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Research Article

Microalgae Biorefinery: Optimization of Starch Recovery for Bioplastic Production

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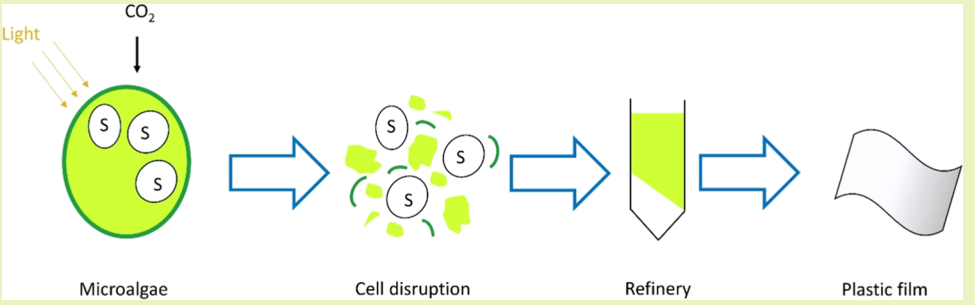

ABSTRACT: Microalgae are a promising source of starch with the potential to reduce land and water footprints as compared to terrestrial plants. However, so far, there are no specific downstream processes to recover microalgal starch. In this work, the development of a lab-scale biorefinery to produce microalgal starch is described. Guidelines are provided on how to set up microalgae cultivation, attaining until 37% starch content and 0.48 g L^{-1} d^{-1} starch productivity in 500 mL lab-scale photobioreactors. Cell lysis by ultrasonication in water and ethanol was studied, obtaining better disruption rates at a lower temperature (≈30 °C) in water. The refinery of the lysate was studied by comparing the conventional Percoll protocol with more potentially scalable methods: aqueous two-phase systems (ATPSs) and ethanol extraction. Ethanol allowed attainment of the best results, separating quantitatively lipids, with reduced pigment degradation, by ensuring higher starch recovery (91%) and starch content (57%) in the refined pellet. Finally, refined starch was used to produce a plastic film that showed mechanical properties comparable to those obtained by using corn starch. This study provides preliminary evidence that microalgal starch could replace conventional starch sources in the biobased industry, possibly with reduced environmental impacts.

KEYWORDS: *starch extraction, Tetradesmus obliquus, bioplastic, water footprint, biopolymers, aqueous two-phase systems, ultrasonication, cell disruption*

INTRODUCTION

Starch is a biodegradable biopolymer that can be used for several industrial applications.[1,2] It may play a relevant role in the replacement of conventional fossil-based polymers in the biobased economy.[3] Currently, starch is produced only from terrestrial plants such as corn and potatoes. However, this production can generate a competition for arable land and water consumption among food production and biomaterial production.[4] Microalgae are promising future alternative sources for starch. As compared to terrestrial plants, starch production from microalgae may have different possible advantages: (i) microalgae may attain starch productivities up to tenfold higher than conventional terrestrial plants;[5] (ii) microalgae may use less water, including wastewaters or saltwater as water source;[6] and (iii) microalgae may be cultivated on nonarable lands. These properties may allow microalgae to potentially produce starch with a lower water and land footprint than conventional terrestrial crops.

Starch content in microalga varies depending on the species and the cultivation conditions. High-accumulating strains can attain up to 40−45% starch as dry weight after few days of cultivation in nitrogen starvation.[5,7,8] In addition to starch, microalgae can be even a source of other biomolecules such as triglycerides (TAGs), pigments (carotenoids, chlorophylls), and high value proteins.[9] Together with starch, these different biomolecules may be extracted in a biorefinery process that is able to produce different end products.

So far, the industry of microalgae has mostly focused on single products from single cultivations. The main products are dry biomass, pigments (astaxanthin, β-carotene, phycocyanin), and omega-3 fatty acids for producing food additives, nutraceuticals, and feed.[10] These applications exploit mainly

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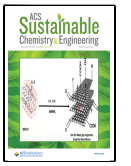

lipids and proteins since they have higher added value than starch when used in food and feed markets. The development of a biorefinery may allow us to valorize different components of the biomass, including starch, especially if it is converted into specific end products such as biomaterials. At present, the biorefinery concept has been studied mostly with experiments at the laboratory scale. The large part of the studies conducted on microalgae biorefinery were focused on TAGs, carotenoids, and proteins,[11] with little attention toward the recovery and refinery of starch. TAGs and carotenoids are conventionally extracted at lab scale with organic solvents from either dried or wet biomass.[12,13] At industrial scale, the company Cyanotech uses supercritical CO_2 to extract astaxanthin.[14] For protein extraction, a common strategy described in scientific studies is cell disruption of wet biomass followed by protein purification using precipitation or ultrafiltration.[15] Some studies also evaluated the extraction of proteins from defatted microalgal biomass, by using alkaline treatments with 0.1−1 M NaOH[16−18] or by using protease enzymes.[19] However, none of these studies evaluated the possibility of coupling these protocols of lipid and protein extraction with starch recovery. Different studies were focused on the extraction of microalgal carbohydrates (that also include starch), but these studies used destructive extraction treatments that involved chemical hydrolysis of polysaccharides to simple sugars.[17,20,21] A main technical issue in starch extraction and purification is its insolubility in almost all solvents. Native starch can be refined only as a result of the removal of nonstarch components, such as lipids, proteins, and other cellular components, by their selective solubilization in suitable solvents. This is the approach followed by the conventional processes used to extract starch from terrestrial plant sources, which include mechanical cell disruption followed by the separation of proteins in a water suspension from which pure starch is obtained by sedimentation or filtration. However, this approach is hardly applicable to microalgae because as compared to terrestrial plants, microalgae have lower cell size (2−10 vs 10−100 μm), lower starch granule size (∼1 vs 10−100 μm), lower starch content (≤45 vs 80−85%), and cell walls are hard to break down due to the presence of recalcitrant polymers such as sporopollenin.[22,23] The studies in which microalgal starch was purified were based on the utilization of a cell disruption method (sonication or bead beating) followed by centrifugation through Percoll gradient.[5,24,25] However, this kind of centrifugation is not applicable at a large industrial scale. Recently, aqueous two-phase systems (ATPSs) have been reported as a promising scalable method to separate microalgal starch from other cellular components after mechanical cell disruption.[26−28] By using ATPS, starch was recovered in the interface or in the pellet phase, with an yield between 67 and 79%.[26,28] However, the reported purity of refined starch obtained was not higher than 50%[28] and no application was tested.

The aim of this work is to contribute to the development of a biorefinery to obtain starch from microalgae to be used for the production of plastic films. In the first part of the work, the evolution over time of starch synthesis by microalgae in a batch reactor was studied in detail to obtain general guidelines to determine the best harvesting moment to maximize starch accumulation and productivity and minimize water footprint. Subsequently, the work investigates different innovative cascade approaches to obtain a pellet phase enriched in microalgal starch in addition to the separation of other biomolecules (proteins, lipids). The conventional protocol based on the Percoll gradient was compared with other protocols that are more easily scalable industrially. Finally, for the first time, a starch-based film using only microalgal starch as a biopolymer is produced and compared to conventional corn starch.

