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Effect of *Arthrospira platensis* microalgae protein purification on emulsification mechanism and efficiency

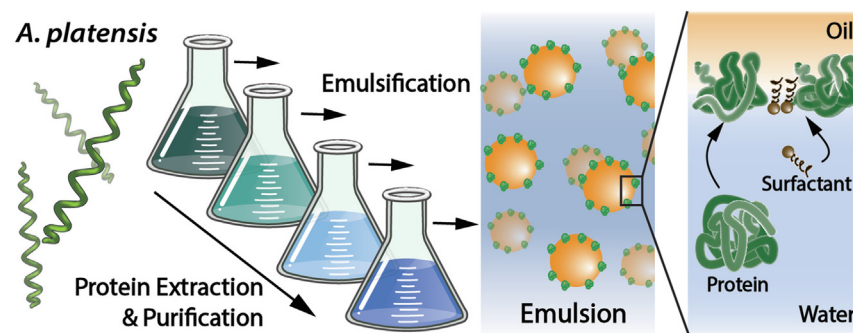
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GRAPHICAL ABSTRACT



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ABSTRACT

In light of environmental concerns and changing consumer demands, efforts are increasing to replace frequently used animal-based emulsifiers. We demonstrate the interfacial network formation and emulsifying potential of *Arthrospira platensis* protein extracts and hypothesize a mechanistic change upon progressing purification. A microalgae suspension of *A. platensis* powder in phosphate buffer solution (pH 7, 0.1 M) was homogenized and insoluble components separated by centrifugation. Proteins were precipitated at the identified isoelectric point at pH 3.5 and diafiltrated. In interfacial shear rheology measurements, the build-up of an interfacial viscoelastic network was faster and final network strength increased with the degree of purification. It is suggested that isolated *A. platensis* proteins rapidly form an interconnected protein layer while coextracted surfactants impede protein adsorption for crude and soluble extracts. Emulsions with 20 vol % medium chain triglycerides (MCT) oil could be formed with all extracts of different degrees of purification. Normalized by protein concentration, smaller droplets could be stabilized with the isolated fractions. For potential applications in food, pharma and cosmetic

Abbreviations: CE, crude extract; SE, soluble extract; PI, protein isolate; DPI, diafiltrated protein isolate; IEP, isoelectric point; WPI, whey protein isolate; NPFC, nitrogen to protein conversion factor; PBS, Phosphate buffer solution; MCT, Medium chain triglycerides.

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1. Introduction

Enforced by the European green deal in the European Union and globally by the Paris Agreement, all facets of society push for more sustainable and resource efficient alternatives to existing animal-based solutions [1,2]. The food system and within it, emulsifiers, are no exception to this. Animal and plant proteins are utilised in manifold occasions to stabilize oil in water systems such as salad dressings, milk, mayonnaise or creams to name a few [3]. Prominent animal protein examples that have extensively been investigated include whey protein isolate, caseins, egg proteins, bovine serum albumin, lysozyme or gelatine [4,5,6]. There are already plant-based solutions provided by soy or pea proteins but alternatives from single cell-based resources such as yeast, bacteria, fungi and microalgae have generated great interest [7,8,9]. In this regard, microalgae present multiple opportunities since they achieve high yields per area and year, do not require arable land for cultivation and have nutritional benefits, including up to 70 % of proteins per dry matter [10,11]. One of the most relevant industrial strains is *Arthrospira platensis*, which is commonly known as *Spirulina* due to its spiral shape. Although, it is phylogenetically a cyanobacteria, it is referred to as microalgae owing to its historic classification as a blue-green alga [10]. It became relevant as a nutritional supplement since its usage is well documented in African and Aztec food chains, allowing a generally recognized as safe (GRAS) status approval by FDA [12]. Its commercial relevance originates as the source of the only natural blue colorant in the food industry, phycocyanins, which are protein-pigment complexes that account for up to 20 % of the protein fraction in *A. platensis* [13]. Isolated from the crude biomass and depending on purity, they achieve market values of up to several thousand € per kg [14,15]. Owing to its proteinaceous origin, processing steps, especially in terms of thermal treatments, have to be harmonized to obtain desired blue products [16,17]. Novel approaches to use *A. platensis* as a source of emulsifiers could build on existing infrastructure, supply chains, and legal frameworks that have already been established to produce, process and sell *A. platensis* products.

