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Method Article

Extracting protein from microalgae (*Tetraselmis chuii*) for proteome analysis ☆

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A B S T R A C T

Microalgae have high potential as a resource for sustainable and green protein for food or bioactive molecules. Nonetheless, despite the high protein content of microalgae (40 - 70% dry weight) progress in the characterization of their protein composition remains challenging. This is due to the highly variable chemical composition of microalgae strains and factors such as their rigid thick cell wall, polysaccharide content, protein stability, pH. The method described herein was developed to optimize protein extraction for proteome analysis of microalgae (*Tetraselmis chuii*) biomass. The effects on protein solubility of solvent type (organic, denaturing, and non-denaturing) combined with three customized microalgae disruption methods were investigated. The proteome targeted high quality protein extracts were for hydro-soluble proteins recovered by cell disruption using bead milling coupled to centrifugation (protein yield ≈ 13%). The developed method is inexpensive, efficient (yielding high-quality protein extracts with a low content of interfering compounds) and from an industrial perspective easily scalable and compatible with other applications. To add value to the end product we additionally propose the use of stabilizing agents to maintain protein solubility during refrigerated storage and a method targeting the fractionation of low molecular weight proteins.

- An inexpensive easy-to-do 5 step protocol for microalgae protein extracts.
- A protein extraction method free from dangerous or highly polluting chemicals.
- Production of high yield aqueous protein extracts suitable for proteomics.

☆ **SECTION:** Biochemistry, Genetics and Molecular Biology

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Introduction

Microalgae are unicellular photosynthetic organisms, classified in more than 11 phyla that inhabit marine and freshwater environments and like plant cells possess a rigid cell wall (this may be absent in some species) and chloroplasts [1,2]. These phytoplanktonic organisms contribute up to 50% of all aquatic productivity. Their high protein content (40-70% dry weight) makes them highly interesting as a potential source of sustainable, green protein for human nutrition, animal feed, aquaculture and to produce functional foods and bioactive peptides [3–9]. Microalgae proteins are currently exploited by industry as natural crude extracts or partially purified extracts [10,11]. However, extraction of high-quality protein from microalgae remains a technological challenge due to, i) limited protein availability caused by the rigid cell wall, ii) the high concentration of anionic or nonpolar polysaccharides and iii) inherent problems linked to protein stability. Therefore, there is an urgent need to develop efficient technologies directed at extraction of microalgae proteins that consider intrinsic and extrinsic factors that influence protein availability and quality. Intrinsic factors include the species/strain, the rigidity of the cell wall, its thickness and composition, the growth phase of the microalgae and the culture conditions [12–14]. Extrinsic factors such as extraction solution characteristics (e.g. pH and salt concentration), contact time and temperature significantly influence protein solubilization and bioactivity, thus affecting the efficiency of protein extraction [13,15,16]. Serious constraints are linked to protein availability, quality, and stability in microalgae extracts and this seems to be dependent on the strain under analysis. Furthermore, protein characterization in microalgae extracts is infrequent and there is a need for systematic analyses of protein profiles to identify potentially exploitable extracts and proteins.

Microalgae vary greatly in their composition even within the same species and this is particularly true regarding the cell wall [18,19], which means that there is no single optimised method for cell disruption and protein extraction for all microalgae [10,20]. The limited progress in protein characterization in microalgae is associated with difficulties in rupturing the cell wall and the poor extraction efficiencies and yields. Since the cell wall, maintains organism integrity under diverse environmental challenges, increasing the permeability of this structure [16] and disrupting the interaction between the cell wall and proteins, can improve protein solubility in extraction solvents and the efficiency of protein extraction [13]. Currently, a variety of cell disruption technologies have been used for microalgae, including bead milling, high-pressure, ultrasonication, microwave, pulse electric field, cavitation, thermal and chemical disruption methods, or alternatively integration of several methods [12,16–19]. High-pressure homogenization is an effective method for both rigid and fragile cell walls [17], but the impact on proteins is not well known. All potential approaches have advantages and disadvantages, and the choice of the extraction solvent is closely linked to protein extraction efficiency. Acids, bases, aqueous solvents, and surfactants increase the permeability of the cell wall, and alkaline treatments have most frequently been used for microalgae cell disruption and protein solubilization [12,16,17]. Osmotic shock assisted liquid biphasic systems [19], ultrasonication and ionic liquid buffer aqueous solutions [20] or autolytic mechanisms (aqueous enzymatic assisted

extraction) [21] are examples of sustainable and interesting cost-effective integrated methods used for the microalgae *Chlorella vulgaris* (one of the species most exploited by industry) and have yielded good rates of protein recovery. Nonetheless, although protein extraction efficiency is an important parameter it is not correlated with protein integrity an important factor for bioactivity and proteomics analysis. Indeed, the reported microalgae protein extraction methods are for analytical and functional purposes, and few have focused on the proteome [12,13,15–17,22–26]. In proteomics studies it is essential to use protein extracts that give representative and consistent, high-quality results. The protein extraction method needs to be adjusted for sample type since the presence and relative concentration of interfering molecules such as lipids, polysaccharides, phenolic compounds and oxidative enzymes (common in microalgae), can affect the efficiency of protein extraction [12,17] and mass spectrometry analysis. Existing microalgae protein extraction methods targeting proteomics analysis are precipitation-based and lead notoriously to protein loss that may be aggravated by the strain under analysis [26].

The *Tetraselmis chuii* (*T. chuii*) strain, CCFM03, which grows fast and has high nutritional value (including a high protein content $\approx$ 35- 40% of dry weight), was the microalgae used in this study. Interest in CCFM03 is high and it was the first strain of the *Tetraselmis* genus to be marketed, for food and nutraceutical applications [27]. However, the potential of the genus is still under explored and limited information exists about the most appropriate protein extraction methods and the proteome profile [22,25,26,28]. Choosing a suitable cell disruption treatment prior to extraction is one of the most vital steps for downstream processing. Furthermore, extraction methods compatible with the final application and mild disruption techniques such as bead milling and ultrasonication [13,28,29], recover proteins with high solubility, bioactivity and function.

The aim of the present study was to explore mild disruption techniques and a variety of extraction solutions to establish an efficient method for extraction of high-quality *native* proteins for proteome studies from the microalgae, *T. chuii*. The effects on cell disruption and protein solubility of a combination of mild cell disruption techniques and extraction solutions/conditions were evaluated. The tested methodological approaches yield high quality protein extracts, use simple and standard technologies that are compatible with downstream processing and have high potential for scalability and are a step-towards unlocking the potential of proteins from the microalgae *T. chuii*.

Method details

Overview

Three different extraction conditions and three different disruption methods (DM) were customized for lyophilized *T. chuii* biomass and protein extraction efficiency was determined (organic, denaturing and non-denaturing extraction conditions, and disruption methods I, II and III - described below and summarized in Fig. 1). After extraction of *T. chuii*, the solubilized proteins were separated from the disrupted microalgae by centrifugation and the quantity and quality of the solubilized proteins in the supernatant was determined (Fig. 1). The Bradford method was used for protein quantification [30] and one-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess protein quality [31]. Enumeration of microalgae before and after extraction was established using a haemocytometer and was performed for disruption method I to analyze the disruption efficiency.