EXPERIMENTAL SECTION

Microalgae Cultivation and Starch Synthesis. A strain of the microalga *Tetradesmus obliquus* (generally known as *Scenedesmus obliquus*) was isolated and identified as previously described.[29] The strain was maintained in solid and liquid BG11 medium, in phototrophic and nonaxenic conditions, as previously described.[29] Microalgae suspension was then transferred to two 500 mL (h = 35 cm, d = 5 cm) column glass photobioreactors (PBRs) at an initial biomass concentration of 0.3 g L^{-1}. The culture medium was filtered through 0.7 μm filters before use. Temperature of the culture was maintained at 28 ± 1 °C and pH at 7.5 ± 0.5 (maintained stable by the CO_2 supply). PBRs were illuminated 24 h with led light lamps (GROWSTAR L-QB1) supplying 500 ± 50 μmol s^{-1} m^{-2} photons (measured by a Gossen Mavolux Digital luxmeter at three different heights of the reactors; conversion factor: 0.0257). Each reactor was fed with 1 L min^{-1} air (filtered by a 0.2 μm filter) and 10 mL min^{-1} pure CO_2. Culture medium composition is reported in the Supporting Information. To study the effect of batch time on starch synthesis, the cultivation was repeated in different batches, under the same conditions, sampled at different times (0−18 d), in two separate column PBRs run in parallel. Collected samples were analyzed for dry weight concentration and starch content inside biomass. The biomass used for the experiments on microalgae biomass processing (cell disruption, extraction) was harvested from different independent batches always at a concentration of 5 ± 1 g/L to ensure homogeneous biochemical composition (starch = 36 ± 3% d.w.). The biochemical composition of the samples used for starch refinery is reported in Table S1. The biomass was harvested by centrifugation at 3000g (10 min), rinsed twice with distilled water, then suspended in distilled water in 20 mL aliquots at 90 g L^{-1}, and frozen at −18 °C until its utilization for the experiments. Dry weight of microalgae suspensions was measured by filtering samples through 0.7 μm glass fiber filters and then dried at 105 °C and weighted. The water footprint for starch was calculated considering the water used for cultivation divided by the amount of starch produced: 1/starch concentration.

Cell Lysis Tests with Ultrasonication. Different kinetic tests were carried out to study the effect of solvent (water and ethanol) with and without cooling for temperature control. For tests with water, microalgal samples (20 mL, 90 g L^{-1}) were defrosted and then directly treated with sonication inside a 50 mL glass reactor (h = 5 cm, d = 4.4 cm), having an external jacket connected to a thermocryostat in which cold water (5 °C) was recirculated. Tests without temperature control were performed only on ethanol to avoid starch gelatinization in water phase.[30]

For tests with ethanol, after defrosting, samples were centrifuged at 3000g (10 min) and the pellet was suspended in 20 mL of ethanol (96%). Tests in ethanol were performed by using the same apparatus used with water, both with and without temperature control. In the test without temperature control, pure ethanol was added at each sampling time to repristinate evaporation losses.

All sonication tests were performed by using the Branson 450 Digital Sonifier (20 kHz, 400 W maximum power) at 60% amplitude (90 μm) and in pulsed mode with t_{on}/t_{off} = 0.3/0.1 s (t_{on} and t_{off} denoting the time interval during which the supply of mechanical energy was active and inactive, respectively). A 12 mm replaceable horn probe was used and immersed inside the suspension at half of the suspension level. Cell concentration was measured at different time intervals throughout the treatment by optical counting in a Thoma chamber by means of an optical microscope. The experiments were carried out in duplicate. Cell disruption yield (Y_L) was measured with eq 1

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$$Y_L = \frac{C_{0,cells} - C_{cells}(T_{on})}{C_{0,cells}} \quad (1)$$

With $C_{0,cells}$ and $C_{cells}(T_{on})$, the initial cell concentration and the concentration that was measured after a certain treatment time T_{on}. The term T_{on} in eq 1 is the sum of the t_{on} times elapsed since the start of the cell disruption treatment ($T_{on} = \sum_{t=0}^{t} t_{on}$). The t_{off} value was not included in the kinetic study because it was assumed that the cell disruption only happened during t_{on} periods, in which the mechanical energy was applied. The t_{off} and t_{off}/t_{on} ratios were set mainly to avoid overheating. Kinetic behavior of the cell disruption treatment is described by a first-order model (eq 2).

$$\frac{dC_{cell}}{dt} = -kC_{cells}(T_{on}) \quad (2)$$

Equation 2 was integrated between $T_{on} = 0$ and T_{on} by setting $C_{cells} = C_{0,cells}$ at $T_{on} = 0$, obtaining eq 3, which was used to estimate the kinetic constant (k) by nonlinear fitting of the experimental data.

$$C_{cells}(T_{on}) = C_{0,cells}e^{-kT_{on}} \quad (3)$$

Refinery of the Microalgae Lysate with Different Methods. Experiments were performed to test different methods to separate lipids and proteins from starch to obtain a pellet phase enriched in microalgal starch.

Conventional Separation through Percoll Gradient. Lysed biomass suspension obtained after sonication in water (with temperature control) at T_{on} 135 min was used. 4.5 mL of lysed biomass suspension was centrifuged at 3000*g* (10 min) in a 15 mL centrifuge tube, and the pellet was washed twice in TE buffer (pH 8; trizma base 10 mM; EDTA 1 mM) and then suspended in 4.5 mL of the same buffer. In the same tube, 9 mL of Percoll solution (17-0891-02 GE Healthcare) was added, and the suspension was vortexed and then centrifuged at 10,000*g* (10 min) at 4 °C. After centrifugation, the supernatant was discarded and the pellet suspended again with 4.5 mL of TE buffer and 9 mL of Percoll for a second cycle of centrifugation. The pellet so obtained was finally dried.

Separation with ATPS. The ATPS comprised polypropylene glycol-400 (PPG-400; Alfa Aesar, ref 40811) and choline chloride (98%, Alfa Aesar, ref A15828) with a 40:14 w/w ratio. For each test, 18 g of distilled H_2O, 7 g of choline chloride, 20 g of PPG-400, and 5 mL (5 g) of lysed microalgal suspension (450 mg d.w. equivalent) were mixed in 50 mL centrifuge tubes. Lysed biomass suspension obtained after sonication in water (with temperature control) at T_{on} 135 min was used. The components were mixed in the dark for 1 h in a rotary shaker at 25 rpm at room temperature, according to the method reported previously.[26,28] Subsequently, samples were centrifuged at 1670*g* (10 min) to favor phase separation, and the upper (PPG-400) and bottom (ChCl) phases were separated with a glass pipet. Tests were performed for 1 extraction cycle and for 3 consecutive extraction cycles. At the end of the extraction, the bottom pellet phase was separated from ATPS, washed once with TE buffer, washed twice with water, and then washed twice with 96% ethanol. The pellet was then dried under a fume hood at an environmental temperature. The experiment was carried out in triplicate.

Separation with Ethanol. Lysed biomass suspensions obtained after sonication in water (with temperature control) and in ethanol (without temperature control) at T_{on} 135 min were used for these tests. 2 mL of lysed biomass suspension was centrifuged at 10,000*g* (5 min), the supernatant was separated, and the pellet was suspended in 1 mL of ethanol (96%), vortexed, and put at 75 °C in a dry block for 10 min, mixing every 2 min. Samples were then cooled and centrifuged at 10,000*g* (5 min), the supernatant was separated, and the pellet was suspended again in ethanol for another extraction cycle. Different consecutive extraction cycles were performed until obtaining a transparent solvent. The extracts were analyzed for pigment concentration as described previously.[28] The pellet was then dried under a fume hood at environmental temperature. The experiment was carried out in duplicate.

For all of the tested methods, the overall mass recovery yield of the pellet obtained (y_X) was calculated by eq 4

$$y_X = \frac{m_P}{m_R} \quad (4)$$

where m_R and m_P are the mass of the raw biomass and the pellet phase, respectively.