Arthrospira platensis's vast protein fraction has been one of the most investigated microalgae protein regarding emulsion and foaming properties and related adsorption studies to the air-water and oil-water interface [18,19,20,21,22,23,24,25,26,27]. However, the mechanism of stabilization and the effective impacts of purification have not been addressed extensively and in combination for *A. platensis*. Similar to studies of other microalgae strains, some research has focused on the performance and interaction of protein isolates with the oil- and/or air-water interface and its potential as an emulsifier and foaming agent [21,28]. Other studies have evaluated the surface activity of different microalgae biomass fractions, highlighting that not only protein isolates could be used as functional fractions from microalgae biomass [29,30]. Law et al. [29] suggest specifically the potential of microalgae derived bioemulsifiers and biosurfactants. The isolation process typically comprises multiple extraction and purification steps with individual recovery rates accumulating to low overall protein yields [31,30,32]. Additionally, purifications require solvents and other chemical resources, such as acids for an acidic precipitation. Energetic resources are likewise needed, e.g. for disruption, pumping and separating fractions. Low yields and high resource con-

sumption adversely impact the environmental sustainability of microalgae, which is indeed not favorable for all applications when compared to established food production systems [33,34]. Promising results were reported for use of the entire biomass in meat alternatives, which was shown to be feasible [35]. Consequently, it should be explored how more of the biomass could be valorized in functional applications such as emulsification and what the implications are on a mechanistic level.

Herein, we present insights into the stabilization of oil-water emulsions by increasingly purified extracts of *A. platensis*. Linked with interfacial adsorption measurements, differences in stabilization mechanism are revealed. Thereby, we show how microalgae biomass can be used most efficiently in providing functional ingredients for multiple industries producing oil in water emulsions. Due to similarities in composition and processing steps, these results are likely to be transferable to other microalgae species and single cell protein sources in general.

2. Materials & methods

2.1. Materials

2.1.1. Microalgae biomass and purification

Spray-dried commercial *A. platensis* biomass was produced in photoautotrophic conditions and acquired from the Institut für Lebensmittel- und Umweltforschung, Nuthetal, Germany (*Spirulina* Premium II, lot SpPRII-1803-280918). The powder was suspended at 20 g/L in 0.1 M phosphate buffer solution (PBS) pH 7 overnight at 4 °C. The suspension with 2 w/w % biomass was homogenized at 100 MPa with a M-110EH pilot scale microfluidizer (Microfluidics Corporation, Westwood, United States) to induce cell disruption and release intracellular components. A Y-shaped ceramic interaction chamber F20Y (75 µm gap width) was used in the microfluidizer with a downstream auxiliary processing module H30Z (200 µm gap width) that provided further treatment of the samples and stabilization of the product flow. At an operating pressure of 100 MPa, the flow rate of the product was 480 mL/min and the shear rates were estimated as $6 \times 10^6 \text{ s}^{-1}$ in the interaction chamber and $2.2 \times 10^6 \text{ s}^{-1}$ in the auxiliary processing module, according to the manufacturer. The disrupted biomass was collected as crude extract (CE). The purification steps and the resulting sample nomenclature are depicted schematically in Fig. 1. After keeping the disrupted cell suspension at 4 °C for 2 h, the separation of the aqueous soluble components and the cell debris was carried out by centrifugation at 10,000g and 4 °C for 30 min with a Thermo Scientific Sorvall LYNX 6000 centrifuge (Thermo Fisher Scientific, Waltham, United States). The supernatant was collected as an aqueous soluble extract (SE). Protein was precipitated by acidifying SE with 1 M HCl to the identified isoelectric point of pH 3.5 (Fig. 2) and collected through centrifugation at 10,000g for 20 min. The protein rich pellet was resuspended in 0.1 M PBS pH 7 and stirred overnight at 4 °C before centrifuging at 24,000g for 30 min to ensure that only resolubilized protein was present in the protein isolate (PI). The PI was further purified by tangential-flow diafiltration using a Vivaflow 200 hydrosart membrane with a 5 kDa molecular weight cut-off (Sartorius AG, Göttingen, Germany) at 2.5 bar transmembrane pressure set with a peristaltic pump (Masterflex

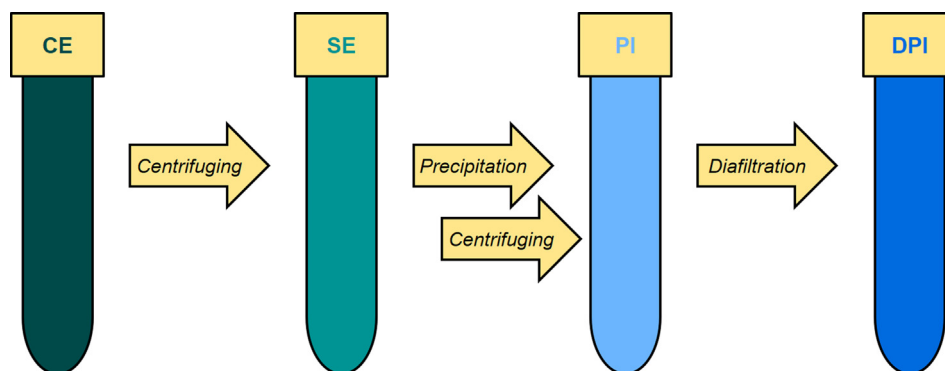


Fig. 1. Overview of purification steps and samples used throughout the study. Homogenized *Arthrospira platensis* suspension is the crude extract (CE). Supernatant of CE yields the soluble extract (SE). Precipitated and resolubilized protein is the protein isolate (PI). After diafiltration it is the diafiltrated protein isolate (DPI). Details on methods are documented in [Section 2.1.1](#).

