeast treated at 10 kV/cm from 80 to 100%, although the concentration of mannoproteins did not increase significantly. This increment could be related to the fact that the fraction responsible for foaming properties that is constituted by mannoproteins with a relative low molecular weight (Núñez et al. 2006) could be larger when aging on the lees is prolonged. In any case, results obtained in this investigation indicated that, for both mannoproteins released from untreated and treated yeast, the wines' foam forming capacity correlated highly with their concentration of mannoproteins ($R^2 = 0.92$), as previously reported by Coelho, Rocha, and Coimbra (2011), who observed that foam capability increased linearly with the



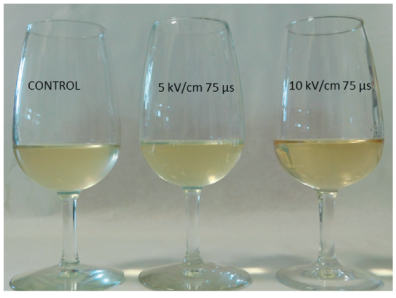


Fig. 3. Turbidity measurements of Chardonnay wine containing *S. cerevisiae* cells either untreated (white bars) or treated by PEF of different intensity: 5 kV/cm 75 µs (striped bars) or 10 kV/cm 75 µs (black bars) and stored in different aging-on-lees periods. The photograph shows Chardonnay wine that had contained untreated and PEF-treated *S. cerevisiae* cells for one month after centrifugation (untreated, 5 and 10 kV/cm, 75 µs).

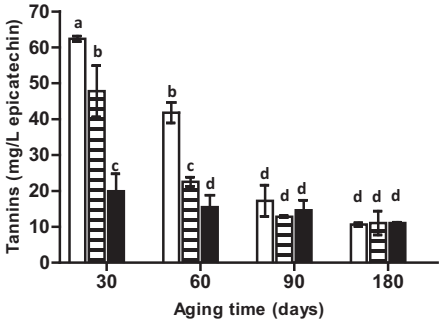


Fig. 5. Total condensed tannins measured in Chardonnay wine containing *S. cerevisiae* cells either untreated (white bars) or treated by PEF treatments of different intensity: 5 kV/cm 75 μs (striped bars) or 10 kV/cm 75 μs (black bars) and stored in different aging-on-lees periods. Data are expressed as epicatechin equivalents.

concentration of mannoproteins. These results support the hypothesis that mannoproteins released from yeast treated by PEF have functional properties in terms of foam capability similar to those of mannoproteins released from natural autolysis, and that the differences observed during varying aging-on-lees periods are a consequence of the varying concentration of mannoproteins.

3.3.4. Tannin concentration measurements

Tannins are polyphenolic compounds with the characteristic ability of complexing with proteins and precipitating them (Sarneckis et al. 2006). Tannins derived from grapes are called condensed tannins (or proanthocyanidins), and their importance in wine quality is essential because they exert an influence on astringency and mouthfeel. Fig. 5 displays the concentration of total condensed tannin values in the wines in the process of the aging-on-lees step. Although the initial concentration of condensed tannins was the same at the beginning of the aging-on -lees period during the two first months thereof, the amount of those compounds was higher in control wine, and lower in the wine containing yeast previously treated at the highest PEF intensity (10 kV). This evolution of the condensate tannins along aging-on-lees seems to be associated with the evolution of mannoprotein concentration along time in the different wines assayed. It is well known that mannoproteins may interact with tannins, thereby decreasing the amount of free tannins and resulting in wines with less astringency and better mouthfeel (Escot & Fuster 2002; Feuillat 2003; Vidal et al. 2004). Therefore, similarly to the effect of mannoproteins on foam capability, the capability of mannoproteins released from yeast treated by PEF was similar to that of those released from untreated yeast, whereby the differences observed reflect the varying concentration of mannoproteins in the wine.

4. Conclusions

The potential of PEF to accelerate the release of mannoproteins of *S. cerevisiae* which participated in the self-fermentation of Chardonnay wine has been shown in this study. Autolysis induced by PEF did not negatively affect the wines' physicochemical properties. The mannoproteins released in a shorter time from PEF-treated cells featured similar functional properties in wine than mannoproteins released during natural autolysis from untreated yeasts. This technique therefore permits to accelerate the “aging on lees” step while avoiding or reducing the problems customarily associated with it. Intense treatments are not required in order to achieve the effect. Wineries could thus process lees using the most economical PEF devices on the market. The gentle PEF treatment parameters required to induce autolysis open up the possibility of processing large volumes in continuous flow with low energy consumption.

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4.2. Extraction of carotenoids from PEF-treated *Rhodotorula glutinis* yeast

4.2.1. Pulsed Electric Fields-assisted extraction of carotenoids from fresh biomass of *Rhodotorula glutinis (Manuscript IV)*

4.2.2. Evaluation of Pulsed Electric Fields technology for the improvement of subsequent carotenoid extraction from dried *Rhodotorula glutinis* yeast *(Manuscript V)*

4.2.1. Pulsed Electric Fields-assisted extraction of carotenoids from fresh biomass of *Rhodotorula glutinis (Manuscript IV)*

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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,

Madrid, Spain) and the Abs_{474} of supernatant of untreated and PEF-treated cells was measured. The carotenoid extraction yield was calculated using the molar extinction coefficient of β-carotene in ethanol (ε = 2592) and was expressed as mg of carotenoids/mL of suspension using Eq. (1).

$$\text{Carotenoids (mg/mL)} = \frac{Abs\ 474 * 10}{2592} \quad (1)$$

2.8. Influence of pH and temperature of storage conditions on R. glutinis after PEF treatment

In order to investigate the influence of incubation media pH and of incubation temperature on the extraction of carotenoids, untreated and PEF-treated cells of *R. glutinis* were incubated in McIlvaine buffer of different pH (5.0, 6.5, 8.0) at different temperatures (15 °C, 25 °C, and 37 °C) for 24 h.

2.9. Statistical analysis

Experiments were performed in triplicate, and the presented results are mean ± 95% confidence interval. *t*-Test and One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences between the means values. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Permeabilization of the cell membrane of R. glutinis to propidium iodide by PEF

The uptake of PI is one of the most widely used techniques to evidence modification of the selective permeability of the cytoplasmic membrane of different microorganisms after the application of diverse chemical or physical treatments (Mackey, 2000). The effect of electric field strength and treatment time on the PI uptake of *R. glutinis* when PI was added before (white bars) or after (striped bars) the PEF treatment is shown in Fig. 1. Electric field strengths lower than 10 kV/cm did not affect the permeability of *R. glutinis* to PI (data not shown). In the range of treatment conditions investigated, the uptake of PI increased with treatment time and with the intensity of electric field strength, independently of whether PI was added before or after the PEF treatment. In the case of the most intense treatments (15 and 20 kV/cm for 150 and 300 μs), no statistically significant differences ($p < 0.05$) were observed between the percentage of stained cells when PI was added before or after treatment. These results indicate that the cells' permeabilization to PI was maintained after the PEF treatment was

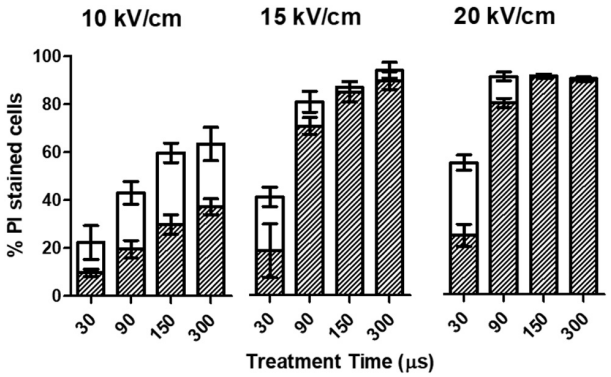

Fig. 1. Influence of the electric field strength and treatment time on the PI uptake when PI was added before (white bars) or after (striped bars) the PEF treatments.

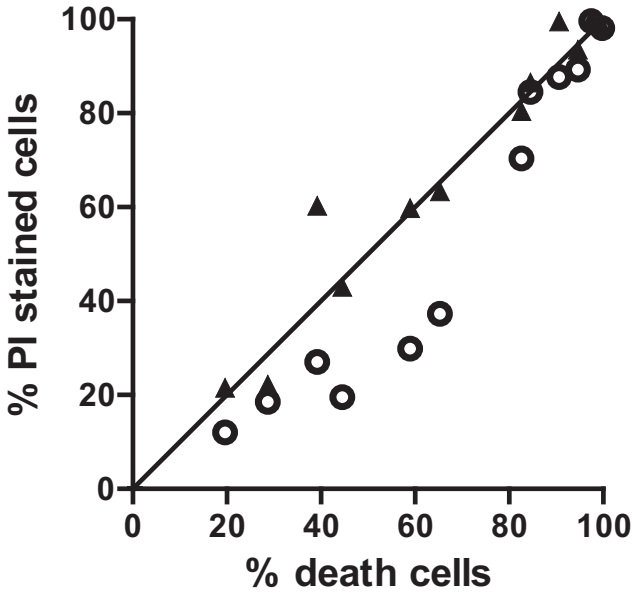

Fig. 2. Relationship between the percentages of the cell irreversible (open circle) and reversible (filled triangles) permeabilizated by PEF assessed by PI against the percentage of death cells treated by PEF. To show the degree to which each treatment causes membrane permeabilization, a theoretical straight line with slope = 1 and intercept = 0, is included.

applied. However, in the remaining treatments it was observed that the percentage of the cells stained by PI was higher when the colorant was added before the PEF treatment. This observation indicates that a percentage of the cells that became permeable to PI in the course of PEF treatment were able to recover selective permeability of their cytoplasmic membrane after treatment, thereby preventing PI uptake. The presence of reversibly electroporated cells of different types of microorganisms such as bacteria, yeast and microalgae after the application of PEF treatments depending on treatment intensity has been previously reported by different authors (Aronsson et al., 2005; Cebrián, Mañas, & Condón, 2015; Luengo, Condón-Abanto, Álvarez, & Raso, 2014; Ulmer, Heinz, Gänzle, Knorr, & Vogel, 2002).

It is well known that the cytoplasmic membrane that protects the cell from its surroundings plays a fundamental role in maintaining microbial homeostasis. Therefore, one of the consequences of the modification of the selective permeability of the cytoplasmic membrane is microbial death. The relationships between the percentage of stained cells when PI was added before or after the PEF treatment and the percentage of dead cells estimated by plate counting after the treatment are shown in Fig. 2. A theoretical straight line with slope 1 and intercept 0 that represents a perfect agreement between electroporation percentage and cell death has been included in the Fig. 2. It can be observed that the % of dead cells is correlated with the % of electroporated cells when PI was added prior to PEF treatment ($R^2 = 0.94$). On the other hand, when PI was added after the PEF treatment, the % of permeabilized cells was lower than the % of inactivated cells when total inactivation was lower than ca. 80%. For example, a treatment of 10 kV/cm for 90 μs inactivated ca. 45% of the population, but only 20% of the cells were irreversibly electroporated. According to these results, a certain amount of cells that died during treatment as a consequence of electroporation were able to recover the integrity of their cytoplasmic membrane: it was not permeable to PI when dye was added after treatment. This observation seems to indicate that the resealing of these dead cells' pores after electroporation is a physical process that does not have biosynthetic requirements.

From these observations it can be concluded that, when a mild PEF

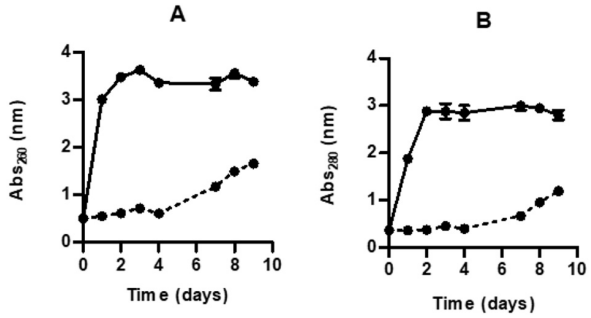

Fig. 3. Evolution along time of the absorbance at 260 (A) and 280 nm (B) of a McIlvaine buffer of pH 7 containing untreated cells (discontinuous lines) and PEF-treated cells (continuous lines).

treatment that causes an inactivation lower than 80% is applied, a certain amount of inactivated cells of *R. glutinis* maintain their cytoplasmic membrane intact. This indicates that the quantification of the number of inactivated cells is not a good index for the estimation of the number of irreversibly electroporated cells. However, when the inactivation caused by PEF is higher than 80%, a good correlation is observed between permanent electroporation and inactivation.

3.2. Release of intracellular compounds from R. glutinis after PEF treatment

In addition to the entrance of molecules such as PI into the cytoplasm, modification of cytoplasmic membrane permeability by electroporation facilitates the release of compounds from the cytoplasm to the surrounding media (Aronsson et al., 2005; Ohshima & Sato, 2004; Saulis, Šatkauskas, & Pranevičiūtė, 2007). The presence in the extracellular environment of compounds absorbing at 260 nm (Fig. 3A) and 280 nm (Fig. 3B) was used to monitor the permeability increment of the cytoplasmic membrane of untreated and PEF-treated cells of *R. glutinis*. The PEF treatment hereby applied to the cells (15 kV/cm for 150 μs) was the less intense treatment in terms of total specific energy (37.12 kJ/kg) capable of inactivating more than the 90% of the population. According to Fig. 2, this treatment irreversibly electroporated all the dead cells. As shown in Fig. 3, the release of specific intracellular compounds absorbing at 260 nm (nucleic acids) and at 280 nm (proteins/peptides) after 10 days of incubation was much more rapid and attained higher values for PEF-treated cells than for untreated ones. The maximum absorbance values attained for the PEF-treated cells were obtained after 2 days of incubation. However, for the untreated cells, no release of those compounds was observed after the first 4 days of incubation. After 10 days of incubation, the concentration of compounds absorbing at 260 and 280 nm in the medium containing the untreated cells was much lower than in the medium containing the PEF-treated cells after 2 days of incubation. The release of intracellular compounds from the untreated cells after 4 days of incubation could be attributed to inactivation and subsequent autolysis that leads to a disorganization of the cytoplasmic membrane, leading to the release of compounds to the extracellular environment. It has been recently demonstrated that PEF accelerates a series of events that occur during *S. cerevisiae* autolysis, such as the release of cell compounds to the extracellular environment (Martínez et al., 2016). Therefore, this effect could also be involved in the faster release and higher concentration of compounds absorbing at 260 and 280 nm in the extracellular media containing PEF-treated cells of *R. glutinis*.

Other effects associated with yeast autolysis are morphological changes at both the structural and the ultrastructural level (Takeo, Yamamura, & Kamihara, 1989). Martínez-Rodríguez et al. (2001) observed that, after 24 h of autolysis initiation, the size of the cells was much smaller than in growth stage. Flow cytometry was used in order to monitor morphological changes in terms of cell size (FS) and complexity (SS) occurring in untreated and PEF-treated yeast across time.

Fig. 4 shows the dot plot of FS versus SS of the control and PEF-treated cells immediately after the PEF treatment (Fig. 4A), and after 1 (Fig. 4B) and 5 days of incubation (Fig. 4C). Differences in the cells' size and complexity are determined by their characteristic position in the plot. PEF treatment did not cause significant morphological changes in the population of *R. glutinis* immediately after treatment. Fig. 3A shows an overlapping of points corresponding to untreated and PEF-treated cells; no statistically significant differences were observed between the averages of FS and SS values for untreated and PEF-treated populations (Table 1). The dot plot after 1 day of incubation shows important differences between both populations. While FS and SS values for the control cells remained practically unchanged, for the PEF-treated cells they decreased to 65 and 63% of the initial values, respectively. These morphological changes could be attributed to the previously described autolysis triggering effect of electroporation (Martínez et al., 2017; Martínez-Rodríguez et al., 2001; Takeo et al., 1989). Finally, Fig. 3B shows that dots corresponding to both populations tend to overlap; Table 1 shows that differences between FS and SS average values for untreated and PEF-treated cells were smaller than after 1 and 2 incubation days. These results seems to confirm that, after a given incubation time, autolysis also started taking place in the untreated cells.

3.3. Extraction of carotenoids from R. glutinis assisted by PEF

The presence of water in *R. glutinis* cells prevents an efficient extraction of carotenoids, owing to the hydrophobic nature of carotenoids and extracting solvents. Since the objective of this investigation was to evaluate whether electroporation of the cytoplasmic membrane of *R. glutinis* by PEF would permit the extraction of carotenoids from fresh biomass (thereby avoiding the costly process of complete drying), ethanol was selected as solvent. Ethanol is a polar water-soluble solvent, and it has proven effective for extracting carotenoids and other polar compounds from fresh biomass pretreated with PEF (Jaeschke, Menegol, Rech, Mercali, & Marczak, 2016; Luengo et al., 2015; Parniakov et al., 2015). Furthermore, ethanol is preferable in terms of environmental, health and safety hazards as compared to other solvents generally used for carotenoid extraction, such as hexane, diethyl ether,

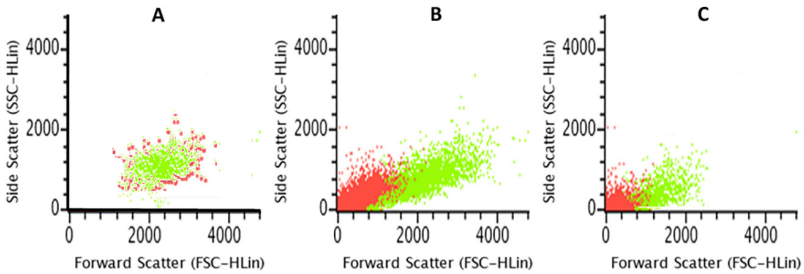

Fig. 4. Dot plot of FS versus SS of untreated (green) and PEF treated cells (red) at 0, 1 and 5 days of storage in McIlvaine buffer of pH 7. Each dot represent a single cell analyzed by the flow cytometer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Evolution along the storage time of FS and SS values of control and PEF-treated cells of *R. glutinis*. The values correspond to the mean ± confidence interval (95%) of three different experiments of 5000 replicates analyzed each one.

Time (day)	Forward scatter		Side scatter	
	Control	PEF-treated	Control	PEF-treated
0	2100.01 ± 55.67(a)	2010.01 ± 44,77(a)	490.25 ± 26,30(a)	450.30 ± 23,47(a)
1	2089.66 ± 57,92(a)	1433.92 ± 46,10(c)	470.93 ± 23,06(a)	285.60 ± 17,91(b)
2	1714.45 ± 48,38(b)	466.52 ± 18,36(d)	478.25 ± 26,02(a)	293.49 ± 8,13(b)
5	509.14 ± 29,16(d)	287.58 ± 12,40(e)	241.06 ± 6,15(c)	244.41 ± 6,77(c)

dichloromethane, or chloroform (Alfonsi et al., 2008).

After treating the *R. glutinis* cells with PEF at 15 kV/cm for 150 µs, ethanol resulted ineffective for extracting carotenoids. Presence of carotenoids was not detected in the solvent even after 48 h of incubation. The inefficacy of ethanol for extracting carotenoids, even after electroporation, could be due to the firm association between carotenoids and other macromolecules that prevented extraction such as proteins and fatty acids, or to the cell wall surrounding the cytoplasmic membrane, which constitutes an obstacle to the mass transfer of carotenoids during extraction (Saini & Keum, 2018). In order to determine if the absence of carotenoid extraction with ethanol as solvent was due to the cell wall obstacle, that barrier was disrupted by bead-beating. After checking by microscopy observation that this treatment caused efficient cell disruption, cell debris was suspended in ethanol. Even after obtaining a complete destruction of the yeast cell, ethanol was inefficient in recovering carotenoids. Therefore, these results suggest that the interaction between ethanol and *R. glutinis* carotenoids is too weak to disrupt the associations between these compounds and other cell macromolecules. The fragile association between *R. glutinis* carotenoids and ethanol is supported by the fact that carotenoids produced by this yeast are polar carotenoids, known to contain a hydrocarbon chain without any functional group (Hernandez-Almanza et al., 2014; Kot, Błażejak, Kurcz, Gientka, & Kieliszek, 2016).

Fig. 5 shows the extraction curves of carotenoids from PEF-treated *R. glutinis* cells, using ethanol as solvent after applying a PEF treatment and incubating the cells for 1, 2, and 5 days in a buffer of pH 7. Extraction curves for untreated cells are also shown for comparison. One can observe that after 1 day of incubation (Fig. 5A) ethanol was effective for extracting carotenoids from PEF-treated cells of *R. glutinis*. When cells were suspended in ethanol, concentration of carotenoids in the solvent increased across time. The highest carotenoid amount extracted (240 µg/g d.w.) was achieved after 1 h of extraction, after which the concentration remained constant. For the untreated cells, a slight increment in the concentration of carotenoids was also observed after the first hour of incubation, but the highest amount of carotenoids extracted from *R. glutinis* cells was four times lower. Increasing the incubation time before carotenoid extraction from PEF-treated cells of *R. glutinis* reduced the amount of time necessary to achieve the highest extraction of carotenoids, but did not increase the overall extracted amount. In the case of untreated cells, no differences were observed in the extraction of carotenoids between 1 and 2 days of incubation. On the other hand, after 5 days of incubation (Fig. 5C), the amount of extracted carotenoids was higher (around 125 µg/g d.w.), but this value was much lower than the amount of carotenoids extracted from PEF-treated cells of *R. glutinis* after 1 h of incubation.

The amount of carotenoids extracted from fresh biomass of our strain of *R. glutinis* is within the range of the values reported in the literature. Buzzini and Martini (2000) achieved a maximum yield of 630 µg/g d.w. when they investigated the production of carotenoids among fourteen strains of *R. glutinis* grown in media containing different carbohydrate sources. Tinoi, Rakariyatham, and Deming (2005) reported a maximum carotenoid extraction yield of 330 µg/g d.w from a strain of *R. glutinis* under optimized conditions using dimethylsulfoxide (DMSO). This solvent is very efficient for the extraction of carotenoids from yeasts. However, the difficulty to be removed by conventional evaporation due to its high boiling point (189 °C) explains because DMSO is not allowed to be used as extraction solvent in the manufacture of food products and their ingredients in the EU.

The efficacy of ethanol for extracting large amounts of carotenoid from PEF-treated cells of *R. glutinis* seems to be a consequence of the autolysis process that is accelerated by PEF. Autolysis is an event associated with cell death, consisting in the hydrolysis of intracellular biopolymers of cells by their own enzymes, followed by the formation of low molecular weight products (Babayan & Bezrukov, 1985; Hernawan and Fleet, 1995). Considering the mechanism of action of PEF, two main events should be involved in the autolysis triggering of *R. glutinis* cells: the death of the cells that causes the disturbance of intracellular structures, and the electroporation of the cytoplasmic membrane, which facilitates the contact of hydrolytic enzymes located in intracellular structures with the cell wall. Therefore, the yeast's hydrolytic enzymes could help disrupt the associations between carotenoids and other molecules of the cell; in this way, the ethanol-carotenoids complex could diffuse across the cell membrane driven by a concentration gradient. On the other hand, the increased speed of carotenoid extraction when incubation time was extended from one to two days could be a consequence of the parallel degradation of the cell

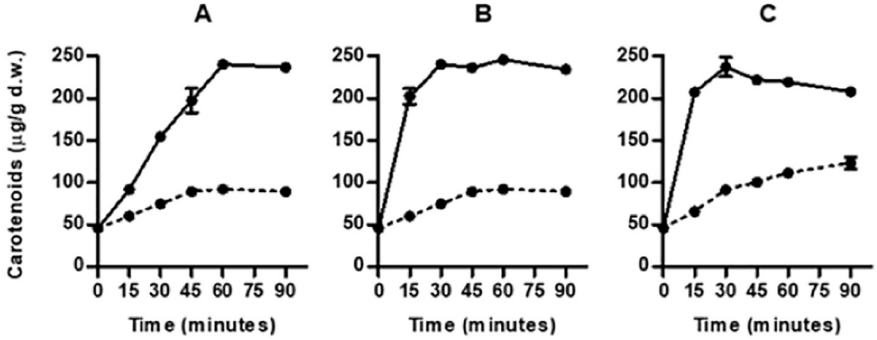

Fig. 5. Carotenoid extraction along time (minutes) in ethanol from *Rhodotorula glutinis* cells after one (A), two (B) or five days (C) of previous storage in McIlvaine buffer of pH 7. Untreated cells are shown in discontinuous lines and PEF-treated cells are shown in continuous lines.

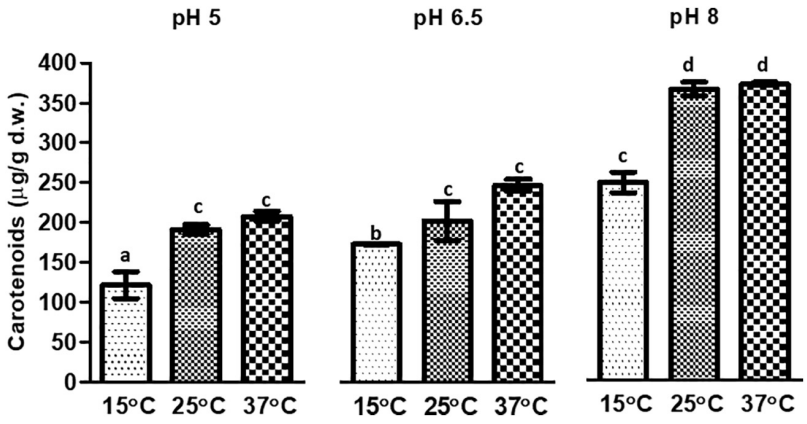

Fig. 6. Influence of pH and temperature of incubation for 24 h on the amount of carotenoids extracted in ethanol from PEF-treated *Rhodotorula glutinis* after 1 h of extraction. The different letters in the graph show statistical significant differences.

wall during autolysis, thereby facilitating the diffusion of carotenoids across the cell wall into the bulk organic solvent. The absence of effect after incubating both untreated cells and PEF-treated cells suspended in ethanol for 48 h immediately after treatment could be ascribed to the fact that ethanol has the property of delaying both natural autolysis and autolysis induced by PEF (Alexandre & Guilloux-Benatier, 2006; Martínez et al., 2017).

As the autolysis process depends on enzymatic activity, and the activity of enzymes is highly dependent on environmental factors, the extraction of carotenoids after 1 h in ethanol from cells of *R. glutinis* treated by PEF and previously suspended in media of different pH at different temperatures during 24 h was investigated. Results of this study are shown in Fig. 6. It is observed that, at the three pH levels investigated, carotenoid extraction increased by raising the incubation temperature from 15° to 25 °C; however, no significant differences were observed between extraction at 25° and 37 °C. On the other hand, the highest amount of carotenoids extracted (375 μg/g d.w.) from the PEF-treated cells of *R. glutinis* was measured after incubation of the cells at pH 8.0 and 25° or 37 °C. The influence of these factors on the extraction of carotenoids, and the morphological changes occurring in the PEF-treated cells evidenced by flow cytometry after 1 day of incubation, both support the hypothesis that the improvement in carotenoid extraction by incubating the *R. glutinis* cells after PEF treatment is a consequence of the autolysis triggered by PEF that causes a disruption of the associations of carotenoids with other molecules, as well as a degradation of the cell wall.

4. Conclusions

In conclusion, this research has revealed that the application of a PEF treatment to cells of *R. glutinis* and a subsequent incubation period permits the extraction of carotenoids from fresh biomass using ethanol as solvent. This procedure could give rise to an innovative and more sustainable method with less environmental impact for extraction of carotenoids from yeast, by eliminating the drying stage from the conventional process, as well as by avoiding non-polar solvents (hexane, diethyl ether, chloroform, etc) generally used for extraction of carotenoids. Existing large-scale PEF equipment already used in the food industry for the treatment of pumpable products in continuous mode could be employed for this purpose.

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4.2.2. Evaluation of Pulsed Electric Fields technology for the improvement of subsequent carotenoid extraction from dried *Rhodotorula glutinis* yeast *(Manuscript V)*

Submitted to Food Chemistry

Submitted

Evaluation of pulsed electric fields technology for the improvement of subsequent carotenoid extraction from dried *Rhodotorula glutinis* yeast

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ABSTRACT

This research aims to evaluate whether the electroporation of *Rhodotorula glutinis* fresh biomass improved the subsequent extraction of carotenoids from dry biomass using supercritical CO_2 and traditional solvent extraction.

Supercritical CO_2 extraction yields were low after all treatments assayed. Similarly, solvent extraction of carotenoids from untreated or PEF treated cells that were immediately freeze-dried after the pre-treatment was neither effective (extraction yield < 20 % total content).

Conversely, PEF-treatment and subsequent intermediate incubation in aqueous buffer for 24 h, followed by freeze-drying and extraction, led to a large improvement with the three solvents assayed (acetone, hexane, ethanol). Ethanol was the most efficient, reaching an extraction yield of 80 % of total carotenoid, which represents a recovery of 267 $\mu g/g_{dw}$. Torularhodin esters constituted the main carotenoid found in the extracts. This is of great interest, as ethanol is eco-friendly solvent and potential applications of torularhodin range from food to medical purposes.

1.-INTRODUCTION

Carotenoids are natural pigments synthesized by plants and microorganisms. The properties of carotenoids as antioxidants, anti-cancer agents, immune response stimulants and colouring agents explain their applications in medical, pharmaceutical, cosmetic, chemical and food industries (Aksu and Eren et al., 2007). Specific types of carotenoids are considered high-value products and market prices of several hundred dollars per milligram are not uncommon (Tinoi et al., 2005).

Rhodotorula yeasts produce not only β-carotene, but also characteristic carotenoids such as torularhodin (3´, 4´-Didehydro-β, Ψ-caroten-16´-oic acid) and torulene (3´, 4´-Didehydro-β, Ψ-carotene) (Moliné et al., 2012). These red carotenoids are the major pigments in *Rhodotorula* species and are almost exclusively produced by these yeasts, except for other minority sources such as ladybird beetles, where they have been isolated in very low amounts. Carotenoid production by red yeasts has advantages over other microorganisms such as adaptation to different environmental conditions, growth under a wide variety of carbon and nitrogen sources, high specific growth rate and production of large quantities of cell biomass (Buzzini and Martini, 2000). Besides, microbial carotenoid production avoids the problem of seasonality of vegetal sources and fits with the growing trend of consumers rejecting compounds obtained by chemical syntheses (Frengova and Beshkova, 2009; Kot et al., 2016; Saini and Keum, 2018).

Carotenoids produced by *Rhodotorula* species are examples of primary carotenoids with structural and functional properties (Frengova and Beshkova, 2009) playing a role in photoprotection

(Moliné et al., 2010). Therefore, it is required to develop efficient selective extraction processes to unlock the full biotechnological potential of these yeasts towards the production of high-value carotenoid pigments.

Traditionally, carotenoids are extracted using organic solvents. However, to obtain an effective extraction it is generally required to previously remove the total water content, as its presence in the cells hinders the efficient extraction of carotenoids due to the hydrophobic nature of carotenoids and organic solvents. Conventional solvent extractions require large amounts of harmful solvents, multiple extraction steps and only possess limited effectiveness, as cell envelopes negatively influence the extraction. Consequently, different physical procedures (heat treatment, ultrasound, high pressure homogenization, bead-milling, vigorous shacking) are applied before or during extraction in order to disrupt these barriers, facilitating the entry of solvents into the cell and the subsequent solubilisation of carotenoids, thus improving extraction yield (Liu et al., 2016; Middelberg, 1995). However, those techniques have several disadvantages such as energy costs, difficulty of industrial up-scaling, isomerization and oxidation of the target compounds and release of cellular debris, leading to the necessity of purification (Liu et al., 2016).

Supercritical carbon dioxide extraction (SC-CO_2) has been presented as an alternative and efficient method for extraction of thermolabile compounds, such as carotenoids, avoiding the use of toxic solvents (Saini and Keum, 2018). A fluid is named supercritical when its temperature and pressure are above the critical point, thereby presenting high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension. These properties explain the greater ability of SC-CO_2 to diffuse into a matrix, compared to conventional organic solvents. Many studies have been conducted to extract carotenoids from higher plants (Döker et al., 2004; Shi et al., 2010), microalgae (Hosseini et al., 2017; Macías-Sanchez et al., 2010), and yeasts (Lim et al., 2002; Park et al., 2007). However, the yields for yeast extraction are still low, due to the presence of intact cell envelopes (Lim et al., 2002).

Pulsed electric fields (PEF) is a physical treatment that causes an increase in cytoplasmic membrane permeability (electroporation) by applying intermittent high-intensity pulses of short duration, typically in the range of µs to ms. Cell membrane perforation induced by PEF treatment can improve the extraction of bioactive compounds, such as pigments, from microorganisms with low energy consumption (Jin et al., 2011, Liu et al., 2013, Luengo et al., 2015, Zbinden et al., 2013). Recently, the enhancement effect of PEF followed by aqueous incubation in the subsequent ethanolic extraction of carotenoids from fresh biomass of *R. glutinis* has been demonstrated (Martínez et al., 2018).

So far, studies carried out with PEF pre-treatment of cells to improve extraction of bioactive compounds were only performed using fresh biomass. However, it is unknown if the previous application of PEF treatment to the fresh biomass still results in the improvement of extraction yields once the biomass is dried before extraction. Depending on the raw material, the target compound, the solvent and the extraction method, it might be desired to use dried biomass as it is the case for supercritical CO2 extraction.