Biochemical Analyses. Starch was analyzed in raw microalgal biomass and on pellets obtained with different treatments. Starch mass was quantified by means of selective enzymatic hydrolysis by using a "total starch assay kit" (Megazyme, ref K-TSTA, Ireland), following the protocol previously described.[28,31] The recovery yield of starch (η_{starch}) in the obtained pellets was calculated by eq 5

$$\eta_{starch} = \frac{m_P C_{S,P}}{m_R C_{S,R}} \quad (5)$$

where $C_{S,R}$ and $C_{S,P}$ are the contents of starch in raw biomass and pellet phases, respectively.

Residual content of lipids in the pellet phase was measured by mixing 30 mg of dried biomass with 3 mL of methanol:dichloromethane at 1:2 ratio for 30 min under magnetic stirring. Samples were then centrifuged at 3000*g* (10 min), the supernatant was separated, and fresh solvent was added for repeated extraction cycles until obtaining a colorless solvent. The whole solvent extract was mixed with 0.25 mL of NaCl 0.6% for each milliliter of the organic phase. The extract was mixed and then centrifuged at 3000*g* (10 min), and the upper aqueous phase was discarded. The residual bottom organic phase was dried under a N_2 flow and at 50 °C overnight, and extracted lipids were quantified gravimetrically.

The yield of lipid extraction from biomass was calculated with eq 6

$$y_{lip} = 1 - \frac{m_P C_{L,P}}{m_R C_{L,R}} \quad (6)$$

where $C_{L,R}$ and $C_{L,P}$ are the content of lipids determined in the raw biomass and pellet phases, respectively. Total carbohydrates inside raw biomass and pellet phases were quantified by acid saccharification followed by spectrophotometric determination of sugars with 3-methyl-2-benzothiazolinone hydrazone (MBTH) method (NREL/TP-5100−60957), as previously described.[32,33] The recovery yield of total carbohydrates (η_{carb}) in obtained pellets was calculated by eq 7

$$\eta_{carb} = \frac{m_P C_{C,P}}{m_R C_{C,R}} \quad (7)$$

where $C_{C,R}$ and $C_{C,P}$ are the contents of total carbohydrates in the raw biomass and pellet phases, respectively.

Proteins were determined by suspending 4 mg of biomass into 25 mL of distilled water in a glass bottle. Then, total nitrogen (TN) was analyzed by using the method for water analyses described by IRSA-CNR 4060,[34] based on the oxidation of organic nitrogen to nitrate followed by spectrophotometric analysis. TN was converted in proteins by considering a conversion factor of 6.25. The yield of protein extraction from biomass was calculated by eq 8

$$y_{prot} = 1 - \frac{m_P C_{P,P}}{m_R C_{P,R}} \quad (8)$$

where $C_{P,R}$ and $C_{P,P}$ are the content of proteins determined in the raw biomass and pellet phases, respectively.

The content of chlorophyll a, chlorophyll b, and carotenoids inside pellets and raw biomass was determined by centrifuging biomass suspension at 10,000*g* (10 min), removing the supernatant, suspending the pellet with 1 mL of ethanol (95%), and then heating for 10 min at 75 °C. This procedure was repeated for different cycles until obtaining a colorless solvent. The absorbance of the collected extracts was measured at 664.2 nm, 648.6 and 470 nm, and pigment concentration was quantified by using the equations reported by Lichtenthaler.[28,35]

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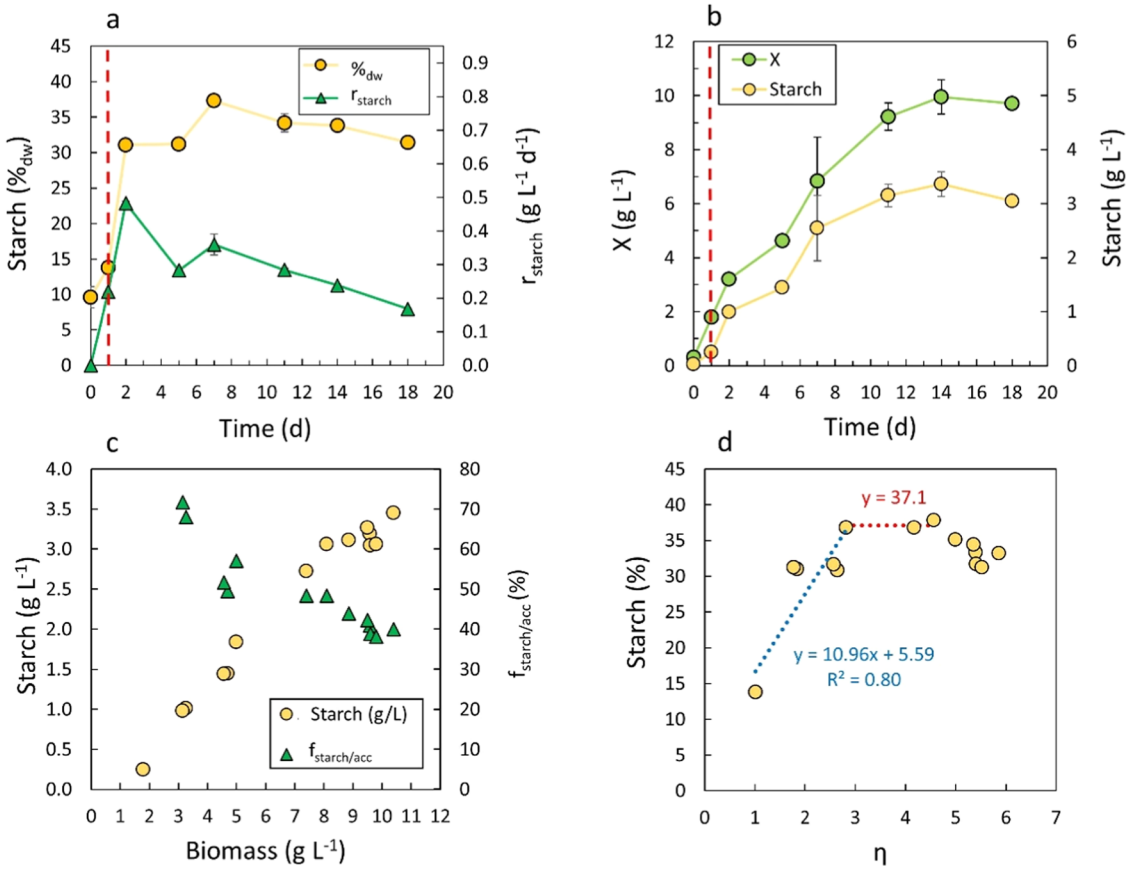

Figure 1. Synthesis of starch by microalgae during cultivation in batch PBRs. The red dashed line indicates the moment of nitrogen depletion in the culture medium. (a) Variation of starch content inside biomass and starch production rate of the batch, for different batch times. (b) Variation of biomass and starch concentration inside the reactor for different batch times. (c) Correlation between starch concentration and biomass concentration (X) and between the fraction of starch accumulated with respect to the total accumulated biomass ($f_{starch/acc}$) and biomass concentration. (d) Correlation between the fraction of accumulated starch inside biomass and the fattening factor (η=X/$X_{N=0}$).

Residual content of solvents in the pellets obtained after extraction carried out to recover starch was determined by drying an aliquot of the sample at 105 °C for 5 h.

Fourier-transform infrared spectroscopy (FTIR) was performed using a Nicolet 6700 (Thermo Fisher Scientific, Waltham, MA) equipped with a Golden Gate ATR accessory (angle of incidence 45°), in a spectral range between 4000 and 650 cm^{-1} with a resolution of 4 cm^{-1} and coadding 200 scans. Absorbance was normalized with respect to the absorbance at 3278 cm^{-1}, corresponding to hydroxyl groups.

