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Ultrasound-assisted Extraction of Carbohydrates from Microalgae

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Microalgae are a promising new source of carbohydrates usable for several industrial applications in the food and biomaterial sector. Previous works on carbohydrate extraction from microalgae were mainly carried out by using destructive chemical hydrolysis, aiming at the extraction of simple sugars. Here in this work, a physical ultrasonication method was investigated to develop a process to extract microalgal carbohydrates in their polysaccharide form, as starch. To this end, different operative parameters were investigated: biomass concentration (3-6 g L^{-1}), microalgae strain (*Tetradesmus obliquus* and *Chlorella* sp.), extraction time, amplitude (21-90 μm) and the configuration of the ultrasonication system (cyclic treatment, pulsed and continuous). The highest extractions were attained with higher amplitude (90 μm). The pulsed ultrasonication (t_{on}/t_{off} = 0.2) worked remarkably better than the continuous one, allowing to attain about 3 folds more carbohydrate extraction yield and consuming 6 folds less kWh per kg of extracted carbohydrates. The higher yield achieved with pulsed ultrasonication was related with a lower drop in the applied power during the ultrasonication treatment, which was -65 % with the continuous system and only -31 % with the pulsed one. The ultrasonication treatment induced a temperature increase up to 70 °C, that caused starch gelatinization and its solubilization in the recovered aqueous solution. Future studies should investigate better the effect of the t_{on}/t_{off} ratio, to limit the dead times (t_{off}) of the process. The specific energy consumption was still too high for many practical applications; however, future optimizations on biomass concentration and operative temperature are expected to reduce remarkably the energy demand of the process.

1. Introduction

Microalgae are a new promising source for several biomolecules, because of their higher productivity as compared to terrestrial plants. Microalgae can be a source of high value proteins, lipids, carbohydrates and carotenoids. However, one of the main limits in the exploitation of microalgae biomass in biorefinery facilities is the need for downstream processes enough efficient to recover valuable compounds in a sustainable way (Ruiz et al. 2016). Microalgae can contain from 10 % to 60 % total carbohydrates as dry weight. These carbohydrates are usually mainly composed of starch (storage compound) and cell walls polysaccharides (cellulose, hemicelluloses). Simple sugars, as glucose, are usually only a negligible fraction of total carbohydrates. Microalgal polysaccharides could be used for different applications in the field of bio-based materials, such as starch-based bioplastics and antimicrobial biopolymers. However, up to date, the extraction of microalgal carbohydrates was mainly focused on the production of simple sugars used as substrate for microbial fermentation (Hernández et al. 2015). In this case, chemical hydrolysis was usually applied, attaining high extraction yields. However, for bioplastic/biomaterial applications, in which the target end products are the polysaccharides, the destructive chemical hydrolysis cannot be applied. For polysaccharides, physical methods, such as ultrasonication, can be more suitable. Ultrasonication can disrupt the cell structures, allowing the extraction of internal compounds directly in water, in an industrial scalable process. However, the extraction of carbohydrates from microalgae with ultrasonication has received few attentions, especially when compared to other biomolecules, such as lipids and carotenoids (Chemat et al. 2017). Therefore, the aim of this work is to develop a process to extract carbohydrates from microalgal biomass by using ultrasonication treatment. To this end, ultrasonication was tested in different extraction configurations, namely cyclic treatment, batch treatment with pulsed ultrasonication and batch treatment with continuous ultrasonication.

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These systems have been applied to different microalgal concentrations, with different amplitudes and for two different microalgae strains.

2. Materials and methods

2.1 Microalgae cultivation

Two different strains of microalgae were cultivated, a strain of *Tetradesmus obliquus* and a strain of *Chlorella* sp., isolated and maintained as described in a previous work (Di Caprio et al. 2018). The microalgae were cultivated in 300 mL flasks in BG11 medium, at room temperature, under light illumination provided by fluorescent lamps (80 μmol m^2 s^{-1}) and under constant environmental air feeding (0.04 % CO_2). The cultures were maintained in sequential batches inoculated every 10-15 days by 1 to 10 dilution in fresh medium.