Biomass for extraction, the microalgae Tetraselmis chuii

The *T. chuii* lyophilized biomass for protein extraction was provided by Fitoplancton Marino, S.L. (FITMAR, El Puerto de Santa María, Spain) [27]. The biomass was produced by initially growing *T. chuii* indoors in 50 mL flasks containing autoclaved seawater (salinity 33 psu) enriched with filter-sterilized f/2 nutrients. The cultures were maintained under standard conditions as previously reported [32]. Small scale microalgae cultures were used to inoculate outdoor photobioreactors (PBRs) and were cultivated under environmental conditions for March. The microalgae were harvested 4 h after sunrise

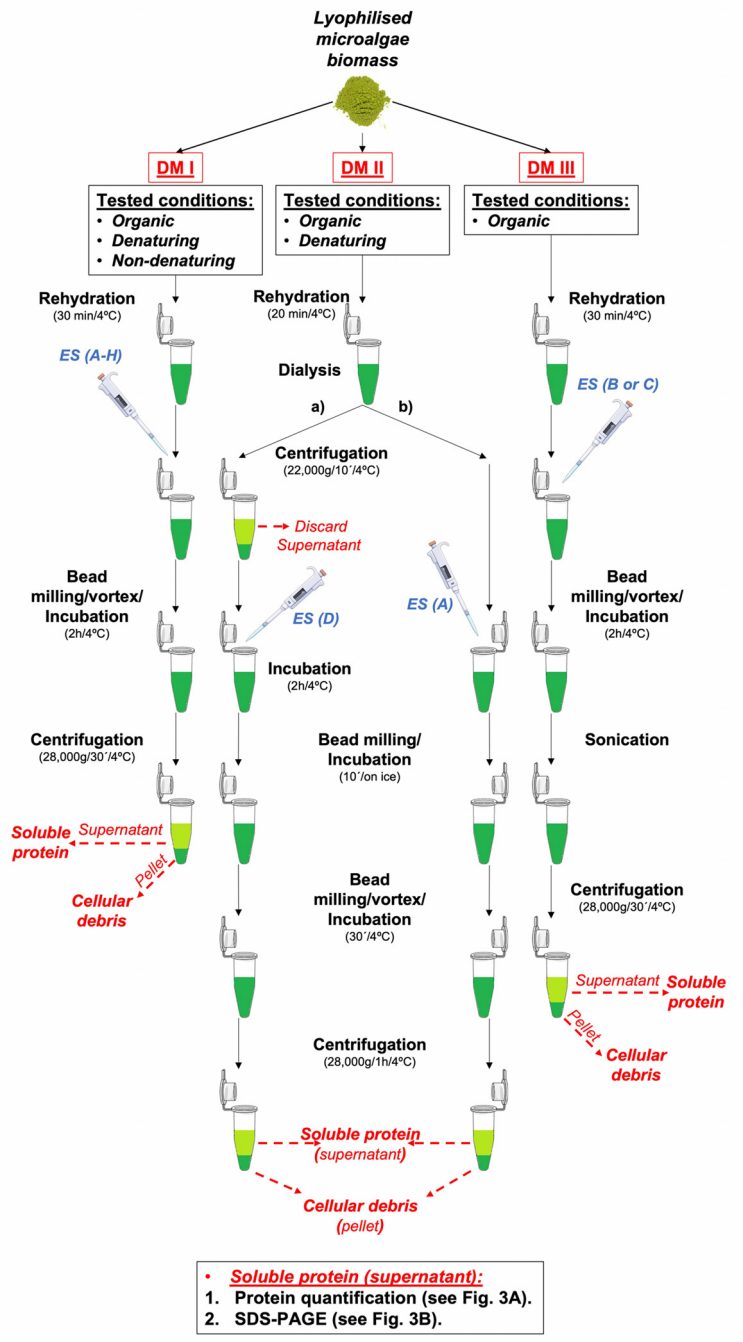

Figure 1. Schematic outline of the experimental design of the protein extraction methods tested using the microalgae *T. chuii*. The disruption methods (DM I, II and III), extraction conditions (organic, denaturing and non-denaturing) and associated technical and analytical methods for the extracted proteins from *T. chuii* microalgae biomass are presented. ES (extraction solution). This figure is linked to Table 1 and 2 of the manuscript.

by centrifugation and thereafter frozen at -20°C, then lyophilized and finally vacuum packed in plastic bags and sent to CCMAR (Faro, Portugal).

Preparation of T. chuii *protein extracts*

Material and reagents

- 2 mL microtube with cap (72.694, SARSTEDT®, Portugal)
- 2 mL microcentrifuge tubes (12034598, AXYGEN®, Portugal)
- MilliQ purified water (Milli-Q® Type 1 Ultrapure Water, Merck, Portugal) (DM I and DM III
- deionised water (Elix® Advantage 10, Merck) (DM II)
- sonicator (Bioblock Vibracell 75186, Spain)
- suspension mixer (SM3000, Digisystem, Portugal)
- Eppendorf refrigerated benchtop centrifuge (M5430R, Eppendorf, Spain)
- iron beads (5 mm, Sigma-Aldrich, Portugal) (DMII)
- zirconia/silica beads (0.5 mm, Sigma-Aldrich) (DM I, II, III)
- stainless-steel beads (0.2 mm, Sigma-Aldrich) (DM I)
- Tissue lyser II (QIAGEN®, Portugal)
- SnakeSkinTM Dialysis Tubing, 3.5K MWCO, 16 mm dry diameter (ID) x 10.7m (Thermo ScientificTM, Portugal) (DM II)
- 1,3-propanediol (Fitoplancton Marino, Spain)
- triton X-100, analytical grade (108603, Merck)
- glycine, analytical grade (104201, Merck)
- sodium hydroxide – NaOH, analytical grade (1064981000, Merck)
- urea (51460, Sigma-Aldrich)
- tris base (MB01601, NZYTech, Lisbon)
- tris hydrochloride (BP153-1, Fisher Scientific)
- sodium chloride - NaCl (137017, Merck)
- ethylenediaminetetraaceticacid - EDTA (324503, Merck)
- dithiothreitol - DTT (D0632, Sigma-Aldrich)
- sodium dodecyl sulphate - SDS (DG092, G-Biosciences, Spain)
- sodium acetate, analytical grade (1062680250, Merck)
- sterile seawater (seawater sterilized by autoclaving and filtered through a 0.22 μm filter)

Note: This list does not include generic laboratory equipment, which is assumed to be available.

Materials are common to all methods reported unless a specific disruption method is specified. A range of extraction solutions with a different composition (A – H) were used for protein extraction from *T. chuii* biomass (Table 1).

Three main disruption methods were tested with different extraction conditions (see Table 1, Table 2 and Fig. 1). In disruption method I (DM I) eight different extraction solutions were tested (**A-H**) [12,25], two different types of beads were used, and two different disruption cycles were applied [29]. In DM II and III different extraction solutions were tested (**A, D** and **B, C**, respectively**)**. Since bead milling is an effective way to release and solubilize intracellular material from microorganisms, this was the main disruptive approach used [22,28,33]. Effectiveness of protein extraction was determined by quantifying the yield and quality of total extracted proteins.

Disruption method I (DM I)

In this method (Fig. 1), all the reported conditions were tested, and organic, denaturing and non-denaturing protein extraction solutions were used (**A**-**H**, Table 1) with two bead milling protocols (4 × 30s pulses or 8 × 30s pulses in case of solutions **C, G** and **H**, Table 2). The lyophilized *T. chuii* was rehydrated in sterile MilliQ purified water (pH ~8.96) at 4 °C. The initial rehydration step was performed in the dark using a suspension mixer (SM3000, Digisystem) with gentle agitation for 30 minutes. This procedure generated a good resuspension of the microalgae (evident from the homogenous green colour of the solution) and minimized the reaction of light sensitive phenolic

Table 1
Chemical composition of the extraction solutions.

