BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Industrial Microbiology

Enhancement of pigment extraction from *B. braunii* pretreated using CO_2 rapid depressurization

Edgar Uquiche[a,b,*], *Ivette Antilaf*[a], *Sonia Millao*[a]

[a] *Department of Chemical Engineering, Center of Food Biotechnology and Bioseparations, BIOREN, Universidad de La Frontera (UFRO), Temuco, Chile*

[b] *Agriaquaculture Nutritional Genomic Center, CGNA, Technology and Processes Unit, UFRO, Temuco, Chile*

ARTICLE INFO

Article history:
Received 6 March 2015
Accepted 25 September 2015
Available online 2 March 2016
Associate Editor: Gisele Monteiro de Souza

Keywords:
Botryococcus braunii
Cell disruption
Chlorophylls
Microalgae
Supercritical extraction

ABSTRACT

Extraction of compounds from microalgae requires cell disruption as a pretreatment to increase extraction yield. *Botryococcus braunii* is a microalga with a significant content of carotenoids and other antioxidant compounds, such as chlorophylls. Cell disruption of *B. braunii* using CO_2 rapid depressurization was studied as a pretreatment for the extraction of carotenoid and chlorophyll pigments. We studied the effect of temperature (21–49 °C) and pressure (6–13 MPa) during static compression on pigment recovery with supercritical CO_2 at 40 °C, 30 MPa and solvent flow of 4.7 L NPT/min. Within the experimental region, the extraction yield of carotenoids and chlorophylls increased by 2.4- and 2.2-fold respectively. Static compression conditions of high pressure and low temperature increased the extraction of carotenoids and especially chlorophylls. We selected 21 °C and 13 MPa as the cell disruption condition, which produced 1.91 g/kg d.s. of carotenoids and 14.03 mg/kg d.s. of chlorophylls. Pretreated microalga gave a 10-fold higher chlorophyll extraction yield compared to the untreated sample. While for carotenoids and tocopherols were 1.25 and 1.14-fold higher, respectively. Additionally, antioxidant activity of pretreated microalga (33.22 mmol TE/kg oil) was significantly higher than the value for the untreated samples (29.11 mmol TE/kg oil) ($p \leq 0.05$). Confocal microscopy images showed morphological differences between microcolonies with and without disruption treatment, suggesting that partial cell disruption by rapid depressurization improved the extraction of microalga compounds.

Introduction

Microalgae are a broad group of autotrophic organisms which grow by photosynthesis and can be cultivated as a source of bioactive compounds with high commercial value.[1] *Botryococcus braunii* is a unicellular green microalga of the class *Chlorophyceae*, characterized by the production of chlorophyll pigments. *B. braunii* cells are held together by an extracellular matrix composed of a cross-linked aldehyde polymer core

* *Corresponding author.*
E-mail: edgar.uquiche@ufrontera.cl (E. Uquiche).
http://dx.doi.org/10.1016/j.bjm.2016.01.020

and are capable of producing large amounts of hydrocarbons, exopolysaccharides and carotenoids.[2,3] These hydrocarbons are largely stored in the extracellular matrix.[4] *B. braunii* is classified into three races A, B and L, depending on the types of hydrocarbons produced.[5] The presence of carotenoids is more pronounced in races B and L.[6] The carotenoids found include β-carotene, lutein, violaxanthin, canthaxanthin, astaxanthin, zeaxanthin.[7,8] *B. braunii* is an interesting microalga for the extraction of high-value compounds for uses in nutraceutical applications.[7,9]

One of the characteristics of microalgae is the rigidity of their cell walls. In *B. braunii* the wall of each cell has an internal fibrillar layer made of polysaccharide and an external trilaminar sheath.[4] Cell wall of *B. braunii* is composed of a cellulose-like polysaccharide (as β-1,4- and/or β-1,3-glucan).[10] Cell disruption is therefore necessary to release intracellular compounds and improve extraction solvent access.[11] The following methods have been used for microalgal cell disruption: sonication,[11] high-pressure homogenizers,[12] chemical disruption,[13] enzymatic degradation,[14] bead milling,[11,15] and microwaves.[16]

Studies comparing methods of microalga cell disruption have been reported in literature. Different methods of cell disruption to identify the most effective method for extracting lipids from microalgae (*Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp.) was investigated.[13] Among the methods tested (autoclaving, bead milling, microwaves, sonication, and treatment with 10% NaCl solution), the microwave oven was the most efficient for lipid recovery. In other study was investigated different cell disruption methods for extracting lipids from microalgae (*Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp.), including autoclaving, bead milling, microwave, sonication and treatment with 10% NaCl solution.[17] The sonication was the most efficient method for lipid recovery. However, the sonication method has been indicated to be unscalable. Bead milling and high-pressure homogenizing are scalable for industrial use. Cell disruption by bead mill is based on subjecting cells to high stress produced by abrasion during rapid agitation with glass or ceramic beads. This method is effective with different types of microorganism.[18] In cell disruption by high-pressure homogenizer, the cell suspension is forced to pass through an adjustable discharge valve with a restricted orifice.[19] Castor and Hong,[20] pointed out that mechanical cell disruption methods are non-selective in cell wall disruption; this leads to the formation of small fragments of cell wall, increasing the downstream purification burden because these fragments are difficult to separate from the process stream.[18]

Gaspar et al.[21] studied the effect of the decompression rate on disruption efficiency in trichomes from origanum bracts. They observed that as the decompression rate increased, the pressure drop across the gland wall also increased, resulting in higher disruption efficiency. Thus, disruption of these glands was caused by a pressure gradient formed across the gland walls during rapid depressurization. During the CO_2 compression stage, glands were slightly permeable to the passage of CO_2 by a process akin to diffusion.