Synthesis and Characterization of the Plastic Film Obtained from Microalgal Starch. Two tests were carried out using microalgal starch and commercial pure corn starch. For microalgal starch, the pellet obtained after extraction with ethanol after sonication in ethanol was used. A 2% (w/v) starch solution in water was prepared and heated at 90 °C for 30 min at a 500 rpm stirring rate. Then, it was cooled and maintained at 40 °C for 20 min, followed by addition of 30% (w/w) glycerol (starch basis), and finally the solution was poured on a PTFE dish (d = 6.1 cm) to obtain a film by solvent casting. Thermogravimetric analysis (TGA) was performed under N_2 flux on 5−8 mg of the sample, by employing a Mettler TG 50 thermobalance, at a thermal scanning interval of 25−500 °C and a scanning speed of 10 °C/min. The soluble fraction was measured by submerging a weighted sample for 2 h in water and then drying it for 24 h at an environmental temperature. Water vapor transmission rate (WVTR) was determined by following ASTM method E96 (Standard Test Methods for Water Vapor Transmission of Materials) with some modifications. Circular films were sealed onto a glass container (d = 0.6 cm; h, 3.2 cm) containing 2 mL of water. WVTR was calculated over time, and the value achieved at the steady state (6 h) was taken for comparison (Figure S1). To determine surface wettability of the film, the static contact angle was evaluated by depositing a drop of water onto the film surface, and a picture was captured. The resulting image was processed by Software Motic Image Plus 2.0 ML to determine the contact angle as previously described.[36] Mechanical properties like tensile strength (σ), Young's modulus (E), and elongation at break (ε) of films were determined by tensile tests by using an ISTRON 4502 instrument (Instron Inc., Norwood, MA). For analysis, films were cut into rectangular specimens with a known size and were fixed between two Instron flat plates. Measurements were carried out at a 100 mm/min deformation rate and with a 2 kN load cell.

Statistical Treatment of Data. Experiments were replicated 2, 3, or 4 times, and the results were reported as mean ± standard error (SE). Significant differences (α = 0.05) among treatments were evaluated by using Student's t test, F-test, one-way analysis of variance (ANOVA), and Tukey's post hoc test, by using R and Office Excel. Nonlinear fitting was performed by the MATLAB function "nlinfit", which performs the minimization of the sum of the squared residuals. The uncertainty in model parameter prediction was indicated with 95% confidence intervals (CIs), calculated with the MATLAB function "nlparci".

RESULTS AND DISCUSSION

Synthesis of Microalgal Starch in Photobioreactors. Starch is a reserve compound that can be accumulated by microalgae under different stress conditions. N-starvation is one of the easiest and most effective strategies to achieve relevant starch accumulation because it can be easily applied

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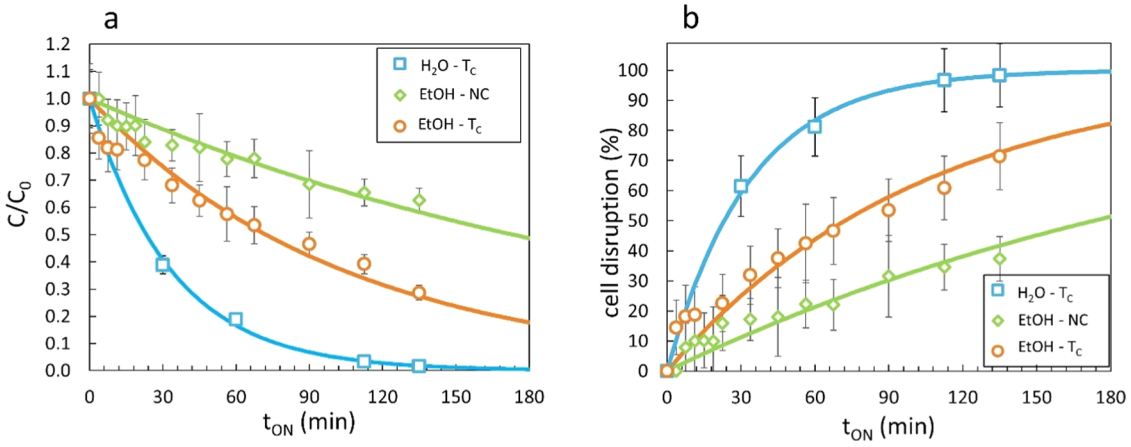

Figure 2. Results of cell disruption kinetic tests performed with ultrasonication. (a) Ratio between cell concentration at different treatment times and initial cell concentration. (b) Cell disruption yield (%) at different treatment times. Tests were carried out with H_2O and ethanol (EtOH) as solvents, with (T_C) and without (NC) temperature control.

even in industrial photobioreactors (PBRs). Cultivation experiments performed in this study were designed based on results obtained in a previous study.[28] Based on these data, we could predict that starting from an inoculum of 0.3 g L^{-1}, N would be depleted after 1 day. After N depletion, all CO_2 uptake is directed toward the accumulation of reserve compounds (starch and lipids), since without nitrogen cells cannot synthesize new functional biomass as proteins and nucleic acids.[37] After 1 day cultivation, biomass increased from 0.3 g L^{-1} to 1.8 g L^{-1} (Figure 1), which is enough for the complete depletion of the 140 mg L^{-1} nitrogen initially supplied (N content in *T. obliquus* grown in balanced growth is 9.6%[28]). Right after N depletion, from day 1 to day 2, the largest increase in starch content was attained, from 13.8 ± 0.1 to 31.1 ± 0.1%, resulting even in the maximum peak of starch productivity (0.48 ± 0.01 g L^{-1} d^{-1}) (Figure 1a). After that moment, starch content inside biomass increased a little more until 37% after 7 days, and then it slowly decreased to 31.4% at the end of the batch (18 d). The maximum starch content attained in this study is comparable with values previously reported for *T. obliquus*.[31] Starch productivity gradually decreased after 2 d, to being 0.168 ± 0.001 g L^{-1} d^{-1} at the end of the batch. After N depletion, biomass increased from 1.8 g L^{-1} to 9.9 ± 0.6 g L^{-1}, remaining stable at the end of the batch (Figure 1b), likely because of the achievement of the maximum accumulation ability (η_{max}).[37] This is a typical behavior for microorganisms grown under N-starvation.[37] The measured increase in biomass under N-starvation is comparable with a value previously reported for *T. obliquus*[31] and among the highest values reported for microalgae.[37] Starch was synthesized until new biomass was produced (Figure 1b,c), achieving a maximum concentration of 3.4 ± 0.2 g L^{-1}, after about 2 weeks of cultivation. This value can be used to make a preliminary estimation of the water footprint that may be obtained for starch production by microalgae cultivation. The 3.4 g L^{-1} concentration obtained in our 500 mL reactor corresponds to 294 L kg^{-1} water consumption, which is about 6-fold lower than the 1671 L kg^{-1} average water footprint of corn starch.[38] However, this comparison, although very promising, is only preliminary. Data obtained at least at the pilot plant scale are needed to make a more reliable comparison.

In addition, it should be considered that microalgal culture medium may be recycled for a certain extent at industrial scale, thus allowing to potentially reduce further the water footprint.[39]

The rate of accumulation of starch became gradually slower than the rate of accumulation of other nonstarch compounds (e.g., triglycerides) (Figure 1c). Indeed, the fraction of starch to the total amount of accumulated biomass ($f_{starch/acc}$) decreased from 68 to 72% (X = 3.1−3.3 g L^{-1}) to 38−41% (X = 9.6−10.4 g L^{-1}). Total accumulated biomass was calculated as the difference between biomass at a certain moment minus the biomass at the point of nitrogen depletion ($X_{N=0}$ = 1.8 g L^{-1}).