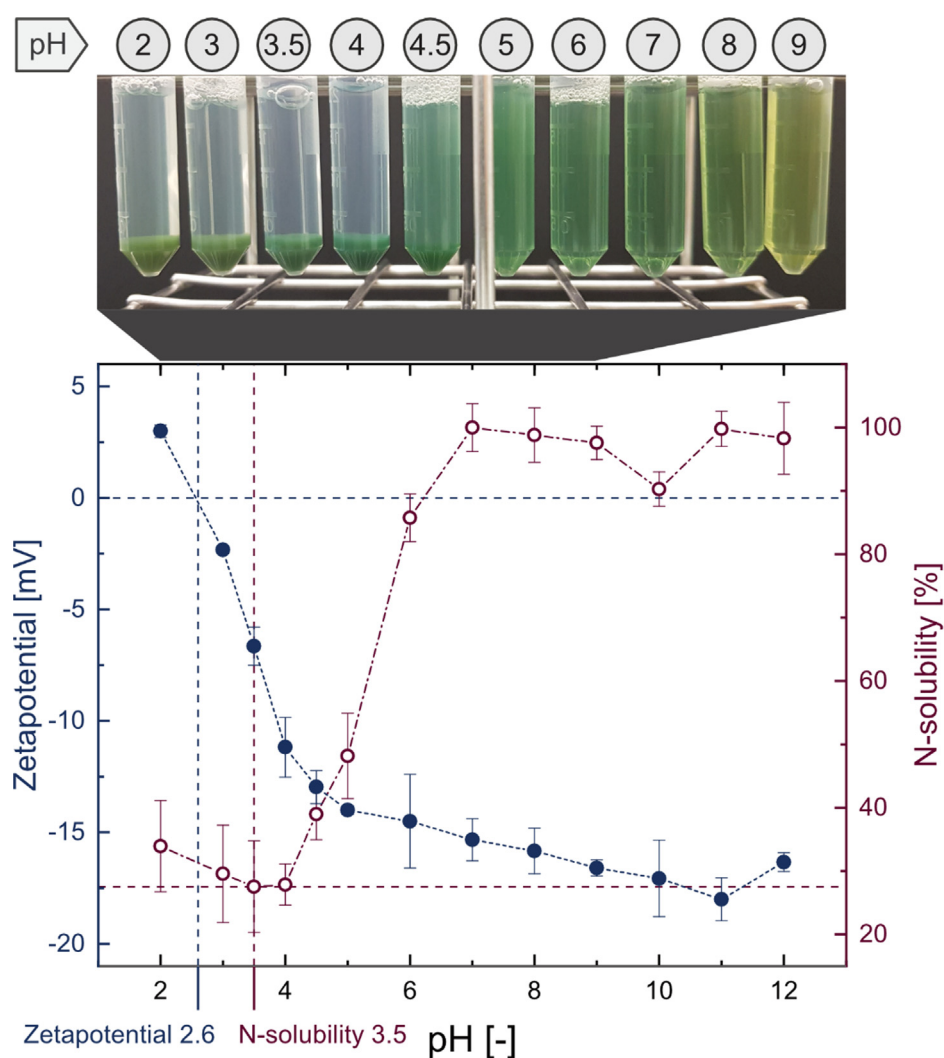


Fig. 2. Pictures of soluble algae extract (SE) with adjusted pH from pH 2 to pH 9 visualizing precipitation at pH ≤ 4 . The graph shows nitrogen-solubility and zeta potential of SE from pH 2 to pH 12 with minimum *N*-solubility at pH 3.5 and zeta potential = 0 mV at pH 2.6. Horizontal and vertical dashed lines guide the eye for minimum *N*-solubility and balanced zeta potential. Measurements were performed in triplicates.

L/S, Cole-Parmer GmbH, Wertheim, Germany). The diafiltration was stopped after a turnover of 4 times the sample volume with fresh 0.1 M PBS (pH 7) to reduce low molecular weight surfactants by 99 % [36]. The diafiltrated sample was handled as diafiltrated *A. platensis* protein isolate (DPI).

2.1.2. Whey protein isolate

Whey protein isolate (WPI) was purchased from Davisco Foods International (BiPro, lot 1000520168, Le Sueur, United States). According to the manufacturer, it contains per 100 g: 93.2 g protein, 4.4 g moisture, 2.3 g ash and 0.5 g fat, with a nitrogen to pro-

tein conversion factor of 6.38. As the microalgae powder, it was stored in dry conditions at room temperature in the dark.

2.1.3. Chemicals

Phosphate buffer solution (PBS) was produced with Milli-Q water from a Merck Millipore system (Merck, Darmstadt, Germany) and $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ purchased from Sigma-Aldrich (Buchs, Switzerland) with purities $\geq 99.0\%$. The PBS was fortified with 10 mM NaN_3 ($\geq 99.5\%$) (Sigma-Aldrich) to prohibit biological growth in prepared samples. 1 M HCl was purchased from Merck. NaOH ($\geq 98.0\%$), *n*-dodecane ($\geq 99\%$) and D-glucose ($\geq 99.5\%$) were purchased from Sigma-Aldrich. Medium chain triglyceride (MCT) oil was obtained as Myritol 318 from BASF AG (Düsseldorf, Germany).