2.2 Ultrasound-assisted carbohydrate extraction

Microalgal suspension was collected from the cultivation flask, centrifuged (4,025 g, 3 min) and suspended in distilled water (dH_2O) to have 20 mL at 3 g L^{-1} or 6 g L^{-1}. In parallel, for each test, another aliquot of the same suspension was centrifuged as well, the pellet washed with water and dried in oven at 105 °C. This latter dried biomass was used to quantify the initial total carbohydrate content (f_C). The microalgae suspension was put inside a 50 mL Falcon tube, with a 12 mm replaceable sonicator horn immersed inside. The horn was connected to a Branson 450 Digital Sonifier (20 kHz, 400 W maximum output power). The ultrasonication was carried out in three different configurations:

- Cyclic treatment (CT): after turning on the sonicator, the ultrasonication was performed in continuous mode for 5 min. After that, the suspension was centrifuged (4,025 g, 5 min), the supernatant collected and stored at -18 °C. The pellet was suspended in 20 mL dH_2O, the absorbance was read at 750 nm and the described treatment repeated for several consecutive cycles.
- Batch treatment with pulsed ultrasonication (BP): the sonicator was operated in pulsed mode (t_{on} = 0.1 s, t_{off} = 0.5 s) up to the end of the test ($\sum t_{on}$ = 5, 15, 30, 60 min). After that, the suspension was centrifuged (4,025 g, 5 min), the supernatant collected and stored at -18 °C.
- Batch treatment with continuous ultrasonication (BC): the sonicator was maintained turned on throughout the test (5, 15, 30, 60 min). After that, the suspension was centrifuged (4,025 g, 5 min), the supernatant collected and stored at -18 °C.

These conditions were tested for both *T. obliquus* and *Chlorella* sp. The CT was tested for two different amplitudes of 10 % and 60 %, corresponding to 21 μm (96 W) and 90 μm (140 W), while BP and BC only at 90 μm amplitude. The apparent cell lysis was calculated with the Eq(1).

$$\text{Apparent lysis } (\%) = \frac{\left(A_{750}(t) - A_{750}(t=0)\right)}{A_{750}(t=0)} 100 \quad (1)$$

The extracted carbohydrate yield was calculated with the Eq(2).

$$\text{Extracted carbohydrates } (\%) = \frac{m_C}{x\ f_C} 100 \quad (2)$$

With m_C the mass of total carbohydrate extracted in the aqueous phase (supernatant), x the initial biomass and f_C the fraction of carbohydrates in the initial biomass.

2.3 Total carbohydrate analysis

Initial total carbohydrates inside biomass were determined by hydrolyzing sugars (acid saccharification with H_2SO_4) from 100 mg of dried biomass and by spectrophotometric analysis of sugars by using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method (NREL/TP-5100-60957). This latter method was chosen because it gave less interference than the conventional Dubois method (Van Wychen et al. 2017). The same procedure was applied to the liquid extracts obtained after the ultrasonication, with only one variation in the saccharification: an aliquot of 72 % H_2SO_4 was added to the liquid sample to attain 4 % final concentration. Then the samples were processed in the same way.

2.4 Extraction kinetic model

The kinetic of the extraction in the BC and BP tests has been modelled as shown in Eq(3).

$$C\ (t) = C_e(1 - e^{-kt}) \quad (3)$$

With C(t) and C_e the concentration of carbohydrates at time t and at the equilibrium, respectively, and k the mass transport coefficient. The results have been reported directly as %, by dividing the concentration of the extracted carbohydrates by the concentration of carbohydrates from initial biomass. Nonlinear fitting was

performed with the MATLAB function 'nlinfit' by the minimization of the sum of the squared residuals. For each estimated parameter, the 95% confidence intervals values were determined with the function 'nlparci'.