Thus, the objective of this research was to evaluate whether the electroporation of fresh *Rhodotorula glutinis* biomass improves the subsequent extraction of carotenoids from dry biomass using supercritical CO_2 and traditional solvents. In addition, the effect of an intermediate incubation between the PEF-treatment and the drying and extraction was evaluated in order to consider secondary effects resulting from the electroporation.

2.-MATERIAL AND METHODS

2.1 Yeast 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 from single colonies 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. 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 on Potato-Dextrose-Agar (PDA, Oxoid, Basingstoke, UK). Dry weight (dw) of the yeast was determined by vacuum drying at 60 °C until constant weight. Stationary growth phase was achieved after 48 h of growing; however, experiments were performed with cells after 72 h of cultivation, which corresponded with the highest absorbance at 474 nm and consequently maximum carotenoid production. Biomass concentration at this time was 10 g_{dw}/mL and 10^8 cells/mL.

2.2 PEF treatment

The PEF equipment used in this study was manufactured by the German Institute for Food Technologies (DIL, Quakenbrück, Germany). The apparatus generates exponential waveform pulses at frequencies of 1 to 4 Hz. Discharge of the capacitor is realised by a spark gap, and provides voltages in the range of 1.5 – 30 kV.

The treatment chamber used in these experiments was a parallel plate batch chamber consisting of two parallel stainless steel electrodes of 77 mm x 61 mm and 1 mm of thickness, separated by a polyoxymethylene (POM) insulator. The volume of the chamber was 40 mL. Distance between the electrodes was 1 cm.

Actual voltage applied were measured using a high voltage probe (Tektronix P6015A, Beaverton, USA) connected to an oscilloscope (Tektronix TBS1102B-EDU, Beaverton, USA). Voltage data registered by the oscilloscope were plotted and voltage decay was fitted using an exponential equation, to calculate the pulse width at 37 % of the maximum voltage. Equation 1 was used to calculate the energy delivered per pulse by the discharge of the capacitor.

$$W = 0.5\, U^2\, C\ [J] \qquad \text{Eq 1}$$

With C being the capacity of the capacitor (0.5 µF), and U being the maximum voltage measured across the electrodes of the treatment chamber. The total specific energy input [kJ/kg] was calculated by multiplying the pulse energy (W) with the number of pulses divided by the mass of sample in the treatment chamber.

Prior to the treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 x g for 5 min at room temperature, and re-suspended in citrate-phosphate McIlvaine buffer (pH 7.0; 5.0 mS/cm) to a final concentration of approximately 10^8 cells/mL.

A previous screening of PEF treatments at electric field strengths of 10–20 kV/cm and 100–400 pulses was performed in order to evaluate the parameters to achieve different levels of inactivation. Afterwards, suspensions were subjected to the selected treatment consisting of the application of 400 exponential decay pulses of a pulse width of 6.8 microseconds at an electric field strength of 15 kV/cm.

2.3 Evaluation of the permeabilisation of *R. glutinis* after the PEF-treatment

After the treatments, PEF treated and untreated cells were stained with propidium iodide (PI) using the protocol described by Martínez et al., 2018. The samples were analyzed by flow cytometry (BD Accuri C6, New Jersey, USA) in order to check the effectivity of PEF-treatment by means of the measurement of percentage of permeabilized cells.

2.4 Intermediate incubation and freeze-drying

After the PEF-treatment, untreated and PEF-treated suspensions were freeze-dried immediately or incubated in the own treatment medium (citrate-phosphate McIlvaine buffer, pH 7.0) for 24 hours at 25 °C in darkness and then freeze-dried. The flash freezing was performed at -40 °C, and samples were subsequently lyophilized (FreeZone6, Labconco, Kansas City, USA) until total dehydration, which was achieved after 48 hours.

2.5 Supercritical CO_2 extraction

The supercritical fluid extraction (SFE) was carried out using a 24 mL extraction column (I.D. 14 mm) filled with freeze dried biomass of *R. glutinis* (4 g of control or 1.5 g of PEF treated sample) placed between layers of glass beads serving as solvent flow distributors. The extraction column was heated in an air-conditioned oven to the desired temperature and then pressurized by a high pressure pump for liquid CO^2 (Applied Separations, USA). A pressure of 50 MPa and a temperature of 80 °C was set as optimal conditions for SFE of carotenoids from yeast as described by Lim et al. (2002). After 15 minutes of static extraction, a heated micrometer valve downstream of the extractor was opened and the extraction in the dynamic mode was started. The CO^2 flow rate was adjusted using the micrometer valve to a low value of 0.8 g/min, ensuring the solvent saturation by a solute. The extract was collected at ambient temperature in pre-weighed glass vials serving as a separator. By gradual weighing of the extract during the experiment, the amount of SC-CO^2 required to obtain the total extract from the control and PEF treated *R. glutinis* was determined as 10.3 and 61.3 g/g_{dw}, respectively. After the SFE with pure SC-CO^2 terminated and the first fraction of extract was obtained, the ethanol was added into the SC-CO^2 as co-solvent. Ethanol was supplied at a constant flow rate by a high-pressure pump LCP 4020.3 (ECOM s.r.o.) and mixed with SC-CO^2 before entering the column to reach a concentration of 20 % (w/w) in the SC-CO^2. The second fraction was obtained at a SC-CO^2-to-feed ratio of 12 g/g_{dw}. Ethanol was evaporated at ambient pressure under a nitrogen stream.

Vials with dry extracts from both extraction steps were weighed, tightly closed, and stored in a refrigerator until the analysis.

2.6 Solvent extraction

Control and PEF treated samples of 0.05 g of freeze dried biomass were placed in tubes and 10 mL of the different solvents were added (Ethanol, Hexane or Acetone). The tubes were placed in a shaking device (PTR-60, Grant Bio Multirotator, Grant Instruments, Cambridge, UK) at 75 rpm, for 12 hours. Samples were taken periodically and the carotenoid content of the supernatants after centrifugation (3000 x g, 5 min) was measured.

2.7 Total extraction

Total carotenoids were extracted by suspending 0.05 grams of freeze-dried biomass in 5 mL dimethyl sulfoxide (DMSO), vortexing for 1 min and incubating while shaking at room temperature for 1 h. This was followed by the addition of 5 mL hexane, after that the mixture was vortexed for 1 min and further shaken for 15 minutes. Then, tubes were centrifuged (4000 x g, 10 min) resulting in a yellow colored hexane upper phase. This hexane phase was collected and the procedure was repeated until the hexane phase collected appeared transparent. This way, the non-polar carotenoids could be collected. The remaining DMSO phase was still pink colored, i.e. it still contained more polar carotenoids. To extract these, 1 mL Diethylether and 1 mL of saturated NaCl solution were added to the DMSO. The tubes were vortexed for one minute and shacke for 15 minutes. After that the tube was centrifuged, as previously described. The pink pigmented upper phase was collected and the procedure was repeated until the collected diethylether phase appeared transparent. These diethylether extracts were evaporated under continuous nitrogen flux. This extract was dissolved in hexane and pooled with the hexane previously collected for spectrophotometric quantification of total carotenoids.

2.8 Photometric carotenoid analysis

The carotenoid content of the extracts was determined using an ultraviolet light (UV) spectrophotometer (UV-1800, Shimazdu, Kioto, Japan) and the UV absorption spectra were recorded. The carotenoid extraction yield was calculated from the measured absorption, using the molar extinction coefficient of β-carotene in ethanol (ε=2620), acetone (ε=2500) or hexane (ε=2592) and was expressed as mg of carotenoids/mL of suspension, using Equation 2.

$$c_{carotenoids} = \frac{Absorption_{454\,nm} \cdot 10}{\varepsilon} \left[\frac{mg}{mL}\right] \qquad \text{Eq 2}$$

2.9 Carotenoid analysis by HPLC

High performance liquid chromatography (HPLC) was accomplished using an Alliance Waters 2695 Separations Module (Waters, Milford, MA, USA) with integrated autosampler and a photodiode array detector. A 4.6 x 250 mm C-30 Carotenoid column of 5 µm of particle size (YMC, Wilmington, USA) was used.

The solvents were HPLC grade methanol (VWR, Paris, France) and methyl-tert-butyl-ether (MTBE; Fisher Scientific, Pittsburgh, PA, USA). A gradient system was used involving two separately mixed mobile phases. Mobile phase A was methanol/MTBE/water (81:15:4) and mobile phase B was methanol/MTBE (9:91). The initial values were 100 % of A and 0 % of B, to 50 % A and 50 % B in 45 min, followed by 100 % B within 25 min. The flow rate was 1.0 mL/min throughout the entire run. All samples were injected via a 20 µL loop using a 100-µL syringe.

On the basis of the absorbance maxima for the carotenoids of *R. glutinis,* detection was done at 450 and 485 nm by a Waters 2998 Photodiode Array Detector. The elution profile of β-carotene standard with the C30 column was obtained and standard curves were constructed by plotting HPLC peak absorbance area versus concentration of the β-carotene in the injected sample.

After the HPLC analyses of the samples, a saponification protocol was performed with the samples, according to Granado et al., 2001, in order to break ester bonds, and chromatograms were repeated.

2.10 Statistical analysis

Experiments were performed in triplicate, and the presented results are mean ± 95 % confidence interval. t-Test and One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences between the mean values. The differences were considered significant at $p < 0.05$.

3.- RESULTS

3.1 Electroporation of *R. glutinis* cells

Before freeze-drying, *R. glutinis* cells were subjected to 400 exponential decay pulses (6.8 µs pulse width) at an electric field strength of 15 kV/cm with a total specific energy input of 84 kJ/kg resulting in an inactivation of 2 log cycles in the population of *R. glutinis*.

Flow cytometry analysis of untreated and PEF treated cells was conducted in order to evaluate if the treatment applied was able to electroporate the cytoplasmic membrane of *R. glutinis* (Figure 1). Results indicated that PI entered around 99 % of the yeast cells in the suspension, confirming that the selected PEF treatment conditions were suitable to permeabilize most of the cells of *R. glutinis*. However, PEF treatment did not cause significant changes in cell morphology or size in the population of *R. glutinis* immediately after the treatment (Figure 1). This might be beneficial in order to facility the solid-liquid separation after solvent extraction and in order to provide suitable bulk properties of the dried material during CO_2 extraction.

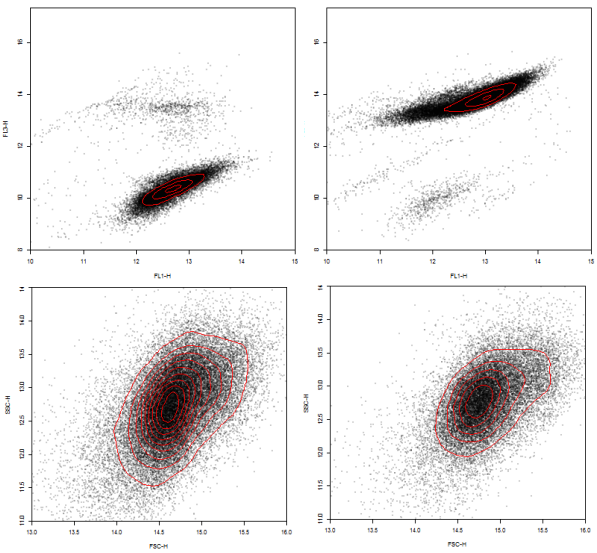

Figure 1. Scatter plots of FL1-H versus FL3-H channels (top) illustrating propidium iodide (PI) uptake and plots of forward scatter versus side scatter (bottom) depicting changes in size and granularity of untreated (**A**) and PEF treated (**B**) cells after PI dying. Each dot represent a single cell analysed by the flow cytometer.

In this research previously to the extraction step cells were dehydrated by freeze-dried to protect from exposure to high temperatures and air that occurs in traditional drying process.

3.2 Effect of PEF on supercritical CO_2 extraction of carotenoids from freeze-dried cells of *R. glutinis*

Supercritical CO_2 (50 MPa, 80 °C) was ineffective for extracting carotenoids from freeze-dried cells of *R. glutinis*. No statistically significant differences were observed in the amount of extracted carotenoids from untreated freeze-dried cells (control) and PEF treated freeze-dried cells, with the total extracted carotenoid levels being lower than 5 μg /g_{dw} in both cases, representing less than 2 % of the total carotenoids content. As the cytoplasmic membrane of the cells treated by PEF was permeabilized before freeze-drying, the penetration of the CO_2 into the cytoplasm does not seem to be the cause of the low extraction efficiency. Table 1 shows the average extraction yields (μg/g_{dw}) for untreated and PEF treated *R. glutinis* cells, incubated for 24 hours at 25°C in McIlvaine buffer (treatment medium) prior to freeze-drying. A previous incubation for 24 hours that was demonstrated to be effective for extracting carotenoids from PEF treated fresh biomass of *R. glutinis* using ethanol as solvent (Martínez et al., 2018) represented a very little positive effect on SC-CO_2 extraction. Taking into account that the total carotenoid content of this yeast was 336.49±18.90 μg/g_{dw}, yields obtained after SC-CO_2 extractions were very low for both untreated and PEF-treated samples, representing only 1.39 and 4.19 % of the total carotenoid content, respectively. After the first extraction step with pure CO_2 at supercritical stage, the remaining biomass was extracted again with SC-CO_2 supplemented with 20 % w/w of ethanol and the second carotenoid fraction was collected. Results showed that the carotenoid extraction could be improved for both untreated and PEF-treated cells when 20 % ethanol was used as co-solvent. Yields increased from 1.39 % to 18.99 % in case of untreated samples and from 4.19 % to 20.00 % for PEF-treated samples (Table 1). These results show that PEF-treatment could neither significantly improve SC-CO_2 ethanol co-solvent extraction of carotenoids from dried *R. glutinis* in comparison to untreated biomass. Although there was a little positive effect of PEF treatment, no statistically significant differences were found between the extraction yields obtained from untreated and PEF treated biomass. Even after a PEF treatment that caused 99 % permeabilization of *R. glutinis* cytoplasmic membranes and the subsequent incubation of the suspension, SC-CO_2 extraction was not efficient. The yield represented less than 25 % of the total content even after two extraction steps without and with co-solvent.

Table 1. Carotenoid extraction from freeze dried untreated or PEF treated (15 kV/cm; 84 kJ/kg) and 24 hours incubated biomass of *Rhodotorula glutinis* by supercritical pure CO_2 (SC-CO_2) and supercritical CO_2 using 20 % of ethanol as co-solvent.

	SC-CO_2	**SC-CO_2 + 20% ethanol**
Control	4.48±1.13	61.34±5.5
PEF	13.54±0.35	64.58±3.7

Data are expressed as μg total carotenoids/g_{dw} and represented as mean ± standard deviation.

3.3. Effect of PEF on solvent extraction of carotenoids from freeze-dried cells of *R. glutinis*

Table 2 shows the effectivity of different solvents in the extraction of carotenoids from untreated and PEF treated cells of freeze-dried *R. glutinis*. In both cases, yeast biomass was immediately freeze-dried after the application of the PEF treatment. It is observed that extraction yield from the freeze-dried biomass in the tested solvents (acetone, ethanol and hexane) was little, even after extraction times as long as 12 h. Among the three solvents assayed, acetone was the most effective, although the highest yield reached did not surpassed 23 % of total carotenoid content. In the case of hexane and ethanol the extraction yield was lower than 10 % of the total content.

Table 2. Carotenoid extraction from untreated or PEF treated (15 kV/cm; 84 kJ/kg) and immediately freeze-dried biomass of *Rhodotorula glutinis* in different solvents.

	Acetone		**Ethanol**		**Hexane**	
Time	*Control*	*PEF*	*Control*	*PEF*	*Control*	*PEF*
1 h	14.64±3.12(a)	15.61±2.50(a)	15.32±1.20(a)	15.06±0.50(a)	8.02±0.72(b)	10±1.13(b)
2 h	18.34±3.10(α)	19.87±3.50(α)	16.44±3.12(α)	17.35±2.72(α)	11.40±3.45(β)	12.44±2.53(β)
12 h	85.50±7.29(i)	75±2.65(i)	26.62±2.41(ii)	25.70±2.72(ii)	22.43±1.48(iii)	18.21±3.45(iii)

Data are expressed as μg total carotenoids/g_{dw} and represented as mean ± standard deviation. Letters in brackets show statistically significant differences ($p < 0.05$) among extracts for each extraction time.

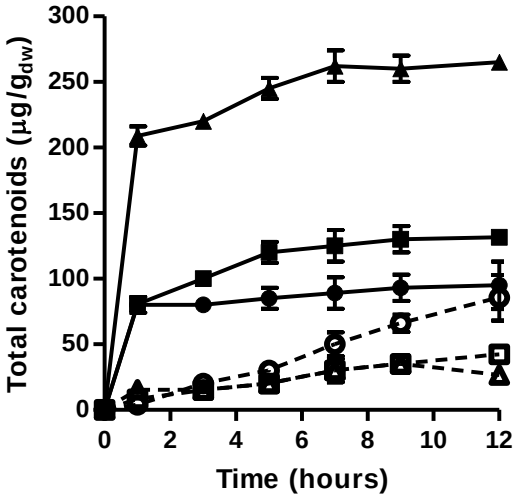

Figure 2. Carotenoid extraction effectiveness over time in different solvents: hexane **(■),** acetone **(●),** or ethanol **(▲)** from dried *Rhodotorula glutinis* cells after PEF treatment (15 kV/cm; 84 kJ/kg) and incubation (solid symbols, continuous line) or untreated before incubation (hollow symbols, dashed line).

Figure 2 shows the extraction curves of carotenoids in the same solvents from untreated and PEF treated cells of freeze-dried *R. glutinis* but in this case the freeze-drying was performed after incubating the cells for 24 h in citrate-phosphate McIlvaine buffer (pH 7.0; 5.0 mS/cm) at 25 °C. It is shown that carotenoid extraction yield from untreated, freeze-dried cells was very low, even after intermediate incubation. Ethanol and hexane extracts contained a carotenoid concentration below 50 µg/g$_{dw}$ and the concentration was below 75 µg/g$_{dw}$ for acetone extracts, even after 12 h of extraction under constant shaking. Extraction yields from untreated, freeze-dried cells after 24 h incubation were higher than those obtained for dried cells without incubation in case of ethanol and hexane (50 %) and similar when the extraction was performed in acetone. On the other hand, electroporation of the cells and subsequent incubation for 24 h before freeze-drying resulted in an improvement of carotenoid release for all the solvents studied. Most of the extraction was achieved during the first hour and then the extraction yield increased more slowly. For all the solvents assayed, after 1 h of extraction the yield from PEF-treated cells was at least 10 fold higher that the yield from untreated cells. Ethanol in combination with a PEF pre-treatment was demonstrated to be the most efficient combination, achieving 62 % of the total carotenoid content in 1 h. After 7 h of extraction, the ethanolic extract from PEF-treated *R. glutinis* contained 79 % of total carotenoids, thus being approximately tenfold higher than the ethanolic extract of untreated cells. For hexane extractions, at this time, the yield from PEF-treated cells was ca. threefold higher compared to the untreated reference, achieving approximately 40 % of total carotenoids. Only in case of acetone, which is usually cited as preferred solvent for effective extraction of polar carotenoids (Moliné et al., 2012; Saini and Keum, 2018), the yield from untreated cells was similar to the yield of PEF-treated cells after 12 h of extraction. However, the amount of carotenoids extracted represented only 25 % of the total content. These results reveal that PEF-treatment of fresh biomass followed by incubation at 25 °C in the treatment medium (McIlvaine buffer, pH 7) before dehydration had a positive effect on the improvement of subsequent extraction from dried biomass. This effect was especially significant when ethanol was used as the solvent.

3.4. HPLC analysis of extracts

Subsequent to the evaluation of efficient combinations of PEF pre-treatment, intermediate incubation time and freeze-drying, specific carotenoids extracted by the three assayed solvents were identified and quantified using HPLC.

As similar chromatograms were obtained for the three solvents, figure 3A exemplarily illustrates the chromatogram of the ethanolic extract, which was shown to be the most effective solvent. Three main peaks were observed, corresponding to retention times of 31, 45.5 and 47.5 min. Considering the use of a reverse-phase HPLC column, it was expected that β-carotene eluted after the more polar carotenoids, such as torularhodin and torulene. However, according to the elution profile of the standard curve, the first peak corresponded to β-carotene and the others, with a maximum absorbance at 490 nm, corresponded to carotenoid esters with a very intense red colour.

Percentages of areas corresponding to each of the peaks related to the total area are presented in table 3. Although the yield of extraction increased significantly after PEF treatment combined with incubation, compared to untreated cells (Figure 2),

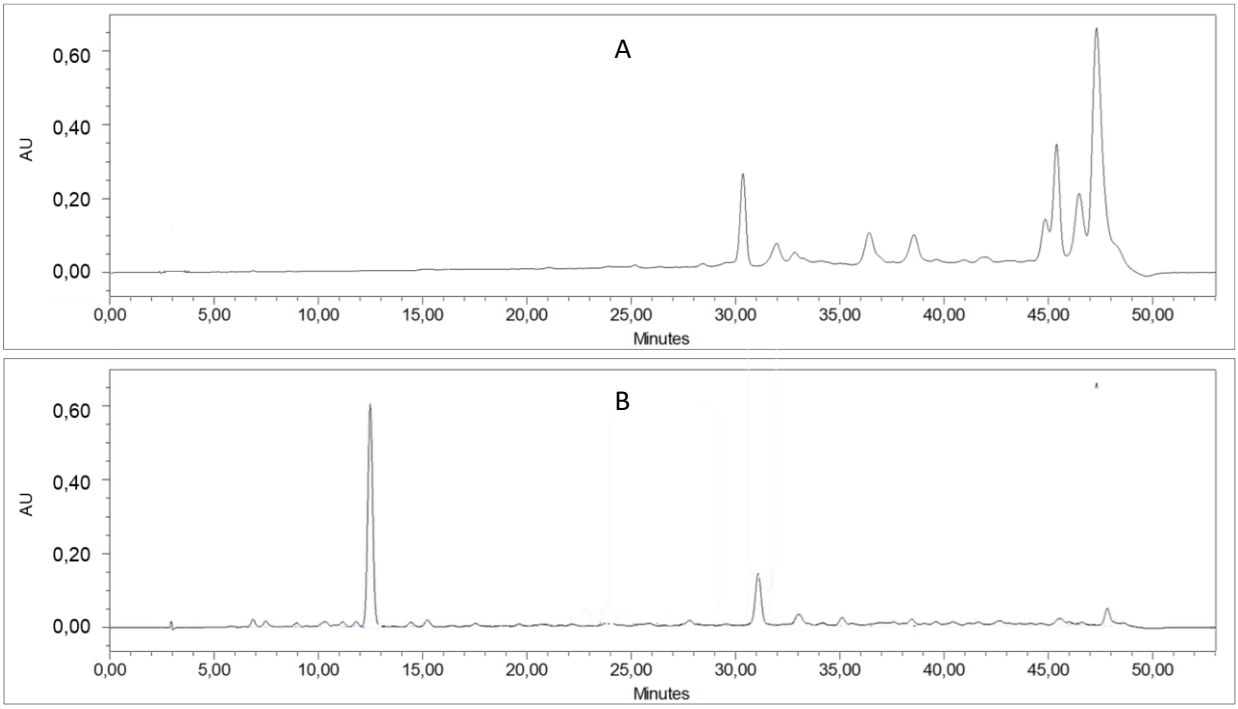

Figure 3. HPLC chromatogram of an ethanolic extract obtained from PEF-treated *Rhodotorula glutinis* incubated before freeze-drying (C-30 column). Detection was done at 485 nm. Chromatograms corresponding to the sample directly after extraction (**A**), as well as after alkaline hydrolysis (**B**).

the composition of different extracts obtained from *R. glutinis* did not vary in the same solvent as a consequence of PEF treatment (Table 3). The percentage of the area that represents each peak was not significantly different due to the pre-treatment of the sample before extraction for any of the solvents assayed.

It is known that the torularhodin molecule possesses a hydroxyl side chain, which may permit the formation of esters with fatty acids. In order to evaluate if the lower polarity of torularhodin with respect to β-carotene was a consequence of a possible link between this compound and fatty acids, the ester bonds were broken by alkaline saponification. After this treatment, a well-defined peak appeared in the chromatogram after 12 min of elution (Figure 3B), which corresponded to a relatively polar carotenoid with an absorbance maximum at 490 nm. This could be compatible with torularhodin being the main carotenoid found in the ethanolic extract. The concentration of torularhodin esters constituted a large percentage (ca. 60 %) of the total carotenoid content extracted in ethanol from PEF-treated cells.

Table 3. Average percentages of the total area of each of the main peaks found in chromatograms obtained after extraction in different solvents from untreated or PEF treated cells.

Peak	RT (min)	Maximum absorption	Identification	Acetone		Ethanol		Hexane	
				Cont	*PEF*	*Cont*	*PEF*	*Cont*	*PEF*
1	31	450 nm	*β-carotene*	28 % (a)	30 % (a)	14 % (b)	15 % (b)	40 % (c)	40 % (c)
2	45.5	490 nm	*Carotenoid ester*	23 % (α)	22 % (α)	23.5% (α)	25% (α)	23 % (α)	24% (α)
3	47.5	490 nm	*Carotenoid ester*	23 % (i)	23% (i)	39 % (ii)	40 % (ii)	22 % (i)	19% (i)

Letters in brackets show statistically significant differences ($p < 0.05$) among extracts for each of the peaks. RT: Retention time.

4.-DISCUSSION

Currently there is an increasing interest in carotenoids obtained by biotechnological processes as an alternative to synthetic production by chemical methods (Kot et al., 2016). However, the envelopes of the microbial cells, the hydrophobic nature of carotenoids, the association of carotenoids with other macromolecules such as proteins and fatty acids, and the sensitivity of carotenoids to heat, light or acids make yeast-derived carotenoids difficult and inefficient to recover. Therefore, this source of carotenoids requires the development of environmentally friendly, simple, rapid, and inexpensive extraction methods (Saini and Keum, 2018). In order to achieve this objective, in this study PEF was selected as an alternative to heavily energy consuming pre-treatments (e.g. ultrasound, high-pressure homogenisation or bead milling) aiming to break down the physical barriers present in the cells. Further, SC-CO_2 extraction was selected as an environmentally friendly alternative to the extraction with organic solvents, which are known to be associated with environmental, health, and safety hazards.

SC-CO_2 extraction technology has been widely used in literature as an alternative to traditional methods for carotenoid extraction from microbial sources with variable results. Some studies showed extraction yields of around 70–90 % of the total content (20–30 mg/g_{dw}) for astaxanthin from the microalgae *Haematococcus pluvialis* (Bustamante et al., 2011; Machmudah et al., 2006; Sanzo et al., 2018). Similarly, studies of carotenoid extraction from selected cyanobacteria species achieved yields of 50 and 90 % of the total content, i.e. 4.93 and 1.355 mg/g_{dw}, respectively (Macías-Sánchez et al., 2007; Montero et al., 2005). However, the effectiveness of SC-CO_2 technology for carotenoid extraction from red yeasts is unclear. Lim et al. (2002) achieved an 84 % (~265 µg/g_{dw}) extraction of the total carotenoid content from *Phaffia rhodozyma*, while Hasan et al. (2015), in contrast, reported an extraction yield of 119.7 µg/g_{dw}, which represented less than 50 % of the total carotenoid content. In this investigation, the extraction of carotenoids from *R. glutinis* using SC-CO_2 was reported for the first time. Results obtained showed that SC-CO_2 extraction is not an efficient method for the recovery of carotenoids from *R. glutinis,* as the maximum extraction yield did not exceed 5 % for the assayed conditions. These results were unexpected, as SC-CO_2 is known to possess dissolving properties similar to hexane, i.e. it is a very effective solvent for relatively non-polar materials (Sahena et al., 2009). Among the main carotenoids produced by *Rhodotorula* species, β-carotene is non-polar and although torularhodin is more polar itself (Frengova and Beshkova 2009), the HPLC analysis of extracts indicated that it was forming esters instead of being present in a free form, thus being less polar. The low effectivity of SC-CO_2 for extracting these carotenoids does not seem to be related with the difficulty of CO_2 to penetrate into the cytoplasm, as other authors suggested (Wang et al., 2012), as the increased permeability of the cytoplasmic membrane of the 99 % of the cells by electroporation did not increase the carotenoid extraction yield. On the other hand, the difficulty of CO_2 to disrupt the association of carotenoids with other macromolecules such as lipids or proteins in the cell membrane neither seemed to be the reason of the low effectivity. The incubation of the electroporated cells for 24 h, hypothesised to contribute to the disruption of carotenoids associated with other molecules of the cells by intrinsic hydrolytic enzymes (Martínez et al., 2018) neither increased the efficacy of SC-CO_2. Efficiency of SC-CO_2 extraction increased significantly when ethanol was used as a co-solvent, however the highest yield obtained was only around 20 % of the total carotenoid content. In this case the enhancement of the solvent effectiveness of SC-CO_2 was neither related to the effect of PEF on the yeast cells. This increase in extraction is attributed to the fact that addition of a polar co-solvent enhances the solubilizing capabilities of SC-CO_2 (Lim et al., 2002; Macías-Sánchez et al., 2008; Montero et al., 2005; Pan et al., 2012) and has even been suggested to affect the cell wall structure including a swelling of the cell that may reduce the resistance against the mass transfer of the solute into the SC-CO_2 (Machmudah et al., 2006).

Solid-liquid solvent extraction was also shown to be ineffective for extracting carotenoids from freeze-dried cells of *R glutinis*, independent of

the fact if they were PEF treated or not. The maximum extraction yield did not exceed 25 % of the total carotenoid content when the extraction with acetone was extended for 12 hours. This extraction yield was much lower than those obtained by other authors on the solvent extraction of carotenoids from dry biomass of yeast using different mechanical, chemical or enzymatic techniques of cell disruption, which liberate the carotenoids from the cellular structures and thus enable the solvents to directly interact with carotenoids, without penetrating into the cell (Buzzini and Martini, 2000; Hasan et al., 2015; Michelon et al., 2012; Park et al., 2007; Tinoi et al., 2005). Although electroporation of the yeast's cytoplasmic membrane could facilitate the penetration of the solvents, the extraction process could be restricted by the interaction of carotenoids with cellular structures and the transfer of the solvent and carotenoids across the cell envelopes resulting in lower carotenoid recovery.

Among the procedures for extracting carotenoids from *R. glutinis* biomass employed in this study, ethanolic extraction of PEF treated cells and subsequent freeze drying after 24 h incubation time achieved the maximum carotenoid yield. It is expected that electroporation is the only occurring effect on the cytoplasmic membrane in the cells when they are immediately freeze-dried after PEF treatment. However, it is assumed that after 24 h incubation time a PEF-triggered autolysis and an associated hydrolysis of links between carotenoids and cell structures is initiated. This additional effect that has previously been demonstrated on the extraction of carotenoids and phycoerithrin from fresh biomass of *R. glutinis* and *Porphyidium cruentum* (Martínez et al., 2018; Martínez et al., 2019) has now also been observed for the first time for biomass that is subsequently dried. It was hypothesized that the osmotic disequilibrium due to uncontrolled molecular transport through the cytoplasmic membrane after electroporation could cause the liberation of hydrolytic enzymes from the organelles into the cytoplasm. During incubation, these enzymes might degrade the structures where carotenoids are bound to cell constituents, facilitating their subsequent extraction by solvents.

Considering the polarity of the carotenoids produced by *R. glutinis,* ethanol unexpectedly was shown to be more effective than other solvents assayed. This observation disagrees with previous research on extraction of carotenoids from dried biomass of microbial cells, in which less polar solvents such as hexane or acetone are preferred due to their higher effectivity (Cerón et al., 2008; Saini and Keum, 2018). While in these studies the cells are completely destroyed by mechanical, chemical or enzymatic methods applied before drying or during the extraction process, the overall structure of the PEF treated *R. glutinis* cells was maintained, even after 24 h incubation and subsequent freeze drying (data not shown). Therefore, solvent extraction required that the solvent penetrated through the cell membrane into the cytoplasm and interacted with the carotenoids. The presence of a little amount of water remaining inside the cells after freeze-drying could complicate the penetration of the most non-polar solvents into the cytoplasm, explaining the higher effectivity of ethanol for extracting carotenoids. Therefore, the application of a PEF treatment to fresh biomass of *R. glutinis* 24 h before freeze-drying may be an effective alternative for extracting carotenoids from *R. glutinis* compared to the use of mechanical methods with high energy costs and organic solvents with higher environmental, health and safety hazards than ethanol. As the overall cell structure is maintained after ethanolic extraction of carotenoids from PEF treated cells, it is expected to obtain a more pure extract compared to those obtained by mechanical methods used for the complete destruction of the cell structure.

The use of ethanol as a solvent did not modify the proportion of the carotenoids extracted from *R. glutinis.* The high valuable pigment torularhodin was found to be the main carotenoid present in the ethanolic extracts. Other authors using other solvents such as acetone also found that torularhodin was the most abundant carotenoid of *Rhodotorula* species, representing between the 60-70% of the total extracted carotenoids *(*Mihalcea et al., 2015; Park et al., 2007).