Tested condition	Extraction solution	Chemical composition[a]	Characteristics
Organic	A	1,3-propanediol 100% and 1,3-propanediol 90% (v/v) with Triton X-100 10% (v/v)	Mild reagents
	B	1,3-propanediol 100% and triton X-100 100% and 250 mM tris buffer[b] (pH 8.3 at room temperature)	Alkaline
	C	1,3-propanediol 100% and triton X-100 100% and 50 mM glycine-NaOH[c] buffer (pH 10.6 at room temperature)	Alkaline
Denaturing	D	8 M urea, 50 mM tris-HCl[d] (pH 8 at room temperature), 100 mM NaCl[e], 10 mM EDTA[f], 10 mM DTT[g], 1.7% (w/v) SDS[h]	Highly denaturing
	E	50 mM Tris-HCl[d] (pH 6.8 at room temperature), 100 mM DTT[g], 1.7% (w/v) SDS[h]	Denaturing
Non-denaturing	F	triton X-100 10% (v/v), 100 mM sodium acetate buffer (pH 4.0 at room temperature) and 100 mM sodium acetate buffer (pH 4.0 at room temperature)	Acidic
	G	MilliQ water (pH 8.96 at 4 °C)	Freshwater
	H	Sterile seawater (pH 8.10 at 4 °C)	Seawater

[a] Composition of the extraction solutions used before addition to the rehydrated biomass
[b] tris buffer (152.5 mM of tris base, 97.5 mM of tris hydrochloride)
[c] glycine-sodium hydroxide
[d] tris - hydrochloride
[e] sodium chloride
[f] ethylenediaminetetraacetic acid
[g] DL-dithiothreitol
[h] sodium dodecyl sulphate. The final concentration of the solutions is indicated throughout the methods text.

Table 2
General overview of the tested protein extraction conditions combined with the different cell disruption methods.

Tested condition	Extraction solutions	Cell disruption		
		Disruption method	Beads	Cycles
Organic	A	DM II - bead milling + dialysis	iron (5 mm) + zirconia/silica (0.5 mm)	3 × 30s + 4 × 30s
		DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s
	B	DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s
		DM III - bead milling + sonication	zirconia/silica (0.5 mm)	4 × 30s
	C	DM III - bead milling + sonication	zirconia/silica (0.5 mm)	4 × 30s
		DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s or 8 × 30s
			stainless-steel (2 mm)	4 × 30s or 8 × 30s
Denaturing	D	DM II - bead milling + dialysis	iron (5 mm) + zirconia/silica (0.5 mm)	3 × 30s + 4 × 30s
		DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s
	E	DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s
Non-denaturing	F	DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s
	G	DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s or 8 × 30s
	H	DM I - bead milling	zirconia/silica (0.5 mm)	4 × 30s or 8 × 30s

(+) - the method combines more than one type of homogenization step/beads/disruption cycles; (or) - the method tested two different types of cell disruption cycles, separately; (s) - seconds.

compounds (e.g. chlorophyll) with photosynthesis-related proteins (e.g. chlorophyll-binding proteins). Bead milling followed by vigorous vortexing (2,400 rpm/minute) for 15 second pulses, at 15-minute intervals over 2 hours in the dark caused effective cellular disruption and produced a viscous cellular lysate. The vortexing was found to be important for the successful solubilization of stable proteins and reduced aggregation and interaction with non-proteinaceous compounds, both of which cause precipitation and reduce the protein yield. To harvest the proteins from the microalgae homogenate and separate them from polysaccharides that are highly abundant in the cell-wall and interact with proteins, a final centrifugation step was included (indicated in the detailed protocol).

Protocol

1. Weigh 50 mg of lyophilized microalgae biomass into a 2 mL microcentrifuge tube and add 1 mL of MilliQ purified water at room temperature (the n° of microalgae biomass samples to be suspended should be in accordance with the number of extraction solutions to be tested). Incubate at 4 °C for 30 min in the dark using a suspension mixer at 50 rpm for rehydration of the biomass. After incubation, place the suspended microalgae biomass on ice and ensure that throughout the extraction process the temperature is maintained at 4 °C.
2. Add 300 mg of zirconia/silica beads (0.5 mm, for extraction solutions **A-H**) or stainless-steel beads (0.2 mm, for extraction solution **C**) to a sterile 2 mL microtube with a cap (#72.694, SARSTEDT®) and then add 0.5 mL of the rehydrated microalgae biomass. Always keep the microalgae suspension in the dark on ice.
3. Add each of the extraction solutions (**A-H**, Table 1 and 2) to a 0.5 mL suspension of microalgae biomass (prepared in point 2) as follows: **A** - 0.25 mL (0.1 mL of 1,3-propanediol 100 % and then 0.15 mL of 1,3-propanediol 90% (v/v) with triton X-100 10 % (v/v)); **B** - 0.25 mL (0.15 mL of 1,3-propanediol 100 % and then 0.1 mL of 250 mM tris buffer pH8.3 with triton X-100 10 % (v/v)); **C** - 0.25 mL (0.15 mL of 1,3-propanediol 100 % and then 0.1 mL of 50 mM glycine - NaOH pH 10.6 with triton X-100 10 % (v/v)); **D** and **E** – 0.5 mL; **F** – 0.25 mL (0.15 mL of 100 mM sodium acetate buffer pH 4.0 and then 0.1 mL of 100 mM sodium acetate buffer pH 4.0 with triton X-100 10% (v/v); **G** and **H –** 0.4 ml.
4. Disrupt the microalgae biomass using a Tissue lyser II (QIAGEN®) with 4 cycles of 30 seconds or 8 cycles of 30 seconds.
5. Thereafter, incubate the samples in the dark at 4 °C, for 2 h and vortex for 15 seconds at 15-minute intervals. Keep the temperature at 4°C throughout the extraction procedure to avoid protein degradation and precipitation.
6. Centrifuge the protein extract at 28,000 g at 4 °C, for 30 min and remove the supernatant to a precooled sterile 2 mL microtube and store the sample at -20 °C (see the preservation protocol).

Disruption method II (DM II)

In this method deionized water was used as the rehydrating agent for the microalgae biomass instead of MilliQ purified water, a dialysis step was introduced [15] and organic or denaturing extraction conditions coupled to bead milling [22] were deployed (Fig. 1). Deionized water was used for microalgae biomass suspension as it is used in subsequent dialysis step for salt removal. After resuspension of the microalgae biomass in deionized water, the slurry was rehydrated by incubation (20 minutes) in the dark using a suspension mixer (SM3000, Digisystem) before dialysis. The removal of salt by dialysis ensured the formation of a homogeneous microalgae suspension, which was dark green and highly viscous. After dialysis, one of two procedures was performed: a) collection of the biomass by centrifugation and re-solubilization in a highly denaturing buffer (**D**) in the dark for 20 minutes, or b) by direct addition of a “mild” organic reagent (**A**) to the dialyzed microalgae suspension.