Studies of cell disruption using CO_2 rapid depressurization to improve the availability of extracted solutes have been reported in literature. This method is based on introducing a pressurized subcritical or supercritical gas into the cells followed by rapid depressurization, causing cell disruption.[18] During the stage of static compression, supercritical CO_2 is very diffusible and can penetrate cells.[21] After the cells are saturated with CO_2, a sudden depressurisation is applied and a pressure gradient across the cell wall is generated. They observed that as the decompression rate increased, the pressure drop across the gland wall also increased, resulting in higher disruption efficiency. Thus, disruption of these glands was caused by a pressure gradient formed across the gland walls during rapid depressurization. The cell disruption occurs due to the expansion of the CO_2, which improves the speed and increases the extraction yield.[22,23] Furthermore, cells are exposed to minimal shear forces, so no heat is generated which might damage heat sensitive compounds, such as carotenoids and chlorophylls.

Temperature, pressure and exposure time during static compression are the operational variables that may affect the efficiency of cell disruption.[24] Juhász et al.[23] studied the effect of CO_2 rapid depressurization on endoglucanase recovery from *Escherichia coli*. The temperature (32–45 °C) and pressure (12–25 MPa) affected the recovery of the enzyme, while exposure time (5–60 min) had no significant effect. Rapid depressurization has been applied to disruption bacterial cells,[25–27] yeast cells,[28,29] goji berry seeds,[30] rape pollen[31] and trichomes.[21,32]

Extraction with solvents is the traditional technique for lipid extraction from microalgae. Lipids are traditionally extracted using nonpolar solvents, commonly n-hexane.[33] However, the United States Environmental Protection Agency listed n-hexane among 187 hazardous air pollutants in the 2002 National-Scale Air Toxics Assessments because of its toxic nature.[34] Supercritical fluid extraction has received increasing attention as an extraction technique, because it can provide high solubility, improved mass-transfer rates, and increased selectivity with small changes in the temperature and pressure of the extraction operation.[35] Carbon dioxide (CO_2) is probably the most widely used supercritical fluid, and has emerged as a substitute for n-hexane for the extraction of nonpolar solutes from biological substrates, due to its inertness, non-toxicity, non-flammability and non-explosiveness.[36] Furthermore, its relatively low critical properties make CO_2 ($T_c = 31.1$ °C, $P_c = 7.38$ MPa) an ideal solvent for the extraction of thermally labile components. We studied extraction from *B. braunii* using CO_2 supercritical fluid at 40 °C and pressures of 12.5, 20 and 30 MPa.[37] The extraction yield and the fraction of the hydrocarbons in the extracts both increased with pressure and at 30 MPa these compounds were obtained rapidly. The authors reported that chlorophylls were not detected in the extracts. Santana et al.[38] studied the extraction of lipids from *B. braunii* for biodiesel production. These experiments were conducted at temperatures from 50 to 80 °C and pressures from 20 to 25 MPa. Lipid extraction yield was found to decrease with temperature and to increase with pressure. Carotenoids and chlorophylls are important antioxidants.[39,40] These pigments can be extracted from *B. braunii* using supercritical CO_2, and can be used as an indicator of cell disruption in the case of photosynthetic microalgae species containing chlorophyll and carotenoids, because they are released when the cell collapse occurs.[41]

Cell disruption using rapid depressurization can be carried out in supercritical extraction equipment, so that after pretreatment, temperature and pressure conditions are set to carry out the supercritical extraction of the lipidic compounds. This procedure saves time and minimizes equipment and labor requirements as well as contamination and extract loss.[24] Also, it can be scaled up to industrial scale.[20] If pretreatment and extraction are conducted in the same extractor, the substrate will be protected from exposure to oxygen and high temperature, avoiding oxidation reactions. Microalga cell disruption using CO_2 rapid depressurization has not yet been reported in literature. In this work, we hypothesized that cell disruption of *B. braunii* using rapid depressurization may enhance the extraction yield of pigments, carotenoids and chlorophylls, by adjusting the temperature and pressure conditions under static compression. The objective of this work was to improve the recovery of pigments from *B. braunii* with supercritical CO_2, through a pretreatment of cell disruption using CO_2 rapid depressurization. Response surface design was used to evaluate the effect of the static compression conditions on pigment recovery from *B. braunii*.

Materials and methods

Substrate

B. braunii UTEX LB572 was supplied by Universidad de Antofagasta (Antofagasta, Chile). This strain corresponds to race B and was cultured under outdoor production in pilot-scale panel reactors.[42] Microalga samples consisted of air-dried microalgae, which were carefully milled with mortar and pestle until a particle size of less than Tyler mesh 40 was obtained. Average particle size was 0.244 mm. The microalga sample had a moisture content of 7.9 ± 0.2/100 g dry substrate (d.s.) (determined gravimetrically by drying in an oven for 10 h at 102 °C) and an oil content of 106 ± 2 g/kg d.s. (determined gravimetrically by extraction with technical grade hexane in Soxhlet apparatus for 10 h at 70 °C). Samples were stored until use in dry, dark conditions, packed in the absence of oxygen.

Substrate pretreatment

The initial sample of microalga samples consisted of air-dried microalgae. This sample was divided into two fractions: a fraction without treatment by rapid depressurization (as control), and the other fraction subjected to treatment by rapid depressurization. The pretreatment using CO_2 rapid depressurization began by loading ca. 5 g of microalgae sample (substrate characterized) into a 50 cm^3 extraction vessel (14-mm internal diameter). The extraction vessel was placed in an air-convection oven of a Spe-ed SFE unit (Applied Separations, Allentown, PA). Prior to pretreatment, air trapped in the extraction vessel was purged by means of a controlled flow of CO_2. The extraction vessel was then pressurized with high-purity (99.95% pure) CO_2 (Linde, Santiago, Chile), and kept under static compression for 1 h, at different combinations of temperature (21–49 °C) and pressure (6–13 MPa). The CO_2 was then quickly released by opening a valve, which allowed the pressure to diminish to normal atmospheric pressure; the extraction vessel was then kept at atmospheric pressure for a further 10 min.