Torularhodin is an uncommon carotenoid of distinct importance. This singlet oxygen quencher exhibits strong antioxidant activity and acts as

Vitamin A precursor (Moliné et al., 2012). Besides, this unusual carotenoid has been proposed as colorant for meat products due to its characteristic red colour (Maldonade et al., 2008; Sakaki et al., 2001; Zoz et al., 2015). Furthermore, the anti-cancer potential of torularhodin has recently been demonstrated in in-vivo mice experiments (Du et al., 2016). These potential applications ranging from food technology to pharmaceutics require the reliable and reproducible production and extraction of torularhodin. However, this pigment is produced by a very small number of sources, mostly by this yeast and a few others sources that are complicatedly scalable for industrial exploitation. Therefore, the presented method would improve the extraction step, facilitating the development of a continuous and economically feasible source of torularhodin from red yeasts.

5.-CONCLUSIONS

The application of a PEF treatment to the fresh biomass of *R. glutinis* and the subsequent incubation for 24 h before freeze-drying resulted in an enhancement of the carotenoid extraction as compared with the carotenoid extraction from untreated or even PEF treated biomass that was freeze-dried immediately after the treatment.

Among the solvents used in this investigation, ethanol was shown to be the most effective agent, permitting the extraction of more than 80 % of the carotenoids from the PEF-treated biomass of *R. glutinis* freeze-dried after 24 h of incubation. Furthermore, the main carotenoid found in this extract corresponded to torularhodin, which is of great importance as it can only be obtained from limited natural sources.

These findings demonstrate the possibility of using PEF as a pre-treatment for the extraction of torularhodin in ethanol from dried *R. glutinis* biomass. This is of great interest, as PEF is an industrially scalable technique, ethanol is considered an eco-friendly solvent, and applications of torularhodin might range from food, cosmetics, and pharmaceutical industries.

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4.3. PEF-assisted extraction of valuable compounds from microalgae

4.3.1. C-phycocyanin extraction assisted by Pulsed Electric Field from *Artrosphira platensis (Manuscript VI)*

4.3.2 Pulsed Electric Field permeabilization and extraction of phycoerythrin from *Porphyridium cruentum (Manuscript VII)*

4.3.3. Pulsed Electric Field permeabilization to extract astaxanthin from the Nordic microalgal strain *Haematococcus pluvialis (Manuscript VIII)*

4.3.1. C-phycocyanin extraction assisted by Pulsed Electric Field from *Artrosphira platensis (Manuscript VI)*

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C-phycocyanin extraction assisted by pulsed electric field from *Artrosphira platensis*

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ABSTRACT

This paper assesses the application of pulsed electric fields (PEF) to the fresh biomass of *Artrhospira platensis* in order to enhance the extraction of C-phycocyanin into aqueous media.
Electroporation of *A. platensis* depended on both electric field strength and treatment duration. The minimum electric field intensity for detecting C-phycocyanin in the extraction medium was 15 kV/cm after the application of a treatment time 150 μs (50 pulses of 3 μs). However higher electric field strength were required when shorter treatment times were applied. Response surface methodology was used in order to investigate the influence of electric field strength (15–25 kV/cm), treatment time (60–150 μs), and temperature of application of PEF (10–40 °C) on C-phycocyanin extraction yield (PEY). The increment of the temperature PEF treatment reduced the electric field strength and the treatment time required to obtain a given PEY and, consequently decreased the total specific energy delivered by the treatment. For example, the increment of temperature from 10 °C to 40 °C permitted to reduce the electric field strength required to extract 100 mg/g d_w of C-phycocyanin from 25 to 18 kV/cm, and the specific energy input from 106.7 to 67.5 kJ/Kg.
Results obtained in this investigation demonstrated PEF's potential for selectively extraction C-phycocyanin from fresh *A. platensis* biomass. The purity of the C-phycocyanin extract obtained from the electroporated cells was higher than that obtained using other techniques based on the cell complete destruction.

1. Introduction

C-phycocyanin is a blue-colored water-soluble protein of great commercial and industrial significance. It is widely used as colorant in both food and cosmetic industries, but also as fluorescent marker in biomedical research. Furthermore, its potential as a therapeutic agent in oxidative stress-induced diseases has been demonstrated (Zhao, Yi-liang, Jiamei, & Wei-min, 2014).

The blue-green microalga *Artrosphira platensis* is an excellent source of C-phycocyanin. This compound that serves as a principal photoreceptor for this cyanobacterium's photosynthesis is arranged, along with other phycobiliproteins, into supramolecular complexes called phycobilisomes, located in the thylacoid membranes (Sekar & Chandramohan, 2008). It has been estimated that this cyanobacterium's protein fraction may contain up to 20% of C-phycocyanin. The commercial exploitation of this colored substance requires its extraction from the phycobilosomes, and subsequent purification. Pigment purity is of utmost importance, particularly for fluorescent applications in clinical and immunological analysis (Sekar & Chandramohan, 2008).

It has been reported that the extraction of phycobiliproteins from cyanobacteria is notoriously difficult because of the extremely resistant cell wall (Wyman, 1992). Although many different methods have been assayed for the extraction of C-phycocyanin from *A. platensis*, none of them are considered as standard procedure.

C-phycocyanin can be extracted from dry or wet *A. platensis* biomass into aqueous media. In order to improve the extraction yield and to reduce extraction time, different procedures that cause the breakage of cell envelopes such as freeze/thaw cycles, homogeneization in mortar and pestle, sonication, bead milling and lysozyme disintegration of the cell wall have been assayed (Duangsee, Phoopat, & Ningsanond, 2009). However, all of these cell disruption methods are characterized by a lack of specificity that causes the release of cell debris or other impurities that could negatively affect the quality and purity of the extracts, as well as downstream recovery and purification operations. On the other hand, it has been reported that the drying of *A. platensis* biomass resulted in approximately 50% loss of C-phycocyanin (Sarada, Pillai, & Ravishankar, 1999).

Pulsed electric fields (PEF) cause the increment of cell membrane permeability (electroporation), via the application of high-intensity electric field pulses of short duration – generally in the order of microseconds to milliseconds (Kotnik, Kramar, Pucihar, Miklavcic, & Tarek, 2012). Several studies have demonstrated that the electroporation of

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bacteria, yeast and microalgae improves the extraction of intracellular compounds of interest, such as lipids, proteins, carbohydrates, or pigments with low energy consumption (Barba, Grimi, & Vorobiev, 2015; Coustets et al., 2015; Ganeva, Galutzov, & Teissié, 2003; Goettel, Eing, Gusbeth, Straessner, & Frey, 2013; Luengo, Martínez, Bordetas, Álvarez, & Raso, 2015; Parniakov et al. 2015a and b; Postma et al., 2016; Zbinden et al., 2013). Treatment of fresh *A. platensis* biomass by PEF could improve the current extraction process of C-phycocyanin from fresh biomass by reducing the release of cells debris, facilitating subsequent purification operations, and minimizing the energetic costs and losses associated with the drying of the biomass.

Temperature of application of PEF has been demonstrated to be a key parameter affecting cell membrane electroporation. Several studies have demonstrated that increasing the temperature decreases the critical electric field required to cause electroporation in both eukaryote and prokaryote cells and enhance effects derived from PEF treatment such as microbial inactivation or improving extraction of intracellular compounds (Lebovka, Praporscic, Ghnimi, & Vorobiev, 2005; Saldaña, Álvarez, Condón & Raso, 2014).

This study aims to evaluate the potential of the application of PEF to fresh *A. platensis* biomass to improve the extraction of C-phycocyanin into aqueous media, assessed both in terms of extraction yield and purity of the extract. Here we have evaluated the effects of PEF electric field strength, treatment time and application temperature, in order to establish which are the optimal conditions for C-phycocyanin extraction.

2. Material and methods

2.1. Culture conditions of Arthropira platensis

Arthrospira platensis (BNA 0007B, National Bank of Algae, Canary Islands, Spain) were grown in a modified *Spirulina* medium (Aiba & Ogawa, 1977).

Cells were cultured photoautrophically in 2-L tubes of 8 cm diameter and 53 cm height, bubbled with air (6 mL/s) at 30 °C, in light:dark cycles (12:12 h) using white fluorescent lamps (15 mmol/m^2 s). The culture medium was initially inoculated at an optical density of 0.1 at 560 nm using a pre-culture. To determine biomass concentration, samples were taken every 24 h. Experiments were performed using cells at the stationary phase of growth after an incubation time between 7 and 9 days. Biomass concentration at the stationary phase was around 1 g_{dw}/L. Dry weight (dw) of microalgae was determined by vacuum drying (GeneVac Ltd., UK) at 60 °C using 1 mL of the cell suspension until reaching constant weight (around 1 h).

2.2. Cell disruption

In order to determine the total amount of C-phycocyanin, an aliquot of 150 μL of wet cell biomass was blended with 1350 μL of distilled water, and the mixture was disrupted by bead-beating using a bead beater (bead diameter 0.1 mm, BioSpec Products INC, USA) at a speed of 4800 rpm (10 cycles of 10 s). Following each cycle, the sample was cooled down in water at 0 °C to avoid overheating of the sample.

2.3. PEF treatments

The PEF equipment used in this investigation was previously described by Saldaña et al., 2010. Fresh biomass of *A. platensis* was treated at different temperatures in a tempered batch parallel-electrode treatment chamber with a distance between electrodes of 0.25 cm and an area of 1.76 cm^2. The inner part of the electrodes was empty and dielectric oil (conductivity: 1.4 μS/cm) tempered was recirculated through both electrodes to temper the treatment medium at (10.0, 25.0, 40.0 °C) and to maintain a constant temperature in the medium during the PEF treatments The temperature of the treatment medium was measured with a thermocouple before and after the PEF treatment; temperature variations were always lower than 2 °C.

Before treatment, fresh biomass of *A. platensis* was centrifuged at 3000 × *g* for 10 min at 25 °C and suspended in distilled water. Three cycles of centrifugation and resuspension in distilled water were conducted to obtain a final conductivity of 1.0 mS/cm at 25 °C. This conductivity did not change at 10 °C mS/cm and increased to 1.1 at 40 °C mS/cm. The *A. platensis* suspension (0.44 mL) was placed in the treatment chamber by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium). The suspension was subjected from 15 to 50 monopolar square 3 μs waveform pulses of 3.75, 5 and 6.25 kV. These voltages resulted in electric field strengths of 15, 20, 25 kV/cm respectively that corresponded with total specific energies that ranged from 13.5 to 110.1 kJ/kg of suspension. The energy per pulse (*W*) was calculated using the following equation:

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

in which σ (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 (*W*) 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. Frequency of application of the treatments was 0.5 Hz.

2.4. C-phycocyanin extraction

For C-phycocyanin extraction, 1 mL of the untreated or PEF-treated *A. platensis* suspension was added to 19 mL of distilled water. The extraction was conducted in a rotary shaker at 20 °C in the dark. In order to obtain extraction curves, samples were gradually collected until 420 min. After centrifugation (6000 × *g* for 90 s), the supernatant's optical density was measured at 615 and 652 nm. C-phycocyanin concentration was calculated according to the following equation (Bennett & Bogorad, 1973):

$$PC = OD_{615} - 0.474 \times (OD_{652})/5.34 \quad (2)$$

in which PC is the C-phycocyanin concentration (mg/mL), OD_{615} is the optical density of the sample at 615 nm, and OD_{652} is the optical density of the sample at 652 nm.

The purity of C-phycocyanin extract was monitored spectrophotometrically by the following equation (Abelde, Betancourt, Torres, Cid, & Barwell, 1998):

$$EP = OD_{615}/OD_{280} \quad (3)$$

in which EP is the protein extract purity and OD_{615} indicates the phycocyanin concentration; OD_{280} is the optical density of the sample at 280, indicating the total concentration of proteins in the solution.

The C-phycocyanin extraction yield (PEY) was calculated as:

$$PEY = PC \times V/d_w \quad (4)$$

in which PC is the C-phycocyanin concentration (mg/mL), V is the volume of solvent (mL), and d_w is the dried biomass (g).

2.5. Experimental design

Response surface methodology (RSM) was used to evaluate the effect of electric field strength (15–25 kV/cm), treatment time (60–150 μs), and temperature (10–40 °C) on the C-phycocyanin extraction yield (PEY) from *A. platensis* after 360 min of extraction.

The data obtained after having treated the cells under the conditions described in Section 2.2 were modeled with the following second-order

polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \quad (5)$$

in which Y is the response variable to be modeled, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear coefficients, β_{ii} is the quadratic coefficients, β_{ij} is the cross-product coefficients, and k is the total number of independent factors. A backward regression procedure was used to determine the models' parameters. This procedure systematically removed the effects that were not significantly associated ($p > 0.05$) with the response until a model with a significant effect was obtained.

2.6. Statistical analysis

Experiments were performed in triplicate, and the presented results are means ±95% confidence interval. One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences between the means values. The differences were considered significant at $p < 0.05$. Multiple regression analysis was conducted for fitting the Eq. (5) to the experimental data and significant terms of the model was determined by ANOVA.

The central composite design and the corresponding data analysis were carried out by using the software package Design-Expert 6.0.6 (Stat-Ease Inc., Minneapolis, MN, USA).

3. Results

3.1. Effect of PEF on the gradual extraction of C-phycocyanin from A. platensis

Extraction curves of C-phycocyanin after subjecting the cells of *A. platensis* to pulsed electric field treatments for 75 μs (25 pulses of 3 μs) and 150 μs (50 pulses of 3 μs) are shown in Fig. 1A and B respectively. Also included in these figures, the extraction curve obtained from untreated cells of *A. platensis* shows that C-phycocyanin was not detected in the extraction medium containing the untreated cells of *A. platensis* after the longest extraction time assayed in this investigation (420 min). This observation indicates that the cytoplasmic membrane, acting as a semipermeable barrier, prevented the exit of C-phycocyanin from the *A. platensis* cells to the extraction medium when water was used as solvent. However, when *A. platensis* cells were exposed to an electric field that caused electroporation of the cytoplasmatic membrane, the C-phycocyanin located in the *A. platensis* cells was released to the extraction medium. The enhancement of the extraction yield of other pigments such as carotenoids and chlorophylls from PEF electroporated microalgae has been observed by other authors (Luengo, Condón-Abanto, Álvarez, & Raso, 2014). The lipophilic properties of these pigments required the use of organic solvents such as ethanol. However, in this research, since C-phycocyanin is a water soluble protein, its extraction was conducted in an aqueous media preventing protein denaturation and permitting a more environmentally friendly extraction process (Chemat, Via, & Cravotto, 2012).

As it is shown in Fig. 1, C-phycocyanin was not released immediately after the application of the PEF treatment. Independently of the PEF treatment's intensity, at least 150 min of extraction were required to detect the presence of C-phycocyanin in the extraction medium. This lag time necessary for extracting C-phycocyanin from *A. platensis* was not observed in the release of other compounds from microalgae previously treated by PEF. Different authors have detected different intracellular compounds of microalgae such as ions, carbohydrates, proteins, carotenoids or chlorophylls after extraction times lower than 60 min (Goettel et al., 2013; Luengo et al., 2015; Postma et al., 2016). This varying behavior could be related to the extracted compounds' molecular

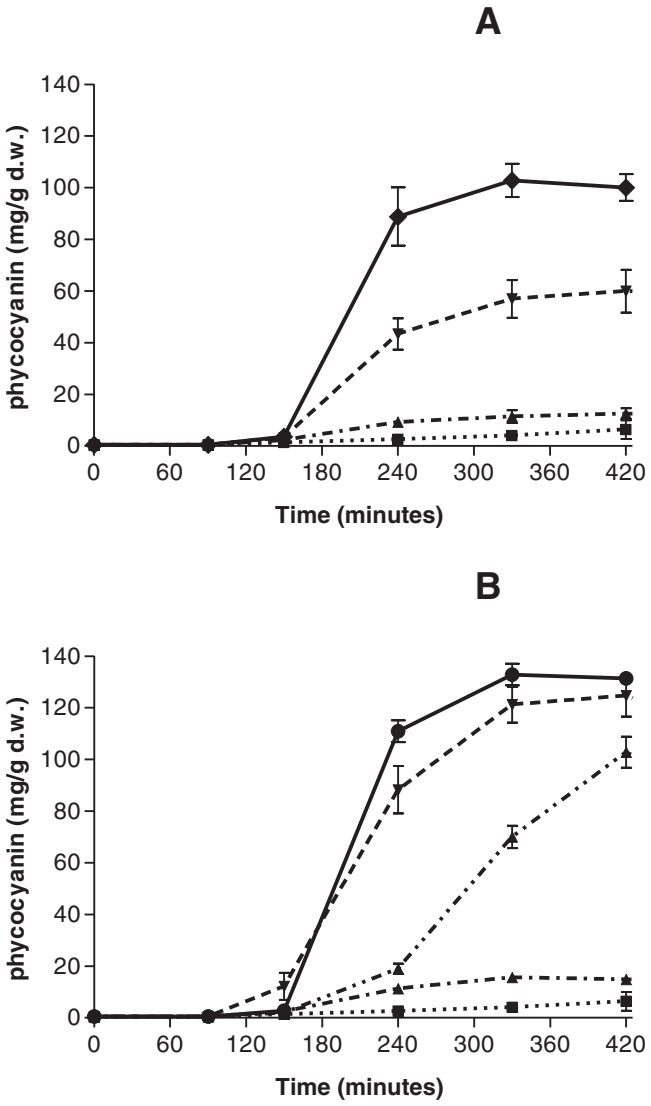

Fig. 1. Extraction curves of C-phycocyanin from *Artrospira platensis* cells treated by PEF at different electric field strengths at 25 °C (■ 0 kV/cm, ● 10 kV/cm, ▲15 kV/cm, ▼ 20 kV/cm, ♦ 25 kV/cm) for 75 μs (A) and 150 μs (B).

weight and to the size of the pores formed by PEF treatment. Whereas low molecular weight compounds could cross the cytoplasmatic membrane immediately after their electroporation, the exit of molecules of higher molecular weight may require that the pores created by PEF treatment enlarge themselves in the course of time. On the other hand, the fact that C-phycocyanin is located in phycobilosomes assembled on the thylakoid membranes rather than free in the cytoplasm could also be the cause of the lag time observed in the extraction of this compound.

Fig. 1 shows that the minimum intensity of the PEF treatment required to electroporate *A. platensis* cells to a level that permitted the extraction of C-phycocyanin depended both on electric field strength and treatment duration. When treatment duration was 75 μs (25 pulses of 3 μs), electric fields higher than 15 kV/cm were necessary to detect the presence of C-phycocyanin in the extraction medium. However, a treatment at 15 kV/cm was sufficient to provoke the release C-phycocyanin when its duration was 150 μs (50 pulses of 3 μs). This observation confirms the results obtained by other authors on the electroporation of bacteria and microalgae, indicating that the critical electric field strength – defined as the lowest electrical field strength required to electroporate a cell – depends on the treatment's duration in addition to the electric field strength applied (Garcia, Gomez, Mañas, Raso, &

Pagan, 2007; Luengo et al., 2014). Treatment duration also influenced the effect induced by the electric field strength at the higher intensities assayed. The increment of electric field strength from 20 to 25 kV/cm almost duplicated the maximum amount of extracted C-phycocyanin when treatment duration was 75 μs. However, the same increment in electric field strength scarcely increased the C-phycocyanin release when treatment duration was 150 μs.

Fig. 2 compares the cells of *A. platensis* treated by PEF (25 kV/cm for 150 μs) and by bead-beating (a cell disintegration technique habitually used for obtaining intracellular compounds). We observed that whereas the bead beater caused the complete destruction of the *A. platensis* cells, PEF treatment only caused the separation of those cells that form the characteristic cylindrical filaments of *A. platensis* called trichomes; the overall structure of the cells, however, was not affected by the treatment. This varying effect of the two described techniques on the cell integrity of *A. platensis* also explains why the purity of the C-phycocyanin extract obtained after the application of PEF treatment (0.51 ± 0.021) was much greater than the purity of the extract obtained after treating the cells with the bead beater (0.21 ± 0.013). The purity of the extract obtained from *A. platensis* cells treated by PEF was similar to that reported by Silveira, Burkert, Costa, Burkert, and Kalil (2007) when the extraction was performed from dry biomass of *A. platensis* in different aqueous media, using extraction times of up to 72 h and without treating the dry biomass by any mechanical disruption technique whatsoever.

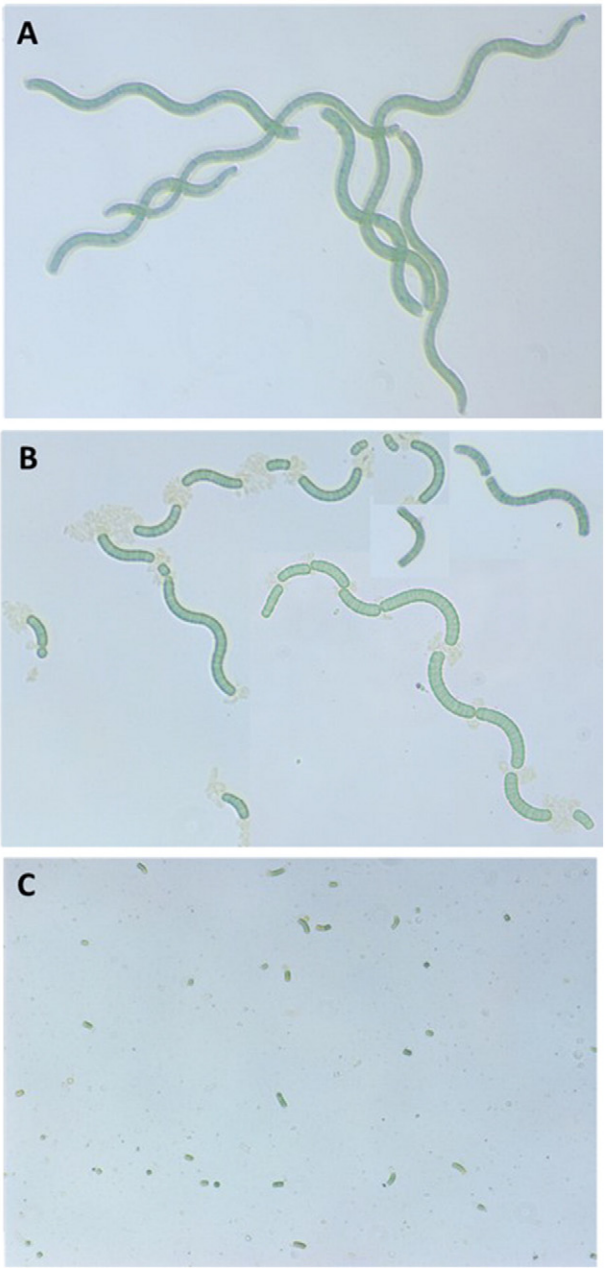

Fig. 2. Optical microscopy observation (400×) of untreated (A), PEF (25 kV/cm, 150 μs) treated (B) and bead-beating treated (C) cells of *Artrosphira platensis*.

3.2. Influence of electric field strength, treatment time and temperature of application of PEF treatment on the extraction of phycocyanin from A. platensis

After demonstrating that PEF is an effective procedure for extracting C-phycocyanin from *A. platensis* using water as solvent, in a second step we investigated the influence of electric field strength, treatment time (number of pulse × pulse width) and temperature in order to find the optimal PEF treatment conditions that maximize extraction. Although electric field strength and treatment time are the main processing parameters of PEF technology, we also included temperature in our experimental design in view of its influence on membrane electroporation. Several studies have demonstrated that PEF treatment above room temperature decreases the critical electric field required to cause cell electroporation, increases microbial inactivation and improves the subsequent extraction of intracellular compounds (Lebovka et al., 2005; Luengo et al., 2015; Postma et al., 2016; Timmermans et al., 2014).

Results from the central composite design constructed to investigate the favorable influence of the three above-mentioned factors on the extraction of C-phycocyanin from *A. platensis* are shown in Table 1. According to the extraction curves shown in Fig. 1, extraction time was fixed at 360 min because longer times did not increase the extracted amount of C-phycocyanin. C-phycocyanin values varied from 6.5 to 159.9 mg/g dw of *A. platensis* culture, indicating that the total amount of extracted C-phycocyanin may increase by a factor of almost 25, depending on the treatment conditions used in the electroporation of the *A. platensis* cells. The maximum amount of extracted C-phycocyanin was obtained when the *A. platensis* cells were previously treated at 40 °C by the most intensive PEF treatment assayed (25 kV/cm, 150 μs). The total content of C-phycocyanin in the *A. platensis* cells, calculated after the complete destruction of the cells by bead-beating was 217.14 ± 4.71. Therefore 70% of the total content was extracted under the most intense PEF treatment applied. The purity of extract (0.46 ± 0.019) obtained under the most intense PEF treatment conditions (25 kV/cm 150 μs) applied at 40 °C was similar to that obtained when the treatment was applied at 25 °C (0.44. 0.034) The higher extraction of C-phycocyanin observed by increasing the three processing parameters

Table 1
Phycocyanin extraction yield (PEY) from *Artrospira platensis* cells treated by PEF at different temperatures, electric field strengths and treatment times.

Temperature (C°)	Electric field strength (kV/cm)	Treatment time (μs)	Phyocyanin extraction yield (mg/g)*
40	25	150	151.94 ± 14.22[a]
40	25	60	118.26 ± 5.11[b]
40	20	105	82.48 ± 8.7[c]
40	15	150	69.1 ± 20.3[cg]
40	15	60	14.44 ± 7.16[d]
25	25	105	98.39 ± 5.87[e]
25	20	150	86.25 ± 5.08[c]
25	20	105	40.08 ± 5.06[f]
25	20	60	17.01 ± 5.02[d]
25	15	105	16.78 ± 7.42[d]
10	25	150	111.14 ± 16.2[be]
10	25	60	44.81 ± 5.82[fg]
10	20	105	49.92 ± 2.28[fg]
10	15	60	6.5 ± 2.85[h]
10	15	150	19.63 ± 5.86[d]

Means followed by the different letters are significnatly different ($p > 0.05$).
* Mean ± 95% confidence interval.

assayed could be caused by an increment in the number of cells that become electroporated, and/or by an increment in the number and/or size of the pores in the electroporated cells. The maximum amount of C-phycocyanin released from the *A platensis* cells was within the value ranges reported in the literature using other extraction procedures, such as suspension of fresh biomass in distilled water for 3–4 days, or the application of 2–3 freezing-and-thawing cycles prior to extraction (Sarada et al., 1999).

In order to quantify the influence of electric field strength, treatment time and temperature on the extraction of C-phycocyanin from *A. platensis* fresh biomass, we performed multiple regression analysis, fitting the experimental data displayed in Table 1 to Eq. (1). Our analysis resulted in the following equation after removing non-significant terms ($p > 0.05$):

$$PEY = -105.56 + 3.50\,E + 0.68\,t - 1.95\,T + 0.16\,E\,T \quad (6)$$

in which PEY is the C-phycocyanin extraction yield (mg/g d_w), E is the electric field strength (kV/cm), t is treatment time (μs), and T the temperature of application of PEF treatment (°C).

Table 2 shows the results of the analysis of variance (ANOVA) for the significant terms of the model and the statistics used to test its adequacy. The model F-value was 30.42 indicating that the model was significant ($p < 0.0001$) and therefore the terms in the model have a significant effect on the response. The determination coefficient (R^2) of the model was 0.92 pointing out that <8% of the total response variation could not be explained by the model. On the other hand, the adjusted R^2 value that corrects the R^2 according to the number of responses and terms in the models was close to R^2 values, thereby indicating that there was a good agreement between the experimental and predicted values.

The F-values of the model parameters displayed in Table 2 are indicators of the significances of the variables' effects. According to those F-values, the electric field strength's linear term ($F = 61.4$), along with time ($F = 40.62$), were the two most significant variables indicating that changes in these factors had the most influence on the PEY. The linear terms of temperature and the interaction between electric field and temperature were also significant, but had lower F-values. The presence of that interaction term means that the effect of electric field strength on PEY depends on the treatment's application temperature.

In order to illustrate the influence of electric field strength, treatment time and temperature on the improvement of extraction of C-phycocyanin from fresh biomass of *A. platensis*, we obtained graphical representations using the regression model (Eq. (6)) considering the responses within the range of experimental conditions assayed (Fig. 3). The effect of treatment duration at 25 kV/cm and different PEF treatment temperatures on PEY is shown in Fig. 3A. PEY increased linearly with the treatment time in the range of 60 to 150 μs. Independently of the PEF treatment temperature, an increase of treatment time of 60 μs (20 pulses of 3 μs) led to an increase in PEY of around 60%. On the other hand, it was observed that the increment of PEF treatment temperature caused a reduction in the number of pulses required to obtain a given PEY and, consequently, a decrease in the total specific energy delivered to the *A. platensis* cells. At 10 °C, in order to extract 100 mg of phycocyanin/g d_w, a treatment for 145 μs was required, corresponding to a total specific energy of 106.7 kJ/kg. However, at 20 and 40 °C, the same PEY was reached with treatments of 115 μs and 60 μs, corresponding to total specific energies of 87.61 and 48.67 kJ/kg respectively.

On the other hand, Fig. 3B shows the effect of electric field strength on PEY at the longest treatment time assayed (150 μs), with different applied PEF treatment temperatures. Similarly to the influence of treatment time, we observed that PEY increased linearly along with electric field strength. However the effect of electric field strength on the increment of PEY gradually increased when PEF treatments were applied at higher temperatures. The increment of the treatment temperature from 10 to 40 °C increased PEY by 25% at 15 kV/cm, and by 60% at 25 kV/cm. Another interesting observation concerning the influence of electric field strength on PEY at different treatment temperatures is that temperature increase causes a reduction in both the electric field

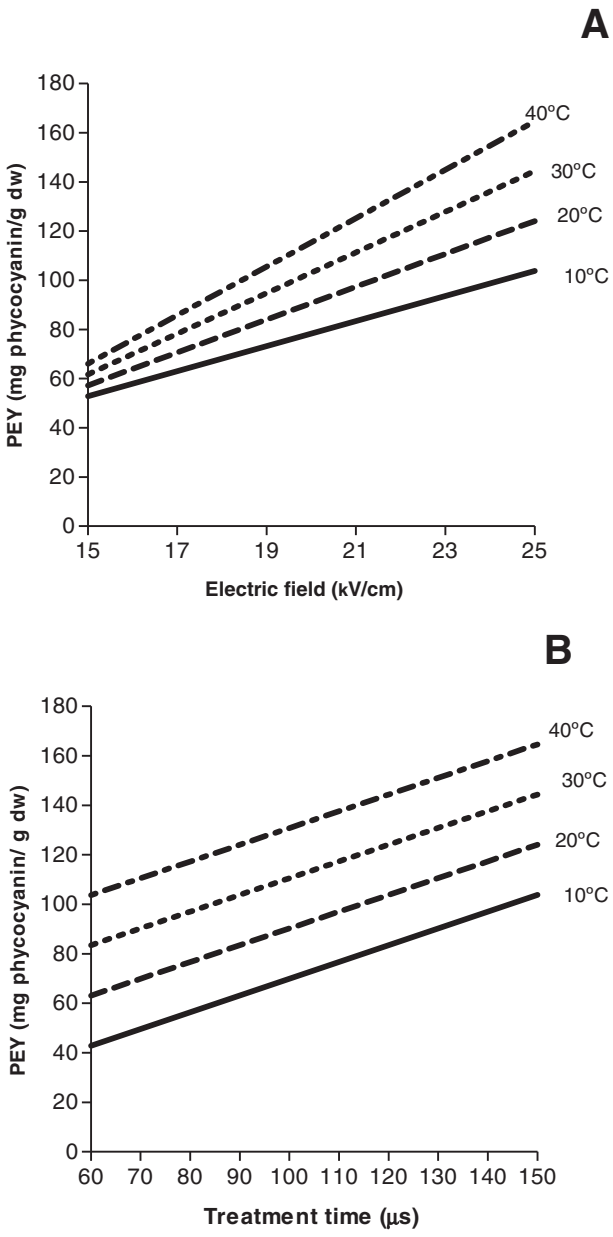

Fig. 3. Influence of treatment time at 25 kv/cm (A) and electric field strength for 150 μs (B) on the phycocyanin extraction yield (PEY) from *Artrospira platensis* at different temperatures.

Table 2
F-values and *p*-values of the ANOVA analysis for the model developed to describe the influence of the temperature (T^a), treatment time (t), and electric field strength (E) on the C-phycocyanin extraction yield from *Artrospira platensis*.

	F-Value	p-Value
T^a	14.96	0.0031
E	61.4	<0.0001
t	40.62	<0.0001
$E.T^a$	4.98	0.0498
Model	30.42	<0.0001
R^2	0.92	
adjusted-R^2	0.89	
RMSE	14.77	

strength and the specific energy required to obtain a given PEY. For example, a temperature increase from 10 to 40 °C reduced the electric field strength required to extract 100 mg/g d_w of C-phycocyanin from 25 to 18 kV/cm, and the specific energy input from 93.7 to 48.6 kJ/Kg. As the increment of the conductivity of the treatment medium was very low when the temperature increased from 10 to 40 °C, the energy input corresponding to the most intense PEF treatment applied (25 kV/cm for 150 μs) was less of a 5% higher than those applied at 10 and 25 °C. Therefore the effect observed was a consequence of the changes caused by temperature in cell envelopes rather than the input energy delivery.