The ratio between biomass produced at a certain point to $X_{N=0}$ is defined as the fattening factor ($\eta = X/X_{N=0}$)[37] and can be used as a general parameter, easily measurable, to predict the fraction of accumulated biomass in every cultivation system. It could allow one to predict the fraction of accumulated biomass just from the measurement of biomass concentration and could be used to easily define the best moment to harvest microalgae biomass rich in starch, even without the necessity to measure the starch content. Such relation is shown in Figure 1d. In the initial phase of accumulation (η = 1−3), starch content can be predicted by using a straight-line eq (Figure 1d), while for η = 3−5, starch content remains stable at 37.1 ± 0.6%, and after that it slightly decreases. Using this approach, the prediction ability was assessed on data obtained by performing a new independent experiment for starch accumulation. The coefficient of variation obtained was between 0.01 and 26%, which can be considered acceptable for an industrial plant monitoring. Further validations are required to verify if this method works even with cultivations performed at larger scales, such as pilot and industrial plants.

The best moment to harvest biomass to obtain the maximum starch content should be at η between 3 and 5, since in this range starch is accumulated at the maximum level (37%) and because it allows one to obtain a more easily reproducible starch content (less susceptible to experimental errors on η determination). Harvesting microalgae at a η close to 5 should be preferable since it allows attainment of higher biomass and starch concentration in the reactor, thus reducing harvesting cost and water footprint. In an industrial production

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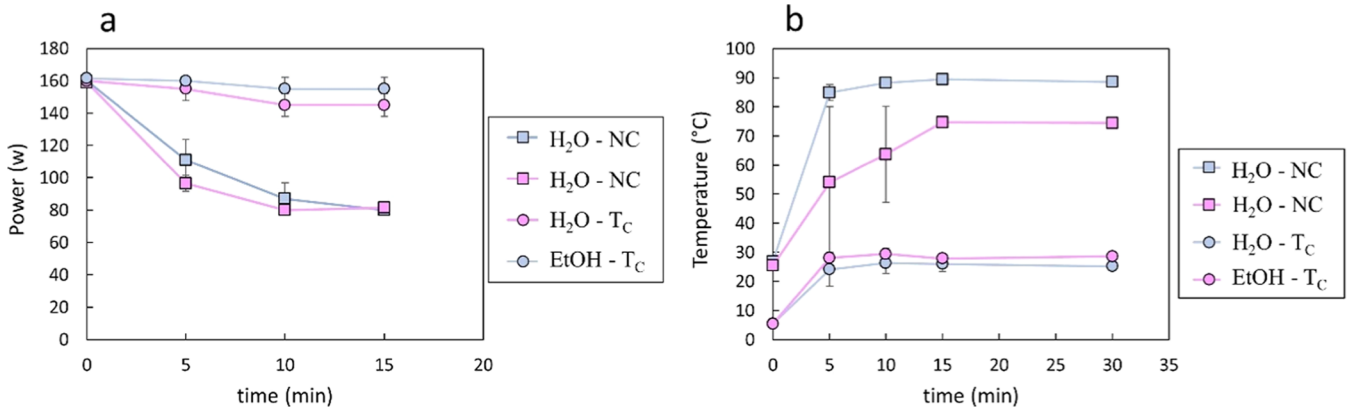

Figure 3. Applied power (a) and temperature profile (b) measured during the sonication tests performed with H_2O and ethanol (EtOH) as solvents, with (T_C) and without (NC) temperature control.

facility, the harvesting phase is usually set to maximize the accumulation or productivity of the most valuable compound. Since other compounds such as carotenoids, lipids, and proteins have larger values than starch, the accumulation of these compounds is expected to take precedence in deciding the harvesting moment. Consequently, harvesting at the optimal moment for starch is expected to be feasible only when it coincides with the best moment for more valuable compounds. This is probably possible for lipid production, since lipids are accumulated along with starch in N-starvation,[31] while it is less feasible with proteins, accumulated in N replete conditions.

Cell Disruption Rate during Sonication in Different Solvents and Temperature. Cell disruption is required to allow starch granules to be released from cellular shells made of cell membranes and cell walls. Different methods can be used for such a purpose, among which sonication and bead milling are some of the most popular for microalgae.[23] In a previous study, we showed that despite sonication having a lower cell disruption rate than bead milling, it allows to recover starch with a higher yield.[28] In another study, it was shown that parameters such as t_{on}/t_{off} ratio in pulsed sonication mode could allow to obtain better extraction rate at lower energy consumption.[33] Solvents can play a fundamental role in optimizing the conditions since physical properties of solvents, as density, can affect the mechanical pressure applied by the sonicator horn.[40] In addition, solvents can play a chemical role by affecting the solubilization of cellular components, thus affecting cell lysis, allowing the extraction of other value-added compounds such as proteins or pigments. The comparison of different solvents indicates that sonication in water at controlled temperature (T_C = 25−30 °C) is much more efficient than that in ethanol (Figure 2). Specific decay rates (k) determined for the first-order model were 0.030 ± 0.002 min^{-1} (τ = 33 ± 2 min) and 0.010 ± 0.001 min^{-1} (τ = 100 ± 10 min), respectively, for water and ethanol ($p < 0.05$). A possible reason for this 3-fold difference could be related to the lower density of ethanol (d = 0.78 g cm^{-3}) than water (d = 1.0 g cm^{-3}), which could affect the applied pressure. However, the difference in applied power was only slightly lower (- 6%) with ethanol (Figure 3a) and not significant ($p > 0.05$). Likely, other reasons, such as the negligible solubility of proteins in ethanol, could be responsible for the different cell disruption rates at controlled temperature. When sonication was carried out in ethanol without any temperature control system, allowing the solvent to attain a temperature close to its boiling point (Figure 3b), the cell disruption kinetics was affected negatively. As shown in Figure 3, the applied power decreases: 48% for water and 44% for ethanol, at increased temperature. For ethanol, the increase in temperature from 28 ± 1 °C to 74 ± 2 °C resulted in a 2.5-fold reduction ($p < 0.05$) of the specific kinetic disruption rate from 0.010 ± 0.001 min^{-1} to 0.0040 ± 0.0009 min^{-1} (τ = 250 ± 50 min). The increase of temperature results in a density decrease of ethanol from 0.78 to 0.74 g cm^{-3}. Such a difference seems too small to justify the difference in the disruption rate. However, it should be considered that when the temperature is close to the boiling temperature, there is a high formation of bubbles that can further reduce the actual density of the solvent, explaining the remarkable reduction in the power applied by the sonicator (Figure 3a). Cell disruption in water was not tested without temperature control because it would induce starch gelatinization,[33] which was outside of the aim of this work (extraction of starch in native form). However, for water, the same trend was observed in terms of applied power (Figure 3a), suggesting the same mechanism involved for ethanol.