Supercritical extraction

It was then re-pressurized and the pretreated substrate was extracted at 40 °C (temperature of the air convection oven containing the extraction vessel, controlled automatically) and 30 MPa (extraction pressure controlled manually with an air-booster pump), with 4.7 L NPT/min of CO_2 with a single superficial velocity of 1 mm/s. In all cases, the extraction was carried out for 60 min (corresponding to a specific consumption of solvent of 101.5 kg CO_2/kg d.s.), after which the expansion valve (kept at 120 °C) was opened. Of this way, rapid depressurization of the microalgae cells and consecutive supercritical CO_2 extraction were carried out with the same substrate charge within the extractor vessel. This procedure is similar to that reported in others studies.[31,32] The oil recovered was assessed gravimetrically by difference using cleaned and dried glass vials, and the extracted oil yield (Y_{oil}, g/kg d.s.) was measured. Each experimental assay was conducted in duplicate. Recovered oil extracts were used for later analysis.

Analysis of extracts

Carotenoid concentration (C_{car}, g carotenoids/kg oil) was quantified in an oil sample dissolved in chloroform p.a. (Merck, Darmstadt, Germany). Absorbance was read at 452 nm by spectrophotometry in a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI).[32] The standard for the analysis of carotenoids (β-carotene type I, ≥95% pure) was obtained from Sigma–Aldrich (Saint Louis, MO). The carotenoid extraction yield (Y_{car}, g/kg d.s.) was obtained from $Y_{oil} \times C_{car}$. Chlorophyll concentration (C_{chlor}, mg chlorophylls *a* and *b*/kg oil) was quantified in an oil sample dissolved in ethyl ether p.a. and absorbance was read at 642 and 660 nm in the spectrophotometer. Chlorophyll *a* and *b* contents were determined using equations reported by Wrolstad *et al.*[43] The chlorophyll extraction yield (Y_{chlor}, mg/kg d.s.) was obtained from $Y_{oil} \times C_{chlor}$. The tocopherol concentration (C_{toc}, g tocopherol/kg oil) of selected oil samples was quantified at 520 nm in α-tocopherol equivalents.[44] The standard for the analysis of tocopherols α-tocopherol was obtained from Sigma–Aldrich. The tocopherol extraction yield (g/kg d.s.) was estimated from $Y_{oil} \times C_{toc}$. Antioxidant activity of selected oil samples was measured using a Trolox equivalent antioxidant capacity assay.[45] Antioxidant activity was expressed as millimole Trolox equivalent/kg oil. Trolox standard was obtained from Sigma–Aldrich (Saint Louis, MO).

Confocal microscopy

Control sample and microalgae pretreated with rapid depressurization were observed with confocal microscopy. A microalga sample (100 mg) was suspended in 1 mL of phosphate buffer (pH 7.5 at 25 °C) in an Eppendorf tube and filtered through a Millipore filter (Millipore Corp., Bedford, MA). A solution of white calcofluor (Sigma–Aldrich, Saint Louis, MO) in dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany) was

Table 1 – Extraction yield of carotenoids (Y_{car}) and chlorophylls (Y_{chlor}) by CO_2 at 40 °C and 30 MPa as a function of temperature and pressure of static compression.

Run	T [°C]	P [MPa]	X_1 [–]	X_2 [–]	Y_{car} [g car/kg d.s.]	Y_{chlor} [mg chlor/kg d.s.]
1	25	7.0	−1	−1	1.12	12.19
2	45	7.0	1	−1	0.73	11.53
3	25	12.0	−1	1	1.46	13.78
4	45	12.0	1	1	1.29	9.97
5	21	9.5	−1.41	0	1.72	10.78
6	49	9.5	1.41	0	1.00	7.44
7	35	6.0	0	−1.41	0.78	12.94
8	35	13.0	0	1.41	1.11	16.03
9	35	9.5	0	0	1.02	13.04
10	35	9.5	0	0	1.01	12.68
11	35	9.5	0	0	0.93	11.91
12	35	9.5	0	0	0.99	12.41

prepared in 1:10 ratio v/v. Suspension of microalgae was marked with 100 μL of calcofluor solution and incubated at room temperature for 15 min. The suspension was centrifuged in a Hitachi centrifuge CT15E (Hitachi Koki Co., Ltd., Tokyo, Japan). Microalgae samples were viewed using a Confocal Laser Microscope FV-1000 (Olympus Corp., Tokyo, Japan). FluoView software FV-10 2.0 (Olympus Corp., Tokyo, Japan) was used for image acquisition (40× magnification).

Experiment design

Central composite rotatable design (CCRD) was used to evaluate the effects of the independent variables: coded temperature (X_1, Eq. (1), where T is temperature °C), and coded pressure (X_2, Eq. (2), where P is pressure in MPa), both expressed in dimensionless units, on the response variables: extraction yield of carotenoids (Y_{car}) and chlorophylls (Y_{chlor}).

$$X_1 = \frac{T - 35}{10} \quad (1)$$

$$X_2 = \frac{P - 9.5}{2.5} \quad (2)$$

The design was based on a two-factor factorial design ($n = 2$), with two levels (coded values −1 and +1. The factors and their levels are shown in Table 1. The CCRD matrix had 4 (2^n) cube points (runs 1–4) and 4 ($2n$) star points (runs 5–8), at an axial distance to the center of 1.41 ($\alpha = 2^{n/4}$), and four replications of the center points (runs 9–12) to determine experimental error (Table 1). Experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to extraneous factors. A second-order model (Eq. (3)) was used to describe the response variable Y as a function of coded temperature (X_1) and coded pressure (X_2),

$$Y = A_0 + A_1X_1 + A_2X_2 + A_{12}X_1X_2 + A_{11}X_1^2 + A_{22}X_2^2 \quad (3)$$

where A_0 is a constant; A_1 and A_2 are linear coefficients; A_{12} is a cross-product coefficient; and A_{11} and A_{22} are quadratic coefficients. Three-dimensional surface response plots were generated by varying the two variables within the experimental range. The model fit was evaluated by analysis of variance (ANOVA). The coefficients of the response surface equation were estimated using Design Expert Design-Expert Software, version 6.0.1 (Stat-Ease, Inc., Minneapolis, MN). The statistical significance was based on the total error criteria with a confidence level of 95%.