The effect of the three PEF processing parameters investigated in this study on the improvement of extraction of C-phycocyanin from *A. platensis* does not agree with their effect on the extraction of lutein from *C. vulgaris* using ethanol as solvent. Luengo et al. (2015) reported that treatments exceeding 100 μs did not improve the extraction of lutein from *C. vulgaris*, and that increases of electric field strength in the range of 10 to 20 kV/cm were less effective on the subsequent extraction of lutein than in the range of 20 to 25 kV/cm. On the other hand, whereas temperature increases in the range of 10 to 30 °C significantly enhanced the extraction of lutein, further increments from 30 to 40 °C did not have a significant effect. This varying behavior supports the hypothesis that the characteristics of cell envelopes and the type of compound to be extracted may have a significant influence on the PEF treatment conditions one should apply in order to improve the extraction of intracellular compounds. As a consequence, defining the optimal PEF processing conditions for the extraction of different compounds of interest, requires to perform specific studies corresponding with each type of application.

4. Conclusions

Results obtained in this investigation have demonstrated PEF's potential for the selective recovery of C-phycocyanin from fresh biomass of *A. platensis* using water as solvent. The purity of the C-phycocyanin extract obtained from electroporated cells was higher than that obtained using other techniques based on the complete destruction of the cells. This higher purity could facilitate the downstream purification operations that are required before C-phycocyanin can be used in certain applications – for instance, as a fluorescent marker in biomedical research.

The efficacy of the PEF treatment on the extraction enhancement depended on the electric field strength, treatment duration and temperature of application the PEF treatment. Optimization of these processing parameters to electroporate microalgae at low energy inputs and electric field strengths it is necessary to introduce the PEF as an economically feasible technology to improve biomass processing for metabolic recovery using low-cost pulse generators and minimal energy consumption.

Acknowledgments

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4.3.2. Pulsed Electric Field permeabilization and extraction of phycoerythrin from *Porphyridium cruentum (Manuscript VII)*

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Pulsed electric field permeabilization and extraction of phycoerythrin from *Porphyridium cruentum*

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A B S T R A C T

This paper assesses the extraction of β-phycoerythrin (BPE) into aqueous media by the application of pulsed electric field treatments (PEF) to the fresh biomass of *Porphyridium cruentum*.

An increase in electric field strength from 2 to 10 kV/cm, or a prolongation of treatment time increased electroporation and inactivation of *P. cruentum* in the range investigated (2–10 kV/cm; 30–150 μs). A perfect agreement between fraction of dead cells and fraction of electroporated cells was observed when more than 20% of the cells were inactivated. Even after 48 h of incubation, BPE was not detected in the extraction medium containing untreated cells of *P. cruentum*: thus, an intact cytoplasmic membrane prevented the exit of BPE. After 24 h of extraction, the entire BPE content (32 mg/g d.w.) was released after treating *P. cruentum* cells at 8 or 10 kV/cm for 150 μs. However, BPE was not released immediately after the PEF treatment, thereby requiring, in most cases, a lag time of over 6 h until the compound could be detected in the extraction medium. This behavior indicates that BPE extraction requires not only the diffusion of the compound across the cell membrane, but also the dissociation of the compound from the cell structures. In this sense, it is hypothesized that *P. cruentum* autolysis triggering by PEF could be the main cause involved in the effectivity of these treatments in BPE extraction. An improved grasp of the kinetics and the mechanism of the enzymes participating in microalgae autolysis, and of the autolysis trigger by PEF, will allow this process to be developed at an industrial scale.

1. Introduction

B-phycoerythrin (BPE) is a water-soluble, red-coloured phycobiliprotein present in nature in different organisms (cyanobacteria, eukaryotic algae, etc.). This pigment is the main component in phycobilisomes of marine microalgae such as *Phorphyridium cruentum*. Industrial demand for natural products has led to increased interest in *P. cruentum* BPE since this pigment can be used as a colorant in the food, cosmetic, and pharmaceutical industries, and as a fluorescent biomarker in immunology [1].

Since *P. cruentum* BPE is an intracellular metabolite assembled on the thylakoid membranes of chloroplast, the procedures generally applied to obtain this pigment involve a disruption of cellular and chloroplast membranes [2]. Several cell-disrupting methods, such as high-pressure homogenization [3], sonication [4,5] or bead-mill, have been assayed for the extraction of BPE from *P. cruentum*. These microalgae, however, are encapsulated within a layer of high-molecular-weight sulfated polysaccharides gel (the red microalgae cell wall) composed of numerous monosaccharides, mainly xylose, glucose, and galactose [6–8]. In this sense, the breakage of the cell wall of *P. cruentum* algae is no trivial task, since cell-attached exopolysaccharides hinder cell destruction and the subsequent extraction processes [3,4]. Another well-known drawback of methods leading to total cell destruction is their lack of specificity, which thereby leads to the release of cell debris, causing a mixture of all intracellular components. Consequently, for some specific BPE applications such as immunology biomarking, it is necessary to apply complex purification methods such as the aqueous two-phase system [4,9,10], selective precipitation [11], membrane separation, or chromatographic techniques [5,12].

Increasing general interest in the use of microalgae to obtain valuable products necessitates the elaboration of cost-effective disruption methods to overcome the problems associated with traditional pretreatment methods.

Pulsed electric fields (PEF) increase the cell membrane permeability (electroporation) due to the application of pulses of high voltage (kV) and duration in the range of microseconds to milliseconds between two electrodes [13]. The improvement of extraction of intracellular compounds of interest such as lipids, carbohydrates, or pigments with low energy consumption thanks to the microalgae electroporation has been demonstrated by several authors [14–19]. However, some authors have observed that PEF is not very effective for enhancing extraction of other intracellular components such as proteins. Postma et al. [20] observed that over 95% of proteins were retained inside microalgal cell after PEF.

This study aims to evaluate whether the increment of cell membrane permeability caused by PEF could result in an effective and cost-competitive technique to improve the extraction of BPE from fresh biomass

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of *P. cruentum*.

2. Materials and methods

2.1. Cultivation conditions of Porphyridium cruentum

Porphyridium cruentum (UTEX 161) was obtained from the Culture Collection of Algae at the University of Texas, Austin (USA). The cells were grown in batch culture in 0.5-l columns 5.3 cm in diameter at 25 ± 1 °C in artificial seawater (ASW) medium [21]. For a solid medium, 1.5 g of technical agar was added to 100 mL of the medium. ASW medium (liquid and solid) was autoclaved at 121 °C for 20 min.

The cultures were illuminated continuously by fluorescent cool-white lamps (15 mmol/m^2 s). The medium was bubbled with air (6 mL/s). The cultures were initially inoculated with 1×10^4 cells/mL. Algae growth was monitored by measuring the number of cells with a Thoma counting chamber (ServiQuimia, Constantí, Spain) and an optic microscope (microscope L-Kc, Nikon, Tokyo, Japan). The biomass concentration was determined every 24 h. Experiments were performed using cells at stationary growth phase, achieved after an incubation time of 15 to 18 days. Biomass concentration at the stationary phase was around 2 g_{dw}/L. Dry weight of microalgae was determined by vacuum drying (GeneVac Ltd., UK).

2.2. PEF treatments

The PEF equipment used in this investigation has been previously described by Saldaña et al. [22]. Prior to treatment, fresh biomass of *P. cruentum* was centrifuged at 1000 ×*g* for 1 min at 25 °C and re-suspended in a citrate-phosphate McIlvaine buffer (pH 7.0; 1 mS/cm) to a final concentration of approximately 10^7 cells/mL. The *P. cruentum* suspension (0.44 mL) was placed in a static parallel electrode treatment chamber (gap: 0.25 cm; diameter: 1.6 cm) by means of a 1 mL sterile syringe (TERUMO, Leuven, Belgium).

The suspension was subjected to 10 to 50 monopolar square waveform pulses of 3 μs at electric field strengths ranging from 2 to 10 kV/cm, and a frequency of 0.5 Hz. These treatments correspond to specific energy inputs ranging from 0.12 to 15 kJ kg^{-1}. The temperature of the treatment medium was measured by a thermocouple before and after the PEF treatment. Initial temperature was 22 ± 1 °C, and after treatment the sample temperature was always lower than 30 °C. Once applied the PEF treatments, microalgal cells suspended in citrate-phosphate McIlvaine buffer of pH 7.0 were incubated at 22 ± 1 °C for 48 h in darkness for monitoring the release of phycoerythrin.

2.3. Cell disruption treatments

Total amount of pigments were determined according to [23]. An aliquot of 150 μL of wet cell biomass was blended with 1350 μL of distilled water, and the mixture was disrupted with a bead beater (bead diameter 0.1 mm, BioSpec Products INC, USA) at a speed of 4800 rpm (10 cycles of 10 s). Following each cycle, the sample was cooled down in water at 0 °C to avoid overheating of the sample.

2.4. Enumeration of viable cells

Viable cells were enumerated by pour plating [24]. PEF-treated and untreated (control) cell suspensions were serially diluted in a sterile solution of peptone water. From the selected dilutions, 20 μL were plated into ASW solid media. The plates were incubated at 25 °C for 8 days with the same light regime used for the liquid culture, and the number of CFU/mL were counted to determine the inactivation rate after treatment Longer incubation times did not increase the colony counts.

2.5. Staining cells with propidium iodide

Quantification of the number of *P. cruentum* electroporated cells was performed according to [24] by measuring the entry of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Barcelona, Spain). PI is a small (660 Da) hydrophilic molecule that is unable to cross through intact cytoplasmatic membranes After the PEF treatments, 50 μL of PI (0.1 mg mL^{-1}) were added to 450 μL of *P. cruentum* suspension, resulting in a final concentration of 0.015 mM. 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. PI trapped inside the cells (permeabilization of individual cells) was checked using an epi-fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan). The percentage of fluorescent cells was calculated after observing a population of at least 150 cells.

2.6. Pigment extraction

For β-phycoerythrin extraction, untreated or PEF-treated cells of *P. cruentum* suspended in citrate-phosphate McIlvaine buffer were kept in a dark location at 25 °C. In order to obtain extraction curves, samples were periodically collected over a period of 48 h. After centrifugation (6000 ×*g* for 2 min), the optical density (OD) of the supernatant was measured at 280, 565, 620 and 650 nm. The β-phycoerythrin concentration was calculated according to the following equations [5]:

$$B-PE\left(\frac{mg}{mL}\right)=\frac{(OD_{565}-2.8\,(R-PC)-1.34(APC))}{12.7}$$

in which B-PE is the β-phycoerythrin concentration, and R-PC and APC correspond with R-phycocyanin and allophycocyanin, respectively.

$$R-PC\left(\frac{mg}{mL}\right)=\frac{(OD_{620}-0.7OD_{650})}{7.38}$$

$$APC\left(\frac{mg}{mL}\right)=\frac{(OD_{650}-0.19OD_{620})}{5.65}$$

The purity of B-PE extract was monitored spectrophotometrically, and the following equation was applied:

$$Extract\ purity=\frac{OD_{565}}{OD_{280}}$$

in which OD_{565} indicates the β-phycoerythrin concentration and OD_{280} is the optical density of the sample at 280 nm, an estimate of the total concentration of proteins in the solution.

2.7. Statistical analysis

Experiments were performed in triplicate, and the present results are means ± 95% confidence interval. Replicates correspond to three biological replicates of different experiments, both for treatments and controls.

3. Results and discussion

3.1. Irreversible membrane permeabilization of P. cruentum by PEF treatments of varying electric field strengths and treatment times

The loss of selective membrane permeability to PI of *P. cruentum* cells after PEF treatments of varying electric field strengths and duration is shown in Fig. 1. After application of PEF treatments in the range of 2 to 10 kV/cm and 30 to 150 μs, 15% to 98% of the *P. cruentum* cells were permeable to PI. Fig. 1 thus shows that the PI permeabilization of *P. cruentum* was enhanced by increasing electric field strength and treatment time. For example, the increment of electric field strength

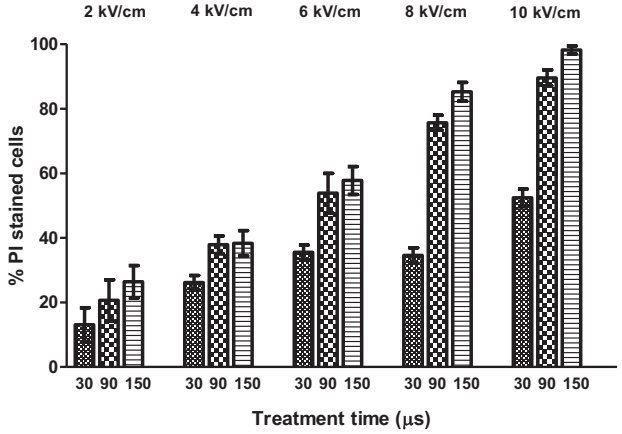

Fig. 1. Influence of the electric field strength and treatment time on the PI permeabilization of *Porphyridium cruentum* cells treated by PEF. Data shown as mean ± 95% confidence interval, n = 3.

from 2 to 10 kV/cm for 150 μs augmented the percentage of permeabilized cells from 27 to 98%. The increment of treatment duration from 30 to 150 μs at the intermediate electric field strength assayed (6 kV/cm) increased the percentage of electroporated cells from 37 to 60%.

Microbial inactivation of PEF has been generally associated with the fact that electroporation modifies the selective permeability of the microbial membrane, thereby preventing cells from maintaining their microbial homeostasis [25]. The relation between the percentage of cells permeabilized to PI and the percentage of dead *P. cruentum* cells estimated by plate counting after PEF treatments applied in this research is shown in Fig. 2. The results show that when the percentage of dead cells was greater than 20%, there was a perfect agreement between the fraction of dead cells and those that uptook PI, represented by the theoretical straight line with slope 1 and intercept 0. In the case of PEF treatment conditions that inactivated less than 20% of the cells, the percentage of PI-stained cells was greater than the percentage of dead cells. These results seem to indicate that when the less intense treatments were applied (2 kV/cm during 30 and 90 μs and 4 and 6 kV/cm during 30 μs), some of the cells affected by the treatment were able to uptake PI, but the increment in membrane permeability was not great enough to affect microbial homeostasis to a degree that would lead to cell inactivation.

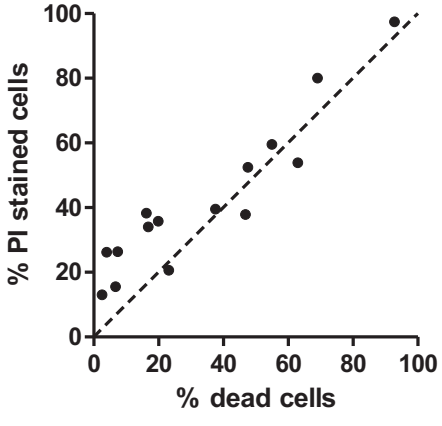

Fig. 2. Relationship between the percentages of *Porphyridium cruentum* cells irreversible permeabilized assessed by PI against the percentage of dead cells treated by PEF. To show the degree to which each treatment causes membrane permeabilization, a theoretical straight line with slope = 1 and intercept = 0, is included. Data shown as mean, n = 3.

The results herein obtained confirm observations made by other authors regarding the ability of PEF to electroporate and inactivate microalgae and other microorganisms [22,24]. In this case, we observe that the electric fields required to electroporate and inactivate *P. cruentum* were much lower than those required for other small microorganisms such as bacteria, or for other microalgae such as *C. vulgaris*, which required minimum electric fields of 10 kV/cm [24,26]. The greater cell volume of *P. cruentum* explains its higher sensitivity to PEF. Currently, it is accepted that an increment in the transmembrane voltage is necessary for electroporation to occur, whereby the external electric field strength required to reach the transmembrane voltage threshold to induce electroporation is greater for smaller cells [27].

3.2. Effect of PEF on the extraction of β-phycoerythrin

The effect of treatment time (75 μs and 150 μs) at two electric field strengths (4 and 8 kV/cm) and of electric field strength (2, 4, 6, 8 and 10 kV/cm) at 150 μs on the extraction of BPE from *P. cruentum* is illustrated in Fig. 3A and B, respectively. The extraction curve obtained from untreated cells shows that BPE was scarcely detected in the extraction medium containing intact cells of *P. cruentum* after the longest extraction time assayed in this investigation (48 h), or even after longer extraction times of one entire week (data not shown). However, when *P. cruentum* cells were exposed to PEF treatments, it was observed that BPE was progressively released to the extraction medium. It is observed that, similarly to the increment in PI uptake, both the release rate and the maximum extraction yield of BPE depended on electric field strength and treatment time. For example, after 12 h of extraction, the increment of treatment time at 8 kV/cm from 75 μs to 150 μs increased the BPE extraction yield from 10.1 to 24.6 mg/g d.w., and the increment of electric field strength from 2 to 10 kV/cm for 150 μs increased the BPE extraction yield from 5.1 to 25.4 mg/g d.w. On the other hand, Fig. 3B shows that the maximum extraction yield of BPE strongly depended on electric field strength. At the lowest electric field strengths assayed (2 and 4 kV/cm), the maximum extraction yield was not achieved even after 48 h of incubation. However, at the greatest intensities (8 and 10 kV/cm), the highest extraction yield was achieved after 24 h, whereas 48 h of extraction were required to achieve the same extraction yield at 6 kV/cm.

The maximum BPE extraction yield within the shortest extraction time (24 h), which was around 30 mg BPE/g d.w., was achieved after treating *P. cruentum* cells at 8 and 10 kV/cm for 150 μs. After those treatments, the supernatant of PEF-treated cells turned strong pink, and the remaining pellets were colorless, thereby indicating that practically 100% of the cells' BPE content had been extracted. This extraction yield, which was comparable to the extraction yield obtained after completely destroying the cells by bead-beating (32 mg/g d.w.), was achieved with PEF treatments featuring total specific energies of as little as 15 kJ kg^{-1} or even lower. The maximum extraction yield obtained after the application of the most intense PEF treatments was higher than that reported by other authors. Román et al. [11], working with an outdoor culture of *P. cruentum* containing 16.6 mg BPE/g d.w., reported an extraction yield of 60% using sonication as a pretreatment. Bermejo et al. [28], studying the effectivity of different cell disruption methods such as osmotic shock, ultrasound, freeze-thawing, lysozyme and liophylization in the recovery of BPE from *P. cruentum*, obtained extraction yields ranging between 30 and 70%. The highest extraction (16.6 mg/g d.w.) was obtained by combining the use of lysozyme and liophylization. On the other hand, Marcati et al. [12] achieved 48% of BPE recovery with a purity index of 2.3 using a two-step membrane process featuring polyethersulfone flat membranes. The purity of the extract obtained from *P. cruentum* cells treated by PEF (0.94 ± 0.01) was much greater than that obtained after bead-beating (0.28 ± 0.03). This purity was similar to that reported by other authors prior to the purification steps [11,28].

As Fig. 3 shows, even after the application of the most intense

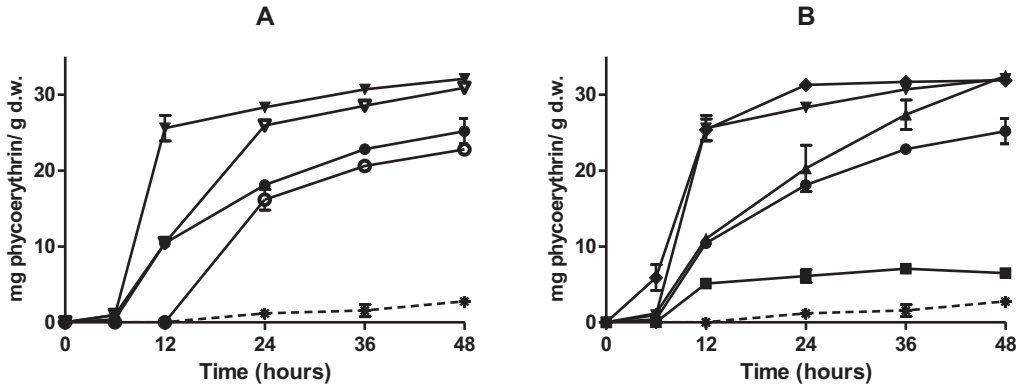

Fig. 3. A Extraction curves in pH 7 McIlvaine buffer of β-phycoerythrin from PEF-treated cells of *Porphyridium cruentum* at 4 (●) or 8 kV/cm (▼) and different treatment times: 75 μs (empty symbols) and 150 μs (filled symbols) (3B) Extraction curves in pH 7 McIlvaine buffer of β-phycoerythrin from PEF-treated cells of *Porphyridium cruentum* at different electric field strengths (■ 2 kV/cm, ● 4 kV/cm, ▲6 kV/cm, ▼ 8 kV/cm, ♦ 10 kV/cm) for 150 μs. In order to compare, extraction curves from untreated *P. cruentum* (*) is also represented in both figures. Data shown as mean ± 95% confidence interval, n = 3.

treatments that caused the PI staining of almost 100% of the *P. cruentum* population, BPE was not released immediately after the PEF treatment. In most cases, an extraction time exceeding 6 h was required to detect BPE in the extraction medium; in the case of the less intense effective PEF treatment applied (4 kV/cm for 75 μs), the presence of BPE in the medium was detected after 12 h of incubation. This kinetics of extraction of BPE from PEF-treated *P. cruentum* cells differs from that of the release of different intracellular compounds such as ions, carbohydrates, proteins, carotenoids, or chlorophylls from PEF-treated microalgae such as *C. vulgaris* [16,17,20]. In these studies, it was observed that release of these compounds did not require a lag time, and that extraction times under 1 h were sufficient in order to achieve maximum extraction yields. In the case of the extraction of C-phycocyanin (a water-soluble phycobiliprotein) in aqueous media from *Arthrospira platensis* previously treated by PEF, a delay of 150 min at the onset of the extraction was observed [23]. This delay was attributed to the molecular weight of C-phycocyanin and to the size of the pores formed by PEF treatments. The hypothesis was that low molecular weight compounds could cross the cytoplasmic membrane immediately after their electroporation, whereas the release of molecules of greater molecular weight might require that the pores created by PEF treatment enlarged over the course of time. The similar molecular weight of C-phycocyanin (240 kDa) and β-phycoerythrin (250 kDa) could explain that the lag observed in the extraction curves is a consequence of the time required to enlarge the pores created by PEF up to a size sufficient to permit the compound's release. However, the considerable amount of time required to detect β-phycoerythrin in the extraction medium seems to indicate that other factors are involved in the release of β-phycoerythrin. For example, the very thickness of the exopolysaccharide cell wall of *P. cruentum* could hinder the extraction of these phycobiliproteins, even if the cytoplasmic membrane was permeabilized. Lam et al. [29] observed a complete release of hydrophilic proteins from cell wall free microalgae mutants of *Chlamydomonas reinhardtii* after the application of a PEF treatment. However the effect of PEF on the protein release from the native cells containing an intact cell wall was lower. However, this does not seem to be the reason for the delay in the extraction of BPE: even after the complete disruption of the *P. cruentum* cells by bead-beating, 24 h were still necessary to achieve the maximum extraction yield.

It is well known that phycobiliproteins such as BPE are assembled into an organized cellular structure, the phycobilisome, which, at the same time, is located in the thylakoids of chloroplast instead of floating free in the cytoplasm [3,30]. Therefore, the extraction of BPE not only requires the diffusion of the compound across the cell membranes, but also a dissociation of the compound from the phycobilosome. The efficacy of PEF for extracting large amounts of BPE after a prolonged extraction time seems to be a consequence of the fact that pulsed electric fields contribute in some way to that disassociation, in addition to the electroporation of the cell membrane which they likewise bring about. It has been recently demonstrated that PEF treatments could accelerate the autolysis process in yeast, thereby enhancing the release of polysaccharides from the cell wall of *S. cerevisiae* [31]. Autolysis consists in the hydrolysis of intracellular biopolymers of cells by their own enzymes after cell death [32,33]. *P. cruentum* autolysis triggered by PEF could be the main cause involved in those treatments' effectivity in BPE extraction. Considering the mechanism of action of PEF, the selective permeability of the cytoplasmic membrane of the *P. cruentum* cells is lost after electroporation and, as a consequence thereof, the entrance of water from the suspending medium to the cytoplasm causes the disturbance of intracellular structures and the release of hydrolytic enzymes located therein. Therefore, the hydrolytic enzymes of *P. cruentum* could help to disassemble the associations between BPE and other structures of the cell; in this way, the water-BPE complex could diffuse across the cell membrane, driven by a concentration gradient. This hypothesis is supported by data presented in Fig. 4 which show the correlation between the percentage of electroporated cells and the percentage of BPE extracted after 24 (Fig. 4A) and 48 (Fig. 4B) hours, respectively. After 24 h of extraction, the perfect correlation observed between the fraction of PI stained cells of *P. cruentum* and the fraction of total BPE released to the extraction medium would indicate that BPE was completely released from the cells that were electroporated by PEF. Nevertheless, after 48 h of extraction, the percentage of extracted BPE was higher than the percentage of permeabilized cells in the case of mild-intensity treatments (Fig. 4B). For example, a treatment that permeabilized 53.8% of the population (6 kV/cm for 90 μs) allowed for an extraction of almost 97% of BPE after 48 h of extraction. Therefore, after longer extraction times, PEF permeabilization of the cytoplasmic membrane of *P. cruentum* did not perfectly correlate with the BPE extraction yield. This increment of the extraction yield in the suspensions containing a large proportion of non-electroporated cells could be caused by the hydrolytic enzymes released from the electroporated cells which, in turn, engender the autolysis of the cells unaffected by the PEF treatment. Recently, the autolysis of microalgae by certain lytic enzymes involved in cell division and programmed cell death such as endopeptidase has been proposed as a low-cost alternative to enzymatic or physical disruption methods in order to facilitate the release of intracellular compounds [34]. However, the self-degradation of the cellular constituents by their own enzymes is a slow process that occurs after cell death. Therefore, it would be highly recommendable to accelerate this process by means of PEF treatments in order to reduce the

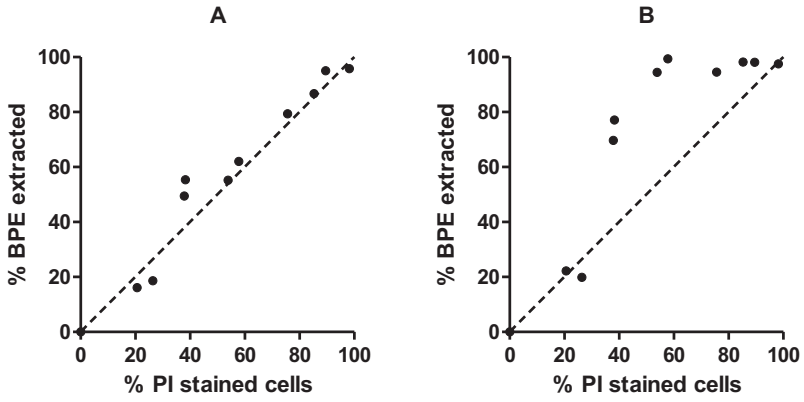

Fig. 4. Relationship between the percentages of *Porphyridium cruentum* cells irreversibly permeabilized assessed by PI against the percentage of BPE extraction after 24 (4A) or 48 h (4B). To show the degree to which each treatment causes membrane permeabilization, a theoretical straight line with slope = 1 and intercept = 0, is included. Data shown as mean, n = 3.

downstream operation duration and avoid degradation of the target products.

The cell destruction stage represents one of the most critical steps that have an impact on costs and extraction yields for obtaining bioproducts from microalgae. PEF could serve as an effective and cost-competitive technique to obtain that objective. In this investigation it has been demonstrated that low-intensity PEF treatment of *P. cruentum* fresh biomass, followed by 24 h of incubation, permits an extraction of the totality of β-phycoerythrin contained in the cells, with a purity greater than that obtained via mechanical cell disruption methods. An improved understanding of the kinetics and the mechanism of the enzymes participating in microalgae autolysis, and of the autolysis trigger by PEF, will allow for the development of this process at an industrial scale in order to extract high-value products from microalgae biomass.

Declaration of authors' contributions

J.M. made substantial contributions to the conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for intellectual content, final approval of the article, statistical expertise and collection and assembly of data. C.D. made substantial contributions to the acquisition and analysis of data, drafting of the article and final approval of the version to be submitted. I.A. and J.R. made substantial contributions to the conception and design of experiments, data interpretation, revision of article, provision of study materials, acquisition of funding and final approval of the version to be submitted.

Conflict of interest statement

Authors declare that there are no conflict of interest neither any potential financial or other interest that could be perceived to influence the outcomes of this research.

Conflicts, informed consent, human or animal rights

No applicable.

Declaration of authors' agreement

All the authors declare their agreement to authorship and submission of this manuscript for peer review.

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4.3.3. Pulsed Electric Field permeabilization to extract astaxanthin from the Nordic microalgal strain *Haematococcus pluvialis (Manuscript VIII)*

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PULSED ELECTRIC FIELD PERMEABILIZATION TO EXTRACT ASTAXANTHIN FROM THE NORDIC MICROALGAL STRAIN *HAEMATOCOCCUS PLUVIALIS*

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ABSTRACT

The Nordic microalgal strain *Haematococcus pluvialis* grown in a multi-cultivator photobioreactor was exposed to various stress conditions such as high light (200-1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$), salt (5-10 $g \cdot L^{-1}$ NaCl), sudden nitrogen starvation and mixotrophic growth in the presence of xylose or glucose to induce astaxanthin accumulation. Highest carotenoid content (19.1 $mg \cdot g^{-1}_{dw}$) was achieved in nitrogen-free culture medium at a light intensity of 1200 $\mu mol \cdot s^{-1} \cdot m^{-2}$. The efficiency of Pulsed Electric Field (PEF) pre-treatment of stressed fresh biomass of *H. pluvialis* followed by aqueous incubation was compared to classical disruption methods (bead-beating, freezing-thawing, thermal treatment or ultrasound) for the subsequent extraction of astaxanthin in ethanol.

The sensibility of cells to treatments and therefore also the extraction yields of astaxanthin were depended on the growth conditions. Mixotrophic growth in the presence of xylose resulted in most resistant cells, independent of the pre-treatment. N-starved cells treated with PEF followed by aqueous incubation for 6 h resulted in extraction of 96 % (18.3 $mg_{car} \cdot g_{dw}^{-1}$) of the total carotenoid content compared to 80 % (15.3 $mg_{car} \cdot g_{dw}^{-1}$) using other physical methods. Immediately after PEF treatment the cell structure remained intact, extraction by ethanol was only effective after incubation in aqueous treatment media. The proportion of free forms of astaxanthin was higher in PEF-treated samples compared to mechanical disruption, suggesting PEF triggering an esterase activity.

PEF pre-treatment of the cells followed by incubation in growth medium improved astaxanthin extraction in the eco-friendly solvent ethanol. Existing large-scale PEF equipment to treat liquid products in continuous mode ($T \cdot h^{-1}$) is already available, up-scaling of algal astaxanthin extraction using PEF therefore is feasable.

1. INTRODUCTION

The unicellular freshwater green microalga *Haematococcus pluvialis* produces the high-value carotenoid astaxanthin, and thereby is one of the most important natural sources for this pigment [1, 2]. Due to its coloring properties astaxanthin is highly attractive in industrial aquaculture as feed additive for salmons, trout, and crustaceans to provide their characteristic pink/red color [3, 4]. Its strong antioxidant properties further are recognized in therapeutic applications, it provides anti-inflammation, immune stimulation, anti-tumors, anti-diabetic, cardio-protective and neuro-protective [5 - 9].

Nowadays, the majority of astaxanthin is synthesized chemically [3], however, the consumer´s demand for natural products provides an opportunity to produce natural astaxanthin [4]. While synthetic astaxanthin costs about 1000 $/kg, the natural one, derived from the yeast *Phaffia Rhodozyma* or the microalga *H. pluvialis*, is far more expensive (2500-7000 $/kg [3]). *H. pluvialis* is considered a promising source for natural

astaxanthin due to its ability to accumulate high quantities in comparison to *Phaffia Rhodozyma* [9].

Astaxanthin accumulates within oil droplets as mixture of mono and di-esters during the transformation of green vegetative *H. pluvialis* cells to red encysted cells; this transformation is induced by exposure to stress, such as nitrogen starvation, photoinhibition, salt stress, phosphate deficiency or high temperatures [4; 10]. The biosynthesis of this carotenoid is associated with the formation of a very thick, three-layered cell wall, which consists of an outer trilaminar algaenan sheath, a middle layer consisting of a homogeneous polysaccharide arrangement containing mannose and cellulose, and an inner layer made out of heterogeneous polysaccharides [11; 12]. This indigestible cell wall limits the bio-availability of astaxanthin, when consumed orally, therefore astaxanthin has to be extracted from the cyst before its use.

The extreme resistance to mechanical and chemical pretreatments of cell wall before disruption processes as well as the lipophilic nature of astaxanthin esters are major challenges during the extraction process [1; 13]. High extraction yields are desired without compromising the quality of the carotenoid due to degradation [14; 15; 16]. Traditionally carotenoids are extracted from dried algal biomass using organic solvents, however, the high energy amount required for drying, oxidation of the products and the use of potentially harmful organic solvents are disadvantageous. Consequently, extraction from moist biomass would be preferred, although it involves previous disruption of the cells.

Over the last years, several cell disruption techniques have been explored, such as mechanical treatments, chemical treatments using solvents, cryogenic grinding, acid or base treatment, high pressure, ultrasounds, microwaves, ionic liquids and enzyme lysis [1; 2; 3; 17; 18; 19; 20; 21]. However, high energy consumption, difficulties in industrial up-scaling, pigment degradation, the need for multiple separation steps and/or the necessity of downstream purification steps are some of the remaining problems.