Urea and SDS added in the first procedure (solution **D**) were used to aid the extraction and isolation of hydrophobic proteins by denaturing the microalgae membrane proteins. The other reagents, such as Tris, NaCl, DTT and EDTA were added to stabilize proteins and decrease protein interactions. The addition to a final concentration of 0.53% (v/v) triton X-100 and of 10% (v/v) 1,3-propanediol in the second procedure (solution **A**) was to enhance microalgae cell wall permeability and solubilization of proteins. Cellular disruption was performed using bead milling with different

beads starting with iron beads (5 mm) and 3 disruption cycles of 30 seconds followed by zirconia/silica beads (0.5 mm) with 4 disruption cycles of 30 seconds. Previous studies of bead milling have revealed bead size and composition influence microalgae disintegration and the release of proteins and carbohydrates [29]. A short incubation of the samples on ice (10 minutes) was performed between the bead milling steps to avoid overheating of the samples and denaturation of proteins. After bead milling the microalgae, homogenates were vigorously vortexed for 15 seconds every 15 minutes over 30 minutes in the dark and a final centrifugation step was included to separate the solubilized proteins (as indicated in the detailed protocol).

Protocol

1. Weigh 100 mg of lyophilized microalgae biomass into a 2 mL microcentrifuge tube and add 1 mL of deionised water at 4 °C. Incubate at 4 °C, for 20 min, in the dark using a suspension mixer at 50 rpm. Prepare two independent samples to test two different extraction solutions/steps. The handling of the samples for method (**A**) and (**D**) follows the same procedure until point 3 of the current protocol.
2. Transfer the rehydrated microalgae biomass to a dialysis tube (SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 16 mm dry diameter (ID) x 10.7 m, Thermo Scientific™) previously prepared following the manufacturer instructions. Dialyze at 4 °C in 3000 mL of deionised water for 24 h, using a magnetic stirrer to keep the water gently moving. The deionised water used for dialysis (3000 mL) should be changed three times during this step.
3. Transfer the dialyzed microalgae biomass to a precooled 2 mL microtube with a cap (#72.694, SARSTEDT®).
4. As indicated in point 1, perform two different procedures for each sample: a) collect the microalgae biomass by centrifugation at 2,200 g, 10 min., 4 °C, resolubilize the pellet in 1 mL of denaturing buffer (solution **D**), and incubate at 4 °C for 20 min, in the dark; b) directly add 0.1 mL of 1,3-propanediol 100 % followed by 0.1 mL 1,3-propanediol with triton X-100 10 % (v/v) (solution **A**) to the sample.
5. From this point onwards the procedure is the same for the two sample tubes. Add one iron bead (5 mm) and disrupt the microalgae with a Tissue lyser II (QIAGEN®) using 3 disruption cycles of 30 seconds and then place on ice for 10 minutes.
6. Transfer the suspended pellet of extracted microalgae biomass to a new 2 mL microtube with a cap (SARSTEDT®) containing 300 mg of zirconia/silica beads (0.5 mm). Disrupt the pellet with a Tissue lyser II (QIAGEN®) using 4 disruption cycles of 30 seconds and then place on ice.
7. Incubate the samples in the dark at 4 °C, for 30 min, vortex for 15 seconds at 15-minute intervals during the incubation. Keep the temperature at 4 °C throughout the extraction procedure to avoid protein degradation and precipitation.
8. Centrifuge the protein extracts (supernatant or pellet) at 28,000 g, 4 °C, for 1 h. Collect the supernatant into sterile precooled 2 mL microtubes on ice and store the sample at -20 °C (see the preservation protocol).

Disruption method III (DM III)

Suspend the lyophilized *T. chuii* biomass in sterile MilliQ purified water (pH ~8.96) at 4 °C as in DM I but using a different ratio of dried biomass/volume of water. Method III is summarized in Fig. 1 and the initial rehydration step occurred in the dark using a suspension mixer (SM3000, Digisystem) and generated a homogeneous, viscous suspension of *T. chuii* biomass. After hydration of the biomass extraction buffer was added to give a final concentration of 1.33 % (v/v) Triton X-100 and 30 mM of Tris buffer, pH 8.3 (**B**) or 1.33 % (v/v) Triton X-100 and 6 mM Glycine-NaOH buffer, pH 10.6 (**C**) to enhance the protein extraction and to stabilize the extracted proteins and reduce protein aggregation. Lastly, 20 % (v/v) of 1,3-propanediol was used as a solvent to improve biomass solubilization and reduce extract viscosity. Disruption of the lyophilized microalgae was performed by bead milling followed by vortexing for 15 seconds at 15-minute intervals over 2 hours in the dark at 4°C. The vortexing contributed to solubilize stable proteins, reduce aggregation and interactions with non-proteinaceous compounds. An extra disruption step using sonication, which has

previously been shown to be effective for microalgae disruption [16], was added after the bead milling and vortexing to increase biomass disruption and minimize protein aggregation and precipitation. A final centrifugation step was performed to recover the solubilized proteins from the supernatant and remove microalgae debris.

Protocol

1. Weigh 50 mg of microalgae lyophilized biomass into a 2 mL microtube with a cap (72.694, SARSTEDT®) and add 0.5 mL of MilliQ purified water at room temperature. Incubate at 4 °C, for 30 min. using a suspension mixer at 50 rpm for rehydration of the biomass. After incubation, place the tubes containing the microalgae biomass on ice and add 300 mg of zirconia/silica beads (0.5 mm). Prepare two independent samples for testing two different extraction solutions.
2. Add 0.01 mL triton X-100 (100%) and 0.09 mL of 250 mM tris buffer, pH 8.3 (solution **B**) or 0.01 mL triton X-100 (100%) and 0.09 mL of 50 mM Glycine-NaOH buffer, pH 10.6 (solution **C**), resuspend by pipetting up and down and add to each sample tube 0.15 mL of 1,3-propanediol 100%. Keep the samples on ice.
3. Homogenize the two tubes containing suspensions of microalgae biomass using a Tissue lyser II (QIAGEN®) at a frequency of 30 s^{-1} and with 4 lysis cycles of 30 seconds. Thereafter, incubate the sample in the dark at 4 °C for 2h and vortex for 15 seconds every 15 minutes. Keep the sample on ice to avoid temperature fluctuations and associated protein degradation and precipitation.
4. Sonicate the homogenized samples using an amplitude of 50 % with 5 cycles of 2 second pulses at intervals of 5 seconds.
5. Centrifuge the protein extract at 28,000 g, 4 °C, for 30 min. Keep the two samples on ice before and after the centrifugation. Collect the supernatant from each sample into a new precooled 2 mL microtube. Store at -20 °C (see the preservation protocol).

Efficiency of microalgae disruption

The efficiency of bead milling (using 2 types of beads and a variable n° of disruption cycles) on microalgae disruption was determined using a haemocytometer and three technical replicates (n = 3) for samples from DM I. Briefly, the microalgae suspension was agitated and a 10 μL aliquot diluted 1:10 and 1 μl applied to a haemacytometer. Slides were observed using a microscope (Leica DM IL LED) connected to a digital camera (VWR® VisiCam® HDMI6). Cell disruption efficiency was assessed by determining the number of intact microalgae before and after bead milling as previously described [21]:

$$D_Y = 1 - \frac{N_T}{N_C} \quad (1)$$

Where D_Y is the index of microalgae disruption, N_T is the number of intact microalgae counted after bead milling and N_C is the number of intact microalgae counted in a suspension before bead milling (DM I). Results are presented as the mean ± SEM of the technical replicates (n = 3).

