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Sequential Extraction of Carbohydrates and Lipids from *Chlorella vulgaris* Using Combined Physical and Chemical Pre-Treatments

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Abstract: A key focus of microalgae-based fuels/chemicals research and development has been on the lipids that many strains generate, but recent studies show that solely recovering these lipids may not be cost competitive with fossil-derived processes. However, if the carbohydrates can also be recovered and ultimately converted into useful chemical intermediates, this may improve the economics for microalgae-based sustainable product technologies. In the present work, physical and chemical pre-treatments were performed on the *Chlorella vulgaris* microalgae strain to recover the carbohydrates from the biomass primarily in the form of glucose and galactose. The effects of temperature, acid concentration, microalgae solid-to-liquid loading, and hydrolysis time on carbohydrate hydrolysis and recovery was explored to identify optimum conditions. The highest recovery of total carbohydrates, 90 ± 1.1 wt% at 95% confidence which represents 40 wt% of the initial biomass, was obtained using temperature-assisted weak-acid extraction. Sequential extraction of carbohydrates and lipids was then explored. The highest recovery of total lipids was 71 ± 1.8 wt%, which represents 22 ± 0.9 wt% of the initial biomass. The sequential extraction of carbohydrates followed by lipids resulted in an overall recovery of 60 ± 1.6 wt% of the initial biomass, which is higher than current single product recovery strategies. These results suggest that adding carbohydrate recovery may be a viable strategy for overcoming a major economic hurdle to microalgae-derived chemical and fuel production by significantly increasing the yield of usable materials from microalgae biomass.

Keywords: algae carbohydrates; algal biorefinery; lipids; extraction; hydrolysis

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1. Introduction

The production of fuels and chemicals from microalgae lipids remains one of the most sought after alternatives to fossil-derived products [1]. Barriers to widespread commercial application include the costs to recover the lipids out of the microalgae [2]. Recovery of microalgae lipids is a multistep process which includes cultivation, harvesting, lipid extraction, and purification [3]. Many of the proposed processes have relatively high input and recovery costs [4], which are major challenges to commercial microalgae-derived fuel production. Therefore, there is a growing interest in finding technologies to reduce processing costs such as mild cell disintegration methods that efficiently facilitate the extraction of the valuable intracellular compounds, including lipids [5] and increase valorization of the biomass by extracting both lipids and carbohydrates from the microalgae. Both lipids and carbohydrates can then be converted into usable fuel and chemical products which may improve economics and encourage more widespread commercial development [2].

Reports on the extraction of individual intracellular components, i.e., lipids, carbohydrates, and proteins, from microalgae are available in abundance [6]. For example, Debnath et al. published a comprehensive review of many studies focused on optimizing condi-

tions to maximize the carbohydrate content in microalgae plus pre-treatment strategies to optimize bioethanol production [7].

Studies for the sequential extraction of lipids and carbohydrates from microalgae are more limited. Wang et al. isolated the proteins, lipids, and carbohydrates from *Nannochloropsis* sp. to study pyrolysis mechanisms [8], while A. Hernandez-Garcia and co-workers investigated the growth of a consortia of microalgae-bacteria on wastewater followed by the recovery of lipids and carbohydrates for subsequent conversion into valuable products [9]. The focus of this second study was on optimizing biomass growth rather than on optimizing carbohydrate and lipids recovery. Other studies have documented strategies to use alternative low-to-medium energy consuming processes for lipid and carbohydrate recovery such as a pulse electric field, ionic liquids, surfactants [10], supercritical CO_2 [11], or organic solvent solutions [12]. These techniques are still under development and subject to further research before they can be optimized for commercial implementation [13].

Lupatini et al. found that multiple intracellular components such as proteins and carbohydrates can be extracted sequentially from the same sample of *S. plantensis* to recover up to 47 wt% of the biomass as usable compounds [14]. However, since only the pre-treatment processes were optimized, higher recoveries of carbohydrates or proteins should be possible. With up to 60% of the biomass in the form of carbohydrates, mostly as glucans [6], high recovery efficiency and low processing cost for conversion to energy or chemical intermediates are likely possible. This may allow microalgae technologies to be competitive with technologies that process lignocellulosic materials into fuels and chemicals [15,16].

In the present work, the sequential extraction of carbohydrates and lipids from *Chlorella vulgaris* was studied. Optimum conditions were identified for carbohydrate extraction by either microwave- or temperature-assisted weak-acid extraction. Multiple pre-treatment methods were also investigated, such as freeze drying, ball milling and sonication prior to extraction, to determine the method that is most efficient and yields the greatest recovery of carbohydrates, similar to work conducted by Izanou et al. [17]. The present work goes further than Izanou, with an optimization of extraction techniques in addition to cell disruption to maximize yield of usable compounds within the biomass. A benefit of weak-acid extraction for the recovery of carbohydrates is the acid simultaneously extracts the carbohydrates out of the biomass while also converting them into simple sugars, i.e., glucose and galactose [18]. Further, using an initial weak-acid treatment can also increase subsequent lipid extraction efficiency through cell wall hydrolysis [19].

For lipids, this work used the optimum extraction conditions identified by Foerster et al. [20], who performed comprehensive studies of lipid extraction methods and conditions with the same microalgae cultures. Based on their results, we have performed lipid recovery by physically pretreating the biomass by ball milling followed by solvent extraction with methanol at elevated temperatures. Finally, the sequence of extraction—lipids followed by carbohydrates or carbohydrates followed by lipids—was optimized.

2. Materials and Methods

2.1. Materials

Freeze dried autotrophic *C. vulgaris* (80–120 mesh, Qingdao Sunrise Trading Co., Ltd., Qingdao, China) with the cell walls intact was obtained for this study. The freeze dried biomass contained approximately 92% solids with the balance as moisture, as determined by the NREL total solids in biomass determination protocol [21]. Experimentation using microwave-assisted carbohydrate extraction and respective analyses was completed at the University of Leeds, UK, whereas the temperature-assisted carbohydrate extraction and lipid extraction work was completed and analyzed at the University of North Dakota, USA.

2.2. Materials for Carbohydrate Recovery Experiments

Microwave-assisted carbohydrate extraction was performed in a 1200 W StartSYNTH MA084 Labstation (Sorisole, Italy). Quartz sample vessels (cat. #: QB00045) placed inside

a polytetrafluoroethylene reaction vessel (cat. #: DM00082A) were used. The bulk solids were separated from the carbohydrate-rich solvent in a Sigma 4–5 L centrifuge (Osterode am Harz, Germany), and the supernatants were filtered using a single-use 0.45-micron filter (Agilent-model, cat. #: 16555-K) and stored in 1.5 mL HPLC vials (Agilent-model, 5182-0864) for analysis.