2.5 Scanning electron microscopy (SEM) analysis

The initial microalgal suspension (1 mL, A_{750} = 0.5) was collected by centrifugation (5,000 g, 5 min) and then suspended overnight at 4 °C in 1 mL PBS with 2 % glutaraldehyde. The pellet was then dried with ethanol at gradient concentrations: 30 %, 50 %, 70 %, 80 %, 90 %, 100 %. Finally, a drop of suspension was put on a glass cover slip and dried in a fume hood. The aqueous suspension supernatant (50 μL), obtained after ultrasonication (60 min, BP), was mixed with 50 μL PBS with 2 % glutaraldehyde. Then, 5 μL of such suspension were put on a glass cover slip, which was dried in a fume hood. The obtained samples were pre-treated for superficial coating with Cr and then analyzed by a Field Emission SEM (Zeiss, Auriga).

2.6 Statistical analysis

Two or three replicates were tested for each test (considered as the repetition of the extraction experiment). Statistical differences were evaluated by two-ways ANOVA.

3. Results and discussion

3.1 Cyclic treatment

The cyclic treatment was applied to two different strains (*T. obliquus* and *Chlorella* sp.) to evaluate the effect of the biomass concentration, applied amplitude and treatment time. Figure 1 reports the absorbance and the corresponding lysis detected for each condition, after each cycle (t_{on} = 5 min). The two strains showed a different ratio between A_{750}/biomass (a_{750}), likely due to their different morphology. *Chlorella* sp. is mainly unicellular with spherical shape, while *T. obliquus* forms 4-8 celled coenobia with ellipsoidal shape. A relevant absorbance reduction was obtained for all the conditions as a result of the ultrasonication treatment, resulting in an apparent lysis between 35 - 63 % at the end of the test, depending on the conditions adopted. The lysis attained a saturation for higher treatment times (> 30 min), which was more evident for *T. obliquus*.

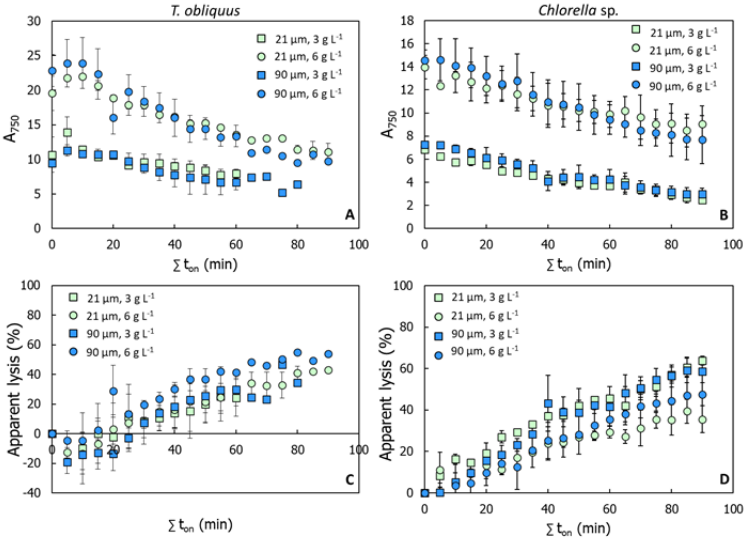

Figure 1. A_{750} and apparent lysis variation as function of treatment time, for the two strains treated with the cyclic treatment. Data for the different initial biomass concentrations and different applied amplitudes are shown.

Only for *T. obliquus,* negative lysis values (up to -20 %) were obtained during the first 5 - 20 min (Figure 1C), due to an increase in the absorbance read (Figure 1A). Such anomalous behavior was probably due to the known property of ultrasonication to separate the cells from coenobia into single cells (Di Caprio et al. 2017). Single cells have higher surface area than coenobia, therefore they should also show a higher a_{750}. Not any relevant variation in the lysis was observed as a result of the different amplitudes applied. For *Chlorella* sp., the increase in biomass concentration from 3 g L^{-1} to 6 g L^{-1} reduced the lysis from 61 ± 4 % to 40 ± 10 % (p = 0.017). The microalgae used for the tests had an initial 31.5 ± 4.7 % total carbohydrate content. Based on previous analyses, about 60-70 % of these carbohydrates should have been composed of starch.