Table 2 – Analysis of variance of regression coefficients and statistical indicators of appropriateness of the second order model selected.

Regression coefficient	Y_{car}		Y_{chlor}	
	Estimate	p-Value	Estimate	p-Value
A_1	−0.1972	0.0003	−1.147	0.0080
A_2	+0.1708	0.0009	+0.543	0.1274
A_1^2	+0.1892	0.0008	−1.682	0.0019
A_2^2			+1.018	0.0234
F-value	29.89*		13.75*	
r^2	0.918		0.887	
Adjusted r^2	0.888		0.821	
Lack of fit	8.00^{ns}		5.30^{ns}	
Signal-to-noise ratio	16.63		13.63	

* Significant at $p \leq 0.001$; ns: non-significant ($p > 0.05$).

Results and discussion

The experimental results of the extraction yields of carotenoids (Y_{car}) and chlorophylls (Y_{chlor}) as a function of the temperature and pressure applied during static compression are shown in Table 1. Experimental data is the average of two measurements. We note that the pressure drop occurred in the first 10 s. For cell disruption of rape pollen collected by bees, was reported that the pressure (treatment at 45 MPa) was quickly released within 1 min.[31] For treatment of microorganism with compressed CO_2, was reported that a rapid release of the CO_2 pressure (1.5–5 MPa) was within 4 s.[26] Oil extraction yield (Y_{oil}) ranged from 84.43 to 103.01 g/kg d.s. (full data not shown). Carotenoid extraction yield (Y_{car}, g/kg d.s.) ranged from 0.73 to 1.72 g/kg d.s., 2.4-fold differences. Chlorophyll extraction yield (Y_{chlor}, mg/kg d.s.) ranged from 7.44 to 16.03 mg/kg d.s., 2.2-fold differences.

Table 2 summarizes the statistical indicators obtained from the analysis of variance applied to the second-order model selected (Eqs. (4) and (5)). The model was considered adequate because of the significance of the model ($p \leq 0.001$), the non-significance of the lack of fit ($p > 0.05$) relative to pure error, the high signal-to-noise ratio (>4) and high coefficient of determination (r^2). For instance, this coefficient indicates that the model explains 91.8% of the variability in the carotenoid extraction yield Y_{car}. The information provided by the statistical indicators was complemented by a good correlation between predicted and experimental responses within the experimental range investigated, for both responses, since the plot shows a close fit of the experimental with the predicted values (data non shown).

Extraction yields of carotenoids and chlorophylls

Analysis of variance was used to evaluate the significance of the model's regression coefficients (Table 2). A large regression coefficient and a small p-value would indicate a more significant effect on the response variable. Significant coefficients ($p > 0.05$) were used to write the second order models. The variable with the largest effect on the carotenoid extraction yield was the linear term of the temperature ($p = 0.0003$), followed by the quadratic term of the temperature ($p = 0.0008$) and the linear term of the pressure ($p = 0.0009$). There was no significant effect of the quadratic term of the pressure ($p = 0.5212$), nor of the interaction term between temperature and pressure ($p = 0.2708$). Thus the second-order model (Eq. (4)) establishes a statistically significant relationship between carotenoid recovery and the temperature and pressure conditions when carrying out rapid depressurization in the selected experimental range ($6 \leq P \leq 13$ MPa; $21 \leq T \leq 49$ °C). Coded variables are important because they give a direct, quantitative indication of the effect of the independent variables on any dependent variable as a function of the selected experimental range. The term A_2 (=+0.1782), which multiplies the linear term X_2, indicates that the carotenoid extraction yield increases by 0.1782 g/kg d.s. when the compression pressure increases by 2.5 [=0.5(12–7)] MPa while operating at 35 °C ($X_1 = 0$).

$$Y_{car} = 0.9705 - 0.1972X_1 + 0.1708X_2 + 0.1892X_1^2 \quad (4)$$

The variable with the largest effect on the chlorophyll extraction yield was the quadratic term of the temperature ($p = 0.0019$), followed by the linear term of the temperature ($p = 0.0080$) and the quadratic term of the pressure ($p = 0.0234$). There was no significant effect of the linear term of the pressure ($p = 0.1274$) or of the interaction term between temperature and pressure ($p = 0.0670$). Thus, a statistically significant relationship between chlorophyll recovery and the temperature and pressure conditions has been established with the following second-order model (Eq. (5)). The linear term of the pressure ($A_2 = +0.543$) was not removed from Eq. (5) to maintain the hierarchy of the model. To better visualize the effect of the temperature and pressure on the recovery of carotenoids