Temperature-assisted weak-acid carbohydrate extraction was conducted in a Consolidated Sterilall Electricall heated double wall sterilizer type autoclave (Boston, MA, USA). Liquid samples were then centrifuged using an IEC model HN-SII centrifuge (Needham HTS, MA, USA) and filtered using an acrodisc syringe filter with a 0.2-micron nylon membrane (Pall Corporation cat. #: PN 4540) and stored in a 2 mL HPLC vial (Agilent cat. #: 15337417) for analysis.

Ball milling pretreatment was accomplished using a Retsch MP100 Planetary Ball Mill (Haan, Germany) without cooling. Sonication pretreatment was performed in a Fisher Scientific 5.7 L ultrasonic bath model 15337417 (Pittsburgh, PA, USA).

2.3. Materials for Lipid-Recovery Experiments

Lipids were extracted by placing the biomass in preweighed ThermoScientific 16 mL clear glass vials (cat#: B7999-4, Rockwood, PA, USA). After methanol addition, the samples were heated in an oven (Blue M Stabil-Therm, Blue Island, IL, USA). The methanol solvent was purchased from Fisher Scientific at histological grade (cat#: A4335-22, Fair Lawn, NJ, USA). Cooled samples were vacuum filtered, and the liquid phase, including the methanol and extracted lipids, was stored in the ThermoScientific 16 mL clear glass vials.

2.4. Microwave-Assisted Weak-Acid Carbohydrate Extraction Methods

A full central composite design of experiments with three replicates was conducted to determine optimized weak-acid extraction conditions for carbohydrate recovery. The main factors under investigation were sulfuric acid concentration, contact time of the solvent and biomass, and liquid-to-solids loading. The levels for each factor are presented in Table 1 and were strategically selected based on previous work completed by Hurun et al. [22]. The levels of each factor were adjusted from previous research due to the addition of biomass pretreatments and the use of microwave irradiation instead of the standard autoclave. Factors that were not optimized during the process were microwave power output and the temperature ramp-up time which were held constant at 1100 W and 10 min, respectively. The results from the experimental work were statistically analyzed using the Minitab software (NIST, v.18).

Table 1. Factors and levels investigated in the design of experiments for carbohydrate extraction.

Factors	Acid Concentration (wt%)	Contact Time (Minutes)	Liquid-to-Solids Loading (mL/$g_{biomass}$)
Low	1.0	10	10:1
Center	2.5	15	15:1
High	4.0	20	20:1

Dried *C. vulgaris* was weighed out as 1 g samples and placed into quartz vessels. Triplicates of each sample were prepared at the sulfuric acid concentration, contact time, and liquid-to-solids loading indicated by the design of experiments schedule. Quartz vessels containing the samples were then inserted into polytetrafluoroethylene reaction vessels and capped. The vessels were then attached and secured to the carousel inside the microwave. The microwave program consisted of a 10 min temperature ramp-up time, the desired hold time at temperature, and a 10 min cool-down period. After the microwave program had finished, the liquid and solid contents were removed from the quartz vessels and centrifuged for 10 min at 2500 RPM. The supernatant of each sample was then collected, filtered, and placed into a HPLC vial for analysis.

After completion of the design of experiments, subsequent experiments were explored in order to fully bound the optimum conditions and to further investigate interactions between factors. Multiple trials were completed at the optimum conditions for verification. Ball milling the biomass prior to extraction was used as a pretreatment process in an attempt to increase cell wall rupture and thus increase extraction efficiency. Ball milling consisted of grinding the biomass for 15 min at a rate of 500 RPM in a planetary ball mill.

2.5. Temperature-Assisted Weak-Acid Carbohydrate Extraction Methods

For experiments to study temperature-assisted weak-acid extraction of microalgae carbohydrates, the optimized conditions for acid concentration and liquid-to-solids loading that resulted from the design of experiments with the microwave-assisted-extraction experiments were used. Solvent-microalgae contact time was reoptimized for this method because an autoclave requires longer temperature ramp up and cool down times compared to a microwave. Dried *C. vulgaris* was weighed out as 500 mg samples and placed into pressure tubes with 10 mL of 4 wt% H_2SO_4. The length of time samples were held in the autoclave for extraction was varied from 20 to 90 min. After extraction, each sample was centrifuged at 2800 RPM for 10 min and filtered. The filtered liquid was placed into HPLC vials for analysis.

Two methods of pretreatment were investigated to increase the recovery of carbohydrates prior to extraction in an autoclave: (1) ball milling the dried biomass and (2) sonicating the sample prior to the temperature-assisted extraction. Ball milling consisted of grinding the biomass for 15 min at a rate of 500 RPM in a planetary ball mill. Sonication was conducted in a 110 W ultrasonic bath at a temperature of 40 °C for a length of 30, 60, or 90 min.

2.6. Lipid Extraction Methods

Lipid extraction followed the method and near optimum conditions determined by Foerster et al. [20]. When lipid extraction was performed first in the sequence, the microalgae was pretreated by ball mill grinding for 15 min at a rate of 500 RPM in a planetary ball mill. Then, 500 mg samples of microalgae were weighed and inserted into the reaction vessel. When lipid extraction followed carbohydrate extraction, the dried biomass recovered from the carbohydrate extraction experiments was weighed and placed in the reaction vessels. For both cases, methanol was added at a microalgae-to-solvent ratio of 1:10 (g/mL). The lipids were extracted into the methanol at a temperature of 180 °C with an extraction time at the target temperature of 20 min. After vacuum filtration, both the liquid samples and residual biomass were placed into an oven at 50 °C to remove residual methanol by evaporation. Extracted lipids were quantified gravimetrically.

2.7. Analytical Methods

Liquid chromatography for microwave-assisted carbohydrate extraction was conducted using a Thermoscientific Dionex UltiMate ACC3000 (Dionex Camberley, Camberley, UK) coupled to a Shodex R-101 refractive index detector (Dionex Germering, Germering, Germany). A Sigma-Aldrich Supercogel C610-H organic acid column (cat. #: 59320-U) was used in conjunction with a Shimadzu CTO-10AC column oven (Milton Keynes, UK) to sustain a temperature of 30 °C. The HPLC used an organic acid column with a mobile phase of 0.1% H_3PO_4 in deionized H_2O that had a flow rate of 0.5 mL/min at a pressure of 8300 kPa, with a total run time of 45 min for the RI detector.