Protein quantification

For protein quantification of the microalgae extracts a colorimetric assay based on the Bradford method can be used [30]. A Quick Start™ Bradford protein assay kit (# 5000201, Bio-Rad) adapted for 96-well microplates (#82.1581, SARSTEDT®) was used following the manufacturer's instructions. Bovine serum albumin was used for the standard curve using a Quick Start BSA Standard Set (#5000207, Bio-Rad) with a linear range of 0.125 - 2 mg/ml. The microalgae protein extracts were diluted 1:2 before use and the absorbance (standards and samples) read using a microplate reader (Tecan Infinite M200) at 595 nm. For protein concentration analyses to eliminate the influence of the extraction solutions and possible reagent incompatibilities a blank control composed of the extraction solution was prepared and quantified under the same conditions. Protein recovery (*Pr, %*) or yield was

determined as described in [19] using the following equation:

$$P_r = \frac{C \times V}{Pi} \times 100\ (\%) \tag{2}$$

where C is the protein concentration (mg/mL) in the final extract, V is the final volume of the protein extract (mL) and Pi is the protein content of the microalgae feedstock (mg). Pi was determined by multiplying the biomass content used in each method by % protein content of the *T. chuii* freeze-dried biomass estimated by elemental analysis to be of 40% (this value was calculated based on total nitrogen (%) of the biomass multiplied by the nitrogen-to-protein conversion factor which corresponds to a constant value of 6.25). Protein recovery is represented as the mean ± standard error of the mean (SEM) of the technical replicates (n = 2 - 8) used in the protein quantification assays.

Analysis of protein quality

One-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the quality of the protein extracts and the proteome profile of microalgae *T. chuii* following the method described in [31]. Protein extracts were run on a vertical gel electrophoresis system (#1658004, Mini-PROTEAN Tetra Cell, Bio-Rad) using standard SDS-PAGE mini-gels (8 × 10 cm) composed of a 5% polyacrylamide stacking phase and 12% polyacrylamide resolving phase. Gels were 0.75 mm thick, and a 10-well comb was used. Samples of *T. chuii* protein extracts (6 μg) were prepared in 2x SDS-loading buffer (100 mM tris-HCl pH 8.6, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200 mM DTT) and run with a PageRuler^{TM} Plus Prestained Protein Ladder (#26620, Thermo Scientific) to permit identification of the molecular weight of extracted proteins. Coomassie blue [staining solution: 40 % (v/v) methanol, 10% (v/v) acetic acid, 0.1 % (m/v) Coomassie brilliant blue R-250; destaining solution: 40% (v/v) methanol, 10% (v/v) acetic acid] and silver staining (#1610449, Silver Stain PlusTM Kit, Bio-Rad, according to the manufacturer's instructions) were used to assess the protein profile of the *T. chuii* protein extracts.

Statistical analysis

Results were analyzed using IBM SPSS Statistics software for Windows, Version 26.0. A non-parametric Kruskal-Wallis unpaired group test was used to identify significant differences in protein yield between the different extraction conditions analyzed. Methods were reproduced between 1 and 4 times and the protein yield was estimated using experimental and technical replicates (n = 2 - 8). One-way Analysis of Variance (ANOVA) was conducted using a Games-Howel *post-hoc* test to identify statistical significance between the cellular disruption index for the DM I extraction conditions (n = 3). The significance cut-off was set at 5% and the results are presented as the mean ± SEM.

Method extension

Protein extract stabilization method (PES)

To obtain high quality proteome results post-extraction, protein stabilization during storage is essential. Microalgae proteins extracted under non-denaturing conditions and solubilized in water tend to aggregate and degrade over time and during thawing. Stabilizers avoid protein degradation by providing a constant concentration in solution. The extracted *T. chuii* proteins were unstable and tended to aggregate and so a method for preservation of the protein extracts was developed. This method involved the addition of stabilizing additives to preserve the intrinsic properties of *T. chuii* proteins before and after freezing. Stabilizers included protease inhibitors to stop protein degradation and buffers to maintain a constant salt concentration and pH, which reduces aggregation and precipitation of proteins. Glycerol was included since it contributes to maintain the native structure of proteins and avoid sample crystallization at low temperatures. Incubation for 2h at 4 °C after addition of the stabilizer improved their action during freezing.

Material and reagents

- 1.75 M tris buffer, pH 8.3 (measured at 4 °C)
- 10x Protease Inhibitor Cocktail powder (#P2714, Sigma-Aldrich) at 4 °C
- 100% glycerol, at room temperature
- MilliQ purified water (Milli-Q® Type 1 Ultrapure Water, Merk) at 4 °C

Protocol

1. Measure the final volume of supernatant from the protein extracts using a 1 mL pipette or by weighing extracts with an analytical balance. After the volume is known, add the following regents to give the final concentration indicated:
    a 50 mM tris buffer, pH 8.3
    b 1x protease inhibitor cocktail
    c 20% of glycerol
    d MilliQ at 4 °C (only to adjust the final volume)

(ii) These reagents favour solubilization of the microalgae protein extracts and limit protein denaturation and precipitation during storage. Keep samples on ice.

2. Vortex for 15 seconds, place the samples on ice and incubate at 4 °C for 2h.
3. Store the samples at -80 °C.

In addition to the optimized procedure outlined above for stabilization of *T. chuii* protein extracts (PES), five solutions containing different combinations of reagents, two different combinations of incubation times and two different combinations of freeze -thaw cycles were tested (Supplementary Table 1).

Protein precipitation using organic solvent (PPOS)

This protocol describes a customized method for separation and concentration of low molecular weight proteins in microalgae extracts (see section: additional information) and is based on a previously published method performed on blood serum [34]. In this method two volumes of acetonitrile containing 0.1% trifluoroacetic acid (TFA) are added to extracts, which are vortexed to generate a dense protein precipitate containing large abundant proteins and to retain in the solvent small proteins and peptides. Since *T. chuii* protein extracts are enriched in proteins of low MW (< 60 kDa) [22] a customized method was developed. The aim was to assess the recovery of low MW proteins in the microalgae protein extracts by substituting the solubilizing solvent of extracted microalgae proteins with acetonitrile. The presence of ion-pairing agents from the TFA dissociate peptides and small proteins from large abundant proteins [35]. Finally, chlorophyll present in the protein extracts are removed from chlorophyll binding proteins by organic phase retention.

In this method, acetonitrile containing 0.1% TFA was added to the soluble microalgae protein extract under non-denaturing conditions (DM I), and immediately vortexed to create protein aggregates. A centrifugation step was performed to separate the protein precipitate from the supernatant. After supernatant removal, the precipitate was dried at room temperature and gently resuspended in water for protein re-solubilization. 1D Tricine-SDS-PAGE [36] was used to assess the quality and profile of the isolated low molecular weight proteins. The acetonitrile precipitation method can also be performed with the stabilized microalgae protein extracts (see preservation method, PES).