In this study, we propose Pulsed Electric Field (PEF) treatment as a sustainable and efficient cell disruption method to selectively extract astaxanthin from *H. pluvialis*. PEF causes cell membrane permeability (electroporation) due to the application of high-intensity electric field pulses of short duration (µs to ms) [22]. Several studies have demonstrated that electroporation of microalgae with low energy consumption improves the extraction of intracellular compounds of interest, such as lipids, proteins, carbohydrates, or pigments [23]. Not the permeabilization of cytoplasmic membrane by PEF treatment, but a long aqueous incubation before extraction seems to cause the improved extraction [24; 25; 26]. This study aims to evaluate PEF treatment combined with aqueous incubation as pre-treatment to extract astaxanthin of biomass from the Nordic strain *H. pluvialis* using sustainable solvents. This technology is compared to classical disruption methods.

2. MATERIAL AND METHODS

2.1 Identification and Cultivation of the Nordic microalgal strain

The microalgal strain used in this work was isolated from freshwater in Umeå, northern Sweden, and initially identified as *Haematococcus pluvialis* (also known as *Haematococcus lacustris*) based on its cell morphology and culture physiology. The taxonomy of the isolate was confirmed by genomic DNA extraction (NucleoSpin Soil DNA extraction kit, MACHEREY-NAGEL, Germany) and PCR amplification of the ITS2 (Internal Transcribed Spacer 2) sequence in the algal rRNA gene, as described in Ferro et al. (2018) [27]. The amplicon was sequenced and a phylogenetic tree (Supplementary Material, Fig. S1) was inferred including ITS2 sequences of 27 *Haematococcus* isolates [28] using Mega7 software (http://www.megasoftware.net/) and a neighbor-joining method validated at 1000 bootstrap replications.

Haematococcus pluvialis was cultivated in a two-step process. In the first step, the required volume of microalgae inoculum was added in 250 mL flasks filled up to 70 % volume with Bold's basal medium (BBM) to an initial optical density OD_{750} of 0.1. These cultures were maintained at 20°C and bubbled with 1 $L \cdot min^{-1}$ of air with 3 % CO_2 for seven days.

After this first step of biomass production, the cultures were harvested and centrifuged under sterile conditions. The pellet was re-suspended and washed twice in culture media used in the second step. The cells then were grown in a Multicultivator MC1000-OD (Photon System Instruments, Czech Republic) and exposed to different stress conditions to induce astaxanthin accumulation: high light intensities (200 and 1000 µmol·s^{-1}·m^{-2}), salt stress (addition of 5 and 10 g·L^{-1} NaCl), sudden nitrogen starvation (media depleted of NaNO3) or N-starvation combined with mixotrophic growth in the presence of xylose or glucose. Each vessel of 85 mL culture volume was independently illuminated by an array of white LEDs. The cultures were bubbled with mixture of 3 % CO2 in air. To compare the disruption efficiency the Nordic strain *Chlorella vulgaris* 13-1 [27] was cultivated in BBM and subjected to the same pre-treatments than *H. pluvialis*.

2.2 Biomass Concentration and Optical Density

pH, maximal photosynthetic efficiency of PSII (Qy), and optical density (measured at 530, 680 and 750 nm) were monitored daily for each algal culture. The biomass concentration of the cultures was determined after filtration of a known culture volume over pre-dried and pre-weighted glass fiber filters (Whatman GC) by measuring the weight increase of the dried filters, as described in Gojkovic et al. (2014) [29]. The biomass concentration was calculated and expressed in g·L^{-1} of culture.

2.3 Cell disruption treatments

The obtained algal biomass was harvested and subjected to different disruption treatments. After each treatment the biomass samples were observed under the light microscope (Leica DMi1, 40x magnification) to determine the degree of intact cells and access the efficiency of the disruption procedure.

2.3.1 PEF treatments

PEF was performed using a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). The equipment consists of a set of capacitors, with a maximum capacitance of 3275 F, which generates square waveform pulses ranging in duration from 0.05 to 5 ms with a maximum output voltage of 3000 V. A parallel electrode treatment chamber composed of a cylindrical methacrylate tube closed with two polished stainless steel cylinders was used to apply the PEF-treatments. The electrode diameter was 10 mm and the gap between the electrodes was 50 mm.

Microalgal biomass was PEF-treated suspended in its own cultivation medium that had a conductivity of 1 mS·cm^{-1}. In a first screening, the sensitivity of the Nordic *H. pluvialis* strain to ms PEF treatment was tested by pulses ranging from 10 to 80 pulses of 5 ms (50-400 ms) with a frequency of 1 Hz at electric field strengths ranging from 0.2 to 1 kV/cm. The specific energy of these treatments ranged from 2 to 400 kJ·kg^{-1}.

Specific energy input (W) per treatment expressed in kJ·kg^{-1} was calculated by the following equation, where m is the mass of microalgae suspension (kg), V is the input voltage (kV), I is the current intensity (A), t is the treatment time (s) and Np is the number of applied pulses:

$$W = 1m \times V \times I \times t \times Np$$

Treatment with 10 pulses of 5 ms at 1 kV·cm^{-1} (50 kJ·kg^{-1}) was selected for extraction. PEF-treated cells were incubated in their own aqueous treatment media from 1 to 12 h at room temperature in the absence of light. Suspensions were then centrifuged and biomass was re-suspended in the extraction solvents.

2.3.2 Bead-beating

Aliquots of 150 µL of wet microalgal biomass was blended with 1350 µL of distilled water, and the mixture was disrupted using a bead beater (Bullet Blender Storm 24, Next advance, Troy, USA) with 12 mg of 0.1 mm diameter glass beads at a speed of 4800 rpm (between 5 and 10 cycles of 60 s). Following each beating cycle, the sample was cooled on ice for 180 s.

2.3.3 Ultrasounds treatment

Aliquots of 5 mL of microalgal biomass was diluted with 45 mL of ethanol. The suspension was sonicated 10 times during 10 seconds at 80 % of the amplitude in an Ultrasound apparatus 450 W Sonifier SFX550 Cell Disruptors (Emerson, San Luis, USA) equipped with a Branson model 102 c (CE) (Emerson). Between cycles, the sample was cooled on ice.

2.3.4 Thermal treatment

Aliquots of 1 mL were placed in 5 mL glass tubes submerged in a Thermostatic Bath (Grant TC120, Grant Instruments, Cambridge, UK) at 70ºC for 1 h.

2.3.5 Freezing-Thawing

Aliquots of 1 mL of microalgal biomass were placed in Eppendorf tubes and centrifuged. The pellet was subjected to fast freezing in liquid nitrogen and after that it was left to melt on ice (slow melting). These freezing-thawing cycles were repeated 5 times.

2.3.6 Dimethyl Sulfoxide treatment

Aliquots of 0.1 mL of microalgal culture were mixed with 0.9 mL of dimethyl sulfoxide (DMSO) in a 2 mL tube. Approx. 12 mg of 0.1 mm glass beads were added and tubes were subjected to repeated bead-beating cycles until the pellet became colorless. This method was used as reference of total content of carotenoids.

2.4 Evaluation of viability of *Haematococcus pluvialis* after PEF treatment

Photosystem II (PSII) maximum quantum yield (Qy) was determined by measuring the chlorophyll fluorescence in a portable pulse-amplitude-modulation (PAM) fluorimeter (AquaPen AP-100, Photon Systems Instruments, Czech Republic) according to the user´s manual. Cultures were dark-adapted for 15 minutes prior to measurement.

After PEF treatment the cells were left for 1 h at room temperature and their photosynthetic efficiency was subsequently measured. Samples with Qy values bellow 0.3 were regarded as considerably affected by PEF.

2.5 Solvent extraction of pre-treated biomass

After the disruption treatments, the algal biomass was suspended in either acetone (99.5 % vol/vol), methanol (99.5 % vol/vol) or ethanol (96 % vol/vol), mixed and incubated for 1 h to extract the carotenoids.

2.6 Chlorophyll and Carotenoids determination

The pigment content (chlorophylls and total carotenoids) was determined using the multi-step methanol extraction method described in Gojkovic et al. (2014) [29]. Chlorophyll and carotenoid content of the extracts were determined spectrophotometrically (T90+UV/VIS spectrometer, PG instruments, Ltd, UK) at 470, 647 and 663 nm. Results were calculated using the modified Arnon's equations [30] and expressed as mg of carotenoids per mL of suspension.

2.7 HPLC analysis of extracts

HPLC analysis was performed as described by Yuan and Chen [31] using a Varian ProStar high performance liquid chromatograph (Varian Inc., Walnut Creek, CA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler and a ProStar 335 photodiode array detector. The system was controlled with a Star chromatography workstation v.6.41 (Varian). Separation was achieved on a reverse-phase column (LC Luna® 100 Å C18 250 x 4.6 mm; 5 µm particle size, Phenomenex) with a pre-column (LC Luna 50 x 4.6 mm; 5 µm particle size, Phenomenex) of the same material. Chromatographic peaks were identified by comparing retention times and spectra against known standards.

2.8 Statistical data treatment

Results represent the mean ± standard error of the mean of three treatment replicates analyzed with technical triplicates. A one-way ANOVA test was conducted to assess significant differences between

the treatments. The differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Evaluation of factors affecting growth and carotenoid production in the Nordic microalgal strain *H. pluvialis*

The Nordic microalgal strain *Haematococcus pluvialis* was cultivated in a two-step process as described in Material and Methods. Step 1 was performed for biomass production, in step 2 the cells were exposed to different stress conditions to induce astaxanthin accumulation. In **Figure 1** optical density and biomass concentration of the cells in step 2 is shown, the microalgae were exposed to a light intensity of either 200 or 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ and grown in BBM (control) or exposed to N-starvation, N-starvation and salt stress (either 5 g L^{-1} or 10 g L^{-1}), N-starvation and xylose (6 g L^{-1}), or N-starvation and glucose (6 g L^{-1}). H. pluvialis continued to grow under stress with varying growth rates. In control medium, no significant difference in the growth rate was observed either at 200 or 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$. The growth rate was diminished compared to the control in cultures containing NaCl or in N-free medium independent of the light intensity. While glucose addition (6 $g \cdot L^{-1}$) to N-free medium accelerated the growth, supplementation of xylose (6 $g \cdot L^{-1}$) to this medium decreased the growth. The photosynthetic efficiency (Qy) of the microalgae exposed to these different conditions decreased over time in cultures exposed to stress confirming the reduction of their metabolic capacity [32].

The accumulation of the total carotenoid content in *H. pluvialis* exposed to stress at the two different light intensities (200 or 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$) is shown in **Figure 2**. The amount of carotenoids per volume of cultures remained constant in control culture throughout the experiment. In N-free medium, however, and in N-free media supplemented with either glucose or xylose the carotenoid concentration increased drastically. The highest carotenoid content expressed per volume of culture (57 $mg_{car} \cdot L^{-1}$) was achieved in N-free culture medium supplemented with 6 $g \cdot L^{-1}$ glucose at a light intensity of 1000 $\mu mol \cdot s^{-1} \cdot m^{-2.}$ Calculating the carotenoid content per dry weight of the biomass, **Figure 2 (lower panel)** shows the carotenoid concentration decrease in cells exposed to both light intensities in control conditions and to 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ in the presence of 10 $g \cdot L^{-1}$ salt.

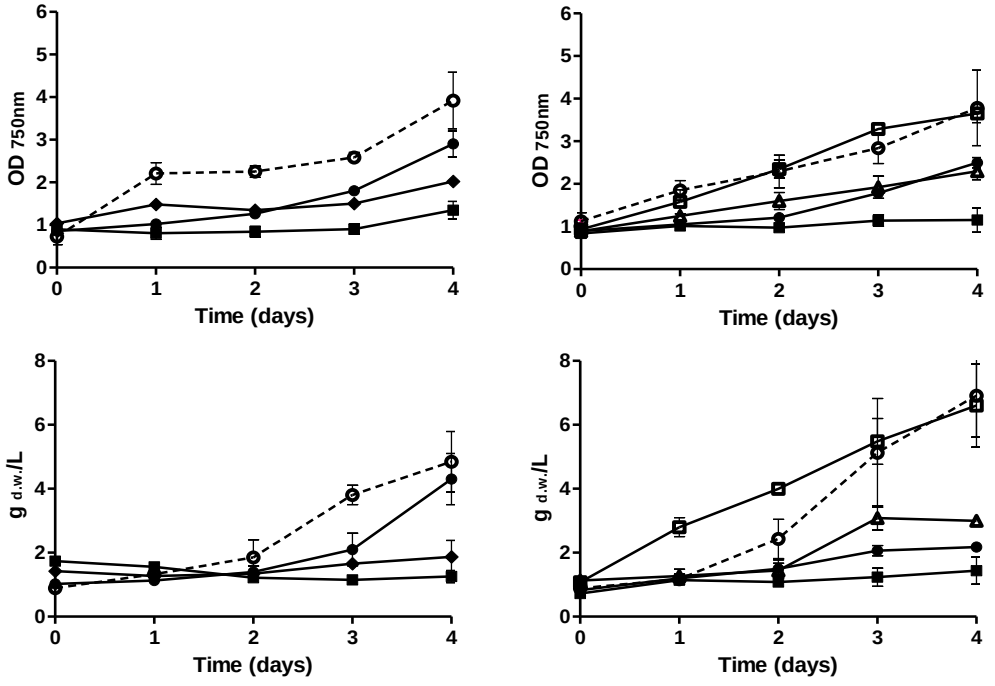

Figure 1. Optical density (750-nm) and biomass concentration ($g \cdot L^{-1}$) increase of *H. pluvialis* under stress conditions in function of the cultivation time for two different light intensities: 200 (A) and 1000 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (B) and a variety of culture media. Normal

culture medium - BBM (○), Nitrogen free BBM (●), Nitrogen free BBM supplemented with: 5 g·L^{-1} NaCl (♦), 10 g·L^{-1} NaCl (■), 6 g·L^{-1} xylose (△) or 6 g·L^{-1} glucose (□).

In all other culture conditions the biomass content of carotenoids increased with prolonged cultivation times. Highest carotenoid content (19.1 $mg_{car}·g^{-1}_{dw}$) was obtained in N-free medium at a light intensity of 1000 µmol·s^{-1}·m^{-2}.

H. pluvialis grows best at high N concentrations, while N starvation induces the formation of reddish, astaxanthin-containing palmella cells. Mixotrophic cultivation improves its growth rate and also stimulates the formation of palmella cells [33; 34]. To achieve optimal carotenoid accumulation both the amount of biomass in a culture and the concentration of astaxanthin per cell are important factors. While the production of biomass is decreased by the stress exposure, at the same time these stress factors are necessary to induce carotenoid accumulation in the cells. Based on the conditions tested the Nordic *H. pluvialis* strain produced highest carotenoid content per culture volume in the presence of glucose (6 g·L^{-1}) combined with N starvation at a light intensity of 1000 µmol s^{-1} m^{-2}. At these conditions the amount of biomass was similar to that at control conditions, while the carotenoid content per dry weight was relatively high. However, one should keep in mind that addition of glucose increases the risk of bacterial contamination in the culture. Sudden N-starvation on the other hand is easily performed, but yields in smaller amount of astaxanthin (37.9 $mg_{car}·L^{-1}$) due to the slower culture growth. Addition of xylose instead of glucose to the N-free medium provides a good alternative, as it improved the carotenoid yield to 41.5 $mg_{car}·L^{-1}$.

Increased carotenoid accumulation in *H. pluvialis* due to salt stress was reported earlier [35]. In our Nordic *H. pluvialis* strain addition of NaCl was not found to be suitable, the induction of astaxanthin accumulation was compensated by decreased growth of the culture resulting in very low final carotenoid yields.

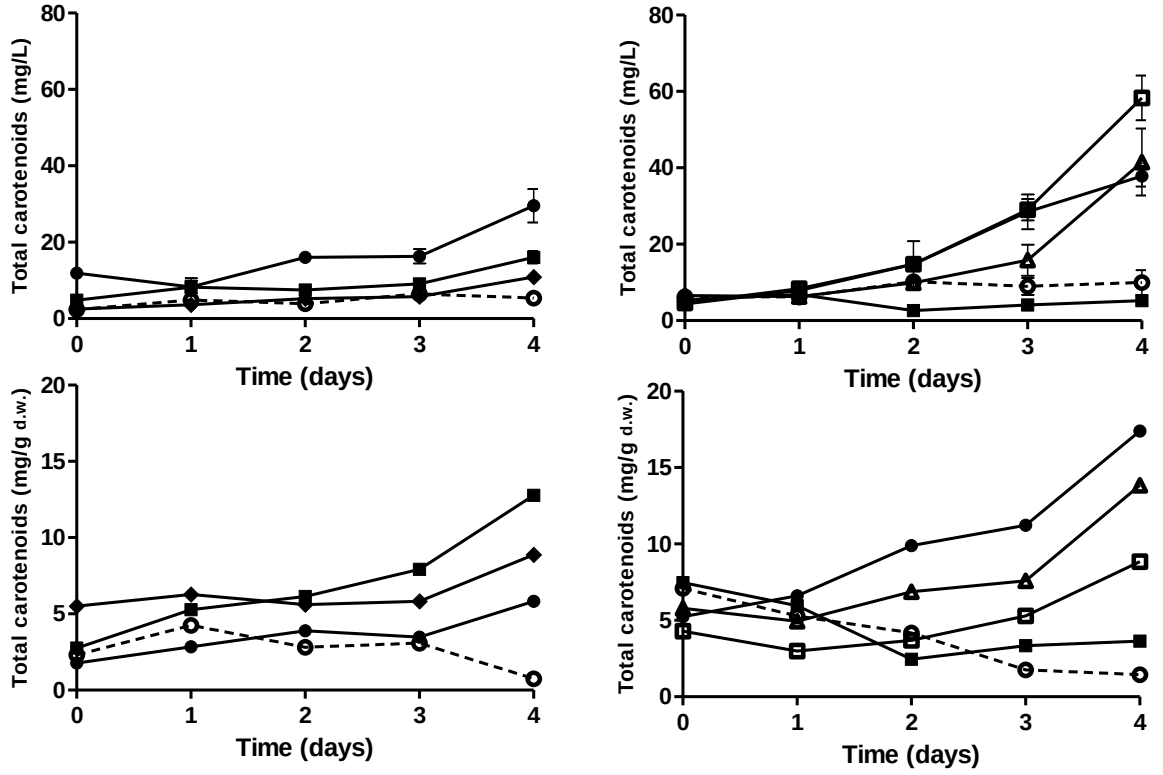

Figure 2. Total carotenoid content of *H. pluvialis* cultures under stress conditions in the function of time in two different light intensities: 200 (A) and 1000 µmol·s^{-1}·m^{-2} (B) and a variety of culture media. Normal culture medium – BBM (○), Nitrogen free BBM (●), Nitrogen free BBM supplemented with: 5 g·L^{-1} NaCl (♦), 10 g·L^{-1} NaCl (■), 6 g·L^{-1} xylose (△) or 6 g·L^{-1} glucose (□). Carotenoid content is expressed in mg·L^{-1} or in mg·g_{dw}^{-1}

3.2. Carotenoid extraction assisted by Pulsed Electric Field

After establishing growth conditions for good carotenoid production, the efficiency of PEF treatment was evaluated on the biomass of *H. pluvialis*. **Table 1** summarizes PEF treatment times and field strengths on *H. pluvialis* cells in the red phase (grown in N-free BBM) in relation to their photosynthetic efficiency 1 h after the treatment. Qy values below 0.3 were considered as dead cells after PEF treatment. With increased electric field or number of pulses (0.2-1 kV cm^{-1}, 10-80 pulses of 5 ms) decreased the Qy values, indicating implications on the cell homeostasis. The minimum electric field strength to affect the cells was 1 kV·cm^{-1} even at the shortest treatment period. The main mechanism to inactivate microbes by PEF is known to be electroporation [22]. The external electric field strength required to reach the transmembrane voltage threshold inducing electroporation is inversely correlated to the target cell size [22]. Given that the diameter of reddish palmella *H. pluvialis* cells is large (≈ 20 µm in diameter), this species is expected to be permeabilized by moderate electric field strengths compared to smaller microalgal cells. While irreversible electroporation of the much smaller *C. vulgaris* cells (2-6 µm in diameter) was observed from 4 kV·cm^{-1} in the millisecond range [24], electropermeabilization of *H. pluvialis* was detected after PEF treatments of 3 kV·cm^{-1} [36]. Our results indicate an electric field strength threshold of around 1 kV·cm^{-1} to affect this particular Nordic *H. pluvialis* strain.

Based on the data shown in **Table 1**, solvent extraction experiments were performed after PEF treatment with the lowest energy input (1 kV·cm^{-1}, 10 pulses, 50 kJ·kg^{-1}) and still effectively affecting the cells. PEF-treated and untreated *H. pluvialis* cells in the red phase were incubated in the corresponding treatment medium for 1, 6 or 12 h and then re-suspended in either acetone (99.5 %, vol/vol), methanol (99.5 %, vol/vol) or ethanol (96 % vol/vol); the extraction yields are shown in **Table 2**. While carotenoid yields from untreated samples were low even after 12 h of intermediate incubation with organic solvents, PEF treatment prior to 6 h of aqueous incubation increased the yield 2.4-fold compared to untreated samples. Shorter incubation times (1 h, **Table 2**) resulted in no statistically significant different yields and prolongation of the aqueous incubation (12 h, **Table 2**) decreased the subsequent solvent extraction in PEF treated cells. Decreased yields after prolonged incubation were not observed in untreated samples. No statistically significant differences were observed between the different solvents used for extraction, independently of the cell treatment. Because ethanol is Generally Recognized As Safe (GRAS) and a food grade solvent, and it also is cheaper than acetone, it was selected for further investigations.

Treatment	Electric field strength (kV·cm^{-1})	Nº pulses (5 ms)	Energy input (kJ·kg^{-1})	Photosynthetic efficiency after PEF (Qy)
Control	-	-	-	0.72
1	0.2	10	2	0.72
2	0.2	40	8	0.72
3	0.2	80	16	0.65
4	0.6	10	18	0.70
5	0.6	40	72	0.70
6	0.6	80	144	0.64
7	1.0	10	50	0.20
8	1.0	40	200	0.20
9	1.0	80	400	0.21

Table 1. Effect of PEF treatments of different electric field strength and treatment time on the photosynthetic efficiency of *H. pluvialis* in red phase after 1 h rest at room temperature.

Table 2. Carotenoid yields ($mg \cdot g^{-1}_{dw}$) from PEF treated (1 $kV \cdot cm^{-1}$,50ms, 50 $kJ \cdot kg^{-1}$) red phase *H. pluvialis* after different aqueous incubation times before solvent extraction in acetone, methanol or ethanol. Data from untreated samples are represented for comparison purposes. Results represent mean ± SD. Symbols in brackets represent significant differences ($p<0.05$).

	Carotenoid yield ($mg \cdot g_{d.w.}^{-1}$)					
Aqueous incubation time	**Untreated**			**PEF**		
	Acetone	*Methanol*	*Ethanol*	*Acetone*	*Methanol*	*Ethanol*
1h	7.27±3.30(a)	6.78±1.50(a)	7.61±2.51(a)	6.46±2.10(a)	6.81±1.31(a)	7.69±2.01(a)
6h	7.03±2.81(a)	7.81±1.98(a)	6.91±1.90(a)	14.93±2.90(b)	18.24±2.94(b)	16.59±2.03(b)
12h	6.17±2.01(a)	6.95±2.15(a)	6.85±1.05(a)	12.77±2.11(c)	13.17±1.61(c)	12.17±1.01(c)

PEF treatment therefore can improve the extraction of carotenoids from *H. pluvialis* after adequate previous aqueous incubation. The benefits of PEF as pre-treatment to extract pigments, proteins, lipids, carbohydrates and other microalgal compounds have been previously demonstrated [23], however, to the best of our knowledge this is the first report of successful extraction of carotenoids from *H. pluvialis* using PEF. PEF is known to improve the subsequent extraction of both water-soluble and non-polar compounds using the appropriate solvent. However, our data show a 6 h pre-incubation of PEF-treated cells to significantly increase the extraction yields in solvents compared to untreated cells (16.6 and 6.9 $mg_{car} \cdot g_{dw}^{-1}$, respectively). While the release of some small intracellular products (ions, small carbohydrates, amino acids) from microalgae after electroporation by PEF is relatively fast and simple, the release of complex molecular compounds is more complicated. *H. pluvialis* cells primarily contain monoesters of astaxanthin linked to palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) fatty acids increasing its solubility and stability in the cellular lipid environment [4]. Treatment of different microalgal species by PEF improved the yields of lipid extraction and decreased the need of harmful solvents [37; 38; 39; 40; 41]. While Liu et al. (2018) [1] reported that (30 $kV \cdot cm^{-1}$) PEF-treatment of *H. pluvialis* suspended in methanol extracted only 6.5 % of the total carotenoid content, in the present study, a 6 h incubation after the PEF treatment was performed before subjecting the cells to the solvents, which improved the extraction considerably in comparison to untreated samples. Luengo et al. (2015), [24] observed a 50% increase in carotenoid extraction of *C. vulgaris* in ethanol, when the cells were incubated for 1 h in aqueous medium after PEF treatment. Incubation after PEF treatment in aqueous media improved also the lipid extraction from *Auxenochlorella* in an ethanol-hexane mixture [26], and Martinez et al. (2017; 2019) [25;42] reported a delay at the onset of the PEF extraction of phycobiliproteins in aqueous media. While the release of these water soluble pigments was undetectable in untreated cells even after long incubation times, total extraction was achieved after incubation of PEF-treated cells. Similarly, a single pulse (3 $kV \cdot cm^{-1}$ and 2 ms) applied to *H. pluvialis* was able to increase four times the yield of extracted proteins after 24 h of incubation at 20°C in comparison to untreated samples [36].

Our results combined with data found in literature indicate that extraction of astaxanthin requires not only the permeabilization of the cytoplasmic membrane to extract the compound, but additionally also a dissociation of the compounds from intracellular structures. Carotenoid release from *H. pluvialis* might be facilitated by an enzyme-driven process that occurs after PEF-triggered cell death and requires incubation time. Based on our data shown in **Table 2** we conclude that a 6 h incubation after PEF treatment in aqueous media is needed to facilitate the subsequent interaction of solvent and carotenoids in *H. pluvialis* and therefore improves the extraction yield. While shorter incubation times are not sufficient for the enzymatic process, longer incubation times decrease the yield due to degradation of the carotenoids. Astaxanthin is a potent biological antioxidant and therefore gets damaged in contact with excited singlet oxygen

molecules [6], but prevents other cell structures from being damaged. We hypothesize that the incubation of PEF treated samples dissociates astaxanthin molecules from its surrounding due to the enzymatic processes, making it more susceptible to degradation. Untreated samples, on the other hand, contain astaxanthin molecules still incorporated into the cell structures, degradation of the carotenoids therefore will proceed slower. Therefore, it is essential to optimize the incubation time after PEF in order to develop an efficient PEF-assisted extraction of carotenoids from *H. pluvialis.*

3.3. Evaluation of the established protocol to extract carotenoids from the Nordic *H. pluvialis* strain

After having established a protocol for the pre-treatment (PEF followed by 6 h incubation) to carotenoid extraction in ethanol, yields received with this protocol were compared to those received with other pretreatments. Biomass produced under control conditions and exposed to stress was harvested and subjected to bead-beating (5 or 10 cycles), freeze-thawing (5 cycles), thermal treatment, ultrasound or PEF followed by incubation* before extracting the carotenoids by ethanol. In order to compare the disruption treatments established on *H. pluvialis* to other algae, biomass of the Nordic *C. vulgaris* strain 13-1 [27] was treated and extracted using the same protocols. The microalga *C. vulgaris* has a small oval cell (2 µm in diameter) with thick cellulose cell wall, which is very resistant to mechanical disruption treatment [43].

In **Figure 3** the carotenoid yields extracted from both microalgae grown in different media and subjected to the various pre-treatments prior to the solvent extraction in ethanol are given. Of untreated *C. vulgaris* cells 54% of the total carotenoid content could be extracted, pre-treatments increased the yields to 75 - 100%. Pre-treatment by PEF followed by incubation in aqueous medium for 6 h resulted in the best results, while the lowest yield was achieved after five cycles of bead beating. Also Luengo et al. (2015) [24] reported an enhancement of 80 % for carotenoid extraction in ethanol after PEF applying pulses of 5 $kV \cdot cm^{-1}$, 40 ms, 150 $kJ \cdot kg^{-1}$ followed by 1h of incubation in the treatment medium.

Extraction of carotenoids from *H. pluvialis* was less efficient, only 7 % to 38 % of the total carotenoid content was extracted from un-pre-treated cells in the red phase after 1 h extraction in ethanol, consistent to previous reports [2; 17; 18; 44; 45; 46]. Pre-treatments improved the extraction, however, the extraction yields (%) were highly depended on the stress applied to the cells. While extractions of cells grown in N-free medium yielded in 38 to 95 % of total carotenoid content, the yields

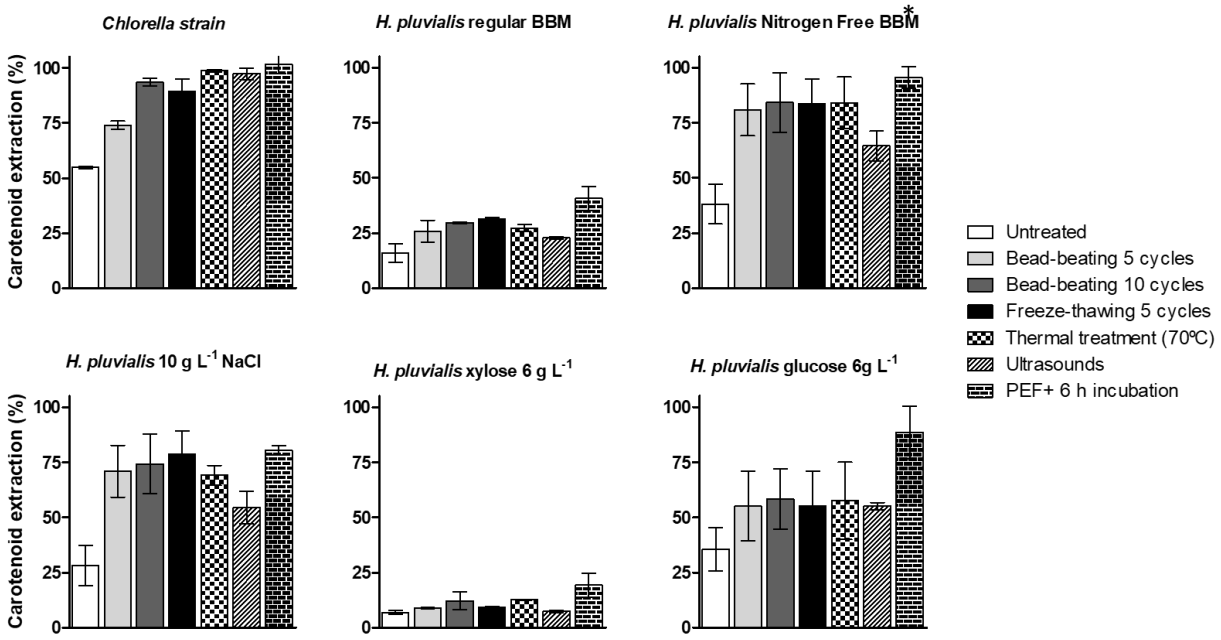

Figure 3. Carotenoid extraction yields (percentage of total content) of *C. vulgaris* and *H. pluvialis* grown in different culture media and subjected to different pre-treatments prior to the solvent extraction in ethanol. (*) means statistically significant differences.

ranged from 7 to 19% in cells exposed to N-starvation in the presence of 6 g·L^{-1} xylose. The best extraction yields were achieved after PEF pre-treatment followed by incubation of 6 hrs, which in the case of cells grown in N-free medium corresponded to 96 % of total content. Statistical significant differences ($p<0.05$) in the extracted carotenoid yields after PEF pre-treatment compared to other physical treatments were observed for *H. pluvialis* cells grown in control BBM medium or in N-free BBM medium supplemented with 6 g·L^{-1} glucose (**Figure 3**).