For the temperature-assisted carbohydrate extraction, liquid chromatography was completed using an Agilent HPLC 1200 series with an Agilent Hi-Plex H organic acid column (cat. #: PL1170-68530, Stockport, UK) using a dilute sulfuric acid mobile phase (EMD Millipure Corporation H_2SO_4 98% for analysis EMSURE, Chicago, IL, USA) coupled to a refractive index detector (Agilent model G1362A, Santa Clara, CA, USA). The HPLC used a Hi-Plex column with a mobile phase of 5 mM H_2SO_4 that had a flow rate of 0.6 mL/min at a pressure of 6500 kPa, with a total run time of 45 min for the RI detector.

A series of aqueous stock solutions were prepared containing five individual *C. vulgaris*-specific sugars to create a calibration curve for each HPLC system [23]. Total carbohydrate composition of the dry biomass was determined following the NREL two-step acid hydrolysis protocol [24]. The stock solutions contained the following four monomeric carbohydrates, all purchased from Sigma-Aldrich with a purity of ≥99%: glucose (cat. #: G8270-5G), galactose (cat. #: G0750-5G), mannose (cat. #: M2069-5G-KC), and arabinose (cat. #: A3131-5G) as well as L-(-)-fucose purchased from Thermo Fisher Scientific (cat. #: A16789, Heysham, UK). The stock solutions were prepared at known concentrations ranging between 0.1 and 4 mg/mL, which were then used for retention time evaluation and calibration of the individual monomeric carbohydrates.

Lipids recovery was measured gravimetrically which provided a simple method for evaluating lipid extraction efficiency. Gravimetric results were obtained by filtering the extraction solution to separate the residual solids from the lipids-rich solvent mixture. The liquids and solids were then dried to evaporate any remaining solvent and then weighed. This method provided sufficient accuracy for determining: (1) if sequential extraction was feasible, (2) the optimum order of extraction, and (3) the near-optimum extraction conditions. More accurate methods for lipids were employed and are discussed in Foerster et al. [20] who demonstrated that gravimetric analysis predicts the same optimum lipids extraction conditions as total carbon analysis.

3. Results and Discussion

The main objective of this work was to identify optimum conditions for the sequential recovery of lipids and carbohydrates from *C. vulgaris* biomass. A prerequisite goal necessary to accomplish this objective was determining the optimum conditions for the recovery of carbohydrates from *C. vulgaris* biomass. Microwave- and temperature-assisted dilute sulfuric acid extraction with biomass pretreatments were studied. Optimization of experimental conditions was conducted in the microwave, and the experimental method was then repeated in the autoclave so that the two methods could be compared.

Temperature showed a significant effect on the yield of carbohydrates during microwave-assisted extraction. A screening study was completed over a temperature range of 100–140 °C prior to running the design of experiments (results not shown). The study resulted in minimal recovery of carbohydrates below a temperature of 120 °C as there was likely not enough energy to break down the biomass and facilitate the extraction. At temperatures between 120 and 140 °C, degradation of carbohydrates into glucose derivative acids and other unknown components began to occur and the recovery dropped significantly. Therefore, the optimum temperature for microwave-assisted extraction was determined to be 120 °C, which is similar to previous carbohydrate extraction research [6]. A microwave temperature of 120 °C was held constant during the investigation of the other factors varied in the design of experiment study.

Table 2 summarizes the results obtained from the design of experiments study with the microwave-assisted weak-acid carbohydrate extraction method. A total of three trials with ball milled biomass were performed at the identified optimum conditions to determine how the pretreatment method affects carbohydrate recovery. Using the microwave-assisted extraction method, 80 ± 1.6 wt% of the total carbohydrates were recovered from this particular strain of dried *C. vulgaris* without pretreatment and 81 ± 0.98 wt% after being ball milled at a 95% confidence level. Thus, ball milling the biomass prior to the microwave-assisted extraction showed no significant statistical improvement and may be an unnecessary additional energy requirement in the process. These results are consistent with Heo et al. who reported up to 82 wt% carbohydrate recovery with microwave irradiation and no significant increase in recovery when additional mechanical agitation was included [25].

Table 2. Results from a complete triplicated central-composite design matrix performed to identify optimum conditions to maximize the total carbohydrate recovery using the microwave-assisted weak-acid-extraction method. The best case conditions, run 8, are highlighted in boldface.

Run Number	Acid Concentration (wt%)	Contact Time (Minutes)	Liquid-to-Solids Loading ($mL/g_{biomass}$)	Fraction of Total Carbohydrates Recovered (wt%)
1	1.0	10	10	50 ± 3.3
2	4.0	10	10	43 ± 4.6
3	1.0	20	10	39 ± 1.5
4	4.0	20	10	76 ± 2.3
5	1.0	10	20	55 ± 0.6
6	4.0	10	20	62 ±1.4
7	1.0	20	20	56 ± 0.4
8	**4.0**	**20**	**20**	**80 ± 1.6**
9	0.0	15	15	1.0 ± 0.1
10	5.0	15	15	54 ± 2.4
11	2.5	7	15	55 ± 0.7
12	2.5	23	15	53 ± 0.3
13	2.5	15	7	51 ± 0.7
14	2.5	15	23	54 ± 1.3
15	2.5	15	15	54 ± 0.4
16	2.5	15	15	56 ± 0.8
17	2.5	15	15	54 ± 0.4
18	2.5	15	15	52 ± 0.5
19	2.5	15	15	54 ± 1.1
20	2.5	15	15	55 ± 0.6

Previous work [22] suggests that glucose accounts for up to 76% of the total carbohydrates in the biomass, but in the present study (Table 3), the glucose composition at the optimum conditions was around 63 wt% with a slightly higher galactose composition than in previous work. It is important to note that less than 10 wt% of the carbohydrates are unknown longer chain polysaccharides that may require further hydrolysis prior to conversion into fuels and/or chemicals. Therefore, even though the glucose yield is slightly lower than previously reported, the resulting carbohydrate solutions are still a very suitable feedstock for biorefinery processes.

Table 3. The composition of total carbohydrates extracted from the *C. vulgaris* biomass for the maximum total recovery case, number 8, Table 2.

Sugar	Fraction of Total Carbohydrates (wt%)	Confidence Interval at 95%
Glucose	63	±3
Galactose	27	±0.9
Arabinose	6	±0.05
Unknown	4	±2

Examining the statistical results from the design of experiments runs in more detail, it was found that only one interaction parameter was significant within the bounds of the experiments, i.e., the interaction between acid concentration and extraction contact time. The interaction is significant because the recovery of carbohydrates did not show a linear trend with increasing extraction time or acid concentration. Instead, the trend showed slight curvature which required further investigation to bound the optimum time and concentration, as seen in Figure 1. The significance of the interaction was expected because, as acid concentration is increased, less time should be necessary to achieve complete extraction. For the other two interactions, an increase in the condition of either factor will

lead to an increase in carbohydrate recovery. Therefore, the levels of each factor were extended beyond the design of experiments in order to bound the optimum conditions.