2.2. Methods

2.2.1. Isoelectric point determination

Protein can be precipitated at the isoelectric point (IEP), where net electrical charge and protein solubility are the lowest [37]. To identify IEP, SE at 2 w/w % was adjusted with 1 M HCl and 1 M NaOH to pH 2–12 using a porotrode (Metrohm Schweiz AG, Zofingen, Switzerland). Net electrical charges of the pH adjusted samples were measured with a Zetasizer Nano ZS using DTS 1070 cells (Malvern Instruments Ltd., Malvern, United Kingdom). Protein solubility was analyzed by centrifuging the pH adjusted solutions for 10 min at 10,000g and measuring the protein concentration of the supernatant (see Section 2.2.2). Measurements were performed in triplicates.

2.2.2. Protein quantification

Protein concentration was measured by quantifying the total nitrogen concentration using a total organic carbon analyzer coupled with a total nitrogen measurement unit (TOC-L with TN, Shimadzu, Japan). Nitrogen concentration was multiplied with an identified specific nitrogen to protein conversion factor (NPCF) of 5.5 for *A. platensis*. NPCF was determined by correlating the nitrogen concentration and the amino acid concentration of *A. platensis* samples. Amino acid analysis was conducted by the Functional Genomics Center Zurich (Zürich, Switzerland) following AccQ-Tag Ultra amino acid analysis (Waters Corporation, Milford, MA, United States). First, 10 to 20 μL of extracted samples were dried and hydrolyzed overnight at 110 °C in 6 M HCl under an argon atmosphere. All hydrolysates were dissolved in 40 μL borate buffer (AccQ-Tag Ultra). A fraction of this was derivatized with 50 μL reagent, which included Norvaline as the internal standard. A 50th was injected into a high-performance liquid chromatography for analysis. Phycocyanins were quantified in the protein isolates [16] by measuring UV-Vis absorbance with a Cary 100 (Agilent Technologies Inc., Santa Clara CA, United States) and applying spectroscopic correlation functions from Yoshikawa and Belay [38]. Measurements were performed in triplicates.

2.2.3. Carbohydrate quantification

Total carbohydrates were quantified by the phenol sulfuric colorimetric method as described by Dubois et al. [39] with D-glucose as standard. Measurements were performed in triplicates.

2.2.4. Emulsion characterization & stability

2.2.4.1. Emulsion formation. Emulsions were formed with emulsifier concentrations between 0.1 and 2.0 w/w % and 20 % (v/v) MCT-oil. MCT-oil was employed as commonly used triglyceride in studies assessing emulsion stabilization. All emulsions were premixed for 2 min with a rotor stator stand-disperser (Polytron PT-MR 6000, Kinematica AG, Luzern, Switzerland) at 10,000 rpm equaling a shear rate of $1.31 \times 10^4 \text{ s}^{-1}$ assuming Couette flow con-

ditions. Pre-emulsions were homogenized by one pass at 100 MPa in the M–110EH pilot scale microfluidizer with the same interaction chamber and processing conditions as described in Section 2.1.1. Emulsions were stored at 4 °C, and measurements were performed in triplicates.

2.2.4.2. Droplet size distribution. The droplet size distribution was measured in triplicates with a laser diffraction particle size analyzer LS 13,320 (Beckman Coulter AG, Nyon, Switzerland). Mie theory was applied to analyze the raw data assuming water as continuous phase (refractive index of $n_{\text{water}} = 1.333$) and MCT-oil as dispersed phase ($n_{\text{MCT}} = 1.449$).

2.2.5. Interfacial rheology

An Anton Paar MCR 501 (Graz, Austria) was employed with a biconical disk geometry [40] and a subphase exchange cup [41]. Interfacial measurements were conducted following the procedure of Bergfreund et al. [42]. In short, the exchange cup was filled with 0.1 M PBS pH 7. The bicone was set at the air-water surface controlled by a normal force sensor and covered gently with *n*-dodecane. A time-sweep experiment with oscillatory shear at strain $\gamma = 0.3\%$ and angular frequency $\omega = 1 \text{ rad/s}$ was started. After ensuring an uncontaminated interface for 5 min by monitoring G'_i and G''_i , 50 mL of subphase was exchanged by simultaneously delivering sample to the cup's bottom and pumping out subphase at the cup's side with a constant exchange rate of 1 mL/min. The slow exchange assured no disturbances were made to the sensitive interfacial measurements. The exchange was administered by 60-mL BD Luer-Lok syringes (Becton Dickinson, United States) actuated by a PHD 2000 syringe pump (Harvard Apparatus, United States). After the 20 h time-sweep experiment, the established interface was exposed to an amplitude sweep at angular frequency $\omega = 1 \text{ rad/s}$ and strain increasing from $\gamma = 0.1$ –100 % with 5 measuring points per decade. The procedure was carried out cautiously to omit any air bubble introduction to the exchange cup that could have potentially falsified measurements. The alkane *n*-dodecane was chosen as hydrophobic phase for its purity over the triglyceride MCT-oil, which was employed for the emulsion tests. The hydrophobic alkane ensures an increased measurement resolution and no interactions with interfacial active impurities. Such impurities are commonly encountered in triglycerides and prohibit the understanding and exploration of competing interactions between surfactants and proteins at the fluid interface. Minor differences in absolute values of the interfacial moduli are expected due to the different polarity of the oils. However, the response of both oils is expected to follow the same trend for the different microalgae fractions [42].