The physical/mechanical methods [48] applied in this study were expected to disrupt the thick cell wall of *H. pluvialis* and its layers. However, microscopic observation revealed that not all cells were totally disrupted. The proportion of disrupted cells in suspension correlated to the percentage of astaxanthin released. Less than 20% of cells grown mixothropic in the presence of xylose during N-starvation were broken, while more than 80% of cells grown in N-free BBM were disrupted. The three-layered cell wall of *H. pluvialis* consists of a mixture of proteins and polysaccharides, but can be highly dynamic in composition depending on the environment of the cell [3, 11]. Based on our results, N-free, mixotrophic growth in the presence of xylose leads to synthesis of a highly resistant cell wall in *H. pluvialis*. Xylose is an abundant sugar in lignocellulose hydrolysates; as a non-fermentable sugar only few microalgae strains are able to use xylose and it can even inhibit the photosynthesis [47]. Little is known about the anabolism of xylose in *H. pluvialis* or its influence on the biosynthesis of the complex cell wall. We observed that N-starvation at autotrophic growth leads to synthesis of a weaker cell wall. While bead-beating, freeze-thawing, ultrasounds or thermal treatments aim to disrupt the cell wall, PEF acts on the cytoplasmic membrane. The overall structure of the cells remains intact immediately after the PEF treatment, but in yeast it has been demonstrated that electroporation accelerates degradation of the cell wall during incubation [49; 50]. Also we observed in the microscope that the cell wall of *H. pluvialis* progressively lost its structure during the incubation, with the shape of the cell remaining unchanged (not shown). Electroporation of the cytoplasmic membrane of yeast has been suggested to induce a decreased osmotic pressure in the cytoplasm, during the incubation water/media will be taken up by the cell leading to a disruption of the lysosomes and a release of hydrolytic enzymes [49; 50]. The increase in permeability of the cytoplasmic membrane will facilitate the contact of these enzymes with the cell wall and its degradation [49; 50]. Considering the

Table 3. The identification of pigments in the extracts of *H. pluvialis* cultivated under different stress conditions after different pre-treatments. Results represent mean ± SD. Symbols in brackets represent significant differences ($p<0.05$).

Peak n°	*Retention time (min)*	*Absorbance maxima (nm)*	*Pigment*	**N free BBM**		**BBM 6 g L-1 Glucose**		**BBM 6 g L-1 Glucose**	
				% total		% total		% total	
				PEF	**Bead-beating**	**PEF**	**Bead-beating**	**PEF**	**Bead-beating**
1	4.8	478	Trans-astaxanthin	30.12± 3.23	18.72± 0.93	32.47± 4.01	17.81± 1.51	37.04± 4.23	19.64± 2.03
2	8.8	476	canthaxanthin	16.23± 2.54	9.66±1. 94	16.29± 2.67	7.71±2. 95	12.73± 2.67	11.61± 2.21
3	11.4	485	Trans-astaxanthin ester	15.63± 1.50	20.09± 2.74	15.58± 2.10	17.35± 4.87	13.45± 5.24	22.83± 3.85
4	13.1	480	Trans-astaxanthin ester	17.66± 1.11	22.55± 2.73	16.84± 2.50	19.81± 3.14	15.27± 2.11	20.29± 3.21
5	15.1	485	Trans-astaxanthin ester	20.34± 1.23	28.96± 3.8	18.80± 2.43	37.31± 4.51	21.51± 2.13	25.62± 3.56

similarities in composition between the cell walls of *H. pluvialis* and yeast [48], our observations could be explained in the same way. PEF treatment followed by incubation would trigger an enzymatic digestion that degrades the microalgal cell wall.

Extracts from stressed *H. pluvialis* cells obtained after PEF with incubation or mechanical treatment were analyzed by HPLC (**Table 3**). Exposure to different stress conditions did not result to significant changes in carotenoid composition, however, so did the pre-treatments of the biomass. The free form of astaxanthin represented less than 20% of the total carotenoid content when extracted after bead-beating, but 30 - 37% using PEF as pre-treatment. Also the proportion of free canthaxanthin present in the extract was higher after PEF pre-treatment compared to mechanical disruption. PEF-treatment and subsequent incubation therefore might induce activity of the enzyme esterase in the cells, which digests the esters from the lipid bodies, facilitating contact with the solvent and releasing astaxanthin in its free form. The free form of astaxanthin is more polar than its esterified form, thus solubilization of the carotenoids in polar solvents like ethanol will be improved. Concomitant degradation of the cell wall during incubation will facilitate rapid extraction.

The astaxanthin yields obtained by our procedure are comparable to previous extractions using high energy consuming physical treatments [1; 2; 3; 19; 20]. Highest extraction yields of xanthophylls (astaxanthin and cantaxanthin) (51.6 $mg_{car} \cdot L^{-1}$) were achieved after cultivating *H. pluvialis* in glucose (6 $g \cdot L^{-1}$) and N-free medium and pre-treat the biomass by PEF followed by a 6 h incubation. The PEF pre-treatment used in this study was quite gentle (50 $kJ \cdot kg^{-1}$) and can easily be up-scaled. Downstream processes to purify the astaxanthin extracts will be facilitated using this method, because PEF is selective and does not induce fragmentation of the cell into small debris. Instead the molecules are released and at the same time the cell structure remains conserved. Thus, after moderate centrifugation, cells are easily separated from the extracts, while harsh disruption treatments cause cell breakage; the small size of cell debris complicates separation by sedimentation.

4. CONCLUSIONS

Here we develop and describe a complete process starting with the production of *H. pluvialis* biomass in the red phase followed by an innovative pre-treatment and extraction of the target carotenoid astaxanthin. Exposure to sudden N-starvation of the Nordic strain *H. pluvialis* resulted in improved carotenoid yields. Mixotrophic N-free growth in the presence of glucose enhanced the growth rate and the total carotenoid content of the cultures. Pre-treatment of the biomass by PEF was compared to physical treatments, the sensibility of cells to these treatments were shown to depend on the growth conditions. Cells grown in xylose-containing medium were highly resistant to breakage by all methods. Fresh biomass pre-treated by PEF followed by an incubation in its own growth medium increased the subsequent extraction yields using food-grade and eco-friendly ethanol as solvent. We hypothesize that the improved extraction of carotenoids from *H. pluvialis* using PEF pre-treatment is due to an enzymatic process triggered by PEF and performed during the incubation. An esterase activity very likely releases carotenoids from the lipid structures, combined with electropermeabilization of cell membrane the ethanol extraction of carotenoids becomes highly efficient. Up-scaling of algal astaxanthin extraction using PEF is feasable, large-scale PEF equipment to treat liquid products in continuous mode ($T \cdot h^{-1}$) is available, its energy requirement is moderate.

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SUPPLEMENTARY MATERIAL

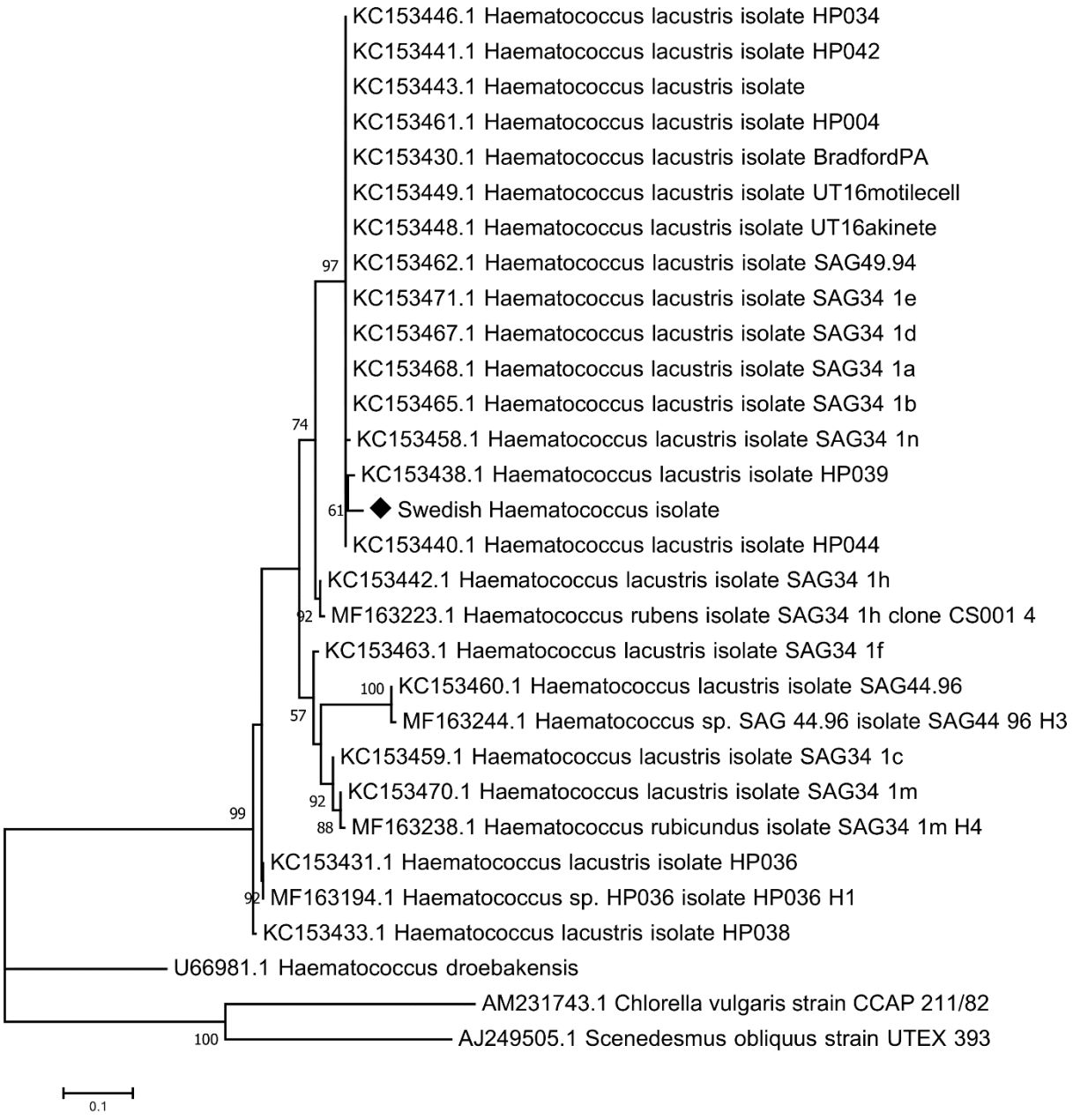

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Fig. S1 Phylogenetic tree based on the ITS2 sequence of 27 different *Haematococcus* isolates and our local isolate (indicated with ◆) inferred using the Neighbor-Joining method (1000 replicates). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ITS2 sequence of microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* were also included as outgroup.

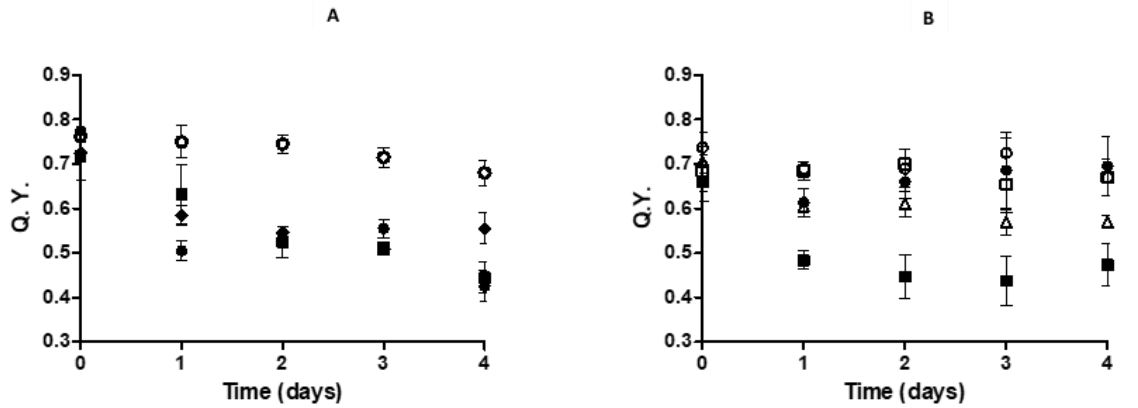

Figure S2. Photosynthetic efficiency (Qy) of H. pluvialis exposed to stress in function of the cultivation time in two different light intensities: 200 (A) and 1000 µmol·s-1·m-2 (B). Control culture medium BBM (○), Nitrogen-free BBM (●), Nitrogen-free BBM supplemented with either 5 g·L-1 NaCl (♦), 10 g·L-1 NaCl (■), 6 g·L-1 xylose (△) or 6 g·L-1 glucose (□).

5. DISCUSSION

Even though the results of this doctoral thesis have been already discussed individually in the manuscripts attached in the results section, the objective of this section is to present an overall discussion of the results with the purpose of obtaining general conclusions of the potential of Pulsed Electric Field technology for the improvement of extraction of valuable compounds from microorganisms. This discussion has been divided in three subsections devoted to the extraction of mannoproteins from *S. cerevisiae*, to the extraction of carotenoids from *R. glutinis* and to the extraction of valuable compounds from microalgae. In this section, new results that are not included in the results section are presented. These results correspond to some experiments conducted in order to get new insights on the mechanism involved in the extraction of mannoproteins and pigments from yeast cells.

5.1. Release of mannoproteins during *Saccharomyces cerevisiae* yeast autolysis induced by Pulsed Electric Field

The autolysis of yeast is a process widely exploited in the food industry for the production of yeast extracts and autolysates, which are used as flavor contributors, flavor enhancers or to improve texture. On the other hand, in the wine industry, yeast autolysis occurs during the production of wines with prolonged yeast contact ("aging on lees"). This process leads to the release of mannoproteins from the yeast cell walls, which have positive technological effects in winemaking and provide positive attributes to the wine such as haze reduction, prevention of tartaric precipitation, mouthfeel contribution, reduction of astringency, and enhancement of the aroma and color of wine (Pérez-Serradilla and De Castro, 2008). However, yeast autolysis in wine is a very slow process lasting from a few months to years. Accelerating this step is highly desirable to reduce production costs and associated problems such as microbial spoiling (Alexandre and Guilloux-Benatier, 2006). Different strategies have been suggested for the acceleration of yeast autolysis, including enzymes capable of hydrolyzing β-glucans from yeast cell walls, thermolysis, or mechanical methods for large-scale disruption of microbial cells (such as ultrasound or high pressure homogenization) (Comuzzo et al., 2015). However, enological procedures aiming to increase the mannoprotein content of wine are not yet fully established; they can be time-consuming, or they can exceedingly augment production costs.

The process of natural autolysis is associated with cell death, which is required to initiate the process of self-degradation. Mannoprotein release requires that enzymes, mainly located in vacuoles of the cytoplasm, achieve access to the cell wall in order to degrade its constituents. The objective of the investigation was to use PEF technology for accelerating the liberation of mannoproteins from the cell wall of yeast. The hypothesis behind this study was that the electroporation of the cytoplasmatic membrane by PEF could facilitate both the release of enzymes involved in the hydrolysis of the cell wall from vacuoles and the subsequent access of these enzymes to the cell wall (Figure 5.1.).

According to our hypothesis, the water inlet to the cytoplasm caused by the loss of the selective permeability of the cytoplasmic membrane could lead to a decrease of the cytoplasmic osmotic pressure and the consequent plasmolysis of vacuoles that could

cause the enzyme release. On the other hand, the pores formed in the cytoplasmic membrane by electroporation could facilitate the contact of these enzymes with the outermost layer of the yeast cell wall where the mannoproteins are located. Therefore, this two simultaneous effects could accelerate the release of mannoproteins to the extracellular environment.

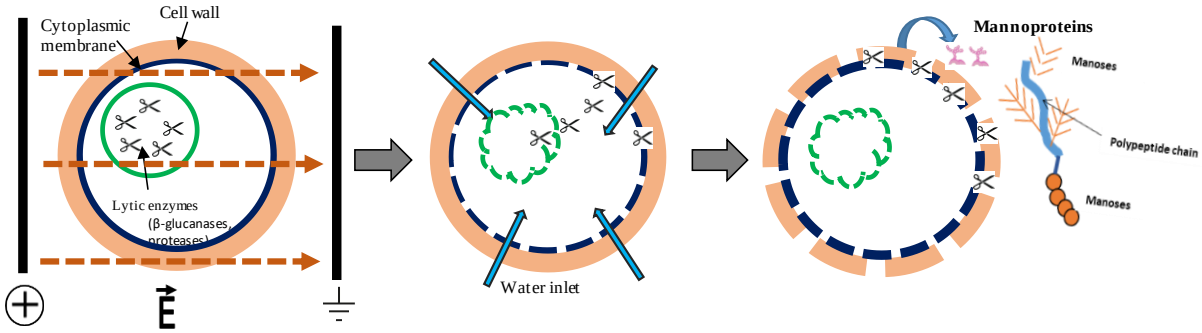

Figure 5.1. Schematic representation of the hypothesis of acceleration of cell wall autolysis by PEF treatment.

In order to demonstrate the proposed hypothesis, the potential application of PEF for triggering autolysis of *Saccharomyces cerevisiae* and accelerating the release of mannoproteins was evaluated (section 4.1.1).

When cells were exposed to a sufficiently strong electric field, cytoplasmic membrane rendered permeable leading to the prompt release to the extracellular environment of nucleic acids and proteins from the cytoplasm to the extracellular enviromment. Furthermore, as it was hypothesized, mannoproteins release was much faster for PEF-treated cells than for untreated cells. Mannose concentration in the extracellular medium increased linearly along time for the samples containing PEF-treated cells. However, mannoprotein release from the untreated cells was negligible for three weeks. After 25 days of incubation, the concentration of mannose in the extracellular medium containing PEF-treated cells of *S. cerevisiae* was 10 times higher.

Once demonstrated the positive effect of PEF on the release of mannoproteins from *S. cerevisiae*, the effect of different incubation conditions on the release of mannoproteins from PEF treated cells was investigated (section 4.1.2). It is well known that incubation conditions have an important influence in the release of mannoproteins during natural autolysis (Fornairon-Bonnefond et al., 2002).

Results obtained in this second study shown that, as in natural autolysis, the release of mannoproteins from PEF treated cells was retarded at low temperature or pH and in the presence of ethanol in the incubation medium. After the same incubation

time, the amount of mannose released from PEF treated cells ranged from 80 mg/L, when they were stored in media with 25 % ethanol, to 190 mg/L when they were stored at 43 ºC without ethanol. Combination of acidic pH and ethanol delayed even more mannoproteins release although PEF-treated yeasts released approximately twice the amount of mannoproteins than untreated yeasts.

It is well known that catalytic activity of enzymes is highly dependent on environmental conditions. Therefore, these results seemed to confirm that similarly to natural autolysis, endogenous enzymes of *S. cerevisiae* were involved in the autolysis triggered by PEF.

In order to go deeper into the mechanisms of PEF-induced autolysis it was investigated if enzymatic activity was detected in the extracellular medium containing PEF-treated cells of *S. cerevisisae*. The corresponding experiments were specifically designed for the discussion of this Doctoral Thesis. According to literature, the main enzymes involved in *S. cerevisiae* autolysis process are proteases and β-glucanases (Alexandre and Guilloux-Benatier, 2006). The presence of activities of these enzymes types in the supernatants of PEF-treated (20 kV/cm; 150 µs) and untreated *S. cerevisiae* cells during one week of incubation at different temperatures (7, 25 and 43 °C) was analyzed. Figure 5.2 shows the evolution along the time of β-glucanase enzymatic activity in extracellular medium of untreated or PEF-treated *S. cerevisiae* cells stored at 7 °C (A), 25 °C (B) and 43 °C (C).

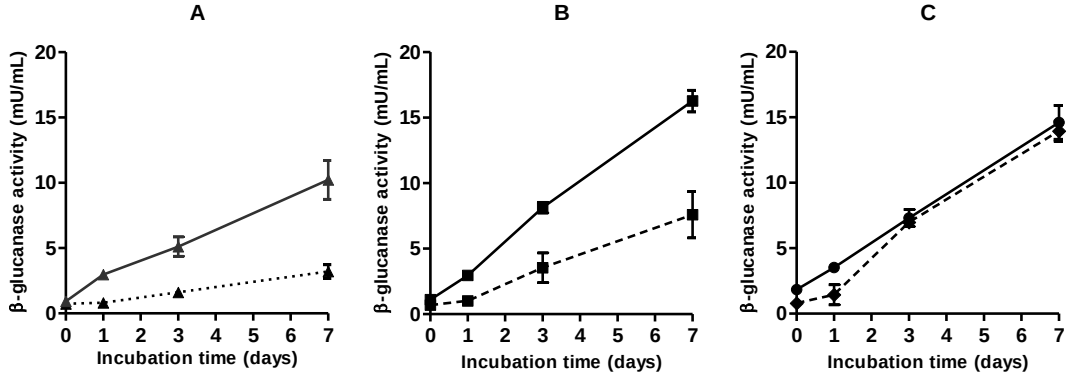

Figure 5.2. Evolution of β-glucanase enzymatic activity in the extracellular media of untreated (discontinuous line) and PEF-treated (20 kV/cm; 150 µs) *S. cerevisiae* (continuous lines) during incubation at different temperatures 7°C (A), 25°C (B) and 43°C (C).

These results shown that, except at 43°C, the release of β-glucanase to the extracellular media was higher when cells were previously PEF-treated. The concentration of β-glucanase in the PEF-treated suspension media increased linearly along time at all the temperatures assayed. At 43°C, statistical significant differences in

the β-glucanase concentration between untreated and PEF-treated suspensions were observed only during the first day of incubation. After that, the β-glucanase concentration in the extracellular medium stored at 43°C was similar from untreated and PEF-treated cells. Besides, this β-glucanase concentration corresponded with that concentration in the extracellular medium that contained PEF treated cells incubated at 25 ºC. These observations agree with the higher release of mannoproteins from PEF treated yeast stored at higher temperatures (section 4.1.2). Although the β-glucanase concentration was similar when PEF treated cells were incubated at 25 ºC than at 43 ºC, the highest catalytic activity of this enzyme at higher temperatures (Figure 5.3.) would explain the greater release of mannoproteins when the incubation was conducted at 43 ºC. The increase of temperature during the reaction of substrate conversion from 25 °C to 43 °C accelerated the enzymatic reactions and the activity increased to 120 and 110 % for β-glucanase and protease respectively. Besides, low temperature (7 °C) greatly slowed down the reactions and for the same incubation times, β-glucanase and protease activity were respectively 4 and 5 % of the activity observed at 25 °C.

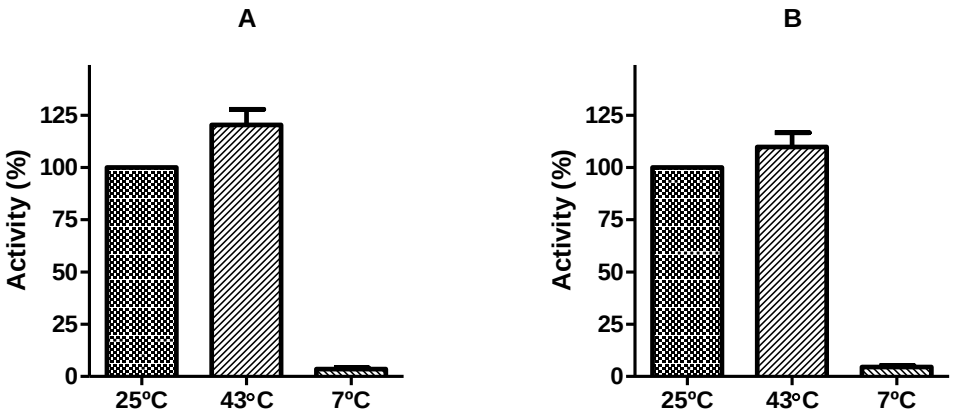

Figure 5.3. Percentage of β-glucanase (A) and protease (B) activity from the supernatants of PEF-treated *S. cerevisiae* analyzed performing the substrate conversion assay under different temperatures. Results are expressed respect a reference that was the performing of the analytical test at 25°C.

On the other hand, the high concentration of mannoproteins in the extracellular medium containing untreated cells at 43 ºC could be caused by the liberation of this enzyme due to the inactivation of the yeast at this incubation temperature (as it was checked) (section 4.1.2). Conversely, the differences observed between the β-glucanase release from PEF-treated cells stored at 7 or 25 °C (Figure 5.2) and the differences in the activity (Figure 5.3.), would support the faster release of mannoproteins at 25 °C in comparison to 7°C. Likewise, Figure 5.4. shows the evolution of the release of protease

enzymes to the extracellular media of untreated or PEF-treated *S. cerevisiae* cells stored at 7 °C (A), 25 °C (B) and 43 °C (C).

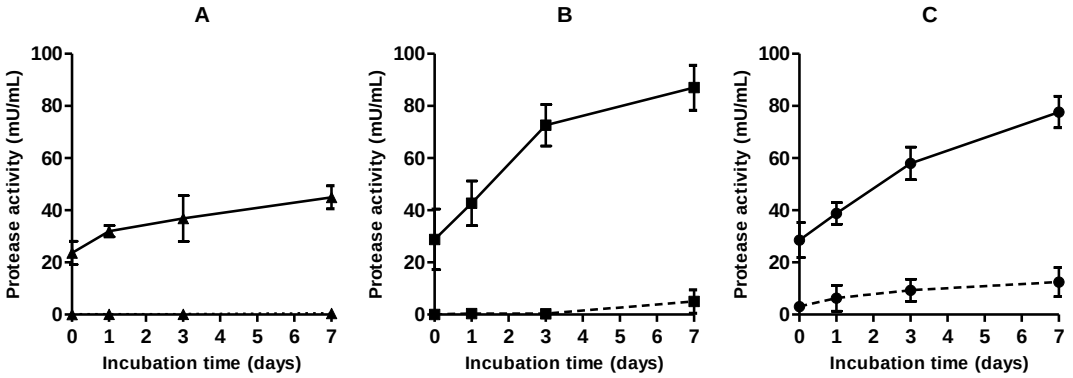

Figure 5.4. Evolution of protease enzymatic activity in the extracellular media of untreated (discontinuous line) and PEF-treated (20 kV/cm; 150 µs) *S. cerevisiae* (continuous lines) during incubation at different temperatures 7°C (A), 25°C (B) and 43°C (C).

In this case, when the cells were treated by PEF, protease enzymes were released in the first moments after the treatment. As it is observed similar protease activity was detected at time 0 at the three incubation temperatures. On the other hand, protease activity in the medium containing untreated cells was very low independently of the incubation temperature and similarly to β-glucanase the liberation of proteases from PEF treated cells increased with temperature. However, no differences were observed between 25 and 43 ºC.

Liberation of mannoproteins has been attributed to β-glucanase and protease activities. However, in our study when kinetic of mannoprotein release is compared with kinetics of enzymatic activity it is observed that mannoproteins release is more associated to the β-glucanase activity rather than protease activity. For example, although the release of mannoproteins in the untreated cells was very high at 43 ºC, at this temperature the protease activity detected was very low (Fig 5.4 C)

As the last objective of our investigation was to demonstrate if the effect observed in buffer could be used for accelerating the mannoproteins release during "aging on lees", the influence of the presence of ethanol on the enzymatic activity was assayed.

Figure 5.5 shows the activity of the enzymes β-glucanase (5.5. A) and protease (5.5. B) at different ethanol concentrations (0, 6, 12 and 25 %, vol/vol) at 25 °C.

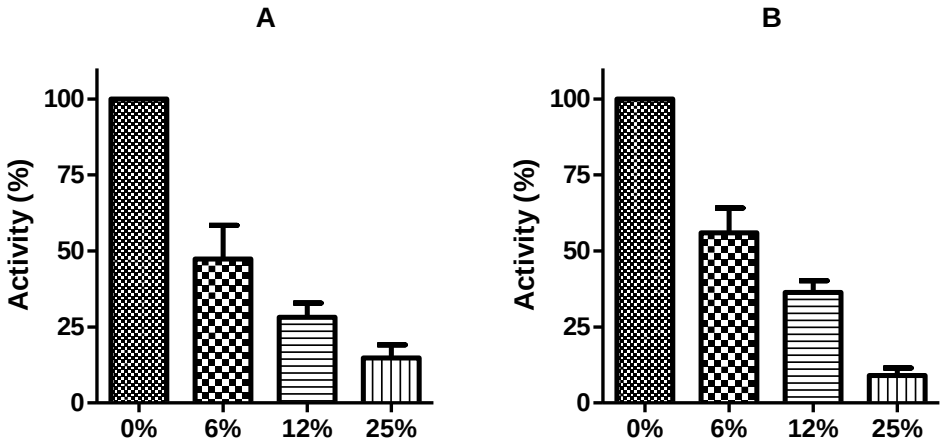

Figure 5.5. Percentage of β-glucanase (A) and protease (B) activity from the supernatants of PEF-treated *S. cerevisiae* analyzed under different ethanol concentrations. Results are expressed respect a reference that was the performing of the analytical test in 0% ethanol.

These results shown that the activity of both enzymes drastically slowed down as the concentration of ethanol in media increased. In the case of β-glucanase, the enzymatic activity decreased by 53, 72 and 85 % when the concentration of ethanol was 6, 12 and 25 % ethanol respectively. On the other hand, the same concentration of ethanol decreased protease activity for 45, 64 and 91 % respectively. The influence of the presence of ethanol on the β-glucanase and protease activities seems to explain the slower release of mannoproteins observed when yeast autolysis was conducted in a media with 10 % of ethanol (section 4.1.2).

The autolysis induced by PEF and the subsequent release of manoproteins from the yeast cell wall are influenced by different well-known factors that likewise affect natural autolysis. This study has demonstrated that these factors mainly influence by affecting the enzymatic activity. However, independently of the conditions in which autolysis occurred, the release of mannose was more rapid when the yeast cells were previously treated by PEF.

Once demonstrated the effect of PEF for triggering autolysis, the potential of PEF for accelerating "aging on lees" step in winemaking was investigated (section 4.1.3). PEF treatment was evaluated for accelerating the release of mannoproteins from *Saccharomyces cerevisiae* during "aging on lees" of Chardonnay wine. The same yeasts which participated in the fermentation of grapes were PEF-treated and wines were aged on presence of these yeasts. After 60 days of incubation, the mannoprotein concentration in wines containing PEF-treated yeast was double of the mannose released from control cells. Wines containing untreated cells required six months of

incubation to reach the maximum release of mannose, thereby confirming that natural autolysis in wine is indeed a slow process. As it has been described, the low pH of wine, along with the presence of ethanol, make the autolytic process more likely to occur at a much slower rate.

The potential of PEF to accelerate the release of mannoproteins from *S. cerevisiae* in white wine was demonstrated. The events occurring would be similar to natural autolysis, involving the enzymatic activity, but accelerated thanks to electroporation. The mannoproteins released in a shorter time from PEF-treated cells featured similar functional properties in wine than mannoproteins released during natural autolysis from untreated yeasts and process did not negatively affect the wines' physicochemical properties. Due to the fact that intense treatments are not required in order to triggering yeast autolysis by PEF, wineries could thus process lees using the most economical PEF devices on the market. However, it has been demonstrated that conditions of incubation are essential for the graduation of the effects of enzymes triggered. Thus, these conditions have to be taken into account for the reshaping of the procedure for different wines varieties. Further studies are required to evaluate the PEF-induced acceleration of “aging on lees” step on red wines to adapt the conditions of treatment and incubation. Once studied the influence of these factors, the gentle PEF treatment parameters required to induce autolysis open up the possibility of processing large volumes in continuous flow with low energy consumption.

5.2. Extraction of carotenoids from PEF-treated *Rhodotorula glutinis* yeast

Carotenoids pigments are extensively used in food product formulations, not only for their coloring properties, but also for their antioxidant and disease-prevention effects. Currently, there is an increasing interest in carotenoids obtained by biotechnological processes as an alternative to synthetic production. *Rhodotorula* yeast carotenoid production has several advantages against other sources. However, the envelopes of the microbial cells, the hydrophobic nature of carotenoids, the association of carotenoids with other macromolecules such as proteins and fatty acids, and the sensitivity of carotenoids to heat, light or acids make yeast-derived carotenoids difficult to be recovered and the current recovery techniques are inefficient. Therefore, the development of simple, rapid, and inexpensive extraction methods are required (Saini and Keum, 2018). In order to achieve this objective, in this thesis (section 4.2.1) PEF was selected as an alternative to heavily energy consuming pre-treatments (e.g. ultrasound, high-pressure homogenization or bead milling) aiming to break down the physical barriers present in the cells.

The potential of PEF for inducing autolysis of *R. glutinis,* was evaluated with the purpose of designing a more efficient and ecofriendly carotenoid extraction process. Propidium iodide uptake and release of intracellular components were used as techniques to reveal the electroporation of *R. glutinis* by PEF. A treatment of only 15 kV/cm for 150 µs irreversibly electroporated the 90 % of the population of *R. glutinis.* Immediately after the PEF treatment, no significant morphological changes were detected in the cells by flow cytometry and ethanol was proved ineffective for extracting carotenoids from fresh biomass of *R. glutinis.* However, after incubating the PEF-treated fresh biomass for 24 hours at 20 ºC in a pH 7 buffer the size of cells became much smaller and ca. 240 $\mu g/g_{d.w.}$ of carotenoids were recovered after 1 hour of extraction in ethanol. The phenomenon was explained by PEF-triggered autolysis, which tended to enzymatically disrupt the association of carotenoids with other molecules present in the cytoplasm. Besides, when cells were broken by mechanical treatment (bead-beating) the subsequent extraction in ethanol was not effective. Therefore, the cell envelopes seems not to be the barrier that prevented carotenoid extraction. On the other hand, the fact that long incubation of PEF-treated cells in ethanol resulted in an inefficient extraction could be explained because this compound

prevented enzymatic activity. Likewise, the increment of the extraction by increasing the incubation temperature from 15 to 25 ºC and by increasing the pH of the media until 8 supported the hypothesis that enzymatic activity was involved in the observed effect. This procedure gave rise to an innovative sustainable method for extraction of carotenoids from fresh yeast, by eliminating the drying stage from the conventional process, as well as by avoiding non-polar solvents (hexane, diethyl eter, chloroform, etc) generally used for extraction of carotenoids.