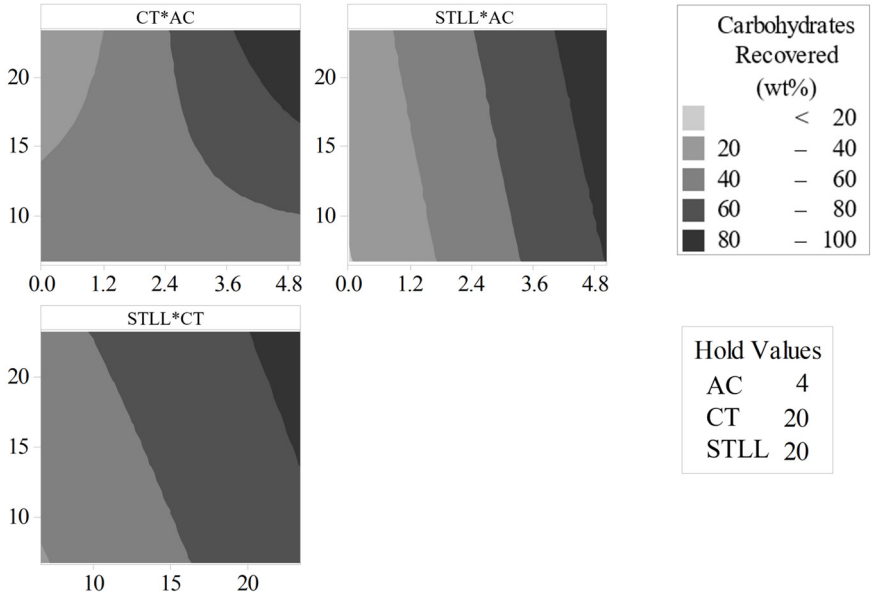

Figure 1. Central-composite design contour plots showing the effect of interactions between significant factors from a study of microwave-assisted carbohydrate recovery and contact time. *AC—acid concentration (wt%); CT—contact time (minutes); STLL—solid-to-liquid loading (mL/$g_{biomass}$).

A second set of microwave-assisted carbohydrate extraction experiments were designed and conducted based off of the results from the initial design of experiments study. The results are summarized in Figure 2. The concentration of sulfuric acid used for extraction and hydrolysis was studied within a range of 1–10 wt% sulfuric acid selected according to Huran et al. [22]. The recovery of carbohydrates increased with an acid concentration up to 4 wt% acid, and then began to decrease (Figure 2a). Therefore, the optimum concentration for the extraction of carbohydrates from the biomass is around 4 wt%. These results are consistent with those reported by Hernandez et al. who observed that carbohydrate extraction decreased with an acid concentration above a 4% solution because of the degradation of monosaccharides into other sugar degradation products (such as furfural, acetic acid, formic acid, and lactic acid) [26].

The extraction contact time was varied from 10 to 40 min (Figure 2b). The recovery of carbohydrates increased until the 20 min mark, where it then began to decrease linearly. Thus, the optimum extraction time is around 20 min. Results for extraction time are 40 min shorter than the results reported by Hernandaz et al. [26] and 5 min shorter than Huran et al. [22] while still maintaining efficient extraction results. The reduced time is most likely due to the optimized liquid-solids loading and the use of microwave technology to increase the cell disruption during contact time.

The liquid-to-solid (solvent-to-biomass) loading was studied over the range of 10–30 mL/$g_{biomass}$. The yield of carbohydrates increased up to 20 mL/$g_{biomass}$ and then began to decrease suggesting an optimum liquid-to-solids loading for efficient extraction of carbohydrates from the biomass of around 20 mL of solvent per gram of biomass (Figure 2c). This optimum is 50 times lower than the ratio reported by Ansari et al. [3], most likely due to the use of a higher acid concentration for the solvent solution in the present work compared to those by the previous research team. A lower liquid loading will reduce the amount of solvent required for the process in addition to reducing the cost of separation of the carbohydrate solution from the solvent.

The optimum conditions (temperature = 120 °C, H_2SO_4 concentration = 4 wt%, extraction contact time = 20 min, liquid solvent-to-solids loading = 20 mL/$g_{biomass}$) deter-

mined for microwave-assisted weak-acid carbohydrate extraction were then used in the temperature-assisted extraction study with the exception of the extraction contact time. The contact time was reoptimized for the autoclave that was used to heat the microalgae/solvent solution because the temperature ramp-up time was different compared to that of the microwave utilized in the previous experiments. In the temperature-assisted extraction experiments, the contact time was varied from 20 to 90 min to determine the optimum length of time for the temperature-assisted carbohydrate extraction method. The results of these experiments are shown in Table 4.