Material and reagents

- soluble protein extracted in DM I
- HPLC grade acetonitrile (#34851, Merck)
- Sequencing grade trifluoroacetic acid - TFA (#28901, Thermo Scientific)
- MilliQ purified water (Milli-Q® Type 1 Ultrapure Water, Merck) at room temperature (referred as freshwater)

- Silver stain PlusTM Kit (#1610449, Bio-Rad)
- Eppendorf refrigerated centrifuge (#5810 R, Eppenforf)
- PageRuler^{TM} Plus Prestained Protein Ladder (#26620, Thermo Scientific)
- 2 mL microtube with cap (#72.694, SARSTEDT®)
- electrophoresis chamber and accessories MiniPROTEAN Tetra Cell® (#1658000EDU, Bio-Rad)
- generic laboratory equipment

Protocol

1. Add 2 volumes (0.7 mL) of acetonitrile containing 0.1% TFA to 1 volume (0.35 mL) of soluble protein extract and vortex vigorously (2,400 rpm/minute).
2. Centrifuge the protein extract at 8,600 g, 4 °C, for 5 minutes.
3. Remove the supernatant and let the pellet dry at room temperature (it takes around 30 minutes).
4. Add 0.15 mL of MilliQ purified water at room temperature to the dried pellet. Resolubilize the precipitated protein (dry pellet) by gently pipetting up and down using a 1 mL pipette.
5. Analyze the low-molecular weight proteins by 1D Tricine-SDS-PAGE electrophoresis, following the methodology described in [36].
6. Cast a mini-Tricine-SDS gel (8 × 10 cm) of 0.75 mm thickness (resolving phase of 16% polyacrylamide with an upper layer of 1 cm of 10% polyacrylamide and a stacking phase of 4% polyacrylamide).
7. The sample preparation method for loading onto the gel is the same as described in [35], except that the reducing sample buffer can be used without dilution as the protein isolate is already resuspended in water. Mix 12 μg of total protein with sample buffer and load onto the Tricine-SDS-PAGE gel, include a protein ladder (PageRuler^{TM} Plus Prestained Protein) for evaluation of the MW.
8. Run the samples at an initial voltage of 30 V for around 25 minutes, followed by a constant amperage of 20 mA for 1 hour and 20 minutes at 4 °C.
9. Stain the gel using the silver nitrate method (Silver Stain PlusTM Kit, according to the manufacturer's instructions) and assess the protein profile.

To assess the efficacy of protein isolation by the PPOS method, the supernatant and pellet colours were observed and compared visually with the protein extract from DM I under non-denaturing conditions (extraction solution **G**, 4 × 30 sec. disruption cycles). Both protein extracts were analyzed in a silver nitrate stained 1D Tricine-SDS-PAGE gel and the protein profiles compared.

Method validation

The efficiency of bead milling with silica or stainless-steel beads was determined by assessing the disruption of the microalgae *T. chuii* in an aqueous solution (Fig. 2). It was evident after rehydration of the lyophilized *T. chuii* biomass that numerous intact microalgae cells remained (Fig. 2A). Inspection of the outcome of the different bead milling approaches did not reveal significant differences between methods and all were effective in disrupting the microalgae biomass with an efficiency in between 50-60% (Fig. 2B-E, Supplementary Table 2). The highest cellular disruption index was obtained with extraction condition **G** and bead milling disruption cycles of 4 × 30s (Supplementary Table 2) using silica beads of 0.5 mm and was similar to what was previously observed (60%) using ultrasound and a closely related strain, *T. suecica* [37]. Even higher disintegration rates were obtained for *T. suecica* using ultrasound and wet fresh biomass [29]. Nevertheless, for microalgae species with a rigid or less rigid cell wall, freeze-drying and milling treatments are effective for cell disintegration (70-80%) and membrane permeabilization [38].

Assessment of the efficiency of bead milling and the extraction solutions as determined by protein quantification and SDS-PAGE (Fig. 3) revealed the highest protein yield (21 - 27%) from freeze-dried *T. chuii* biomass occurred with the organic and alkaline extraction conditions of DM I and III (solution **B** with DM I and solution **C** with DM I and III, Fig. 3A). The second highest protein yield (≈ 13%) was obtained in aqueous solutions (solution **G** with DM I). In fact, the combination of mild mechanical

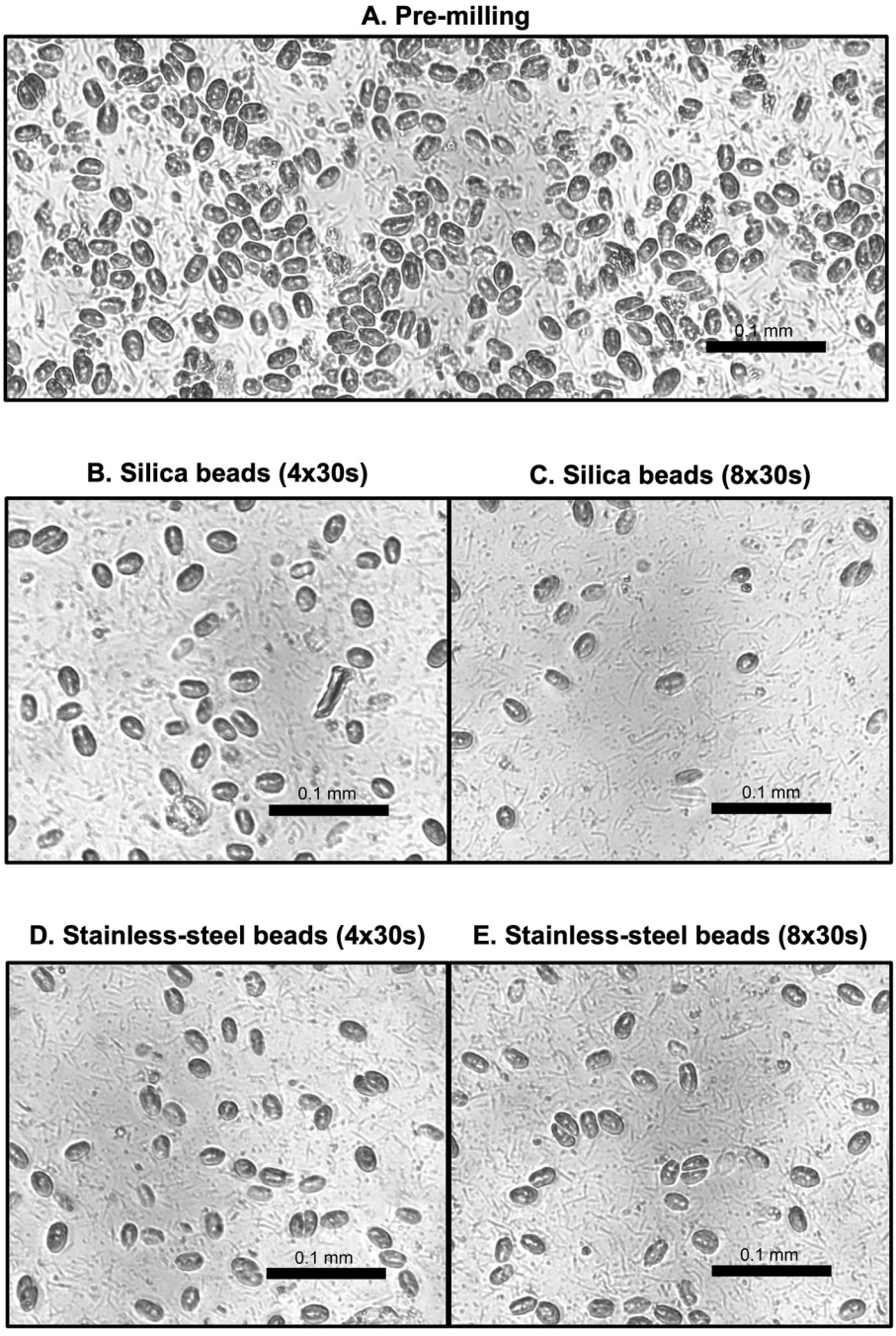

Figure 2. Photographs of lyophilized and rehydrated (with solution **G)** microalgae *T. chuii* before (pre-milling) and after cellular disruption by bead milling (post-bead milling). The biomass was disrupted using the **DM I** protocol and silica or stainless-steel beads using 4 × 30s or 8 × 30s disruption cycles. Image magnification, 20x. This figure is linked to Supplementary Table 2 of the manuscript.