However, not always is preferable to use fresh biomass. Depending on the raw material, the target compound, the solvent and the extraction method, it might be desired to use dried biomass as it is the case of supercritical CO_2 extraction. Therefore, after demonstrating the enhancement effect of PEF followed by aqueous incubation in the subsequent ethanolic extraction of carotenoids from fresh *R. glutinis*, it was evaluated whether the electroporation of fresh *R. glutinis* biomass improved the subsequent extraction of carotenoids from dry biomass (section 4.2.2).

Both supercritical CO_2 and solvent extraction of carotenoids from untreated or PEF treated cells that were immediately freeze-dried after the pre-treatment were ineffective (extraction yields lower than 20 % total content). However, as it was observed when extraction was performed with fresh biomass, if before freeze-drying the PEF-treated cells were incubated in aqueous buffer for 24 h, a large improvement of extraction yields with the three solvents assayed (acetone, hexane, and ethanol) was achieved. Thus, the positive effect of PEF-treatment followed by aqueous incubation is maintained when the biomass is dried after incubation. These findings demonstrated the possibility of using PEF as a pre-treatment for the extraction of carotenoids in ethanol from dried *R. glutinis* biomass. The possibility of drying the biomass would permit to delay the extraction of the target compound facilitating transportation of the biomass. Furthermore, this method would allow to perform extraction procedures that exclusively use dry material (utilizing PEF-treated and incubated biomass) and still have the benefits of permeabilization and autolysis on extraction yields.

Either in the case of continuing with fresh or dried biomass, it was assumed that after 24 h aqueous incubation time a PEF-triggered autolysis is initiated. The enzymes released might degrade the structures where carotenoids are bound to cell constituents, facilitating their subsequent extraction by solvents and/or degrade the cell wall. In order to verify the hypothesis and identify the enzymes involved, it was evaluated if there

was enzymatic activity in the medium in which the PEF treated cells were incubated after electroporation. The presence of β-glucanase, protease and esterase activity were analyzed in the supernatants of PEF-treated (15 kV/cm, 150 μs) and untreated *R. glutinis* cells during one week of incubation at 25°C in pH 7 McIlvaine buffer. According to our hypothesis, the esterase and protease activity would be related with the lysis of the association of carotenoids with other macromolecules such as proteins and fatty acids and the β-glucanase activity would degrade the cell wall facilitating the mass transfer. While β-glucanase and esterase activity were detected in the supernatants, protease activity was negligible.

Figure 5.6 shows the evolution of the β-glucanase (A) and esterase (B) activity in extracellular medium that contained untreated or PEF-treated *R. glutinis* cells. It is observed the presence of much more enzymatic activity in the medium that contained PEF treated cells.

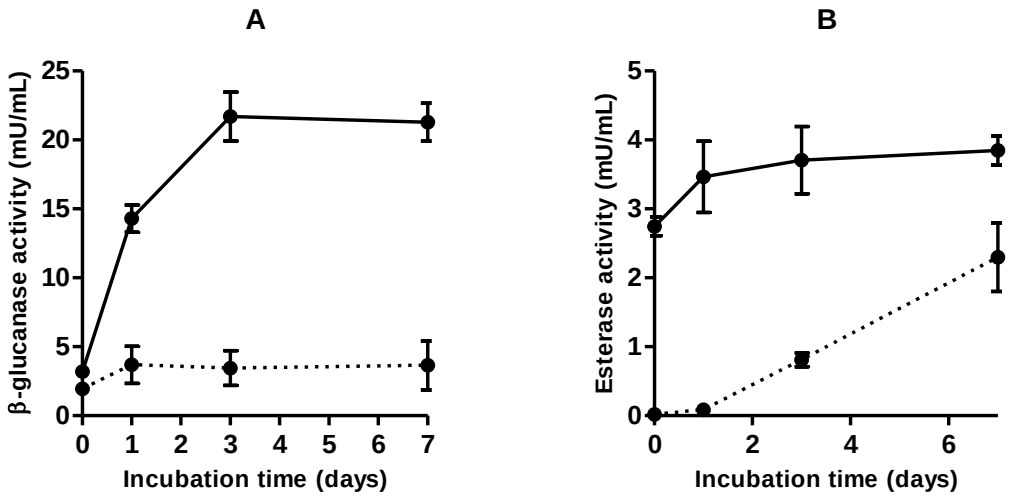

Figure 5.6. Evolution of β-glucanase (A) and esterase (B) enzymatic activity in the extracellular media of untreated (discontinuous line) and PEF-treated (15 kV/cm; 150 μs) *R. glutinis* (continuous lines) during incubation at 25°C in pH 7 McIlvaine buffer.

However, while β-glucanase activity increased progressively in the medium that contained PEF-treated cells until the third day of incubation, the esterase activity was very high just after the application of the PEF treatment. These results seem to indicate a higher release of β-glucanase from *R. glutinis* in comparison to *S. cerevisiae* (Figure 5.2) or that the catalytic activity of the enzymes released from *R. glutinis* is higher. As consequence, while the maximum extraction of carotenoids from *R. glutinis* was achieved after 24 hours of incubation, longer times were required in the case of mannoprotein release from *S. cerevisiae.*

When *R. glutinis* cells were subjected to cycles of mechanical disruption like bead-beating until the complete destruction (checked microscopically), the extraction of carotenoids resulted inefficient. Therefore, it seems that although both enzymes could be involved in the improvement effect of extraction, the role of β-glucanase against the cell wall in the release of carotenoids is minor since the mechanical breakage of cell wall did not improve extraction. Therefore, the main enzyme responsible in the improvement of release of carotenoids seems to be esterase.

The esterase activity was highly dependent of the ethanol concentration (figure 5.6). The activity of esterase enzymes obtained from *R. glutinis* supernatants was analyzed in different ethanol concentration. As the ethanol concentration increased, the activity of this enzyme was extremely reduced. Therefore, a previous incubation in an aqueous medium was required for esterase to liberate the carotenoids attached to other cell compounds. As it was hypothesized, incubation of just PEF-treated *R. glutinis* cells in ethanol resulted in ineffective extraction because this alcohol avoids the activity of the enzymes involved.

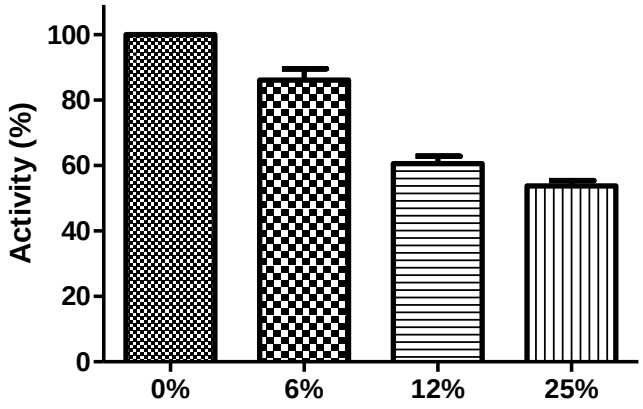

Figure 5.6. Percentage of esterase activity from the supernatants of PEF-treated *R. glutinis* analyzed under different ethanol concentrations. Results are expressed respect a reference that was the performing of the analytical test in 0% ethanol.

The inefficient extraction after long incubation in ethanol of PEF-treated or mechanical disrupted cells in combination with the enzymatic activity analysis of supernatants during incubation ratify our hypothesis. The results confirm the main role of esterase in the extraction of carotenoids supporting the hypothesis that the improvement in carotenoid extraction by incubating the *R. glutinis* cells after PEF

treatment is a consequence of the autolysis triggered by PEF that causes a disruption of the associations of carotenoids with other molecules.

This research has revealed that the application of a PEF treatment to cells of *R. glutinis* followed by an aqueous incubation period permits the subsequent extraction of carotenoids. The mechanism seems to be related with an enzyme-driven process in which esterase plays the main role. The moderate energy delivering required for triggering the disassembling of the carotenoids do not provoke a significant rise in the temperature of the product, thereby preventing the undesirable consequences of heat on the quality of pigments extracted. Besides, this extraction could be carried out not only from fresh but also from dried biomass, because the improvement effect is maintained after freeze-drying. Further studies of the autolysis triggering effect of electroporation in different yeast species that produce compounds of interest are required in order to extent the application of this methodology to other species in which natural autolysis has been scarcely investigated until now.

5.3. PEF-assisted extraction of valuable compounds from microalgae

Microalgae are a natural, renewable, continuously available and abundant source of high-value products, including polyunsaturated fatty acids (PUFA), proteins, amino acids, pigments or vitamins. However, there exist some obstacles that impede the full exploitation of this source, like the lack of sufficiently profitable extraction processes. Similarly to what occurs with yeast, usually these processes requires long extraction times and the use of large amount of organic solvents, which results problematic due to the high costs and environmental impact. Although several authors in literature have reported that PEF technology could represent a viable method for microalgae permeabilization, allowing a relevant release of valuable intracellular compounds (Luengo et al., 2015; Parniakov et al., 2015; Pataro et al., 2017; Poojary et al., 2016), parameters of processing have not been always optimized.

In this thesis, the potential of PEF technology for improving the extraction of valuable compounds produced by microalgae has been studied in three different species (section 4.3.). Table 5.1. shows the required electric field strength to electroporate these microalgae cells in comparison to the widely studied *Chlorella vulgaris,* which is small and spherical. The external electric field strength required to reach the transmembrane voltage threshold to induce electroporation is inversely correlated to target cell size (Kotnik et al., 2012) as it is shown in the Table 5.1. The moderate electric field strengths required to permeabilize palmella cells of *Haematococcus pluvialis* is not only consequence of its large diameter but also of the use of longer pulses of millisecond duration. The size is not the only parameter that influences electroporation because the electric field strengths to electroporate these microalgae cells was higher than those for electroporating yeast cells, which size is smaller (section 4.1. and 4.2.). The orientation of cells in the electric field and their shape, which is also showed in table, may vary the electric field strength required to produce electroporation phenomenon. Beyond the critical electric field strength, permeabilization generally increases along with more intense electric field strength and longer treatment durations.

Table 5.1. Electric field strength to electroporate the different types of microalgae cells studied in this thesis and comparison with *Chlorella vulgaris* cells.

Microalga specie	Shape and size	Electric field strength (kV/cm)	Effect indicating electroporation
C. vulgaris	Spherical, 3-8 µm in diameter	20 kV/cm	90 % PI uptake
A. platensis	Helical trichomes, 2-12 µm size	20 kV/cm	Release of proteins
P. cruentum	Spherical to ovoid, 10-16 µm diameter	10 kV/cm	90 % PI uptake
H. pluvialis	Spherical, 30-60 µm diameter	1 kV/cm	Extraction of astaxanthin after 6 h intermediate aqueous incubation

As a summary, treatment parameters and energy requirements to obtain the different compounds of interest from microalgae studied are compared in table 5.2.

As observed in Table 5.2., microalgae of larger size are electroporated at low electric field strengths, which results in the decrease of energy consumption. On the other hand, it is observed that although *H. pluvialis* required low electric field strength, treatment resulted in considerable energy input because pulses in the millisecond order were used. The reduction of pulse duration from milliseconds to microseconds, combined with an increase of electric field strength has been reported to maintain or even increase the extraction yield while reducing the energy requirements (Luengo et al., 2015). However, when large volumes are treated in continuous flow, the frequency of the PEF generator limits the number of pulses that can be applied to the suspension. Therefore, to achieve a sufficient treatment time to electroporate the cells, the width of pulses has to be enlarged.

Table 5.2. Electric field strength, treatment time and total specific energy to improve the extraction of compounds of interest from the different types of microalgae cells studied in this thesis and comparison with *Chlorella vulgaris* cells.

Microalga specie	Compound extracted	Electric field strength (kV/cm)	Treat-ment time	Total specific Energy (kJ/kg)	Improvement
C. vulgaris	Lutein	20 kV/cm	100 µs	61.38	4 fold in comparison to untreated
A. platensis	c-phycocyanin	20 kV/cm	150 µs	60	Total content extracted after 6 h incubation
P. cruentum	B-phycoerythrin	10 kV/cm	150 µs	9	Total content extracted after 24 h incubation
H. pluvialis	Astaxanthin	1 kV/cm	50 ms	50	96 % extraction of astaxanthin after 6 h intermediate aqueous incubation

Treatments were performed in media of 1 mS/cm conductivity and therefore energy values are comparable.

The application of PEF to the fresh biomass of *Artrhospira platensis* in order to enhance the extraction of the pigment C-phycocyanin into aqueous media was assessed (section 4.3.1). This water soluble phycobiliprotein of great commercial and industrial significance is arranged, along with other phycobiliproteins, into supramolecular complexes called phycobilisomes, located in the thylakoid membranes (Sezar and Chandramohan, 2008). While extraction of phycocyanin from untreated cells was undetectable, PEF treatment permitted the extraction of this pigment. However, a delay of 150 min at the onset of extraction was observed for all conditions. This delay was attributed to the evolution of the size of pores. Low molecular weight compounds could cross the cytoplasmic membrane immediately after electroporation, whereas the release of molecules of larger molecular weight might require that the pores created by PEF treatment enlarge over the course of time. However, the enzymatic activity could be involved in the disassembling of phycobiliproteins from phycobilisomes, thus this aspect requires further investigation.

Phycocyanin extraction from *A. platensis* depended on both electric field strength and treatment duration. The minimum electric field intensity for detecting C-phycocyanin in the extraction medium was 15 kV/cm after the application of a treatment time of 150 μs (50 pulses of 3 μs). However higher electric field strengths were required when shorter treatment times were applied. The increment of the temperature of PEF treatment reduced the electric field strength and the treatment time required to obtain a given phycocyanin yield and, consequently decreased the total specific energy delivered by the treatment. For example, the increment of temperature from 10 °C to 40 °C permitted to reduce the electric field strength required to extract 100 mg/g_{dw} of C-phycocyanin from 25 to 18 kV/cm, and the specific energy input from 106.7 to 67.5 kJ/Kg. This temperature increment within ranges that do not cause thermal degradation of the compounds (<40°C) would be a useful strategy to reduce the electric fileld intensity for the PEF-assisted biorefinery from microalgae. Results obtained in this investigation demonstrated PEF's potential for selectively extraction of C-phycocyanin from fresh *A. platensis* biomass. Furthermore, the purity of the C-phycocyanin extract obtained from the electroporated cells was higher than that obtained using other technique based on the cell complete destruction.

A second study (section 4.3.2) aiming to assess the extraction of another water-soluble phycobiliprotein, phycoerythrin (BPE), into aqueous media by the

application of PEF to fresh *Porphyridium cruentum* microalgae was conducted. An increment in electric field strength or a prolongation of treatment time, increased electroporation and inactivation of *P. cruentum* in the range investigated (2-10 kV/cm; 30-150 µs). A perfect agreement between percentage of dead cells and percentage of electroporated cells was observed when the inactivation percentage was greater than 20 %. While the release of this water-soluble protein was undetectable in the untreated cells even after 48 h of extraction, the entire content was released from PEF-treated cells (8 or 10 kV/cm for 150 µs) after 24 hours of extraction. However, this water-soluble protein was not released immediately; a lag time of over 6 hours was necessary until the compound could be detected in the extraction medium. This behavior seems to indicate that BPE extraction requires not only the diffusion of the compound through the cell membrane, but also the disassembling of the compound from the cell structures. In this respect, it was postulated that PEF could trigger the release of hydrolytic enzymes from the *P. cruentum* organelles that would lyse the bonds between the pigment and other compounds of the cell; thus, the water-BPE complex could diffuse through the membrane, carried by a concentration gradient. This hypothesis is supported by the differences in the correspondence between the percentage of electroporated cells and the percentage of BPE extracted after 24 and 48 h (Figure 4 in section 4.3.2). While after 24 h was observed a perfect correspondence, after 48 h, the percentage of extracted BPE was higher than the percentage of permeabilized cells in the case of mild-intensity treatments. This increment of the extraction yield in the suspensions containing a large proportion of non-electroporated cells could be caused by the hydrolytic enzymes released from the electroporated cells which, in turn, engender the autolysis of the cells unaffected by the PEF treatment. The evaluation of different enzymatic activities in the supernatants of this specie during incubation would be interesting in order to demonstrate this hypothesis.

These two studies of PEF-assisted phycobiliproteins extraction from *A. platensis* and *P. cruentum* in combination with other studies of literature (Luengo et al., 2015; Silve et al., 2018) in which PEF treatment was followed by long aqueous incubation before extraction concurred in suggesting that permeabilization of cytoplasmic membrane was not the direct cause that improves the extraction. Other effects in addition to the cytoplasmic membrane permeabilization might be involved in the

improvement of extraction after PEF treatment. Therefore, it was decided to continue investigating under this approach with another microalgae specie.

Haematococcus pluvialis is a unicellular freshwater green microalga that produces the natural carotenoid astaxanthin, which possesses interesting properties, such as food grade, coloring and antioxidant agent. Under stress conditions, *H. pluvialis* green vegetative cells transforms to red encysted cells and astaxanthin is accumulated within oil droplets as mixture of mono and di-esters (Lorenz et al., 2000; Wayama et al., 2013). This is associated with the formation of very thick cell wall which made necessary the extraction of astaxanthin from the cyst before to use. PEF treatments combined with aqueous incubation were evaluated on the subsequent extraction of astaxanthin from *H. pluvialis*. This study was performed during an internship at Umea University (Sweden), in which laboratory only a millisecond pulses generator was available, thus pulses of millisecond duration and lower electric field strengths (< 1 kV/cm) were applied. This technology was compared to classical cell disruption methods (section 4.3.3). The minimum electric field strength to affect the cells was 1 kV/cm even at the shortest treatment period (50 ms). This low electric field strength could be related to the utilization of pulses of millisecond order. As it was shown in table 5.2. the long treatment time compensated the low electric field strength applied and the energy input to electroporate this microalga was similar to those for the other species.

After the PEF-treatment of fresh biomass, incubation in different solvents resulted ineffective. Thus, PEF-treated cells were subjected to an intermediate incubation in the treatment medium for different times and after that re-suspended in different extraction solvents: acetone, methanol and ethanol. While carotenoid yields from untreated samples were low even after 12 h of previous incubation, the PEF treatment followed by 6 h aqueous incubation resulted in a great increase of the yield. As it has been explained during discussion of this Doctoral thesis, this pre-incubation of PEF treated cells was essential to detect significant extraction. *H. pluvialis* cells, primarily contain monoesters of astaxanthin linked to fatty acids increasing its solubility and stability in the cellular lipid environment (Lorenz et al., 2000). As it has been observed in other studies, it seems that astaxanthin release requires not only the permeabilization of cytoplasmic membrane for the diffusion of the compound, but also additional dissociation of the compounds from the structures where are assembled. It

was suggested that carotenoid release from *H. pluvialis* was facilitated by an enzyme-driven process that occurs after PEF-triggered cell death which requires incubation in a medium and conditions that allows this enzymatic degradation. This hypothesis was supported by the fact that proportion of free astaxanthin was higher in PEF pre-treatment-incubated extracts (37 %) in comparison to extracts obtained by mechanical disruption (20 %, bead-beating). Thereby, PEF-treatment and subsequent incubation would induce esterase activity in the cells that would digest the ester link and release astaxanthin in the free form. Contrarily, in case of mechanical treatment, pigment release is only produced by a physical effect and astaxanthin maintains its esterified form.

Results obtained on improvement of the extraction of valuable compounds from microalgae by PEF seems to indicate that the effect is related not only with the improvement of mass transfer by electroporation. As in the case of yeast, enzymatic activity triggered by electroporation seems to be involved. Although the specific enzymatic activities involved have not been identified, in the case of astaxanthin extraction, it could be hypothesized that the release of carotenoids from the lipid bodies due to esterases would facilitate astaxanthin release and its subsequent dissolution in the solvent. In parallel, β-glucanase activity could be involved in the cell wall degradation detected by microscopy observations. On the other hand, in the case of *A. platensis* and *P. cruentum* the mechanisms and the type of enzymes participating remain unknown. Therefore, it is required to go deeper in the understanding of this mechanism and of the enzymes involved. This would permit the search of new applications in other species and the development of the process for an industrial implementation.

In general, PEF pre-treatment allowed the selective extraction of various microalgal compounds achieving high yields. However, PEF parameters, incubation conditions and the selection of the proper solvent depends on the specie and thus should be adjusted specifically and optimized. The key benefit of PEF pre-treatment compared with other methodologies that involve heating is that PEF do not provoke a significant rise in the temperature of the product, thereby preventing the undesirable consequences of heat on the quality and purity of the extracts. Conversely, severe cell disruption procedures such as milling, bead-beating, or ultrasound result in total cell destruction, thus causing a non-specific leakage of cell compounds. Furthermore, the PEF-assisted extraction of high-value compounds from microalgae presents an advantage over

standard techniques: the opportunity of performing the extraction with fresh biomass instead of after dehydration, leading to the replacement of detrimental chemicals with greener solvents. As a final point, PEF is a process that is low in energy consumption (as it is shown in table 5.2.) and can be implemented in continuous flow, allowing for a feasible scale-up to processing capacities in the magnitudes of thousands liters per hour.

5.4. Future perspectives

For the first time it has been demonstrated that the effect of PEF in the improvement of extraction of intracellular compounds is not only due to the enhancement of mass transfer through the electroporation of the cytoplasmic membrane. In addition, it has been proven that PEF treatment may induce the triggering of the activity of endogenous enzymes of the cells during subsequent incubation.

This original discovery opens up the possibility of new applications of PEF for the facilitation of extraction of microbial compounds that are bounded or assembled in structures. The search of new microorganism species in which application of electroporation for triggering autolysis would improve the release of a compound of interest could result in profitable innovative implementations of PEF technology. PEF parameters, together with suspension incubation conditions, must be optimized to reach the desired effect. Further research will lead to a more precise understanding of the mechanisms implied, and of how the process can be applied on an industrial scale.

Finally, PEF could result a useful tool for accelerating processes of food, biotechnological or pharmaceutical industries based on catalytic effect of endogenous enzymes.

6. CONCLUSIONS

CONCLUSIONS

I. In this thesis it has been demonstrated for the first time that the improvement of extraction of intracellular compounds by PEF is not only caused by the enhancement of mass transfer through the cytoplasmic membrane. PEF triggering activity of some endogenous enzymes plays a very important role in this effect.

II. The electroporation of cytoplasmic membranes of *Saccharomyces cerevisiae* by pulsed electric fields treatment triggered autolysis and accelerated the release of mannoproteins from the yeast cell wall during incubation.

III. Autolysis induced by pulsed electric fields and the subsequent release of mannoproteins from the yeast cell wall was influenced by the incubation temperature, pH and presence of ethanol. The mannoprotein release was faster at higher temperatures and pH, and lower ethanol concentration.

IV. The improvement of the mannoprotein release by PEF was associated to the fact that the treatment facilitated the enzyme liberation from vacuoles and the subsequent access of these enzymes to the cell wall. A high concentration of β-glucanase and protease was detected in the extracellular medium containing PEF treated cells of *S. cerevisiae* as compared with the control.

V. Pulsed electric fields accelerated the release of mannoproteins during the "aging on lees" of *Chardonnay* wine without negatively affecting its physicochemical properties. The mannoproteins released from PEF treated cells of *S. cerevisiae* featured similar functional properties in the wine than mannoproteins released during the natural autolysis.

VI. The electroporation of fresh biomass of *Rhodotorula glutinis* yeast was ineffective for extracting carotenoids using ethanol as solvent. The incubation of electroporated cells of *R. glutinis* for 24 hours in an aqueous medium permitted the extraction of carotenoids in ethanol. This effect was also observed when the cells were freeze-dried after incubation in an aqueous medium.

VII. The necessity of the incubation in an aqueous medium for carotenoid extraction was explained by the action of esterases that caused the disassociation of carotenoids attached to cell structures. A high concentration of esterases, which activity decreased in presence of ethanol, was detected in extracellular medium containing PEF treated cells of *R. glutinis*.

VIII. Pulsed electric fields treatment permitted selective recovery of C-phycocyanin from fresh biomass of *Artrosphira platensis* microalga using water as solvent. The purity of the extract obtained from electroporated cells was higher than that obtained using bead-beating. The efficacy of PEF treatment on the extraction yield was highly depended on temperature of application.

IX. Electroporation of *Porphyridium cruentum* microalga was influenced by electric field strength and treatment time and allowed the extraction of phycoerythrin. The release of this pigment from PEF-treated cells to the aqueous medium always required a lag time that would indicate the necessity of dissociation of the phycobiliprotein from the cell structures, which was hypothesized to be related to the autolysis PEF triggering effect.

X. Similarly to *R. glutinis*, the electroporation of fresh biomass of *Haematococcus pluvialis* microalgae was ineffective for extracting astaxanthin carotenoid using ethanol as solvent. PEF-treatments of fresh biomass followed by incubation in its own growth medium permitted the extraction of this compound in ethanol. The graduation of the effect depended on the growth conditions being greater when the cells were cultivated in a nitrogen free medium. Since mainly astaxanthin in the free form rather than forming esters was identified in the extracts, it was hypothesized that also esterase activity was involved in the observed effect.

CONCLUSIONS

I. En esta tesis ha sido demostrado por primera vez que la mejora de la extracción de los compuestos intracelulares por PEAV no es solamente causada por la mejora de la transferencia de masa a través de la membrana citoplasmática. La actividad de algunos enzimas endógenos desencadenada por los tratamientos de PEAV juega un papel muy importante en este efecto.

II. La electroporación de la membrana citoplasmática de *Saccharomyces cerevisiae* por el tratamiento de PEAV desencadenó la autolisis y aceleró la liberación de manoproteinas de la pared celular de la levadura durante la incubación.

III. La autolisis inducida por PEAV y la subsiguiente liberación de manoproteinas de la pared celular de la levadura estaba influenciada por la temperatura de incubación, el pH y la presencia de etanol. La liberación de manoproteinas fue más rápida a mayores temperaturas y pHs básicos y a más bajas concentraciones de etanol.

IV. La mejora de la liberación de manoproteinas por PEAV se asoció al hecho de que el tratamiento facilitó la liberación de enzimas de las vacuolas y el subsiguiente acceso de estas enzimas a la pared celular. Una alta concentración de β-glucanasa y proteasa fue detectada en el medio extracelular que contenía células de *S. cerevisiae* tratadas por PEAV en comparación con el control.

V. Los pulsos eléctricos de alto voltaje aceleraron la liberación de manoproteinas durante la "crianza sobre lías" del vino *Chardonnay* sin afectar negativamente sus propiedades físico-químicas. Las manoproteinas liberadas de las células de *S. cerevisiae* tratadas por PEAV proporcionaron similares propiedades funcionales a los vinos que las manoproteinas liberadas durante la autolisis natural.

VI. La electroporación de biomasa fresca de la levadura *Rhodotorula glutinis* fue inefectiva para extraer carotenoides usando etanol como solvente. La incubación durante 24 horas de las células electroporadas de *R. glutinis* en un medio acuoso permitió la extracción de carotenoides en etanol. Este efecto fue también observado cuando las células fueron liofilizadas después de la incubación en el medio acuoso.

VII. La necesidad de la incubación en un medio acuoso para la extracción de carotenoides fue explicada por la acción de las esterasas que causaron la disociación de los carotenoides unidos a estructuras celulares. Una alta concentración de esterasas, cuya actividad decreció en presencia de etanol, fue detectada en el medio extracelular que contenía células de *R. glutinis* tratadas por PEAV.

VIII. Los tratamientos de PEAV permitieron la recuperación selectiva de C-ficocianina de biomasa fresca de la microalga *Artrosphira platensis* usando agua como solvente. La pureza del extracto obtenido de las células electroporadas fue mayor que la obtenida usando molino de perlas. La eficacia de los tratamientos de PEAV en el rendimiento de extracción fue altamente dependiente de la temperatura de aplicación.

IX. La electroporación de la microalga *Porphyridium cruentum* estuvo influenciada por la intensidad del campo eléctrico y el tiempo de tratamiento y permitió la extracción de ficoeritrina. La liberación de este pigmento de las células tratadas por PEAV al medio acuoso requirió en todos los casos un tiempo de demora que indicaría la necesidad de la disociación de la ficobiliproteina de las estructuras celulares, que se asumió que estaba relacionada con el efecto de la autolisis desencadenada por los PEAV.

X. De forma similar a *R. glutinis*, la electroporación de la biomasa fresca de la microalga *H. pluvialis* fue inefectiva para extraer el carotenoide astaxantina usando etanol como solvente. El tratamiento de PEAV de la biomasa fresca seguido de la incubación en el propio medio de crecimiento permitió la extracción de este compuesto en etanol. La graduación del efecto dependió de las condiciones de crecimiento siendo superior cuando las células fueron tratadas en un medio libre de nitrógeno. Como se encontró principalmente astaxantina en forma libre en los extractos en vez de formando ésteres, se pensó que también la actividad esterasa estaba involucrada en el efecto observado.

7. ANNEX

7.1. Solvent-free extraction of carotenoids from yeast *Rhodotorula glutinis* by application of ultrasound under pressure *(Manuscript IX)*

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Solvent-free extraction of carotenoids from yeast *Rhodotorula glutinis* by application of ultrasound under pressure

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ABSTRACT

The extraction of *Rhodotorula glutinis* carotenoids by ultrasound under pressure (manosonication) in an aqueous medium has been demonstrated. The influence of treatment time, pressure, and ultrasound amplitude on *R. glutinis* inactivation and on the extraction of carotenoids was evaluated, and the obtained data were described mathematically. The extraction yields were lineal functions of those three parameters, whereas inactivation responded to a more complex equation. Under optimum treatment conditions, 82 % of carotenoid content was recovered. Extraction of carotenoids in an aqueous medium was attributed to the capacity of ultrasound for cell disruption and emulsification. Cavitation caused the rupture of cell envelopes and the subsequent formation of small droplets of carotenoids surrounded by the phospholipids of the cytoplasmic membrane that would stabilize the emulsion. Analysis of the dispersed particle size of the extracts demonstrated that a fine, homogeneous emulsion was formed after treatment (average size: 230 nm; polydispersity < 0.22). This research describes an innovative green process for extracting carotenoids from fresh biomass of *R. glutinis* in which only two unit operations are required: ultrasonic treatment, followed by a centrifugation step to discard cell debris. The extract obtained thanks to this procedure is rich in carotenoids (25 mg/L) and could be directly incorporated as a pigment in foods, beverages, and diet supplements; it can also be utilized as an ingredient in drugs or cosmetics.

1.-INTRODUCTION

Carotenoids are liposoluble pigments naturally synthesized by plants and microorganisms, and they have industrial applications in food, cosmetic, and pharmaceutical product formulations [1], [2]. In addition to their coloring properties, carotenoids have been shown to prevent cancer, macular degeneration, and cataracts when they are ingested in human diet [3]. Moreover, carotenoids can act as an antioxidant agent, and protect cells against oxidative damage [4].

Many carotenoids exploited in the industry are currently obtained through chemical synthesis [1], [5]. However, unfavorable reports published by regulatory agencies (FDA, EFSA), combined with growing consumer concerns regarding artificial additives, are leading to an increased search for natural colorants, which may be healthier than synthetic colorants [6], [4], [7].

Compared with plants as a carotenoid source, microbial carotenoid production only requires a small production area, and is independent of changes in climate, seasonality, and soil composition [8]. Apart from microbial sources of carotenoids such as algae including the *Dunaliella* or *Haematococcus* species, yeast such as *Phafia rhodozyma* and *Rhodotorula glutinis* are likewise of commercial interest [9], [10], [11], [12]. These yeasts have been considered as potential sources of natural carotenoids because they can produce high yields while growing in low-cost substrates such as agro-industrial waste [13], [14].

The carotenoids produced by *R. glutinis* are synthesized intracellularly and remain inside the cell, where they have structural and functional properties [10], [15]. Therefore, the recovery of carotenoids from yeast requires a series of downstream operation units in which extraction is critical [4].

Owing to the hydrophobic nature of carotenoids, they are traditionally extracted after dehydration of yeast biomass by applying a mixture

of organic solvents [16]. Moreover, to achieve effectiveness, treatments designed to disrupt cell walls and other physical barriers are required before or during the extraction process [17], [18], [19], [20]. However, drying out produces thermal degradation, while conventional solvent extraction requires the utilization of a large amount of harmful solvents in multiple extraction steps [16]. After extraction, the toxic solvents (benzene, ether, hexane, etc.) are evaporated and the carotenoids are re-suspended in food-grade solvents, thereby generating a considerable amount of pollutants [21]. Many of these solvents have been shown to be highly toxic and detrimental to the environment; the European Union is therefore implementing stricter rules for their use, resulting in increased costs for storage and disposal, even downright prohibition [22], [23].

In view of these environmental regulations and health concerns, the search for more ecological extraction methodologies has become imperative to ensure the sustainable development of industrial processes designed to exploit yeast as a promising source for pigments.