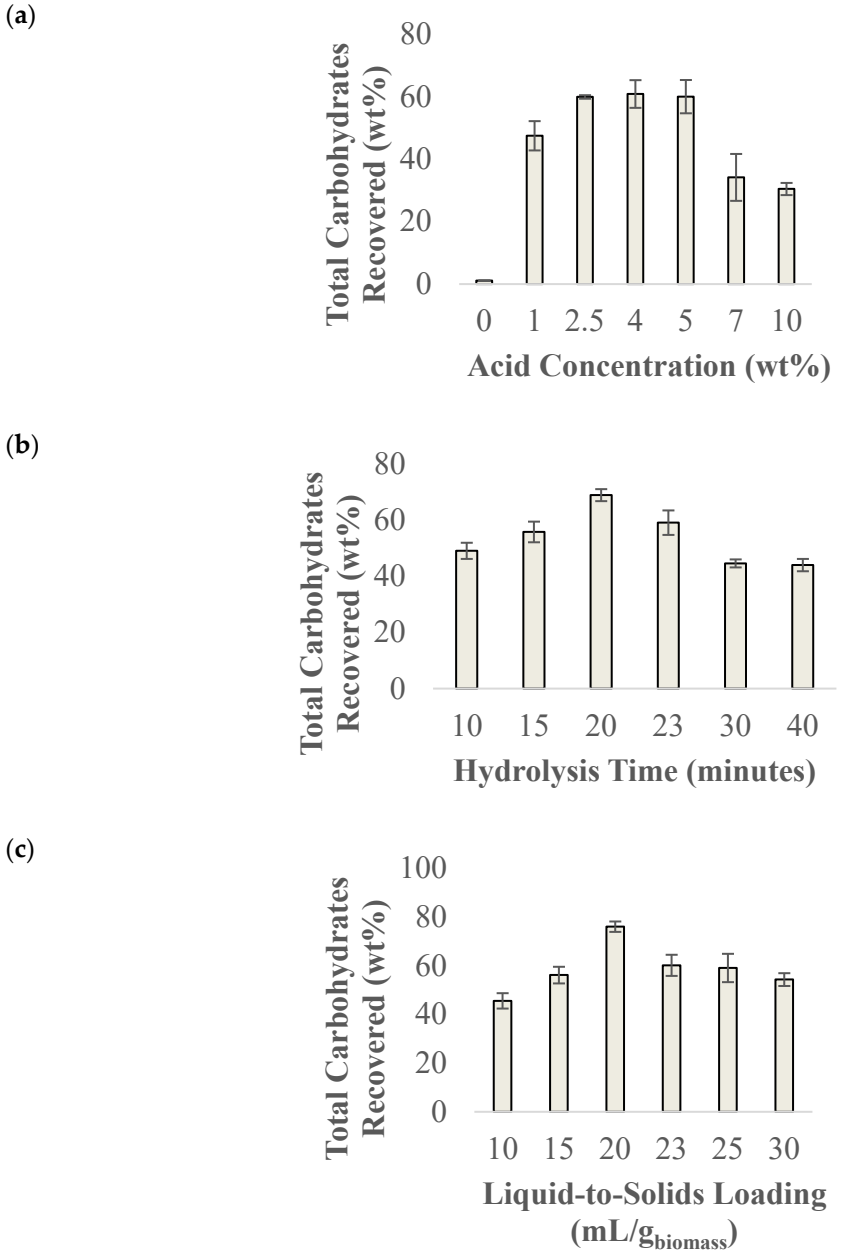

Figure 2. Total carbohydrate recovery results from subsequent experiments to bound the optimum conditions during microwave-assisted extraction at a solid-to-liquid loading of 20 mL/g of biomass and a 20 min contact time: (**a**) varying sulfuric acid concentration, (**b**) varying extraction contact (hydrolysis) time, and (**c**) liquid solvent-to-solids loading.

Table 4. Results from experiments to optimize the contact time for temperature-assisted carbohydrate extraction performed in an autoclave.

Contact Time (Minutes)	Fraction of Total Carbohydrates Recovered (wt%)	Confidence Interval at 95%
20	87	±1
30	90	±1
60	82	±0.5
90	84	±2

The optimum contact time was determined to be around 30 min. The lower carbohydrate recoveries at contact times shorter than 30 min are likely due to incomplete extraction, whereas lower recoveries above 30 min are likely due to carbohydrate degradation into other unwanted or unknown byproducts.

The use of ball milling and/or sonication to pretreat the microalgae prior to temperature-assisted carbohydrate extraction was also studied. The ball milling conditions were not optimized in this study while sonication was varied from 30 to 90 min at a temperature of 40 °C. The results of these studies are summarized in Figure 3.

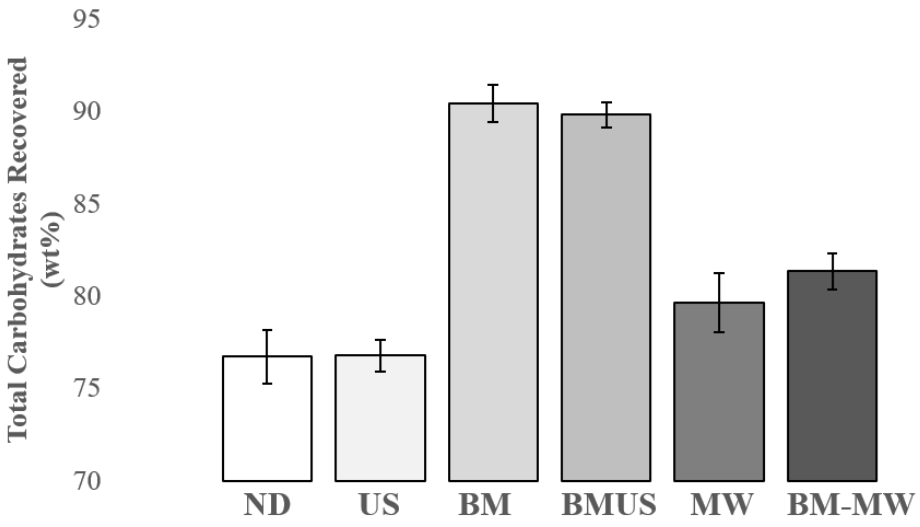

Figure 3. Total carbohydrates recovered for different pre-treatment options combined with extraction at a temperature of 120 °C, H_2SO_4 concentration of 4 wt%, and a liquid solvent-to-solids loading of 20 mL/$g_{biomass}$ for temperature-assisted extraction at an extraction contact time of 30 min for ND—no disruption, US—ultrasonication only, BM—ball-milled only and BMUS—ball-milled and ultrasonication; compared to microwave-assisted extraction at a temperature of 120 °C and an extraction contact time of 20 min for MW—microwave with no pre-treatment and BM-MW—ball-milled followed by microwave-assisted extraction.

In general, the sonication pretreatment with or without ball milling provided no significant statistical improvement (Figure 3) and, therefore, is most likely an unnecessary additional step and energy requirement. Ball milling, followed by a 30 min temperature-assisted acid solvent-microalgae contact time, resulted in the highest total carbohydrate recovery of 90 ± 1.1 wt% at 95% confidence (Table 4). The next best result was obtained using the microwave-assisted acid extraction method with a recovery of 80 ± 1.6 wt% of the total carbohydrates at 95% confidence (Table 2). When the samples were not ball milled prior to temperature-assisted extraction, total carbohydrate recovery reached 71 ± 2.3 wt% at 95% confidence. Thus, there were significant differences in recoveries with each method of extraction and additional pretreatment, as presented in Figure 3.

Previous studies suggest the carbohydrates in *Chlorella's* biomass are primarily stored in the form of starch prior to hydrolysis, which can be partially destroyed and lost when

ball-milling the biomass but can in turn yield more lipids [27]. Although there are some difficulties with the scalability of ball milling, there are significant advantages to the process including simplicity, reproducibility, and low labor requirements [28]. The amount of lost carbohydrates was determined by performing an NREL total carbohydrate determination on samples taken before and after ball milling the biomass. The biomass samples of *C. vulgaris* contained 44 ± 4 wt% of the biomass as carbohydrates, and approximately 10% were destroyed during the ball milling pretreatment process. Table 5 compares the composition of the total carbohydrates extracted for the best of the various carbohydrate extraction methods studied. The composition of carbohydrates from the temperature-assisted acid extraction without pretreatment case resulted in the highest concentration of unknown longer chain carbohydrates most likely due to the lack of increased rupture from either ball milling or microwaves. Therefore, even though some of the carbohydrates are destroyed in the ball milling process, there is still a significant enough increase in carbohydrate recovery and reduction of unknown carbohydrates to justify the use of ball-milling in the optimal method.