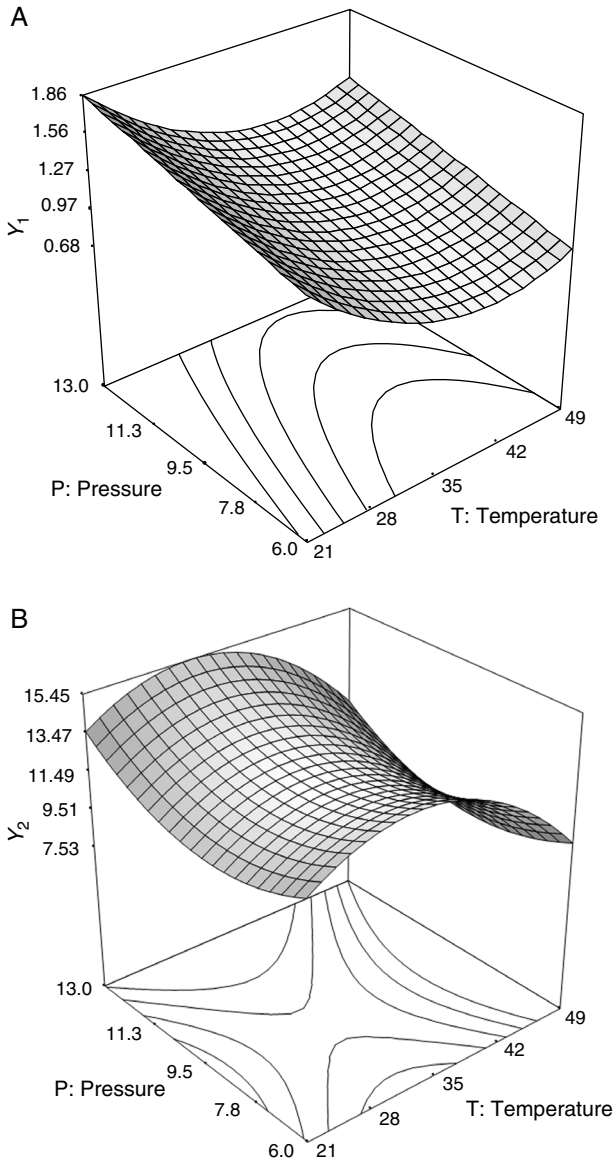

Fig. 1 – Surface plot of extraction yield at 40 °C and 30 MPa with CO_2, for: (A) carotenoids (Y_{car}, g car/kg d.s.) and (B) chlorophylls (Y_{chlor}, mg chlor/kg d.s.), as a function of temperature (T, °C) and pressure (P, MPa) of the rapid decompression.

and chlorophylls in the experimental range, surface response graphs were generated using the second-order models

$$Y_{chlor} = 12.502 - 1.147X_1 + 0.543X_2 - 1.682X_1^2 + 1.018X_2^2 \quad (5)$$

Effects of temperature and pressure

Carotenoid recovery decreased with increasing temperature in the lower temperature range (<40 °C). There was a negative linear effect ($A_1 = -0.1972$) (Eq. (4)) of temperature. Thus, we observed that carotenoid recovery decreases from 1.86 to 1.16 g/kg d.s. when temperature increases from 21 to 40 °C at 13 MPa. In the upper temperature range, over 40 °C, the positive non-linear effect of temperature ($A_1^2 = +0.1892$) becomes important and carotenoid recovery increases slightly with temperature. Similar behavior for Y_{car} was observed in the lower pressure range.

To chlorophylls recovery, the negative linear effect of temperature ($A_1 = -1.147$) (Eq. (5)) was observed in the upper temperature range (over ~35 °C). When the temperature increased from 35 to 49 °C at 13 MPa, Y_{chlor} decreased from 15.26 to 10.36 mg/kg d.s. The negative non-linear effect of temperature ($A_1^2 = -1.682$) becomes important for temperatures below 35 °C. This was reflected in the plateau for temperatures between 30 and 35 °C, where there was a minimal change in Y_{chlor} and 15.41 and 15.26 mg/kg d.s. respectively were obtained. Similar behavior for Y_{chlor} for temperature changes was observed in the lower pressure range. According to analysis of variance, the quadratic term of temperature contributed 28% and 37% to explaining the behavior of Y_{car} and Y_{chlor}, respectively. The quadratic term of pressure contributed 13% to explaining the behavior of Y_{chlor}.

Carotenoid recovery increased with pressure ($A_2 = +0.1708$) (Eq. (4)) to whatever temperature during static compression. There was a positive effect of pressure on Y_{car}. For instance Y_{car} increased from 1.38 to 1.86 g/kg d.s. (1.3-fold increase) when the pressure increased from 6 to 13 MPa at 21 °C. Similar behavior was observed for Y_{chlor} for pressure increases, in the upper pressure range over 9.5 MPa. There was a positive effect of pressure on Y_{chlor}. For instance Y_{chlor} increased from 10.81 to 13.57 mg/kg d.s. (1.3-fold increase) when the pressure increased from 9.5 to 13 MPa at 21 °C. For pressures below 9.5 MPa, the non-linear effect of pressure ($A_2^2 = +1.018$) becomes important, and we observed slight changes in Y_{chlor} with pressure. Supercritical extractions were performed under constant temperature and pressure conditions, therefore the solvent power of CO_2 should not change within the extraction assays. However, microalga extracts are complex mixtures of several interacting compounds. Probably the effect of quadratic terms would be explained by the existence of solute–solute interactions (anti-solvency and co-solvency effects) and/or solute–matrix interactions that affect the solubility behavior of a compound of the mixture in the supercritical phase and its recovery yield[46] (Fig. 1).

Cell disruption studies using CO_2 rapid depressurization have been reported in literature. The cell disruption of *Ralstonia eutropha* to extract poly (β-hydroxybutyrate) (PHB) was studied.[27] A multipurpose SFE-SFC system was used for the cell disruption, and its efficiency was measured as PHB recovery. Extraction yield increased with increasing pressure from 15 to 20 MPa, but decreased at 30 MPa. The authors attribute the negative effect of high pressure to the fact that disruption of the cytoplasm membrane occurs faster than disruption of the cell wall at 30 MPa, resulting in cell wall shrinkage, which hinders the extraction of intracellular compounds. In a similar study on the recovery of PHB from *R. eutropha* cells, it was reported that PHB recovery decreased with increasing pressure from 25 to 35 MPa at constant temperature.[47] Was studied the cell disruption of *E. coli* using CO_2 rapid depressurization for endoglucanase enzyme recovery.[23] The pressure during static compression (10–25 MPa) had a significant positive effect on enzyme recovery. Higher pressure resulted in better enzyme recovery. Therefore, the positive effect of pressure during static compression on cell disruption has been observed at pressures below 20 MPa. With respect to the compression temperature, Hejazi *et al.*[27] reported that biopolymer recovery decreased when the compression temperature rose from 40 to 70 °C. The authors attributed this effect to the supercritical CO_2 being closer to its liquid state at lower temperatures: when rapid depressurization occurs, the CO_2 reaches its gas state with maximum volume change, which in turn causes more extensive cell wall disruption. Also, Khosravi-Darani *et al.*[47] reported that PHB recovery decreased with increasing temperature from 30 to 40 °C at constant pressure. Thus a negative effect of temperature during static compression on cell disruption using rapid depressurization has been observed.