During the cyclic treatment, a relevant fraction of carbohydrates moved from the biomass pellet to the aqueous supernatant. This behavior was expected to be given by the starch gelatinization in the hot water, with some contribution by glycosylated proteins and soluble sugars. During the treatment, the temperature increased, for each cycle, from 21 °C to 76 ± 4 °C, which should be enough high to induce starch gelatinization. However, it should be underlined that no relevant previous information was found about the gelatinization temperature of microalgal starch. Such temperature increase could give some issues in the case the carbohydrate extraction would be integrated with the extraction of thermolabile molecules, such as carotenoids.

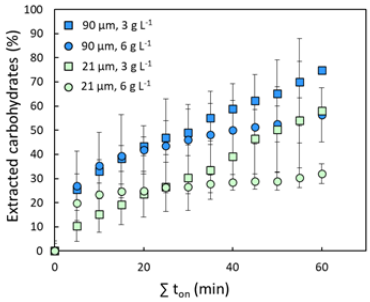

Figure 2. Yield for extracted carbohydrates as function of treatment time for T. obliquus treated with the cyclic treatment.

The kinetic behavior of the extraction was assessed for *T. obliquus* (Figure 2), showing a higher extraction rate in the first 20-30 min, followed by the gradual achievement of an equilibrium phase. Statistical analyses did not indicate any relevant influence of the investigated parameters on the % extracted carbohydrates after 60 min for *T. obliquus*, despite the values ranged between 32 - 75 % extraction yield (Figure 3B). For *Chlorella* sp., the increase in the amplitude from 21 µm to 90 µm induced an increase ($p = 0.02$) on the extracted carbohydrates after 90 min of treatment from 61.3 ± 9.9 % to 79.8 ± 7.6 % (Figure 3A).

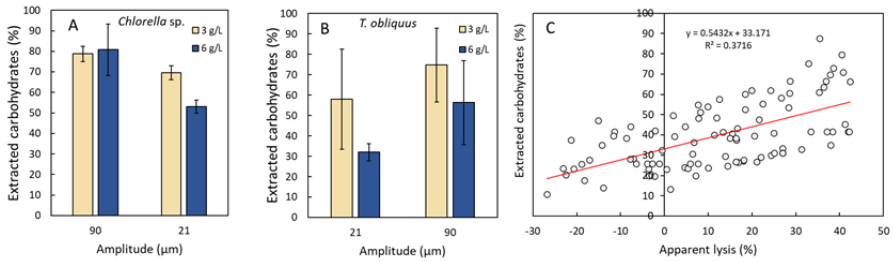

Figure 3. A-B) Extracted carbohydrates (as %) for T. obliquus (after 60 min) and Chlorella sp. (after 90 min) treated with the cyclic treatment. C) Correlation between the extracted carbohydrates and apparent lysis for T. obliquus treated with the cyclic treatment.

To verify the possibility to use the apparent lysis as a quick and easy way to predict the extracted carbohydrates, a correlation between these values was assessed and reported in Figure 3C. A significant linear regression was obtained ($p = 5\ 10^{-11}$), however the standard deviation of the angular coefficient was ± 130 % its value. Therefore, the relation was not considered applicable for accurate predictions of the extracted carbohydrate yield.