3. Results & discussion

3.1. Protein solubility and zeta potential in soluble algae extract

The *A. platensis* biomass contained $58.0 \pm 2.5\%$ protein per dry matter. This is less than indicated by the supplier with 66.8% protein due to application of a specific nitrogen to protein conversion factor (NPCF) of 5.5 instead of the default NPCF 6.25. The protein isolate was extracted to shine light on the effectiveness of protein isolates in the soaring single cell protein industry and to elucidate the stabilization mechanism at the oil-water interface. It is common practice to isolate proteins by precipitation at the isoelectric point (IEP). Protein solubility is strongly related to their surface charge; i.e., the ζ -potential that denotes the net charge of particles in aqueous solution. Proteins are charged negatively or positively above or below the IEP, respectively. At the IEP, proteins carry no net charge and are least soluble due to protonated and deprotonated side chains balancing each other. To evaluate the solubility

of proteins in an *A. platensis* extract, ζ -potential measurements were combined with soluble nitrogen analysis, as depicted in Fig. 2. The ζ -potential as a function of pH suggests an IEP of pH 2.6. However, the lowest nitrogen solubility was observed at pH 3.5. This mismatch can be attributed to coextracted polysaccharides which typically carry negative charges and result in an underestimation of the IEP by ζ -potential [43].

Thus, the IEP of *A. platensis* proteins was concluded to be at the nitrogen solubility minimum of pH 3.5. Comparable values have been reported previously in the literature: IEP = pH 3 by Benelhadj et al. [20] and IEP = pH 2.8–3.5 by Chronakis et al. [24]. A picture of the pH series visualizes the precipitation and emphasizes that the algal pigments coprecipitate with the proteins, which is expected due to the proteinaceous nature of phycocyanin complexes, the major protein pigment system in *A. platensis* (Fig. 2, [12]). Note that IEP = pH 3.5 corresponds to the overall IEP of all protein fractions contained in the *A. platensis* extract, and the IEP of individual protein fractions may differ.

3.2. Recovery of protein in purified algae extracts

The efficiency of isolating protein from the initial algae biomass with intermediate steps is visualized in Fig. 3. *Arthrospira platensis* was dispersed at 2 w/w % in PBS. After homogenization, the disrupted suspension was taken as crude extract (CE) and contained all substances present in the microalgae, including 58.0 ± 2.5 % protein and 8.0 ± 1.2 % carbohydrates. CE was stirred for 2 h, allowing aqueous soluble compounds to be extracted before centrifuging. The supernatant served as the soluble extract (SE) and was translucent (see Fig. 2; pH 7). An amount of 69.1 ± 4.5 % of initial protein could be retained in the SE before it was acidified to IEP (see Section 3.1). Precipitated protein was retrieved by centrifugation, and only the resolubilized fraction was used as protein isolate (PI). This was ensured by centrifuging the solubilized pellet at $24,400g$ for 30 min after solubilization for 24 h at 4°C . A limited protein recovery was obtained with just 13.6 ± 0.7 % of initial protein in PI or 0.8 g/L considering 1 w/w % initial algae concentration. Approximately a third of the protein in PI and DPI could be attributed to phycocyanins, the blue protein pigment complexes in *A. platensis*. Precisely, 32.9 ± 3.0 % and 38.8 ± 3.9 % of protein in PI and DPI was related to phycocyanins, respectively.

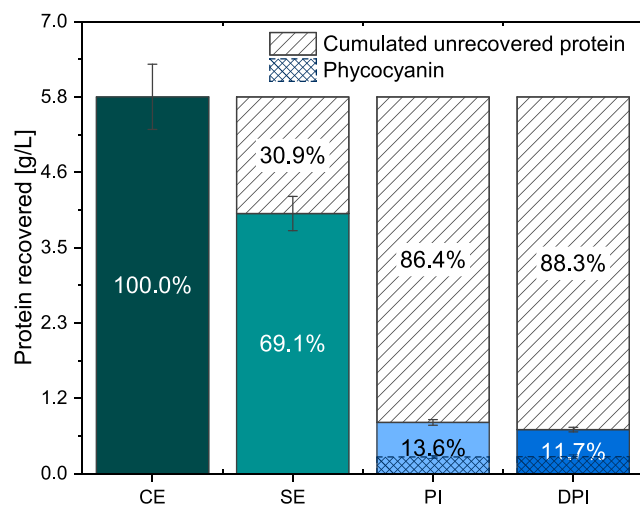


Fig. 3. Absolute and relative amount of protein in the crude extract (CE), soluble extract (SE), protein isolate (PI) and diafiltered protein isolate (DPI) in relation to protein in CE. For PI and DPI, the fraction of phycocyanin complexes to the entire purified protein concentration is marked. Measurements were performed in triplicates.