Ultrasound is a non-thermal technology that has been shown to be very effective in improving the extraction of heat-labile compounds owing to the phenomenon of acoustic cavitation [24], [25]. Cavitation consists in the formation, growth, and collapse of microbubbles inside a liquid submitted to high-frequency sound waves (>20 kHz) [26]. As a consequence, molecules violently collide with one another, giving rise to shock waves and creating spots of very high temperature (5500°C) and pressure (up to 50 MPa) for short periods of time (10^{-9} s) [27]. Ultrasound-assisted extraction does not act through one mechanism alone, but by different independent or combined mechanisms stemming from cavitation such as fragmentation, erosion, capillarity, detexturation, and sonoporation [25]. These mechanical effects may enhance the release of intracellular compounds by disrupting the cell, and by facilitating the penetration of the solvent. The effect of ultrasound on the extraction of lipids [28], [29], [30], carotenoids [31], [32], and other high-value components [33] from different microalgae has been investigated. Likewise, the ultrasound-assisted extraction of lipids [34], carotenoids [35], and polysaccharides [36] from yeast has been reported. Moreover, the extraction of lipophilic compounds in a hydrophilic media thanks to ultrasound was described by Adam et al. (2012) [28].

In the present investigation, ultrasound treatment was applied under pressure (manosonication). This combination has been shown to increase the effect derived from cavitation [37]. Manosonication drastically increases the inactivation effect of ultrasound on microorganisms [38], [24], [39], and it has also proven to be effective in the extraction of carotenoids from tomato pomace [40]. However, the effect of combining ultrasound and pressure on the extraction of biocompounds from yeast has not yet been investigated.

The aim of this study was to evaluate the potential of ultrasound under pressure for extracting carotenoids from *R. glutinis* in an aqueous medium. Response surface methodology was used to evaluate the potential of manosonication for the optimization of the carotenoid-extraction yield. The ultimate objective was to design an ecofriendly and sustainable process for obtaining carotenoids from fresh yeast cells while avoiding the use of organic 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 cultures 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 culture growth was monitored by measuring absorbance at 600 nm (correlated with cellular density) and the number of cells, using a Thoma counting chamber and the plate-counting method in Potato-Dextrose-Agar (PDA, Oxoid, Basingstoke, UK). Dry weight (d.w.) of yeast was determined by vacuum drying (GeneVac, Ltd, UK) at 60 °C until constant weight.

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2.2 Ultrasound under pressure treatment (Manosonication)

Manosonication (MS) treatments were carried out in a specially designed resistometer similar to one previously described in the literature [38]. However, in our case, a 100 mL treatment chamber pressurized with nitrogen was used for the extraction experiments. The equipment allowed us to monitor the effect along time of ultrasound treatments on carotenoid extraction at different pressures and amplitudes. The tip of a sonication horn (13 mm diameter) connected to a 2000 W Digital Sonifier® ultrasonic generator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) with a constant frequency of 20 kHz was used. The wave amplitude values of this equipment range from 34 to 145 µm. Once treatment amplitude has been selected, the equipment supplies the required power: therefore, the greater the applied pressure, the higher the power supplied by the ultrasonic generator to maintain the amplitude of the selected vibration. A cooling coil placed in the treatment chamber was used to dissipate the heat generated by ultrasound and to maintain the temperature below 30 °C by circulating a cooled water-ethylene glycol mixture.

Extraction experiments were performed on cells after 72 h of culture, which corresponded with the highest carotenoid content. Prior to treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 x g for 5 minutes (MiniSpin Plus, Eppendorf Ibérica, Madrid, Spain) at room temperature and re-suspended in a citrate-phosphate pH 7.0 McIlvaine buffer to a final concentration of approximately 10^8 cells/mL (10 $g_{d.w.}$/L). The chamber was filled with the suspension through the valve arranged for this purpose. Manosonication treatments were performed at three different amplitudes of 70, 90, and 120 µm at an atmospheric pressure, 100 or 200 kPa. Samples of 3 mL were collected each 30 seconds along 180 seconds. These treatments correspond to energies between 36.2 and 376.56 kJ/kg based on calorimetric measurements of power output [41].

2.3 Evaluation of yeast inactivation after treatments

After ultrasound treatments under pressure, serial decimal dilutions in peptone water (Oxoid, Basingstoke, UK) of the suspensions were pour-plated in PDA. The number of viable cells, expressed in colony forming units (CFU), corresponded to the number of colonies counted after 72 h of incubation at 25 °C. Longer incubation times did not affect the number of survivors (data not shown).

2.4 Evaluation of carotenoids extraction

2.4.1. Total content of carotenoids in suspensions

Throughout all the procedures, samples were protected from light as much as possible. An aliquot of 5 mL of suspension was centrifuged, and the pellet was re-suspended in 5 mL dimethyl sulfoxide (DMSO), vortexed for 1 minute, and incubated in a shaking incubator (Unimax 1010; Heidolph, Schwabach, Germany) at a velocity of 200 rpm at room temperature for one hour. After that, 5 mL of hexane and 1 mL of diethylether were added to the tubes, vortexed, and incubated in shaking for 30 minutes. Then, 1 mL of NaCl saturated solution was added to the mixture, and tubes were vortexed for 1 minute. Finally, tubes were centrifuged (4000 x g, 10 min) and the colored upper phase was collected. This procedure was repeated until the collected hexane phase became transparent. Pooled together, the collected extracts were evaporated under a continuous nitrogen flux and dissolved with a known volume of hexane for spectrophotometric quantification of carotenoids at 474 nm as described in Martínez et al. (2018) [42].

2.4.2. Quantification of carotenoids extracted

Carotenoid extraction was carried out in the treatment medium itself (McIlvaine buffer) in the course of manosonication treatment. For subsequent quantification, 1 mL of the untreated or US-treated suspension of *R. glutinis* was centrifuged at 10.000 x g for 2 minutes at room temperature in order to separate the pellet-containing cells and the supernatant. Carotenoid extraction was calculated by the difference between the total carotenoid content of the suspension and the carotenoids remaining in the pellet.

2.5 Microscopic observation after treatments

Untreated cells and cells subjected to a manosonication treatment were observed under optical microscopy (Nikon Eclipse 6400, Nikon, Tokyo, Japan) in order to monitor morphological changes and effective cell disruption.

2.6 Experimental design and statistical analysis

Response surface methodology (RSM) was used to evaluate the effect of MS parameters: amplitude (70-120 µm), time (30-180 seconds) and pressure (0-200 kPa) on the inactivation of *R. glutinis* and on the associated carotenoid extraction yield.

The data obtained after treating the cells were fitted to the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i>j}^{k} \beta_{ij} X_i X_j \qquad \text{(Equation 1)}$$

in which Y is the response variable to be modeled, Xi and Xj are independent factors, β_0 is the intercept, βi is the linear coefficient, βii is the quadratic coefficient, βij is the cross-product coefficient, and k is the total number of independent factors. A backward regression procedure was applied to determine the models' parameters. It systematically removed the effects that were not significantly associated ($p > 0.05$) with the response until a model with a significant effect was obtained.

Experiments were performed in triplicate, and the presented results are means ± standard deviation. One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences among the mean values. Differences were considered significant at $p < 0.05$. Multiple regression analysis was conducted to fit Equation 1 to the experimental data, and significant terms of the model were determined by ANOVA. Root-mean-square error (RMSE) was used to measure differences between values predicted by the model and the values observed. The RMSD represents the square root of the second sample moment of the differences between predicted values and observed values, or the quadratic mean of those differences.

Central composite design and the corresponding data analysis were carried out with the software package Design-Expert 10 (Stat-Ease Inc., Minneapolis, MN, USA).

2.7 Analysis of emulsions

The supernatants were analyzed using the dynamic light scattering (DLS) technique with Zetasizer Nanoseries equipment (Malvern, Worcestershire, UK). The size of the particles dispersed in the liquid was analyzed, and results were expressed in Z-Average Size and polydispersity (Pd). The Z-Average Size term is defined as the harmonic intensity averaged particle diameter. Pd is a parameter calculated from an analysis of the DLS-measured autocorrelation function. In that analysis, a single particle size mode is assumed, and a single exponential fit is applied to the autocorrelation function. Pd describes the width of the assumed Gaussian distribution, and a Pd lower than 0.25 indicates that the sample is monodisperse. Three biological replicates of each treatment condition were performed, and each sample was measured twice. Results represent the mean ± standard deviation.

3.- RESULTS AND DISCUSSION

3.1 Carotenoids extraction from *R. glutinis* by application of ultrasound under pressure.

Figure 1 illustrates the effect of pressure along time on the extraction of carotenoids from *R. glutinis* assisted by ultrasound. Independently of the external pressure applied, an exponential kinetic of extraction was observed, and the ultrasound treatment was more efficient when applied at 200 kPa rather than at atmospheric pressure. For example, after 120 seconds of treatment, extraction efficiency increased by 65 %. At 200 kPa, 231 µg of carotenoids/$g_{d.w.}$ were extracted in comparison with 149 µg of carotenoids/$g_{d.w.}$ extracted at atmospheric pressure.

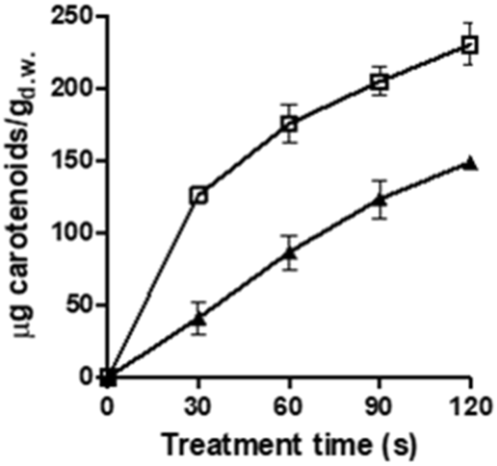

Figure 1.- Carotenoid extraction curve from *Rhodotorula glutinis* cells treated by ultrasound (96 µm amplitude) under different pressures: 0 kPa (▲) and 200 kPa (□).

The application of ultrasound with the purpose of improving the extraction of compounds from microbial cells has been widely investigated [43], [44], [45], [46]. The positive effect of ultrasound on extraction yields is attributed to the mechanical breakage of cells, and to heightened mass transfer produced by cavitation, causing high shear stresses, microstreaming, and turbulence [28], [47]. In this investigation, ultrasound was applied under moderate pressure (manosonication), because it is well known that if ultrasound is applied at sufficiently high intensity, external pressure increases the effects of cavitation [38], [48]. Although the improvement of extraction thanks to the application of ultrasound under pressure has been previously demonstrated in substrates such as dried tomato pomace [40], the positive effect of this strategy on the extraction of carotenoids from yeast is demonstrated here for the first time.

It is worth noting that in this investigation, as previously reported by Adam et al. (2012) [28], ultrasound enables the extraction in aqueous medium of lipophilic compounds such as carotenoids (Figure 2). Therefore, in addition to facilitating extraction by breaking up the cell envelopes of *R. glutinis* cells observed microscopically (data not shown), cavitation likewise permits the formation of a stable mixture of immiscible compounds such as carotenoids and water. It is well known that the main carotenoids produced by *R. glutinis* (torularhodin, torulene and β-carotene) are highly soluble in organic solvents, but do not dissolve in water [12]. A stable mixture of immiscible compounds requires the formation of an emulsion. Emulsification involves the formation of small droplets of the dispersed phase in the continuous phase, and the subsequent stabilization of the droplets by applying surface-active substances (emulsifiers). The formation of small droplets of carotenoids requires a certain amount of mechanical energy that could be supplied by the cavitation brought about by ultrasound: the emulsifying capacity of ultrasound technique has been widely described [49], [50], [51]. Generally, however, when ultrasound is used to form an emulsion, emulsifiers are added to stabilize the system. For example, Amiri-Rigi and Abbasi (2016) [52] extracted lycopene from tomato pomace treated enzymatically in an aqueous medium by applying ultrasound, and they used saponin as an emulsifier. In our investigation, a stable mixture was obtained without having to add an external emulsifier, which indicates that some of the yeast's own compounds could exert that function. It is well known that phospholipids, which are the main components of cytoplasmic membranes, are good emulsifiers due to their amphiphilic structure [53], [54]. Therefore, in a first step, cavitation would cause the breakage of the cell and the release of carotenoids, and, in a second step, it would lead to the formation of small droplets of carotenoids, which would be stabilized by the phospholipids of the cytoplasmic membrane acting as emulsifiers.

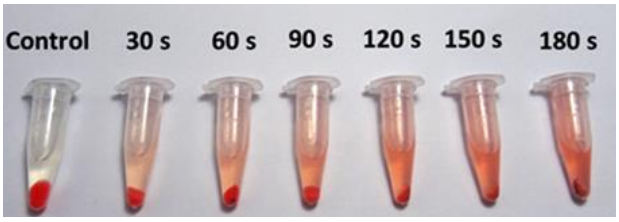

Figure 2.-Supernatant observation after manosonication (96 µm amplitude, 200 kPa) of *R. glutinis* suspension along treatment time.

3.2 The influence of pressure, amplitude, and treatment time of manosonication on the extraction of carotenoids from *R. glutinis*

After having demonstrated that manosonication treatment allowed carotenoid extraction in aqueous medium from *R. glutinis*, we

evaluated the influence of sonication hydrostatic pressure (0-200 kPa), amplitude (70-120 µm), and treatment time (0-180 s) on cell inactivation and on the extraction of carotenoids from fresh biomass of *R. glutinis*.

Response surface methodology (RSM), a widely accepted statistical tool for the optimization of extraction processes [55], was used to study the influence exerted by those factors. Experimental conditions corresponding to a central composition design, as well as results obtained from the inactivation of *R. glutinis* and the extraction of carotenoids, are shown in Table 1. Inactivation response is expressed as Log_{10} cycles of survival fraction, and extraction response is listed as extraction percentage of total carotenoids. Depending on the intensity of the applied treatment, the percentage of extracted carotenoids ranged from ca. 16 % when a treatment of 30 seconds, ultrasonic amplitude of 96 µm, and hydrostatic pressure of 100 kPa was applied, to ca. 82 % when treatment time was increased to 180 seconds, and amplitude and pressure were increased to 120 µm and 200 kPa, respectively. As shown in Table 1, the conditions that produced the highest and lowest carotenoid extraction also led to the highest and lowest inactivation of *R. glutinis* respectively. The relation between the percentage of extracted carotenoids and the percentage of dead cells is shown in Figure 3. The locations of the dots below the equivalence line show that extraction did not match with inactivation. This observation seems to indicate that the extraction of carotenoids not only depends on the destruction of *R. glutinis* cells by ultrasound, but also on other effects generated by ultrasound, such as the disassembling of carotenoids from the yeast structure, as well as emulsification. For that reason, carotenoid extraction continued to increase, even when manosonication treatments that destroyed more than 99% of the population had been applied. For example, a 65 % rate of extraction was achieved with a treatment that inactivated around 99 % of the population (70 µm, 200 kPa, 180 seconds), while with a treatment that inactivated 99,9 % of the population (96 µm, 200 kPa, 180 seconds) extraction increased to 82%. Therefore, in order to maximize carotenoid extraction, it is necessary to extend the duration or increase the intensity of the manosonication treatment to bring about the release of carotenoids attached to yeast structures and/or emulsify the extracted carotenoids

Table 1.- Inactivation of *R. glutinis* and carotenoid extraction yield after ultrasound under pressure treatments of different hydrostatic pressures, amplitudes and treatment times. Mean ± standard deviation

Pressure (KPa)	Amplitude (µm)	Time (s)	Log_{10} survival fraction		Extraction (%)	
			Mean	*SD*	*Mean*	*SD*
0	70	30	-0.15	0.11	23.21	6.00
0	70	180	-0.83	0.04	49.12	4.51
0	96	105	-0.75	0.26	43.42	2.20
0	120	30	-0.26	0.13	24.71	6.42
0	120	180	-1.17	0.09	58.54	7.76
100	70	105	-0.67	0.20	40.85	4.55
100	96	30	-0.14	0.09	15.94	13.41
100	96	105	-0.70	0.09	43.96	7.11
100	96	180	-1.31	0.06	62.19	1.67
100	120	105	-1.14	0.21	63.52	14.32
200	70	30	-0.67	0.46	26.61	4.23
200	70	180	-1.94	0.41	65.35	2.05
200	96	105	-1.44	0.09	59.36	2.89
200	120	30	-0.54	0.06	36.58	0.25
200	120	180	-2.41	0.21	81.80	2.82

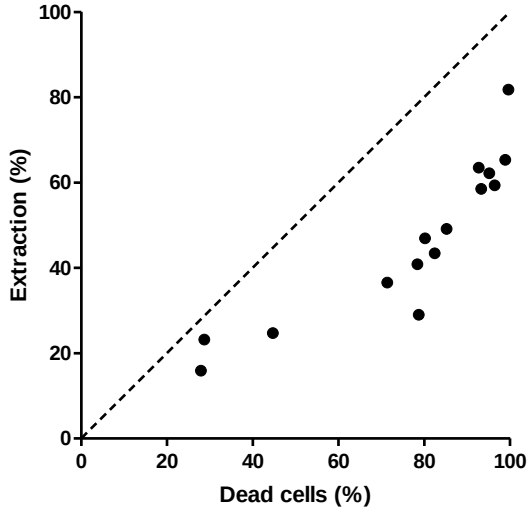

Figure 3.-Relationship between the percentages of extraction of carotenoids from *R. glutinis* cells against the percentages of dead cells. To show the degree to which each treatment causes carotenoid extraction, a theoretical straight line with slope = 1 and intercept = 0, is included. Data shown as mean, n=3.

In order to quantify the effect of hydrostatic pressure, amplitude, and treatment time on the inactivation and extraction of carotenoids from *R. glutinis,* data presented in Table 1 were fitted to a quadratic mathematical equation using multiple regression analysis. After removing non-significant terms ($p>0.05$), the relation between independent variables (hydrostatic pressure, amplitude, and time) and dependent variables (Log_{10} cycles of survival fraction and carotenoid extraction yield) are shown in equations 2 and 3, respectively.

$$(\boldsymbol{Eq\ 2})\ I = -0.1472 + 3.3422 \times 10^{-3} \times P + 7.61 \times 10^{-4} \times A - 3.8667 \times 10^{-5} \times t - 2.2490 \times 10^{-5} \times (P)^2 - 2.565 \times 10^{-5} \times P \times t - 5.54 \times 10^{-5} \times A \times t$$

$$(\boldsymbol{Eq\ 3})\ CEY = -8.7443 + 0.0731 \times P + 0.2295 \times A + 0.2501 \times t$$

in which I is the inactivation expressed in Log_{10} cycles of survival fraction; CEY is the carotenoid extraction yield; P corresponds to the hydrostatic pressure (kPa); A to the amplitude (μm); and t to the treatment time (seconds). Stepwise regression with backward elimination removed the squared terms of amplitude and time, as well as the interaction between pressure and amplitude, from the quadratic equation of inactivation (Eq. 2**)**. Squared terms and interactions were removed from the extraction equation (Eq. 3). In order to show the two equations' goodness of fit, Table 2 shows the results of the analysis of variance (ANOVA) for the significant terms of the two models obtained, along with the statistics used to test their adequacy. In both cases, the obtained F-values of the equations indicate that the equations were significant ($p<0.0001$); therefore, the terms in the equations have a significant effect on the response. The determination coefficient (R^2) of the inactivation equation (Eq. 2) was 0.98, thereby indicating that <2 % of the total response variation cannot be explained by the model. In the case of the extraction equation (Eq. 3), the determination coefficient (R^2) was 0.90, indicating that the percentage of total variation observed in dependent variable parameters not explained by the equation is around 10 %. On the other hand, the adjusted R^2 values that correct the R^2 according to the number of responses and terms in the equations were close to R^2 values, thereby indicating that there was good agreement in both equations between experimental and predicted values.

Yeast inactivation was described by a more complex equation that included not only the linear relationships between inactivation and the evaluated factors, but also some of their interactions. Thus, amplitude and time, pressure and time, and the square of pressure were significant factors. However, in the case of extraction, only the linear effects of the factors were significant. Based on the linear effect of hydrostatic pressure, ultrasound amplitude, and treatment time on the extraction of carotenoids from *R. glutinis* in aqueous medium, it could be considered that ultrasound, within the investigated range of conditions, would not cause an observable carotenoid degradation, because increased intensity of treatment led to greater extraction yield.

Evaluating in more detail the F-values of the Evaluating in greater detail the F-values of the equation parameters displayed in Table 2, the significance of the variables´ effects can be reported. Thus, according to those F-values, in the case of inactivation, the manosonication time linear term (F=254.38) and the hydrostatic pressure linear term (F=108.26) were the two most significant

variables, thereby indicating that changes in those factors exerted the greatest influence on inactivation (Log_{10} cycle of survival fraction). The fact that the squared hydrostatic pressure term (F=12.32) was also a significant term indicated that, beyond a certain pressure value, inactivation significantly increased within the studied range. Although this increment occurs between certain pressure values, the pressure increment would probably hinder cavitation beyond a critical value, and therefore the effect of ultrasound would remain constant or might even decrease thereafter. The fact that further increments in pressure do not increase the percentage of inactivation has been observed by other authors [56], [57]. Finally, the influence of interaction terms (P x t; A x t) was also significant, but had lower F-values. The presence of those interaction terms implied that the effect of pressure and amplitude on inactivation depended on treatment time. With respect to F-values of the terms in the extraction equation (Eq. 3), treatment time (F=82.31) was the most significant parameter, followed by hydrostatic pressure (F=12.50) and amplitude (F=7.70).

Figure 4 shows the response surface plots illustrating the influence of the most significant parameters (treatment time and hydrostatic pressure) on the inactivation (4A) and extraction of carotenoids (4B). In both cases, it is represented the influence of those two factors when sonication amplitude corresponds to the intermediate value of the assayed experimental range (96 μm). As indicated, these figures illustrate the conclusions derived from the analysis of the parameters of multiple regression equations 2 and 3. Figure 4A shows that when time increases independently of pressure, *R. glutinis* inactivation increases linearly, whereas when pressure increases within any time value, the increment in response is not linear. It is thus necessary to apply hydrostatic pressures above 100 kPa in order to be able to observe that this parameter meaningfully increases the inactivation of *R. glutinis*. On the other hand, Figure 4B shows that time and hydrostatic pressure linearly increase carotenoid extraction. Thus in the assayed range, the greater the pressure and the more extended the time, the more efficient is the release of these compounds to the aqueous medium.

Table 2.- F-values and p-values of the ANOVA analysis for the mathematical equations developed to describe the influence of hydrostatic pressure, amplitude and treatment time on the inactivation and extraction of carotenoids of *R. glutinis*. R^2: determination coefficient; RMSE: root mean square error.

	Eq. 1 (Inactivation)		**Eq. 2 (Extraction)**	
	F value	*p value*	*F value*	*p value*
Equations	69.10	<0.0001	34.17	<0.0001
Pressure	108.26	<0.0001	12.50	0.0047
Amplitude	11.68	0.0091	7.70	0.0181
Time	254.38	<0.0001	82.31	<0.0001
Pressure $_x$ Time	21.64	0.0016		
Amplitude $_x$ Time	6.31	0.0364		
$(Pressure)^2$	12.32	0.0080		
R^2	0.9811		0.9031	
Adjusted R^2	0.9669		0.8767	
RMSE	0.0857		5.6723	

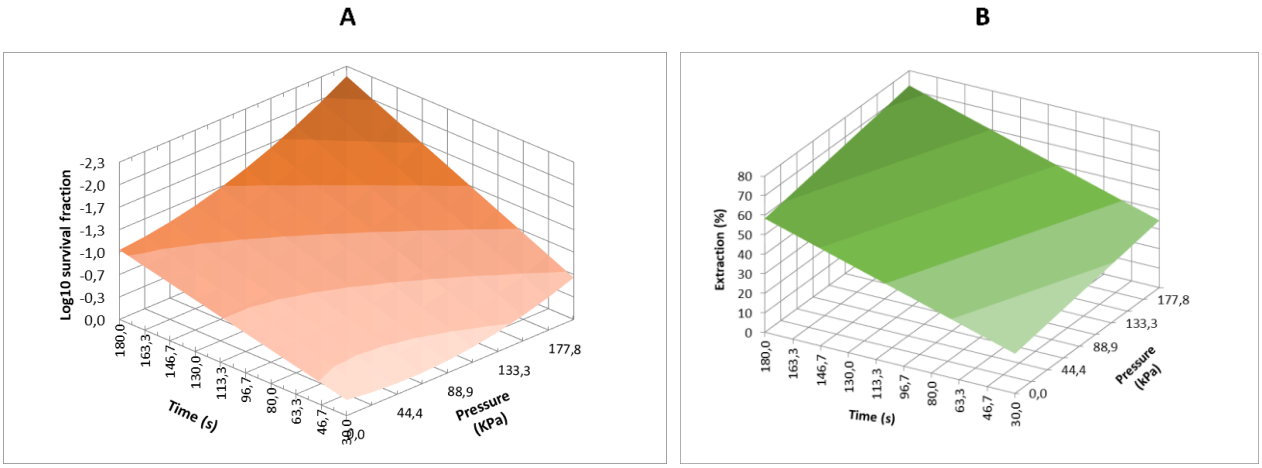

Figure 4.-Three-dimensional response surface plots of the influence of the treatment time and the pressure of manosonication treatments at 96 µm amplitude on the inactivation (A) and the carotenoid extraction (B) from *R. glutinis.*

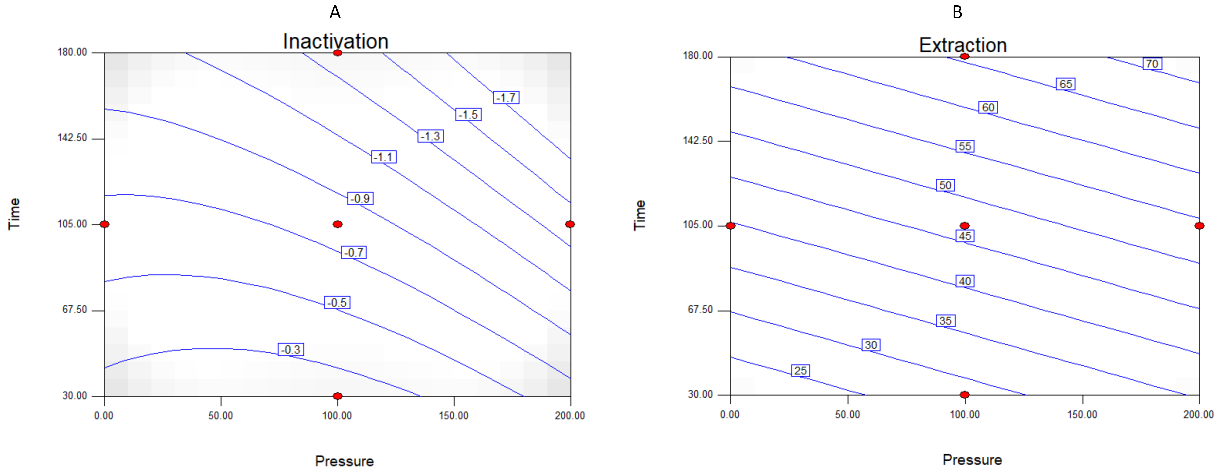

Figure 5.-Fitted iso-log$_{10}$ cycles of cell inactivation (A) and carotenoid extraction percentages (B) contours plots of *R. glutinis* after manosonication treatments of 96 µm of amplitude at different pressures and times.

Figure 5 shows combinations of time and pressure of ultrasound treatments to obtain different Log$_{10}$ cycles of inactivation (A) and carotenoid extraction yields (B) according to equations 2 and 3. An increase in treatment pressure allowed for a significant reduction of the treatment time required for *R. glutinis* inactivation and for carotenoid extraction, but in different ways. In the case of inactivation, pressure increments hardly reduced processing time when they were lower than 100 kPa during the first moments of the treatment; however, with longer treatment times, pressure increments reduced processing time almost linearly. For example, the increment of pressure from 0 to 200 kPa reduced the time required to inactivate 1 Log$_{10}$ cycle *R. glutinis* population by 60 % (from 170 to 70 seconds). Regarding carotenoid extraction yields, any increment in pressure linearly reduced processing time. For example, the application of sonication treatments under 200 kPa, instead of at atmospheric conditions, allowed a reduction of treatment time from 145 to 87 seconds (40 % reduction) for the extraction of 50 % of carotenoid content.

Similarly to our results, Adam et al. (2012) [28] observed a linear influence of ultrasound treatment time on the extraction of lipids from fresh microalgae cells in aqueous medium. Time was identified as the second most significant term, only preceded by the biomass/solvent ratio, which inversely correlated with the yields. However, little was known until now regarding the effect of hydrostatic pressure in ultrasound treatments on the extraction of compounds of interest from

microorganisms. In our results, pressure was identified as the second most influential parameter on inactivation of *R. glutinis* and on carotenoid extraction. Luengo et al. (2014) [40] similarly observed the improvement of carotenoid yields from tomato waste by increasing pressure from 0 to 100 kPa when applying ultrasound. The effect of vibration amplitude was the less significant term affecting *R. glutinis* inactivation and carotenoid extraction. However, amplitude did exert a certain amount of influence, which can be explained by the circumstance that, at higher vibration amplitudes, the effective size of the zone of the liquid undergoing cavitation and the range of bubble size undergoing cavitation also increase [27].

3.3. Analysis of dispersed particles size of the carotenoid extracts

In order to characterize the emulsion obtained after manosonication treatments, the particle size of droplets dispersed in aqueous medium was evaluated. Table 3 shows the Z-Average size and polydispersity of the droplets in the aqueous supernatants of *R. glutinis* suspensions treated by ultrasound under pressure at different conditions. The histograms representing size mainly exhibited a single peak. For all treatment conditions, the deviations in Z-Average size were very low, indicating that particle size was quite homogeneous. Likewise, the Pd values of approximately 0.20 indicate the existence of a single particle size mode with Gaussian distribution of narrow width (monodisperse) in the extracts. Droplet size for all conditions assayed was around 230 nm, and no statistically significant differences were found among sizes after the application of treatments of different intensities. The formation of carotenoid emulsion in aqueous medium after ultrasound treatments had been previously reported by several authors [49], [51], [58]. The small size of the droplets formed in the *R. glutinis* extracts (around 230 nm) would explain the emulsion's notable stability along time. Kanafusa et al. (2007) [59] reported an oil-in-water emulsion containing β-carotene particles of similar size (93-310 nm) after microfluidization under pressure, and de Paz et al. (2013) [60] described a micellar particle size of less than 200 nm obtained by ultrasound emulsification of β-carotene.

Although no significant differences were observed in Z-Average size when amplitude, pressure, or time were varied, parallel tendencies among processing parameters and Z-Average size could be observed. Thus, when increasing pressure, size decreased from 238 to 223 nm, and Z-Average size increased with processing time from 220 to 235 within the range of the conditions investigated. These tendencies could be connected with the mechanisms of action of ultrasound and their interaction with processing parameters, as discussed above. An increment in pressure would limit cavitation; however, when it occurred, a great amount of energy would be released, thereby reducing droplet size. On the other hand, longer processing times would result in increased liberation of carotenoids and phospholipids, thereby incrementing the possibilities of coalescence and enlarging the size of the droplets.

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Table 3.- Z-Average Size and polydispersity (Pd) values of the supernatants of *Rhodotorula glutinis* suspensions treated by ultrasounds under pressure at different conditions.

Pressure (KPa)	Amplitude (µm)	Time (s)	Z-Average Size (nm)		Pd	
			Mean	SD	Mean	SD
0	96	105	238.2	3.31	0.221	0.01
100	96	105	228.0	11.53	0.191	0.02
200	96	30	220.4	3.54	0.213	0.01
200	96	105	223.8	5.36	0.201	0.02
200	96	180	235.1	3.61	0.214	0.01

Despite the different carotenoid concentrations of the *R. glutinis* extracts in the present research, droplet size was similar after different manosonication treatments. Therefore, it seems that the higher effectivity of more intense treatments (higher pressure, time, and amplitude) in terms of extraction yields could be due to the greater number of droplets formed, and not to the increment in droplet size. Furthermore, it is important to point out that a large amount of carotenoid emulsification was achieved in a very short time, especially when ultrasound was combined with the application of pressure. This is of great interest, since carotenoids are easily degraded in the presence of light, heat, and oxygen. Emulsions would protect the active compound and overcome its low bioavailability due to its low solubility in aqueous media. Furthermore, the use of carotenoids as colorants in beverages requires an appropriate formulation in order to stabilize the carotenoid particles in water suspensions and to provide the desired color. The addition of the extracts containing the carotenoid emulsions obtained after manosonication treatment would solve this issue.

.4.-CONCLUSION

Conventionally, carotenoid production from yeast involves cultivation, harvesting, extraction, and purification. In addition to the environmental, health, and safety hazards associated with the solvents usually applied, the cost of several required unit operations including drying, solid-liquid extraction, filtration, and solvent evaporation hamper the upscaling and the economic viability of *R. glutinis* as a source of carotenoids.

This research describes an innovative green-solvent extraction process assisted by ultrasound under pressure in which only two unit operations are required: the treatment of the yeast solution, followed by a centrifugation step to discard cell debris. This treatment enables the extraction of carotenoids from *R. glutinis* yeast in aqueous medium while avoiding the drying of biomass and the use of organic solvents. Cavitation leads to the formation of small droplets of carotenoids surrounded by the phospholipids of the cytoplasmic membrane. An analysis of dispersed particle size of the extracts supports the assumption that a considerably fine, homogeneous, and stable emulsion is formed after treatment.

The extract obtained by this procedure could be directly incorporated as a pigment in foods, beverages, and diet supplements, and can be used as an ingredient in drugs or cosmetics. The emulsion would protect the active compound against degradation, and increase its bioavailability.

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