3.2 Batch treatment with pulsed and continuous ultrasonication

The described cyclic treatment allowed to attain high yields in carbohydrate extraction. However, the application of several cycles (up to 18) for solid-liquid separations, make such a process hardly scalable for an industrial application, because of the high additional energy required to perform so many centrifugation operations. On the other hand, it should be underlined that such an extraction system was used because in a first attempt to conduct the whole extraction in a single batch, not any relevant improvement in the extraction yield was observed after few minutes of treatment. A previous study reported the possibility to significantly increase the reaction yield by applying a pulsed ultrasonication in place of a continuous system (Martinez-

Guerra and Gude, 2015). Therefore, a pulsed ultrasonication with t_{off}/t_{on} = 0.5/0.1 was tested in a batch system and compared to the continuous one. For both the strains the continuous ultrasonication attained a limit in the extraction yield after just 5 min (Figure 4), corresponding to only 22 % carbohydrate extraction for *T. obliquus* and 34 % for *Chlorella* sp., without any remarkable increase for longer times. Pulsed ultrasonication increased 2-4 folds the final extraction yield ($p < 0.05$), attaining up to 74 % carbohydrate extraction for *Chlorella* sp. and 87 % for *T. obliquus* (Table 1). This positive effect by pulsed ultrasonication was related with a lower drop in the output applied power by the sonicator. When continuous ultrasonication was used, the applied power dropped from 144 W to 50 W (-65 %) within 5 min (Figure 4C), that also corresponded to the saturation in the carbohydrate extraction (Figure 4A-B). With pulsed ultrasonication the drop was less intense, from 144 W to 100 W (-31 %). For both the configurations, the power remained stable after the initial drop. The registered drop in applied power could be a result of a change in the viscosity of the solvent, that changes the energy required by the sonicator to maintain the fixed amplitude. It may be deduced that the continuous ultrasonication could lead to a higher accumulation of water vapor, which is known to make cavity bubbles collapse less violently, reducing in turn the ultrasonication yield (Chemat et al. 2017). A higher concentration of water vapor also reduces the viscosity of the solvent and in turn reduces the required applied power to maintain the fixed amplitude.

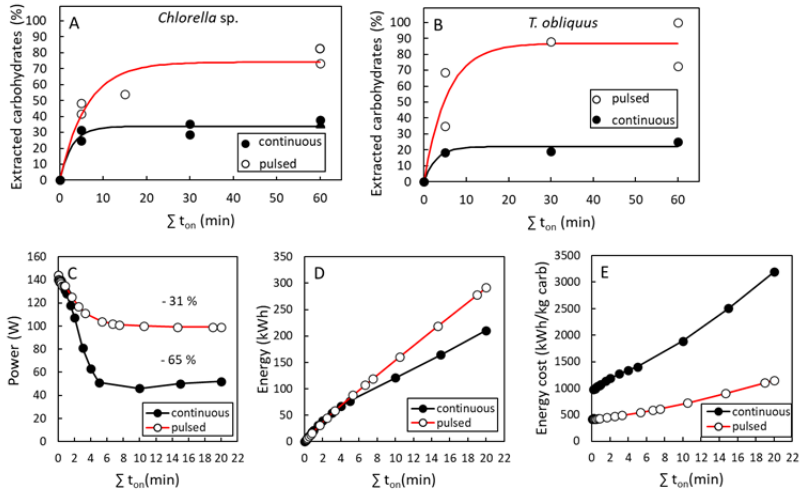

Figure 4. A-B) Extracted carbohydrates as function of time for pulsed and continuous ultrasonication. With solid lines, the fittings obtained with the first order model are shown. C) Applied power. D) Registered drop in applied energy. E) Specific energetic cost for carbohydrate extraction. Amplitude: 90 μm; biomass: 6 g L^{-1}.

Table 1: Model parameters estimated for kinetic tests. Average values ± 95 % CI are reported.