Many publications do not report protein yields. An exception is Schwenzfeier et al. [31], who reported a protein yield of 7 % for an isolate obtained by a multistep protein isolation from *Tetraselmis* sp. Protein purification approaches from microalgae differ widely based on specific methods and targeted degree of purification. However, low efficiency in protein recovery is not surprising, as most of the refinements encompass multiple processing steps with individual efficiency factors each reducing the overall protein recovery. In the case of commonly applied IEP precipitation, it has to be recognized that only solubilized proteins can be precipitated at IEP [44]. Additionally, precipitation at IEP leads to partially irreversible changes in protein structure. Due to neutral net electrical charges, protein-protein hydrophobic attraction surpasses the protein-water electrostatic attraction [45] and electrostatic repulsive forces between proteins decrease. Thus, interactions between proteins increase. Ultimately, the three-dimensional protein structure collapses and leads to precipitation. This aggregation process is part of a denaturation which is limited in its reversibility constraining protein recovery as materialized in protein yields of 13.6 and 11.7 % for PI and DPI, respectively [46,47].

3.3. Network formation at oil-water interface

The protein isolates and soluble extract have been studied at the oil-water interface by interfacial shear rheology. Interfacial moduli developed in oscillatory shear experiments over time. The evolution of this physical response at the interface enables insights into the adsorption, network formation and network stability of surface active compounds present in the *A. platensis* extracts. The alkane *n*-dodecane had been employed as the hydrophobic phase to ensure a satisfying measurement resolution and to omit the introduction of interfacial active impurities, which are to be expected e.g. in triglycerides. A time sweep oscillatory shear experiment was conducted followed by an amplitude sweep. The experiments started with a clean oil-water interface assured by initial interfacial moduli of nondetectable G'_i and $G''_i < 10^{-4}$ Pa·m. The sample was introduced by exchanging the aqueous subphase within the first 50 min at a constant exchange rate of 1 mL/min [42]. Amplitude sweeps have been performed at the end of the time sweeps. Crossover points at which the interfacial network structures broke down were similar for all samples; numerically the strains for crossover points were 14.2, 12.9, 14.9 and 19.0 % for DPI, PI, SE and diluted SE, respectively (Fig. 4B). No differences were observable in the form of the breakdown such as a strain hardening or the like. The amplitude sweeps identify a linear viscoelastic regime up to a strain of 2 % for all extracts. Hence, the preceding time sweeps at a strain of 0.3 % have been performed within the linear viscoelastic regime.

In the case of PI and DPI, the interfacial storage G'_i and loss G''_i moduli picked up almost immediately (Fig. 4A). Surface-active compounds quickly adsorbed to the interface. The elastic modulus surpassed the viscous one after 1 h 15 min for PI and after 2 h for DPI. The crossover indicates the establishment of a viscoelastic network at the interface, since more energy is stored in interfacial structures than dissipated by viscous interactions. The direct establishment of a viscoelastic network by the isolated protein extracts is comparable to other interfacial protein systems characterized by interfacial shear rheology [42,48]. Adsorbed proteins tend to spatially reorganize at the interface facilitating the adsorption of further proteins, which explains the increase in viscoelasticity for hours [49,50]. The network strength increased with measuring time towards final values of $G'_i = 1.8 \times 10^{-2}$ Pa·m for PI's elastic component and $G''_i = 0.7 \times 10^{-2}$ Pa·m for PI's viscous component, i.e., $G'_i = 2.0 \times 10^{-2}$ Pa·m and $G''_i = 0.8 \times 10^{-2}$ Pa·m for DPI. The rearrangement of proteins at the interface holds also