Table 3 – Comparison of supercritical CO_2 extraction (40 °C and 30 MPa) between microalgae untreated and pretreated with rapid decompression (21 °C and 13 MPa).

Characteristics	Untreated	Pretreated	Fold increase
Oil extraction yield (g/kg d.s.)	76.97 ± 1.51[a]	82.37 ± 0.90[b]	1.07
Concentration (mg/kg oil)			
Carotenoids	17.02 ± 0.93[a]	19.75 ± 0.78[b]	1.16
Chlorophylls	19.36 ± 1.68[a]	181.26 ± 3.62[b]	9.36
Tocopherols	8509 ± 55[a]	9059 ± 39[b]	1.06
Extraction yield			
Carotenoids (g/kg d.s.)	1.53 ± 0.15[a]	1.91 ± 0.09[b]	1.25
Tocopherols (g/kg d.s.)	0.91 ± 0.01[a]	1.04 ± 0.01[b]	1.14
Chlorophylls (mg/kg d.s.)	1.38 ± 0.35[a]	14.03 ± 0.91[b]	10.2
Antioxidant activity(mmol TE/kg oil)	29.11 ± 0.51[a]	33.22 ± 0.31[b]	1.14

Different letters in same row indicate significant difference at $p \leq 0.05$.

According to our results, the extraction of carotenoids was best at 21 °C and 13 MPa with Y_{car} at 1.87 g/kg d.s., while the extraction of chlorophyll was best at 32 °C and 13 MPa, with Y_{chlor} at 15.45 mg/kg d.s. In other words higher pigment extraction was obtained in the experimental range of high pressure and low temperature, which agrees with the observations of previous authors.[23,27,47] This range is characterized by higher CO_2 density (about 810–880 kg/m^3). When the CO_2 is denser, its solvent power increases and in consequence so does the permeation capability of CO_2 within the cell. Furthermore, when the CO_2 density increases, the net amount of gas absorbed into the cells increases and this increases the force caused by rapid depressurization.[47] A major change in the volume occurs when the CO_2 returns to the gaseous state due to rapid depressurization.[27] This sudden volume change results in more effective cell disruption and improves the availability of pigments by supercritical extraction.

Comparison between untreated and pretreated microalgae

Table 3 shows a comparison of supercritical CO_2 extraction (40 °C and 30 MPa) using untreated microalgae and microalgae pretreated using rapid depressurization. For rapid depressurization pretreatment, we selected 21 °C and 13 MPa conditions to favor recovery of carotenoids and chlorophylls. The static time was kept constant at 60 min. The extraction yields for carotenoid and chlorophyll reported in Table 3 confirm the predictive power of the selected second-order models (Eqs. (4) and (5)). Table 3 shows that there was significant difference ($p \leq 0.05$) in compound recovery and antioxidant activity between oils extracted with supercritical CO_2 from untreated and pretreated samples using rapid depressurization. We included the measurement of antioxidant activity and quantified total tocopherols, which are important antioxidants. The highest difference was observed in the extraction of chlorophylls, 1.38 and 14.03 mg/kg d.s., for untreated and pretreated samples respectively. Chlorophyll pigments are produced and stored intracellularly in the chloroplasts.[48] Mendes *et al.*[37] reported that in supercritical extracts (at 40 °C and 12.5–30 MPa) obtained from freeze-dried samples of *B. braunii*, chlorophylls were not detected. Therefore, the higher extraction of chlorophylls from pretreated samples with rapid depressurization would be a very good indicator of cell disruption. To support these results, observations were made under confocal laser microscopy.

Confocal microscopy images in Fig. 2 show the effect of CO_2 rapid depressurization pretreatment on the morphology of microalgae cells. Fig. 2A shows a micro-colony of *B. braunii* not treated with rapid depressurization as control sample. Intact cell walls can still be distinguished in the micro-colony. This is clear when we observe the walls surrounding the cells stained blue (emission 450 nm), due to the reaction of calcofluor with the cellulose components of the cell wall. Fig. 2B shows major destruction of cell walls in the micro-colony to which CO_2 rapid depressurization was applied: large cell wall integrity was lost. Cell units are not clearly differentiated, since some cells have fused together. In addition, red areas appear due to an increase in the release of chlorophyll pigments (emission 650–750 nm), which would permit higher extraction of chlorophyll pigments with supercritical CO_2 as compared to the control sample (without treatment with rapid depressurization). There was therefore an observable difference in cell wall integrity between the untreated and pretreated samples, which resulted in increased extraction of carotenoids, chlorophylls and tocopherols, and in higher antioxidant activity in pretreated samples. These observations suggest that pretreatment using CO_2 rapid depressurization improved pigment extraction due to partial cell disruption of the microalgae.