Sample (test)	k (min^{-1})	Extracted carbohydrates (%)
Chlorella sp. (BP)	0.16 ± 0.12	74 ± 16
Chlorella sp. (BC)	0.35 ± 0.26	34 ± 5
T. obliquus (BP)	0.18 ± 0.19	87 ± 25
T. obliquus (BC)	0.35 ± 0.76	22 ± 9

For both the strains and extraction conditions (BP and BC), the extraction kinetic was well described by the first order model. The kinetic constants (k) obtained for the pulsed ultrasonication were almost half than those from continuous, for both the strains. The characteristic time ($\tau = k^{-1}$) varied between 6.25 min for pulsed ultrasonication to 2.85 min for the continuous one, resulting in the achievement of an equilibrium after 9-19 min (3 τ). A relevant aspect to be considered for the pulsed ultrasonication is the $\sum t_{off}$, that contributed at determining the whole process time, even if without an energy supply. As a result of the t_{on}/t_{off} used, for a complete extraction (t_{on} = 19 min), 115 min were in total required. Future studies should investigate how different t_{on}/t_{off} affect the extraction yield, to reduce dead times. The pulsed condition maintained a higher power supply per biomass (Figure 4D), that was about 300 kWh kg^{-1} after 20 min. At least 9.3 kWh per kg were missed for water heating, because the temperature increased quickly, for both BP and BC, up to 70.3 ± 1.5 °C within 5 min and remained stable throughout the extraction. The energy consumption is too expensive for large part of the possible applications, underlining the necessity to find a more sustainable configuration.

The utilization of higher biomass concentrations (50-100 g L^{-1}) and the scale up to higher volumes could allow to attain a more sustainable process, reducing energy losses. Pulsed ultrasonication allowed to reduce 3-6 folds (at 5-20 min respectively) the consumed kWh per kg of extracted carbohydrates (Figure 4E). The increase in temperature supported the hypothesis that, in such conditions, the starch was extracted because completely solubilized by gelatinization in the aqueous phase. To verify this, the initial microalgal sample and the dried aqueous phase were analyzed morphologically with SEM. The morphology of *T. obliquus* (Figure 5A) was like that one expected from previous literature data, showing the characteristic coenobia with wrinkled cell walls (Di Caprio et al., 2017). A completely different morphology was found in the extracted sample, that showed the presence of a uniform layer, like a gel, covering the entire surface (Figure 5B-C). Some empty cell walls submerged in such a layer can be observed (Figure 5B) together with some salt crystals and some white granules. These latter granules were likely starch granules still incompletely gelatinized (Figure 5C).

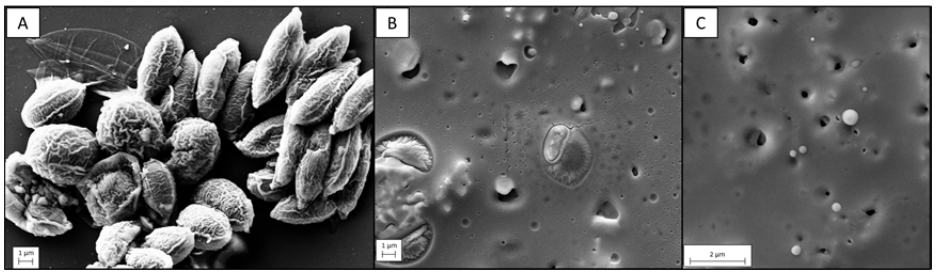

Figure 5. SEM analysis of initial T. obliquus cells (A) and extracted phase (B-C) obtained after ultrasonication treatment (70 °C).

4. Conclusions

This work studied different ultrasonication configurations to extract carbohydrates from microalgal biomass. The most promising was the pulsed extraction in batch, that allowed to attain up to 87 % extraction yield with $\sum t_{on}$ = 19 min, and t_{on}/t_{off} = 0.2. The higher yield obtained with pulsed ultrasonication was related with a reduced drop in the applied power, which was only -31 %, while it was -65 % for continuous ultrasonication. Pulsed ultrasonication also reduced up to 6 folds the kWh used per kg of extracted carbohydrates. The increase in temperature caused starch gelatinization in water, which was likely fundamental for the starch extraction yield. The energy consumption was still too high for a large part of practical applications; however, these results indicate large room for process optimization. The utilization of higher biomass concentration is expected to be a promising strategy to increase process sustainability in the future.

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