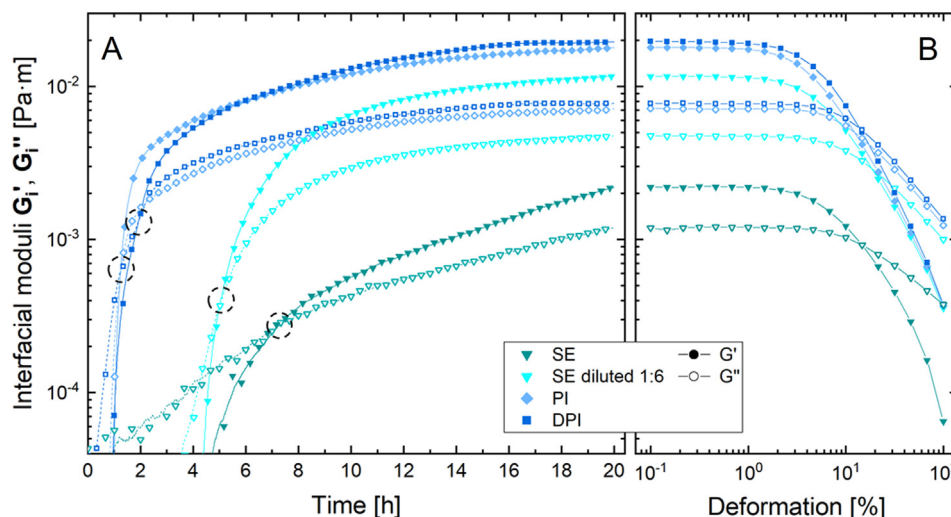


Fig. 4. Viscoelastic shear moduli (G'_i – full and G''_i – empty symbols) of the interface between algae extracts and dodecane from interfacial oscillatory shear-rheology measurements. A: Time sweeps at constant amplitude of 0.3 % and frequency of 1 rad/s. Every 40th measuring point is plotted; Lines display smoothed data by adjacent-averaging; The dotted circles indicate the crossover points, where $G'_i = G''_i$. B: Amplitude sweeps of established interfacial networks after 20 h. Tested algae extracts were soluble extract (SE) at 1 w/w % with 4.0 g/L protein and SE diluted 1:6 to 0.7 g/L protein as well as PI and DPI at 1 w/w % with 0.8 and 0.7 g/L protein, respectively.

true for protein complexes [51,52]. PI's protein was composed to 32.9 ± 3.0 % of phycocyanin, which is the protein pigment complex responsible for *A. platensis*'s blue color. The fraction of phycocyanin enhanced to 37.9 ± 4.1 % of protein in DPI. Phycocyanins arrange at neutral pH in hexameric and trimeric supramolecular complexes [53]. Their rearrangement upon adsorption might take longer than for individual proteins. Consequently, an increased fraction of phycocyanins in DPI may explain partially why DPI established a viscoelastic network slightly slower than nondiafiltered PI (Fig. 4A).

The soluble extracts showed a different adsorption behavior to the interface. SE did not induce any considerable viscous or elastic forces until 4 h after exchanging the subphase (Fig. 4A). The following strengthening of the interfacial moduli was moderate in comparison to the isolated proteins. After 7 h, elastic forces overtook viscous forces indicating a much slower build-up of an interfacial network. Moreover, also after 20 h, the network had not reached plateau values and was weaker, with interfacial moduli a decade below the values for PI and DPI, respectively; G'_i (20 h) = 0.2×10^{-2} Pa·m and G''_i (20 h) = 0.1×10^{-2} Pa·m. Overall, surface active substances in SE formed more slowly a network at the interface with weaker strength than PI and DPI. To exclude a protein concentration effect, SE was diluted 1:6 for a comparable protein content as PI and DPI. Following a similar lag phase as for undiluted SE, the network formation of the diluted SE was faster and established stronger viscoelasticity (Fig. 4A). Interfacial moduli were one order of magnitude higher compared to the undiluted SE after 20 h and almost in the same range as the final network strengths recorded for PI and DPI. The higher network elasticity despite 1:6 dilution suggests the presence of surfactants that impede the formation of a protein network especially in the undiluted case. In mixtures of surfactants and proteins, surfactants are known to dominate the interface owing to their faster adsorption kinetics. Moreover, surfactants may even induce the desorption of already adsorbed proteins [54]. Surfactants do not induce viscoelastic networks with recordable network strength. They stabilize emulsions and interfaces mostly by lowering the interfacial tension. Proteins, on the other hand, form viscoelastic layers at the interface that prevent emulsion droplets from coalescence and aggregation [55]. The formation of this interconnected protein layer is measured by the interfacial moduli, proving that also in the case of soluble algae extracts, eventually, an interconnected pro-

tein network is established. However, the surfactants present in SE delay the formation of protein networks by a competitive adsorption to the oil-water interface. Hence, the isolation of microalgae proteins, e.g. by IEP precipitation, yields proteins with enhanced functionality regarding the formation kinetics and viscoelasticity of interfacial protein networks.

The possible role of coadsorption of polysaccharides should also be considered. Schwenzfeier et al. [31] reported a polysaccharide content of 24 % for *Tetraselmis* sp., which was still present in the protein isolate after IEP precipitation. Hence, considerable amounts of polysaccharides are present even in microalgae protein isolates. Oppositely charged polysaccharides impede the adsorption of proteins due to aggregation prior to adsorption [56]. As most polysaccharides are anionic, no interactions are expected for PI or DPI due to the low IEP of the isolated *A. platensis* proteins. The SE may however contain other protein fractions with higher IEP which aggregate with anionic polysaccharides, impeding the proteins' adsorption. Generally, polysaccharides are not interfacially active themselves [57]. Nevertheless, polysaccharides interact electrostatically with adsorbed protein layers or may be present as glycoproteins, i.e. covalently bound to proteins [58,59,60]. Studies of microalgae extracts have identified polysaccharides that exert surface activity as part of glycoprotein moieties in isolates; Schwenzfeier, Wierenga, Eppink, & Gruppen [61] for *Tetraselmis* sp. and Grossmann et al. [62] for *Auxenochlorella protothecoides*. They argue that glycoproteins improve protein solubility over a wide pH range [62] and they lessen the isolate's pH-dependency of emulsion and foam stabilization [61]. Both verify glycoproteins in microalgae isolates, suggesting that polysaccharides could be an integral component of microalgae adsorption layers.