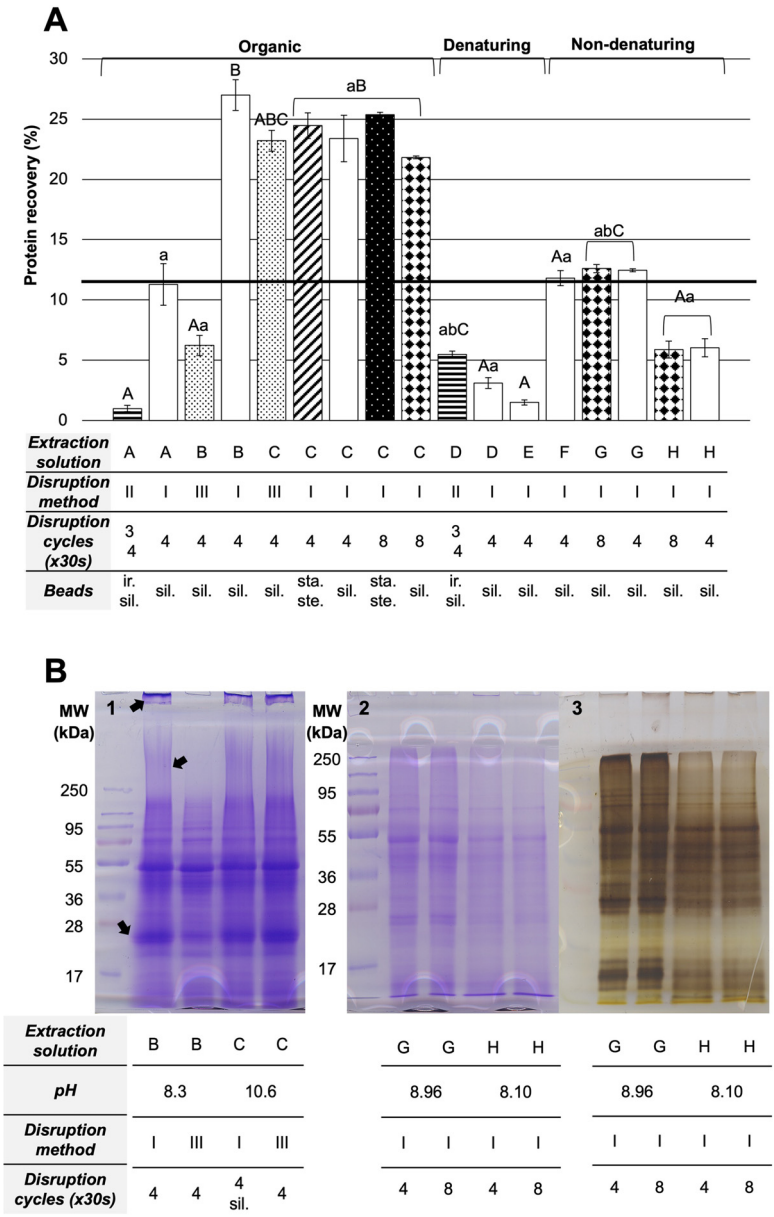

Figure 3. The protein extraction efficiency of the tested extraction conditions and disruption methods with lyophilized biomass of the microalgae *T. chuii*. (**A**) Graphical representation of the protein recovered (*Pr*) as a percentage of the total protein content of *T. chuii* calculated using equation (2) in the manuscript. The protein extracted from *T. chuii* biomass was quantified using a Bradford assay and the results are presented as the mean ± SEM of the technical replicates (n = 2-8). Extraction conditions are grouped into "Organic", "Denaturing" and "Non-denaturing". The extraction methods were repeated between n = 1 – 4 times and significant differences ($p < 0.05$) in the concentration of protein extracted is indicated. Bar patterns represent tested disruption conditions for all grouped extraction conditions. The horizontal line represents the global mean of the protein yield from all protein extracts. (**B**) SDS-PAGE gels (12%) of *T. chuii* protein extracts (6 μg) stained with Coomassie blue (1 and 2) or silver nitrate (3). Images 2 and 3 are the same gel. Black arrows indicate protein aggregation, precipitation, band distortion and vertical streaking. MW - the molecular weight marker (kDa). In panels **A** and **B** the different extraction conditions are detailed in Table 1 and 2 of the manuscript. Ir- iron, sil.- Zirconia/silica, sta. ste.- stainless steel.

disruption with aqueous alkaline or ionic buffers were most efficient for cell disruption and protein recovery (≈ 30%) in the *Chlorella* genus [16,17,20]. The strength of the microalgae cell wall is reduced by alkaline treatments and together with the incorporation of a surfactant TritonX-100 [20] facilitates cell wall breakage and probably also protein solubilization as was shown in the present study with *T. chuii*. Depending on the microalgal strain under analysis, other studies have shown a variety of protein yields using mild protein extraction methods, e.g. 28 - 43% of total proteins from *Chlorella vulgaris, Nannochloropsis oculata* and *Haematococcus pluvialis* [17] or 7-12 % from *Tetrasselmis impellucida* and *suecica*, respectively [15,22,28].

In contrast, denaturing (**D** and **E**) and non-denaturing (**H**) extraction conditions in DM I and II did not result in a high extraction efficiency as the protein yield was less than 6%, compared to the best methods that yielded 21 - 27% ($p<0.05$). The deionized water dialysis step introduced in DM II, before bead milling together with the extra cycle of disruption using a larger bead size (1 mm) did not improve the protein extraction efficiency under, "mild" organic (**A**) or highly denaturing (**D**) conditions (Fig. 3A). The process of salt removal affects the ionic strength surrounding hydrated microalgae changing the ionic balance inside the cells which may cause protein instability leading to precipitation/aggregation [39] of lipid and membrane interacting proteins [40], even before cell disruption. Nevertheless, it was expected that most of the proteins would be soluble under highly denaturing conditions (**D**).

Since a good protein yield is favourable for the detection of proteins by SDS-PAGE the extracts from high protein yielding methods were analyzed further. Protein extracts prepared using the organic extraction buffers (**B** and **C**) and DM I and III or under non-denaturing conditions (**G** and **H**) with DM I were analysed by 1D SDS-PAGE. Coomassie blue staining was used to visualize the proteome profile of the extracts (Fig. 3B) and a range of high to low molecular weight proteins were visible with the total protein extracts of *T. chuii* prepared under organic conditions (**B** and **C**) with DM I and III (Fig. 3B-1). In addition to the well resolved soluble proteins in the organic based extracts, a high content of aggregated/precipitated proteins was evident as a strongly staining region at the top of the stacking gel of the SDS-PAGE, and by vertical streaking of proteins throughout the gel (Fig. 3B-1). A further concern in organic extracts was an excess of salts in the samples, which led to distortion of the protein bands during electrophoresis. Nonetheless, despite the high salt content of the extraction buffers **B** and **C**, they had a good protein yield presumably due to the increased cell wall solubilization and the associated release of proteins caused by triton X-100 and 1,3-propanediol, as explained above. The higher protein yield obtained may explain the higher levels of protein aggregation and precipitation.