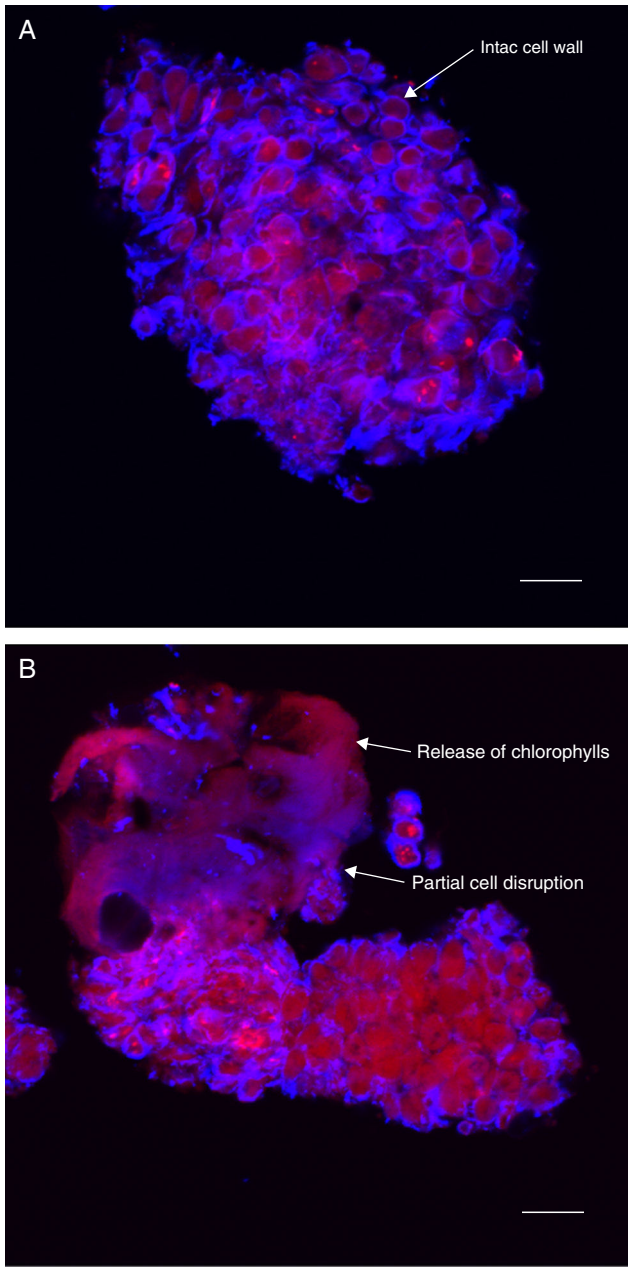

Fig. 2 – Confocal microscopy images of micro-colony of *B. braunii*: (A) not treated with CO_2 rapid depressurization, as control sample and (B) treated with rapid depressurization (bar = 20 μm).

Conclusions

This work investigated cell disruption using CO_2 rapid depressurization as a substrate pretreatment for pigment extraction from the microalga *B. braunii*. According to the response surfaces we suggest a region of low temperature and high pressure for the static compression condition, which favors the extraction of carotenoids and especially chlorophylls. The combination of high pressure (13 MPa) and low temperature (21 °C) during static compression was selected for the pretreatment of microalgae with purposes of comparison with untreated microalgae. Oil from pretreated microalgae presented better antioxidant activity due to the higher concentration of carotenoids, chlorophylls and tocopherols. From confocal microscopy images, it was observed that partial cell disruption of microalgae improved pigment extraction. CO_2 rapid depressurization is a simple and efficient method that can be employed to recover important intracellular components, such as the pigments from *B. braunii*. Moreover, this pretreatment is performed at moderate temperatures which would protect these heat sensitive compounds. This is the first report on cell disruption using CO_2 rapid depressurization of a microalga.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This research was funded by the Universidad de La Frontera (project DI11-0055) and Innova-CORFO (project 09CTEI-6860). Our acknowledgments to Agriaquaculture Nutritional Genomic Center, CGNA (R10C1001).

REFERENCES

1. Ibañez E, Cifuentes A. Benefits of using algae as natural sources of functional ingredients. *J Sci Food Agric.* 2013;93:703–709.
2. Metzger P, Rager MN, Fosse C. Braunicetals: acetals from condensation of macrocyclic aldehydes and terpene diols in *Botryococcus braunii*. *Phytochemistry*. 2008;69:2380–2386.
3. Samori C, Torr C, Samori G, et al. Extraction of hydrocarbons from microalga *Botryococcus braunii* with switchable solvents. *Bioresour Technol*. 2010;101:3274–3279.
4. Largeau C, Casadevall E, Berkaloff C, Dhamliencourt P. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. *Phytochemistry*. 1980;19:1043–1051.
5. Sato Y, Ito Y, Okada S, Murakami M, Abe H. Biosynthesis of the triterpenoids, botryococcenes and tetramethylsqualene in the B race of *Botryococcus braunii* via the non-mevalonate pathway. *Tetrahedron Lett*. 2003;44:7035–7037.
6. Grung M, Metzger P, Liaaen-Jensen S. Algal carotenoids, primary and secondary carotenoids in two races of green alga *Botryococcus braunii*. *Biochem Sys Eco*. 1989;17:263–269.
7. Rao AR, Sarada R, Ravishankar GA. Enhancement of carotenoids in green alga *Botryococcus braunii* in various autotrophic media under stress conditions. *Int J Biomed Pharmaceut Sci*. 2010;5:87–92.
8. Volova TG, Kalacheva GS, Zhilo NO, Plotnicov VF. Physiological and biochemical properties of the alga *Botryococcus braunii*. *Russ J Plant Physiol*. 1998;45:775–779.
9. Rao AR, Sarada R, Baskaran V, Ravishankar GA. Antioxidant activity of *Botryococcus braunii* extract elucidated in vitro models. *J Agric Food Chem*. 2006;54:4593–4599.
10. Weiss TL, Roth R, Goodson C, et al. Colony organization in the green alga *Botryococcus braunii* (Race B) is specified by a complex extracellular matrix. *Eukaryotic Cell*. 2012;11:1424–1440.
11. Ryckebosch E, Muylaert K, Foubert I. Optimization of an analytical procedure for extraction of lipids from microalgae. *J Am Oil Chem Soc*. 2012;89:189–198.
12. Doucha J, Livanský K. Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers. *Appl Microbiol Biot*. 2008;81:431–440.
13. Lee J, Yoo C, Jun S, Ahn C, Oh H. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour Technol*. 2010;101:S75–S77.
14. Gerken HG, Donohoe B, Knoshaug EP. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. *Planta*. 2013;237:239–253.
15. Halim R, Rupasinghe TWT, Tull DL, Webley PA. Mechanical cell disruption for lipid extraction from microalgal biomass. *Bioresour Technol*. 2013;140:53–63.
16. Biller P, Friedman C, Ross AB. Hydrothermal microwave processing of microalgae as a pre-treatment and extraction technique for bio-fuels and bioproducts. *Bioresour Technol*. 2013;136:188–195.
17. Prabakaran P, Ravindran AD. A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Lett Appl Microbiol*. 2011;53:150–154.
18. Geciova J, Bury D, Jelen P. Methods for disruption of microbial cells for potential use in the dairy industry – a review. *Int Dairy J*. 2002;12:541–553.
19. Chisti Y, Moo-Young M. Disruption of microbial cells for intracellular products. *Enzyme Microb Technol*. 1986;8:194–204.
20. Castor TP, Hong GT. *Supercritical fluid disruption of and extraction from microbial cells*. US 5380826 A; 1995.
21. Gaspar F, Lu T, Santos R, Al-Duri B. Modelling the extraction of essential oils with compressed carbon dioxide. *J Supercrit Fluids*. 2003;25:247–260.
22. Nakamura K, Enomoto A, Fukushima H, Nagai K, Hakoda M. Disruption of microbial cells by the flash discharge of high-pressure carbon dioxide. *Biosci Biotech Biochem*. 1994;58:1297–1301.
23. Juhász T, Székely E, Simándi B, Szengyel Z, Réczey K. Recovery of a recombinant thermostable endoglucanase from *E. coli* using supercritical carbon dioxide cell disruption. *Chem Biochem Eng Q*. 2003;17:131–134.
24. Khosravi-Darani K, Mozafari MR. Supercritical fluids technology in bioprocess industries: a review. *J Biochem Tech*. 2009;2:144–152.
25. Lin HM, Yang Z, Chen LF. An improved method for disruption of microbial cells with pressurized carbon dioxide. *Biotechnol Prog*. 1992;8:165–166.
26. Debs-Louka E, Louka N, Abraham G, Ghabot V, Allaf K. Effect of compressed carbon dioxide on microbial cell viability. *Appl Environ Microb*. 1999;65:626–631.
27. Hejazi P, Vasheghani-Farahani E, Yamini Y. Supercritical fluid disruption of *Ralstonia eutropha* for poly(-hydroxybutyrate) recovery. *Biotechnol Prog*. 2003;19:1519–1523.
28. Lin HM, Chan ECh, Chen Ch, Chen LF. Disintegration of yeast cells by pressurized carbon dioxide. *Biotechnol Prog*. 1991;7:201–204.
29. Isenschmid A, Marison IW, von Stockar U. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. *J Biotechnol*. 1995;39:229–237.