Starch Refining. In this study, different methods were tested and compared to refine starch after cell disruption. The aim was to obtain a refined starch sample with yield and purity as high as possible. So far, the reference protocol reported in literature to obtain pure starch from microalgae is based on cell disruption followed by double centrifugation through Percoll gradient.[5,24,25] Using this protocol, authors often stated to obtain pure starch but usually without reporting its purity.[24,25] Only in one study from *Chlorella sorokiniana*, 87% purity was reported.[5] The extraction yield obtained with the Percoll protocol was never reported. In this study, with *T. obliquus*, the yield and purity of starch recovered with the Percoll gradient were 48.5 ± 0.4 and 72 ± 2%, respectively. The purity was lower than that reported for *C. sorokiniana*,[5] possibly due to the fact that *Tetradesmus* species have more recalcitrant cell walls.[23] With an incomplete cell wall breaking, some starch granules can remain embedded inside cell walls, reducing the purity of the recovered pellet. Centrifugation through Percoll gradient is hard to scale up in an industrial scale; therefore, alternative methods have to be found to develop an industrial process. Following preliminary good results obtained in a previous study,[28] ATPSs were tested to separate starch granules from other cellular components. The purity of starch in the pellets obtained after ATPS was 8−10% higher than that

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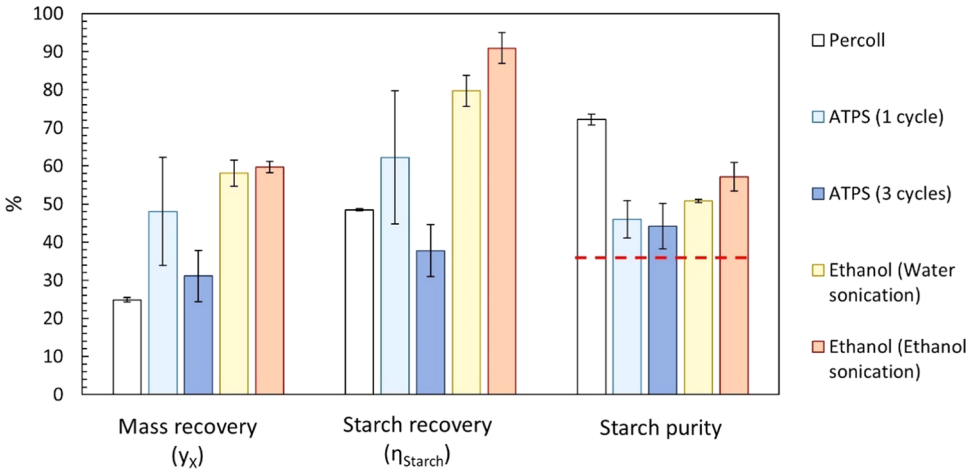

Figure 4. Recovery yield of total biomass (y_X) and starch (η_{starch}), and starch purity in the pellet phases obtained with different treatment methods. The dashed line indicates the purity of starch in the raw biomass. Extraction with Percoll and ATPS was conducted on biomass lysate with sonication in water. Error bars indicate standard errors (n was 4 for ethanol extraction after ethanol sonication, 3 for ATPS, and 2 for the other methods).

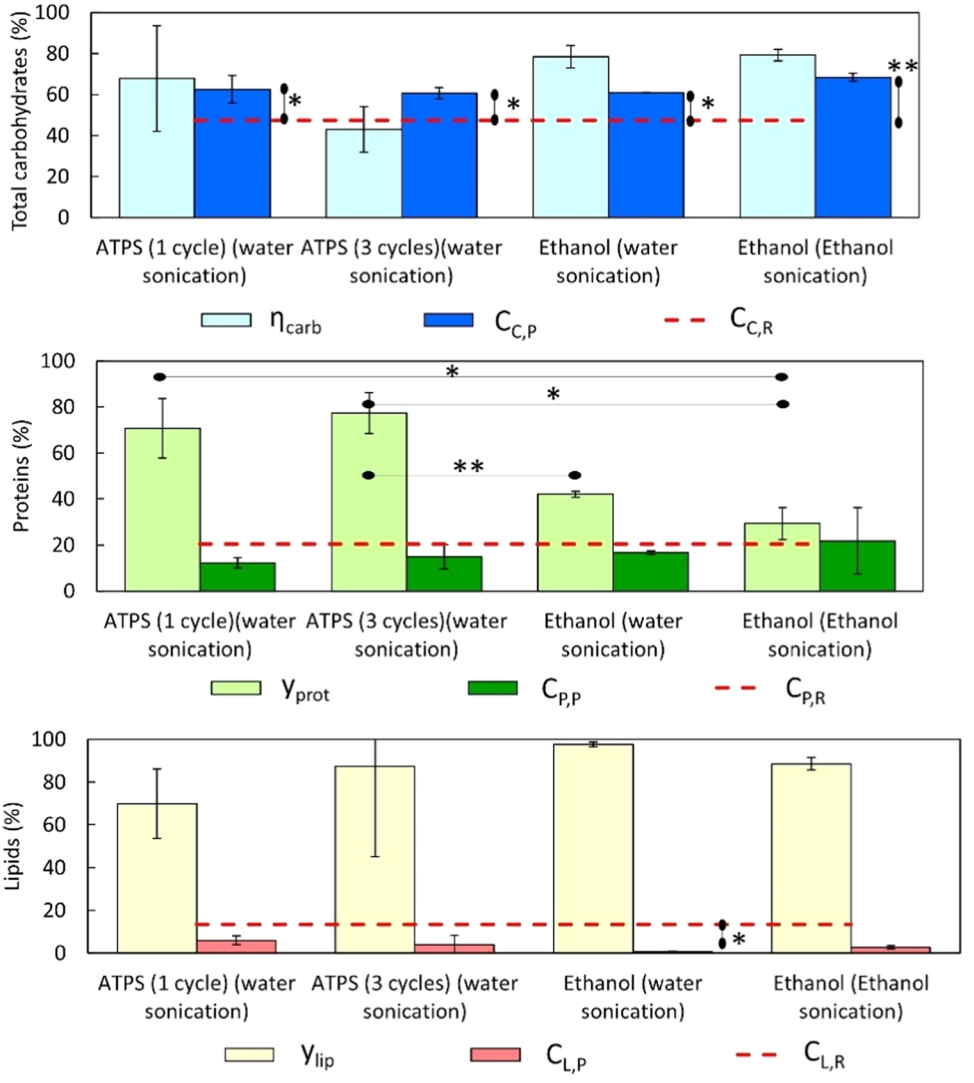

Figure 5. Recovery of total carbohydrates in the pellet phase obtained with different treatments (η_{carb}). Extraction yield of proteins (y_{prot}) and lipids (y_{lip}) from raw biomass obtained with different treatments. Content of total carbohydrates ($C_{C,P}$), proteins ($C_{P,P}$), and lipids ($C_{L,P}$) in the pellet phase obtained with different treatments. Content of total carbohydrates ($C_{C,R}$), proteins ($C_{P,R}$), and lipids ($C_{L,R}$) in the initial raw biomass. Lines and asterisks indicate significant differences (* for $p < 0.05$; ** for $p < 0.01$).