In contrast, with non-denaturing extraction conditions (**G** and **H**) using DM I almost no protein aggregation and low precipitation of proteins occurred, but the protein yield was lower (≈ 13%, $p<0.05$). In SDS-PAGE gels stained with Coomassie blue the proteins in the extracts were well resolved and included high to low molecular weight proteins (Fig. 3B-2). Since the non-denaturing extraction conditions excluded detergent and salts and had a slightly basic pH this may have favoured protein stability when extracted. To better analyze *T. chuii* proteins extracted under non-denaturing conditions (**G** and **H** and DM I), silver staining was applied [41]. The silver nitrate staining of the SDS-PAGE gels of non-denaturing *T. chuii* extracts revealed a high number of well resolved proteins of high to low molecular weight (Fig. 3B-3) and differences in protein abundance were also evident between method **G** and **H,** which agrees with the results of protein yield (Fig. 3A). The results obtained with non-denaturing extraction conditions, and DM I corroborate a previous study showing 0.5 mm zirconia-silica beads were effective in highly viscous samples [13]. Sonication has previously been shown to be an efficient means of protein extraction [16,42]. In the present study the addition of a sonication step after bead milling did not cause a substantial increase in protein yield or quality (Fig. 3A and Fig. 3B) as observed in previous studies [38] with other microalgae strains.

Analyzing the protein profile of the microalgae extracts obtained using DM I and non-denaturing conditions (**G** and **H**), if efficient, should yield proteins previously identified. For example, microalgae contain proteins such as Rubisco responsible for light harvesting which are associated with the light harvesting complexes. These proteins have a molecular weight (MW) range of 22 - 44 kDa in *Tetraselmis sp. (T. sp.)*. Rubisco is composed of several subunits with a maximum MW of 50 kDa [15]. The rubisco 50 kDa subunit may correspond to the intense band identified in the present study of approximately 50 kDa and presumably the light harvesting proteins are represented between 25 - 45

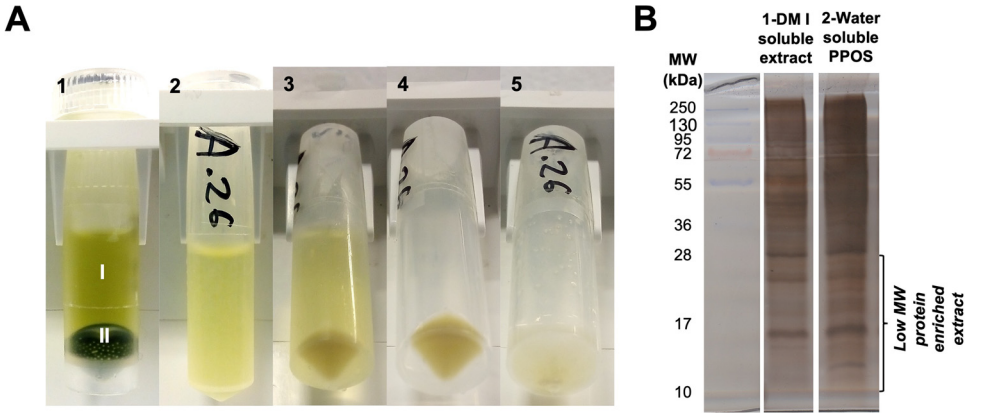

Figure 4. Efficiency of the protein precipitation method using an organic solvent (PPOS) added to a total protein extract of *T. chuii*. (**A**) Analysis of the PPOS method: 1-I, soluble protein extracts; 1-II, cellular debris from DM I; 2, organic solvent added to the soluble protein extract; 3, protein precipitate; 4, dried protein precipitate; 5, solublized protein isolate with MilliQ water. (**B**) Silver nitrate stained Tricine-SDS-PAGE gel of *T. chuii* protein extracts: 1-soluble proteins extracted with DM I (extraction solution **G**, 4 × 30 sec. disruption cycles) and 2- water soluble proteins after organic solvent precipitation. MW - molecular weight marker (kDa).

kDa (Fig. 3B). A similar protein profile was described by Schwenzfeier et al [22]., and 38% [w/w] of the extracted proteins in *T. impellucida* were of low MW.

Microalgae are known to have a high content in phenolic compounds and physical disruption (eg. bead milling) coupled with organic and alkaline solutions recovered the phenolic compounds that can further react with proteins via covalent and non-covalent bonding. If this occurs protein solubility drops and this becomes evident as protein extracts are dark and intensely coloured with decreased protein yield and quality [18]. Under non-denaturing conditions the *T. chuii* extracts were light green, which indicates chlorophyll was present in a micellar structure [12,43] and they were enriched in proteins of low MW (< 60 kDa, Fig. 3B). An additional, procedure for elimination of chlorophyll and for the isolation of low MW proteins from *T. chuii* extracts prepared under non-denaturing conditions using DM I is described in the Method extension (*see section Protein precipitation using organic solvent-PPOS*, based on the method described in [34]). A light green supernatant was obtained for DM I under non-denaturing conditions, contrasting with the dark green pellet of the microalgae cellular debris (Fig. 4A, tube 1). After precipitation with acetonitrile containing 0.1% TFA, the supernatant became slightly less green and the pellet was white and was easily resolubilized in water (Fig. 4A, tube 2-5). These observations suggest green colored interfering phenolic compounds such as chlorophyll were removed from the extracted proteins by acetonitrile precipitation [9,11. A higher number of low MW protein bands were visible in the protein isolates after the PPOS method compared to extracts prepared with DM I (Fig. 4B) indicating that a broad range of MW proteins can be recovered from microalgae extracts with this methodology.

Conclusion

Few studies exist using mild protein extraction methods for microalgae and those that do reveal extreme differences in protein recovery between species. The present study established an efficient method for preparation of high-quality *native* protein extracts for proteome studies using freeze-dried *T. chuii* microalgae biomass. Mild isolation techniques were tested to avoid possible negative effects on proteins. The optimized procedure developed included: 1) bead milling using silica beads of 0.5 mm for effective mechanical disruption of freeze-dried *T. chuii* biomass; 2) for this uncharacterized strain, mild protein extraction methods in organic and alkaline solvents had the highest protein yield of ≈ 27%, presumably due to the dual effect on microalgae disruption and higher solubility of the

predominantly acidic microalgae proteins in alkaline solutions. The combination of a non-denaturing solution with bead milling efficiently broke the microalgae cell wall and released intracellular proteins without changing their native state and originated high quality protein extracts suitable for whole proteome analysis and potentially other applications. Based on the outcome of the present and previous studies it is concluded that the most suitable extraction technique must be selected for each microalgae strain and will depend on the final application.

Additional information

Background - Protein precipitation using organic solvent (PPOS) method

Even though the developed methodology gave good quality protein extracts for proteome analysis, low molecular weight proteins were not recovered. This protein fraction includes antioxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, that repair or prevent cellular damage caused by reactive oxygen species, [44–47]. Microalgae produce antioxidant molecules when exposed to nutrient-limiting conditions [35] and can improve survival and resistance to salinity stress of prey organisms such as the Pacific white-leg shrimp post-larvae [48]. A previously reported method for precipitation and concentration of low molecular weight proteins using organic solvents [34] was customized in the present study (see, method details section).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2022.101637.

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