30. Guoliang L, Junyou S, Yourui S, et al. Supercritical CO_2 cell breaking extraction of *Lycium barbarum* seed oil and determination of its chemical composition by HPLC/APCI/MS and antioxidant activity. *LWT-Food Sci Technol*. 2011;44:1172–1178.
31. Xu X, Sun L, Dong J, Zhang H. Breaking the cells of rape bee pollen and consecutive extraction of functional oil with supercritical carbon dioxide. *Innov Food Sci Emerg Technol*. 2009;10:42–46.
32. Uquiche E, Huerta E, Sandoval A, del Valle JM. Effect of boldo (*Peumus boldus* M.) pretreatment on kinetics of supercritical CO_2 extraction of essential oil. *J Food Eng*. 2012;109:230–237.
33. Johnson LA. Theoretical, comparative, and hystorical analysis of alternative technologies for oilseeds extraction. In: Wan PJ, Wakelin PJ, eds. *Technology and Solvents for Extracting Oilseeds and Nonpretroleum Oils*. Champaign, IL: AOCS Press; 1997:4–47.
34. United States Environmental Protection Agency. http://www.epa.gov/ttn/atw/nata2002/tables.html. Accessed Jan, 2014.
35. Brunner G. *Gas Extraction. An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes*. NewYork, NY: Springer; 1994.
36. del Valle JM, Aguilera JM. Extracción con CO_2 a alta presión. Fundamentos y aplicaciones en la industria de alimentos. *Food Sci Technol Int*. 1999;5:1–24.
37. Mendes RL, Fernandes HL, Coelho JAP, Cabral JMS, Palabra AMF, Novais JM. Supercritical carbon dioxide extraction of hydrocarbons from the microalga *Botryococcus braunii*. *J Appl Phycol*. 1994;6:289–293.
38. Santana A, Jesus S, Larrayoz MA, Filho RM. Supercritical carbon dioxide extraction of algal lipids for the biodiesel production. *Procedia Eng*. 2012;42:1755–1761.
39. Fiedor J, Burda K. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients*. 2014;6:466–488.
40. Hsu CY, Chao PY, Hu SP, Yang CM. The antioxidant and free radical scavenging activities of chlorophylls and pheophytins. *Food Nutr Sci*. 2013;4:1–8.
41. Gerde J, Montalbo-Lomboy M, Linxing Y, Grewell D, Tong W. Evaluation of microalgae cell disruption by ultrasonic treatment. *Bioresour Technol*. 2012;125:175–181.
42. Bazaes J, Sepulveda C, Acién G, et al. Outdoor pilot-scale production of *Botryococcus braunii* in panel reactors. *J Appl Phycol*. 2012;24:1353–1360.
43. Wrolstad RE, Acree TE, Decker EA, et al. *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavours, Texture, and Bioactive Food Components*. Hoboken, NJ: John Wiley & Sons, Inc; 2005.
44. Wong ML, Timms RE, Goh EM. Colorimetric determination of total tocopherols in palm oil, olein and stearin. *J Am Oil Chem Soc*. 1988;65:258–261.
45. Pellegrini N, Serafini M, Colombi B, et al. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three in-vitro assays. *J Nutr*. 2003;133:2812–2819.
46. del Valle JM, Urrego FA. Free solute content and solute-matrix interactions affect apparent solubility and apparent solute content in supercritical CO_2 extractions. A hypothesis paper. *J Supercrit Fluids*. 2012;66:157–175.
47. Khosravi-Darani K, Vasheghani-Farahani E, Shojaosadati SA, Yamini Y. Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for poly(R-hydroxybutyrate) recovery. *Biotechnol Prog*. 2004;20:1757–1765.
48. Granick S. Biosynthesis of chlorophyll and related pigments. *Annu Rev Plant Physiol*. 1951;2:115–144.