Table 5. Composition of total extracted carbohydrates at 95% confidence for microwave- and temperature-assisted acid extraction with and without pre-treatment.

Sugar	Microwave Method (wt%)	Ball-Milled Autoclave Method (wt%)	Autoclave Method without Pre-Treatment (wt%)
Glucose	63 ± 1	58 ± 3	53 ± 0.4
Galactose	27 ± 3	25 ± 0.9	23 ± 1
Arabinose	6 ± 0.5	6 ± 0.02	4 ± 1
Fructose	-	3 ± 0.2	3 ± 0.7
Unknown	4 ± 2	8 ± 1	16 ± 0.9

Identification and quantification of the carbohydrate solutions were performed using a HPLC with a refractive index detector and an organic acid column. A minor issue with this approach is that the retention times for galactose and mannose are sufficiently similar such that they co-elute and are reported as a single peak. Previous work has concluded the presence of mannose in *C. vulgaris* biomass to be less than 2% of the dry weight [23]. Therefore, this peak has been reported herein as galactose in Table 5. Also, up to 17 wt% of the carbohydrate composition has been reported as unknown longer chain sugars that require further work to identify.

The second goal of this work was to identify optimum conditions for the sequential recovery of lipids and carbohydrates from *C. vulgaris* biomass. The order of sequential recovery was included in these studies to determine if there is a difference in recoveries if lipids are extracted first or carbohydrates are extracted first. Foerster et al. [20] have shown that using methanol at a liquid-to-solids loading of 10 $mL/g_{biomass}$ with a 20 min extraction time provided the highest recovery of lipids from among a suite of candidate solvents. Aguirre and Bassi et al. suggest there is a significant difference in extraction efficiency of lipids from *C. vulgaris* when the temperature is increased above 110 °C and that the optimum recovery occurs within the range of 110–200 °C [29]. Foerster et al. determined that the recovery of lipids increased until a temperature of 180 °C, but decreased at higher temperatures [20]. Using the complete optimized conditions (temperature = 180 °C, extraction time = 20 min, liquid-to-solids loading = 10 $mL/g_{biomass}$) resulted in a total lipid recovery of 71 ± 1.8 wt%, which represents 22 wt% of the initial biomass weight at 95% confidence.

The maximum yields obtained for separately extracting lipids and carbohydrates from *C. vulgaris* were found to be 71 ± 1.8 wt% of the total lipids and 90 ± 1.1 wt% of the total carbohydrates in the biomass at 95% confidence, respectively. These values provide an upper limit of the recoveries that could be expected from a sequential extraction method.

In the case of secondary carbohydrate recovery, extraction of carbohydrates from the lipid extracted biomass resulted in a significant decrease of total carbohydrate recovery. The recovery of carbohydrates from the lipid extracted biomass using the optimum extraction conditions defined above was 68 ± 2.8% of the total carbohydrates initially in the biomass (30 wt% of the total initial biomass). Up to 30% of the total carbohydrates initially in the biomass are lost during the pretreatment (ball milling) and lipid extraction processes. However, up to 98 ± 1.3% of the carbohydrates that remained in the biomass after the lipid extraction step were recovered, an 8% increase in recovery efficiency compared to the primary extraction of carbohydrates.

The lipid extracted biomass makes the carbohydrates more available because the lipids contained in the cell wall are removed, allowing better access to the carbohydrates during extraction. Figure 4 shows a simplified mass flow diagram for the loss and recovery of carbohydrates throughout the sequential extraction process. By sequentially recovering lipids followed by carbohydrates, 47 ± 3.1 wt% of the initial biomass weight is recovered as useable lipids and carbohydrates.

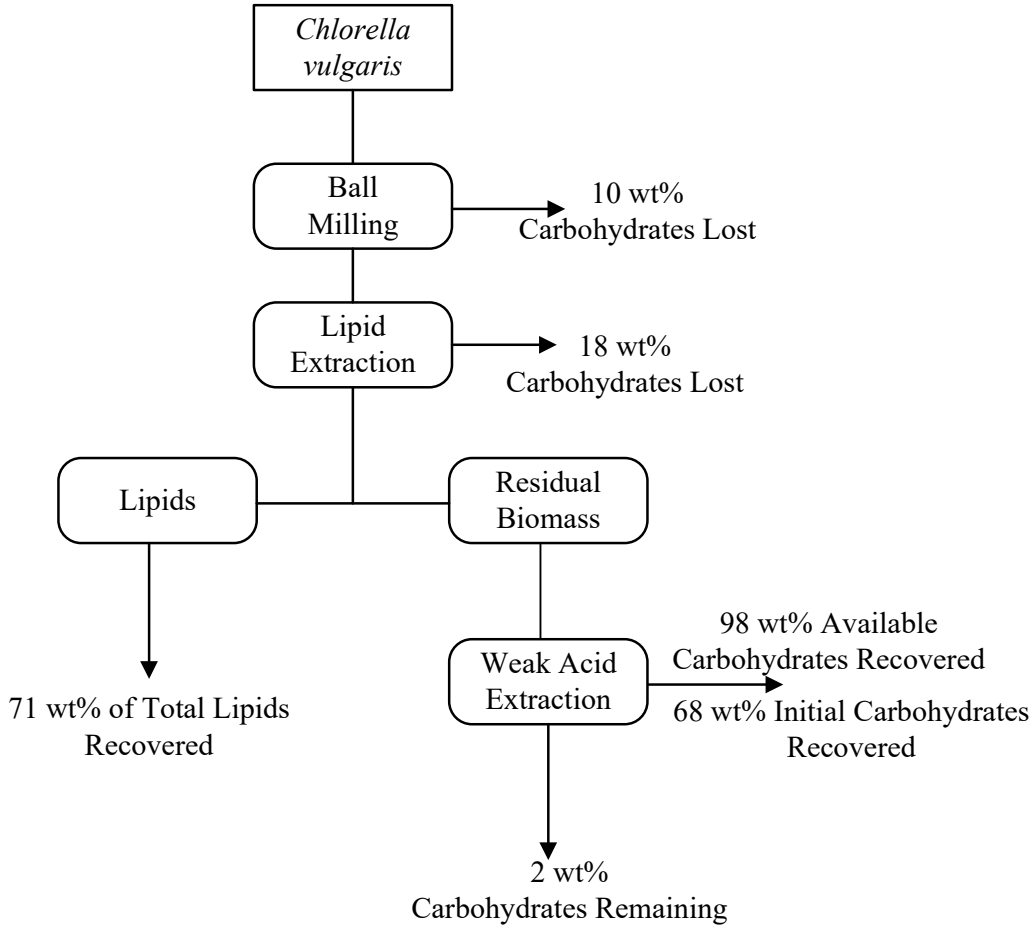

Figure 4. Mass flow diagram showing loss of carbohydrates during each step of the process and recovery of available and initial total carbohydrates.