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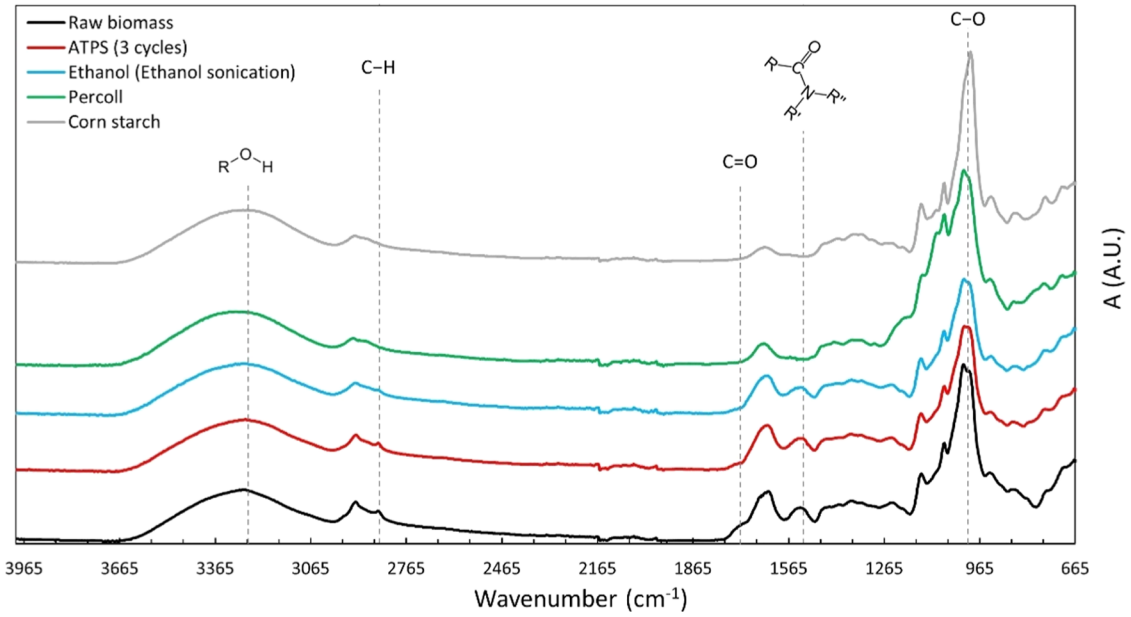

Figure 6. FTIR spectra for different pellets obtained with different treatments compared with raw biomass and pure corn starch.

in the raw biomass (36%), independently of the number of ATPS cycles, but significantly lower than the purity obtained with Percoll (Figure 4). ATPS extraction exhibited higher variability in the recovery yield of starch than those with other methods (F-test, $p < 0.05$), mainly due to the difficulty to reproduce the phase separation and recovery after centrifugation. Increasing ATPS cycles from 1 to 3 reduced starch and mass recovery yield, without improvements in starch purity in the obtained pellet phase. Ethanol extraction after sonication in ethanol resulted in 91 ± 4% starch recovery yields, which was the highest value among the different tested methods. The same method also produced the highest starch content (after Percoll) in the obtained pellet, corresponding to 57 ± 4% (+21% than raw biomass) (Figure 4). This value is higher than the previously reported values with processes alternative to Percoll gradient.[28,41] Optical microscopy confirmed that the refined sample obtained by ethanol extraction after sonication in ethanol was mostly composed of starch granules (Figure S4).

To understand more in detail the mechanisms and the applicative potential of the extractions carried out with ATPS and ethanol, the repartition of total carbohydrates, lipids, and proteins was assessed and is shown in Figure 5. The analyses were conducted for ATPS (1 cycle and 3 cycles) after water sonication and for ethanol after sonication in water or in ethanol. For all conditions, total carbohydrate content in the pellet phase obtained after extraction increased significantly (one-way ANOVA, $p < 0.05$) with respect to that in the raw biomass. As for starch, the highest purity and recovery yield in terms of total carbohydrates was obtained after ethanol extraction from the sample sonicated in ethanol. For this sample, the total carbohydrate content obtained was 68 ± 3% of dry weight, which indicated that in such samples the amount of nonstarch carbohydrates was 11% of dry weight. The protein content in the obtained pellets was lower than that in the raw biomass, but the differences were not significant for all of the tested methods (one-way ANOVA, $p > 0.05$). The extraction yield of proteins with ATPS was 74 ± 10%, remarkably higher than that with ethanol extraction after sonication in both water (42%) and ethanol (29%). The difference is likely due to the higher affinity of the choline chloride water phase of the ATPS for proteins with respect to ethanol, since in ethanol proteins are usually insoluble. Lipids were well extracted by all of the tested methods, with values between 70 and 98%, without any statistically significant difference. This result is given by the good affinity for hydrophobic molecules for both ethanol and polypropylene glycol. Although the difference was not significant, the highest lipid extraction yield (98%) was obtained by ethanol extraction on biomass pretreated by sonication in water. Ethanol extraction after sonication pretreatment in ethanol allowed to achieve 89% lipid extraction yield, which can be considered a satisfactory result, with the advantage to perform both cell lysis and extraction with the same solvent. The content of residual lipids in the pellet phase obtained at the end of the treatment was, for all methods, lower than that in the raw biomass, with a significant difference only for the ethanol extraction after sonication in water. Ethanol extraction allowed to extract even pigments such as chlorophylls (a and b) and carotenoids, which were quantified. Extraction from the sample pretreated with sonication in water allowed to recover 0.30 ± 0.04 mg/g of total carotenoids and 3.5 ± 0.2 mg/g chlorophylls ($a + b$), while extraction from the sample pretreated with sonication in ethanol allowed to recover higher amounts of carotenoids (1.83 ± 0.03 mg/g) and chlorophylls a and b (9.81 ± 0.04 mg/g). The recovery yield of pigments after sonication in water was 25%, remarkably lower than that with sonication in ethanol (84−94%) (Figure S2). This difference was likely due to a higher formation of free radicals in water, as $\dot{O}H$, which are well known to be formed from water thermal decomposition with ultrasound.[42] Free radicals in turn can quickly degrade pigments.[43] Regardless of the solvent used for sonication, all pigments were extracted after about two extraction cycles in hot ethanol (Figure S2).

The chemical composition of the pellet phases obtained with different methods was analyzed with FTIR and compared to raw biomass and pure corn starch (Figure 6) to confirm the purity of starch by comparing the characteristic peaks of

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organic functional groups. From FTIR spectra, starch contribution can be identified by the characteristic large band of hydroxyl groups between 3000 and 3600 cm^{-1}, with a maximum peak at 3278 cm^{-1}.[44] The presence of lipids is indicated by the characteristic bands of $-CH_2$ and $-CH_3$ groups (3000−2800 cm^{-1}) and −C ═O groups (1750−1700 cm^{-1}) from fatty acids.[45,46] Proteins can be identified by amide I and amide II adsorption bands between 1700 and 1500 cm^{-1}.[47] FTIR confirms that the pellet obtained from treatment with Percoll is the one with the highest microalgal starch purity, since the characteristic peaks of lipids and proteins are largely reduced as compared to raw biomass and very similar to the spectrum of corn starch. Comparing the spectra of the pellet phases obtained from ATPS and ethanol extraction, FTIR data confirms that ethanol extraction allowed to obtain a starch purer than raw biomass and ATPS extraction, allowing to separate well lipids, while allowing to separate less well proteins. The FTIR spectrum of the pellet obtained after ATPS extraction was more similar to that of the raw biomass.

Overall, from the comparison of the different methods tested, the extraction with ethanol after cell lysis performed by sonication in ethanol appeared to be the most efficient method to refine starch, lipids, and carotenoids in a biorefinery process. It allows us to obtain higher starch extraction yield, starch purity, and good extraction yield of lipids and carotenoids. Furthermore, because the ethanol evaporation point is used as an intrinsic temperature control system in this setup, external cooling systems can be avoided during cell disruption. Furthermore, ethanol is a solvent quite safe, allowed even for food application, that can be easily recycled by distillation, while the recovery of solvents used in ATPS is still challenging. Moreover, a major problem on the scale-up of such a process is the preservation of biomass quality because microalgae might quickly degrade the accumulated starch after harvesting if the metabolism is not fixed. A quick biomass suspension in ethanol just after the harvesting should easily fix microalgal metabolism allowing avoidance of starch degradation. This method to fix biomass may be applied even on a larger scale.

Synthesis of Films with Microalgal Starch. So far, the utilization of microalgae biomass as feedstock to synthesize biobased materials has been focused mainly toward the utilization of whole biomass, proteins, lipids, or extracellular metabolites, such as lactic acid.[6] Although starch can be up to 40% of the whole microalgal biomass, its industrial valorization has been largely focused on hydrolysis and conversion to glucose monomers to be used as feedstock for further conversion to ethanol or other chemicals.[6] Despite the wide interest in using microalgal starch as a biopolymer for biomaterials,[5,6,48] no bioplastic films made of microalgal starch have been reported so far. Only one study reported the plastification of samples of whole microalgal biomass, with 18% starch content, by extrusion at 120 °C with 30% glycerol.[48] The main limitation that hindered the investigation of microalgal starch as a biopolymer for film production was the lack of protocols to obtain relevant amounts of samples of refined microalgal starch. In this work, we could obtain a sample with 57% starch that was used to synthesize for the first time a starch-based film using microalgal starch as the main polymer, with 30% glycerol as a plasticizer. The material so obtained showed physical and mechanical properties comparable to those obtained with conventional corn starch, for all of the parameters measured (Table 1). For both microalgal and corn starch, the soluble fraction was close to 30%, indicating that it mainly resulted from the leaching of glycerol added as a plasticizer. Tensile strength was in line with typical values previously reported for films made with only corn starch and glycerol.[49] The film obtained with corn starch was white-transparent, while the film with microalgal starch was brown-green. This color might have originated by the denaturation of residual proteins during film preparation at high temperature, as the starting refined starch pellet powder was white (Figure S3). Elongation at break was not statistically different between the two samples (p = 0.052); however, data suggest that the film obtained with microalgal starch was less deformable, possibly because of imperfections inside the film due to residual cell walls. This hypothesis was supported by the images obtained with optical microscopy (Figure S5), in which residual empty cell walls are visible in the refined starch sample. After starch gelatinization, such empty cell walls remained entrapped in the film as heterogeneous components (Figure S6).

Table 1. Physical Parameters Measured for Films Obtained with Corn Starch and Microalgal Starch with 30% Glycerol as a Plasticizer[a]

	corn starch film	microalgal starch film
soluble fraction (%)	33.7 ± 0.1	40 ± 10
contact angle (θ°)	93 ± 8	80 ± 10
WVTR (g h^{-1} m^{-2})	14.5 ± 0.7	12.5 ± 0.8
elongation at break, ε (%)	40 ± 10	9.5 ± 0.7
tensile strength, σ (MPa)	4.0 ± 0.4	5.0 ± 0.8
Young's modulus (MPa)	80 ± 20	93 ± 1

[a]Data are reported as mean ± SD of two replicates.

Thermogravimetric analysis (TGA) of the two films indicated similar behaviors (Figure 7). A first degradation peak was observed between 60 and 100 °C due to residual moisture. For both the starch-based films, the most relevant degradation was observed between 300 and 320 °C, which corresponds to the degradation of starch.[50] Finally, while the corn starch sample attained a complete degradation at 500 °C, about 15% residual mass remained up to 500 °C for the microalgal starch sample, likely due to residual ashes and sporopollenin. The latter is a recalcitrant polymer present in cell walls that requires a temperature of 550 °C for complete degradation.[51] A comparable residual amount (10%) of mass after 500 °C remained even for the raw biomass, confirming that it was mainly due to ashes and sporopollenin. Sporopollenin residues could not be separated since they are insoluble in solvents and remained in the pellet tighter with starch granules.

CONCLUSIONS

The results of this work report the optimization of a process to produce microalgal starch suitable for the production of biobased films. The study covered the entire process from microalgae cultivation to film production.

Microalgae cultivation in a batch reactor is described showing how to find the best harvesting moment to obtain high starch content and productivity. To this end, the fattening factor (η), which is determined from the sole dry weight measurement, allowed us to predict the starch content before harvesting, avoiding costly and long starch analysis.

Among the different conditions tested to purify starch from microalgae, the most promising one (higher starch content and yield) includes an ultrasonication treatment in ethanol without

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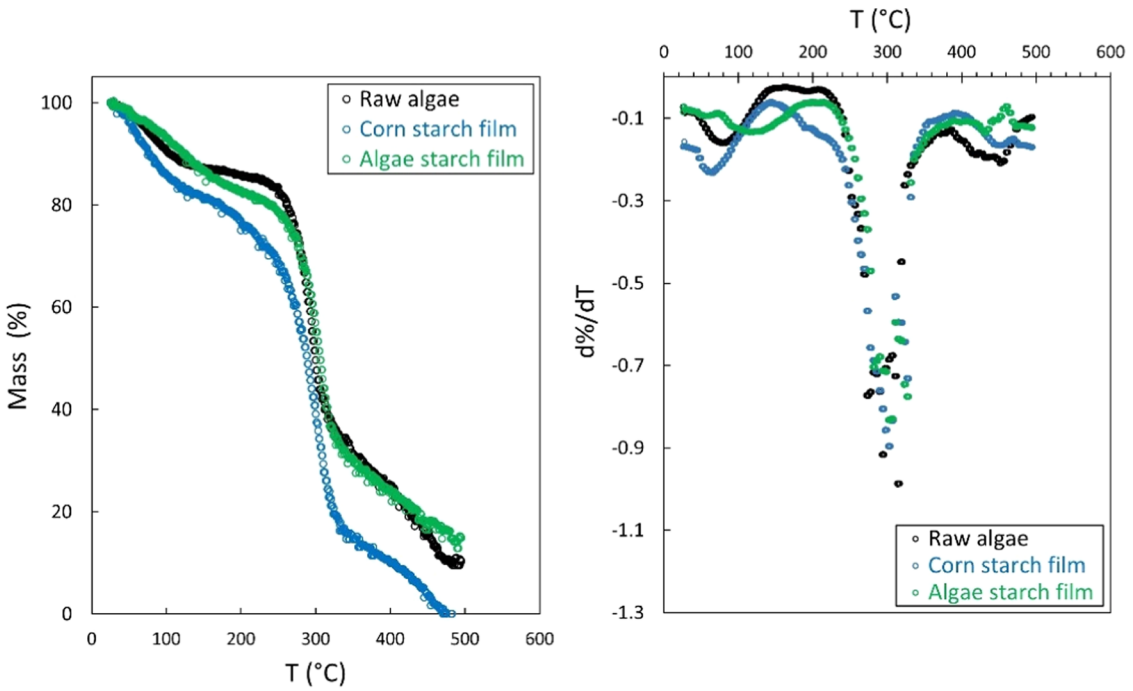

Figure 7. Thermogravimetric analysis (TGA) of raw microalgal biomass and the films obtained with microalgal starch and corn starch (both with 30% glycerol).

temperature control, followed by further ethanol extraction to remove residual lipids and pigments. A final refined white powder with a 57% starch content was obtained. Further optimization work is still required to increase the purity of the microalgal starch.

The refined microalgal starch powder was used to produce, for the first time, a plastic film made of microalgal starch. It was compared with the film obtained with corn starch, finding comparable mechanical properties. These results indicate that microalgal starch may, in the future, replace corn starch in the Bioplastic sector, possibly with lower environmental impacts related to its production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c04133.

S0: Culture medium composition; Figure S1: WVTR kinetics results; Figure S2: Extraction of pigments during ethanol extraction performed after the cell disruption treatment; Figure S3: Refined starch pellet and film obtained with microalgal starch. Figure S4: Lugol staining of the refined starch sample. Figure S5: Images obtained with optical microscopy of the refined starch sample obtained with ethanol extraction after sonication with ethanol. Figure S6: Image obtained with optical microscopy of the refined starch sample after incubation at 95 °C. Table S1: Biochemical composition of the samples employed for the experiments for starch refinery (PDF)

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Notes

The authors declare no competing financial interest.

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