Multiple attempts were made to recover the carbohydrates that were lost during lipid extraction such as using a hexane/water wash, a hexane/water wash followed by acid extraction, or an acid extraction of residual oils. Neither of the hexane/water wash methods recovered measured quantities of carbohydrates out of the residual oils. Using weak-acid extraction, 50 ± 1.3 wt% of the carbohydrates were recovered from the residual oils (results not shown). The procedure was a simple screening study to see if the carbohydrates could be recovered and will require further work to optimize this method. However, even though some of the carbohydrates can be recovered through a second acid extraction

step, this may be destroying or changing the composition of the oil and could make it unsuitable for further use. To our best knowledge, there are no published works that have looked into recovering lost carbohydrates during the lipid extraction process, so it may be advantageous to research this further.

In the case of secondary lipid recovery, the yield of total lipids decreased from 79 wt% to 59 ± 1.5 wt% (a loss of around 4 wt% of the initial biomass weight) which agrees with research completed by Ansari et al. who reported lipid yield reductions of roughly 20% during secondary extractions [3]. There is potential to further improve lipid yield through a multistage liquid–liquid extraction of lipids post acid hydrolysis as described by Martins et al. [30], but this was not investigated in the current study.

Even though the recovery of total lipids was reduced, 60 ± 1.5 wt% of the initial biomass weight was recovered as carbohydrates and lipids, which is higher than current single product recovery strategies. Sadukha et al. studied the sequential recovery of major biochemical compounds from *Chlorella variabilis* biomass and showed 76.4% recovery of lipids but only 54.6% of carbohydrates which results in a lower overall recovery from the initial biomass [31] than reported here. The remaining 40 wt% of biomass is likely rich in proteins and other bioproducts that also have the potential to be recovered even further as proven by Izanlou et al. [17] and Hildebrand et al. [32] which may further decrease the economic risk of microalgal-based fuels and chemicals.

4. Conclusions

In this study, methods for carbohydrate recovery using microwave- and temperature-assisted weak-acid extraction were investigated to optimize the extraction conditions for *C. vulgaris* microalga for subsequent use in a sequential carbohydrate/lipid extraction scheme. We conclude that a ball milling pretreatment released more of the carbohydrates contained in the cell wall and allowed for a higher recovery in the temperature-assisted weak-acid carbohydrate extraction method but was not necessary for microwave-assisted weak-acid carbohydrate extraction. Optimizing the sulfuric acid concentration and solids-to-liquid loading results in a lower optimum extraction contact time compared to biological treatment and other novel processes, while still yielding comparable carbohydrate recoveries. Up to 91 wt% of the total carbohydrates initially in the biomass can be recovered using the optimized weak-acid-extraction method.

The residual carbohydrate-lean biomass after extraction can be used as a feedstock for lipid recovery using a temperature-assisted methanol solvent extraction method. The secondary total lipid recovery was 20 wt% lower than when lipids were the primary extraction product. However, even though the total lipid recovery was reduced, the sequential extraction of carbohydrates, followed by lipids, resulted in the best overall recovery with 60 ± 1.6 wt% of the initial biomass recovered as carbohydrates and lipids. This relatively high overall yield suggests that transformations into fuels and/or other higher value chemicals may be more economically attractive compared to single product recovery strategies.

Some suggestions for future work include: (1) quantifying the residual sugars content entrained with the biomass after filtration, (2) determining whether the carbohydrates "lost" during ball milling are still in a recoverable form, and if not, what compounds were generated by the process, (3) whether the grinding method affects carbohydrate recovery, such as comparing a ball mill to a knife mill with and without cooling, and (4) the impact of sequential extraction on the composition of the recovered lipids.

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References

1. Griffiths, M.J.; Harrison, T.L. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *J. Appl. Phycol.* **2009**, *21*, 493–507. [CrossRef]
2. Knothe, G.A. A technical evaluation of biodiesel from vegetable oil vs. algae. Will algae-derived biodiesel perform? *Green Chem.* **2011**, *13*, 3048–3065. [CrossRef]
3. Ansari, F.A.; Shriwastav, A.; Gupta, S.K.; Rawat, I.; Bux, F. Exploration of Microalgae Biorefinery by Optimizing Sequential Extraction of Major Metabolites from Scenedesmus obliquus. *Ind. Eng. Chem. Res.* **2017**, *56*, 3407–3412. [CrossRef]
4. Slade, R.; Bauen, A. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. *Biomass Bioenergy* **2013**, *53*, 29–38. [CrossRef]
5. Zhang, R.; Chen, J.; Zhang, X. Extraction of intracellular protein from Chlorella pyrenoidosa using a combination of ethanol soaking, enzyme digest, ultrasonication and homogenization techniques. *Bioresour. Technol.* **2018**, *247*, 267–272. [CrossRef] [PubMed]
6. Visca, A.; Caprio, F.D.; Spinelli, R.; Altimari, P.; Iaquaniello, G.; Toro, L.; Pagnanelli, F. Microalgae Cultivation for Lipids and Carbohydrates Production. *Chem. Eng. Trans.* **2017**, *57*, 6.
7. Debnath, C.; Bandyopadhyay, T.; Bhunia, B.; Mishra, U.; Narayanasamy, S.; Muthuraj, M. Microalgae: Sustainable resource of carbohydrates in third-generation biofuel production. *Renew. Sustain. Energy Rev.* **2021**, *150*, 111464. [CrossRef]
8. Wang, X.; Sheng, L.; Yang, X. Pyrolysis characteristics and pathways of protein, lipid and carbohydrate isolated from microalgae *Nannochloropsis* sp. *Bioresour. Technol.* **2017**, *229*, 119–125. [CrossRef]
9. Hernandez-Garcia, A.; Velasquez-Orta, S.; Novelo, E.; Yanez-Noguez, I.; Monje-Ramirez, I.; Ledesma, M. Wastewater-leachate treatment by microalgae: Biomass, carbohydrate and lipid production. *Ecotoxicol. Environ. Saf.* **2019**, *174*, 435–444. [CrossRef]
10. Ursu, A.V.; Marcati, A.; Sayd, T.; Lhoutellier, S.V.; Djelveh, G.; Michaud, P. Extraction, fraction and functional properties of proteins from the microalgae chlorella vulgaris. *Bioresour. Technol.* **2014**, *157*, 134–139. [CrossRef]
11. Nobre, B.P.; Villalobos, F.; Barragan, B.E.; Oliveira, A.C.; Batista, A.P.; Marques, P.A.S.S.; Mendes, R.L.; Sovova, H.; Palavra, A.F. A biorefinery from Nannochloropsis sp. Microalga—Extraction of oils and pigments. Production of biohydrogen from the leftover biomass. *Bioresour. Technol.* **2013**, *135*, 128–136. [CrossRef] [PubMed]
12. Silva, G.N.; Cerqueira, K.S.; Silva, K.M.; Rodrigues, J.R.S.; Souza, R.R. Extraction of total carbohydrates by solvents from the microalgae Chlorella vulgaris and analysis of the influence of $NaNO_3$ and photoperiods on microalgae cell growth. *Braz. J. Dev.* **2023**, *9*, 2473–2487. [CrossRef]
13. Gouveia, L. From Tiny Microalgae to Huge Biorefineries. *Oceanography* **2014**, *2*, 120.
14. Lupatini, A.L.; de Oliveira Bispo, L.; Colla, L.M.; Costa, J.A.V.; Canan, C.; Colla, E. Protein and Carbohydrate Extraction from S. Platensis Biomass by Ultrasound and Mechanical Agitation. *Food Res. Int.* **2017**, *99*, 1028–1035. [CrossRef] [PubMed]
15. Chen, C.-Y.; Zhao, X.-Q.; Yen, H.-W.; Ho, S.-H.; Cheng, C.-L.; Lee, D.-J.; Bai, F.-W.; Chang, J.-S. Microalgae-Based Carbohydrates for Biofuel Production. *Biochem. Eng. J.* **2013**, *78*, 1–10. [CrossRef]
16. Harun, R.; Danquah, M.K.; Forde, G.M. Microalgal biomass as a fermentation feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* **2010**, *85*, 199–203. [CrossRef]
17. Inzanlou, Z.; Mahdavi, M.A.; Gheshlaghi, R.; Karimian, A. Sequential extraction of value-added bioproducts from three Chlorella strains using a drying-based combined disruption technique. *Bioresour. Bioprocess.* **2023**, *44*, 10.
18. Schulze, C.; Strehle, A.; Merdivan, S.; Mundt, S. Carbohydrates in Microalgae: Comparative Determination by TLC, LC-MS without Derivatization, and the Photometric Thymol-Sulfuric Acid Method. *Algal Res.* **2017**, *25*, 372–380. [CrossRef]
19. Fu, C.C.; Hung, T.C.; Chen, J.Y.; Su, C.H.; Wu, W.T. Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. *Bioresour. Technol.* **2010**, *22*, 8750–8754. [CrossRef]
20. Foerster, I.; Seames, W.; Oleksik, J.; Kubatova, A.; Ross, A. Optimization of the Extraction of Triglycerides and Free Fatty Acids from the autotrophic Strain of the Microalgae Chlorella Vulgaris. *Life* **2023**, *13*, 1997. [CrossRef]
21. Sluiter, A. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples: Laboratory Analytical Procedure (LAP)*; Technical Reports; National Renewable Energy Laboratory: Golden, CO, USA, 2008; p. 9.
22. Harun, R.; Danquah, M.K. Influence of Acid Pre-Treatment on Microalgal Biomass for Bioethanol Production. *Process Bio-Chem.* **2011**, *46*, 304–309. [CrossRef]
23. Ortiz-Tena, J.G.; Rühmann, B.; Schieder, D.; Sieber, V. Revealing the Diversity of Algal Monosaccharides: Fast Carbohydrate Fingerprinting of Microalgae Using Crude Biomass and Showcasing Sugar Distribution in Chlorella Vulgaris by Biomass Fractionation. *Algal Res.* **2016**, *17*, 227–235. [CrossRef]

24. Van Wychen, S.; Laurens, L.M.L. *Determination of Total Carbohydrates in Algal Biomass*; NREL/TP-5100-60957; National Renewable Energy Laboratory: Golden, CO, USA, 2013. Available online: Http://www.nrel.gov/docs/fy14osti/60957.pdf (accessed on 20 December 2023).
25. Heo, Y.; Lee, H.; Lee, C.; Kang, J.; Ahn, J.W.; Lee, Y.; Kang, K.Y.; Choi, Y.; Kim, J.J. An integrative process for obtaining lipids and glucose from Chlorella vulgaris biomass with a single treatment of cell disruption. *Algal Res.* **2017**, *27*, 286–294. [CrossRef]
26. Hernández, D.; Riaño, B.; Coca, M.; García-González, M.C. Saccharification of Carbohydrates in Microalgal Biomass by Physical, Chemical and Enzymatic Pre-Treatments as a Previous Step for Bioethanol Production. *Chem. Eng. J.* **2015**, *262*, 939–945. [CrossRef]
27. To, T.Q.; Procter, K.; Simmons, B.A.; Subashchandrabose, S.; Atkin, R. Low Cost Ionic Liquid–Water Mixtures for Effective Extraction of Carbohydrate and Lipid from Algae. *Faraday Discuss.* **2018**, *206*, 93–112. [CrossRef] [PubMed]
28. Ghaly, M.A.H.A. Microalgae Oil Extraction Pre-treatment Methods: Critical Review and Comparative Analysis. *J. Fundam. Renew. Energy Appl.* **2015**, *5*, 4.
29. Aguirre, A.M.; Bassi, A. Investigation of high pressure steaming (HPS) as a thermal treatment for lipid extraction from Chlorella vulgaris. *Bioresour. Technol.* **2014**, *164*, 136–142. [CrossRef] [PubMed]
30. Martins, L.B.; Soares, J.; Silveira, W.B.; Sousa, R.C.S.; Martins, M.A. Dilute sulfuric acid hydrolysis of Chlorella vulgaris biomass improves the multistage liquid-liquid extraction of lipids. *Biomass Convers. Biorefinery* **2021**, *11*, 2485–2497. [CrossRef]
31. Sadukha, S.; Mehta, B.; Chatterjee, S.; Ghosh, A.; Dineshkumar, R. Sequential Downstream Process for Concurrent Extraction of Lutein, Phytol, and Biochemicals from marine Microalgal Biomass as a Sustainable Biorefinery. *ACS Sustain. Chem. Eng.* **2023**, *11*, 547–558. [CrossRef]
32. Hildebrand, G.; Poojary, M.M.; O'Donnell, C.; Lund, M.N.; Garcia-Vaquero, M.; Tiwari, B.K. Ultrasound-assisted processing of Chlorella vulgaris for enhanced protein extraction. *J. Appl. Phycol.* **2020**, *32*, 1709–1718. [CrossRef]