In the current study, surfactants have delayed the build-up of a protein dominated interfacial network in soluble algae extracts. Prior to the protein layer, a surfactant layer was established due to the surfactant's fast adsorption kinetics. It is proposed that co-extracted components, including surfactants and potentially polysaccharides, remained enclosed as impurities in the later proteinaceous interfacial network. Thereby, final network elasticity for SE stabilized oil-water interfaces was inferior to those stabilized by isolated protein fractions. Thus, stabilizing ability against droplet aggregation in formed emulsions was expected to increase with

the degree of protein purification. This relationship could effectively be measured and quantified by the evolution of mean emulsion droplet size over employed protein concentration as described in Section 3.4.

3.4. Formation of stable emulsions regardless of purification

Resource efficiency directly influences sustainability measures [33]. Thus, all algae protein extracts (CE, SE, PI and DPI) with decreasing protein recovery efficiencies along purification have been utilized to prepare emulsions. Box plots for emulsion droplets stabilized by the least and most purified samples, CE and DPI, are displayed in Fig. 5A. The box accounts for the 25–75 % quantile of emulsion droplet sizes from volume based particle size distributions. For both extracts, the box plots display a narrowing droplet size distribution; i.e., a smaller mean diameter d_{43} by increasing the employed initial algae biomass concentration per emulsion. This relation shows that the algae extracts act as stabilizers. Since CE represented the least refined algae extract and DPI the most purified one, the algae extract possesses an emulsification potential generally independent of its degree of purification. This was expected as soluble extracts and protein isolates proved to be interfacial active.

In Fig. 5A, the comparison of stabilized droplet sizes by CE and DPI is based on the initial dry biomass that was used to prepare the extracts and subsequently the emulsions. The graphic shows the emulsion droplets that could be stabilized investing a certain initial biomass concentration. In practical terms, when initially dispersing and homogenizing 5 g microalgae biomass per liter solution, an emulsion could be prepared with a mean droplet size $d_{43} = 5.0 \pm 0.1 \mu\text{m}$; equalling 0.5 w/w % CE. If the crude extract was purified into a diafiltrated protein isolate, an emulsion could be prepared with the same initial biomass with droplets $d_{43} = 10.6 \pm 0.2 \mu\text{m}$; 0.5 w/w % DPI. The comparison accounts for efficiency losses alongside the entire purification by plotting the initial dry biomass instead of the actual biomass employed in the final emulsification step. With droplets in DPI emulsions being larger than in CE stabilized emulsions at equal initial biomasses, the plot highlights that on the basis of initial biomass, purification did not lead to an improved emulsification potential. Smaller emulsion droplets could be stabilized by directly employing CE instead of purifying proteins into DPI. It should be noted that the purification process could be further optimized and that the droplet size is only one criteria in emulsifying applications, e.g. odor

and color of CE could render a protein isolation process indispensable.

Contrary conclusions regarding protein functionality can be drawn from plotting mean droplet size d_{43} against the emulsion's actual protein concentration (Fig. 5B). When normalizing for protein concentration in the emulsion, the algae extracts with an increased degree of purification (PI, DPI) proved more functional at reducing emulsion droplet size than the crude and soluble extract (CE, SE). Whey protein isolate (WPI) was added as a benchmark for protein-based food emulsifiers. The reduction in mean droplet size against protein concentration followed a power law. Precisely, an allometric fit describes the progressions effectively with R^2 -values above 0.98 attesting high goodness of fit:

$$d_{43} = a \cdot C_{\text{protein}}^b \quad (1)$$

with d_{43} denoting the mean diameter of the volume based droplet size distribution in $[\mu\text{m}]$, C_{protein} the protein concentration in emulsion in $[\text{g/L}]$ and a, b being fitting coefficients. Fitted coefficients and R^2 -values for all algae extracts and WPI as comparison are displayed in Table 1.

The mean droplet size decreased similarly with protein concentration for WPI and the protein isolates PI and DPI. Their fitting functions have comparable power exponents and coefficients underlining the similar dependency of mean droplet size and proteins in WPI, PI and DPI. Hence, emulsion stabilization by the isolated proteins is comparable to the benchmark food protein emulsifier WPI. On the other hand, CE and SE could be fitted with smaller power exponents. This quantifies that their emulsions' mean droplet size decreased to a lower extent with increasing protein concentration. At given protein concentration, the emulsion droplets decreased in diameter with increasing degree of purification. The increased functionality per protein matches with the network formation studied at the oil-water interface. The protein isolates adsorbed to the interface and formed a network that stabilizes emulsion droplets from aggregating. In case of the crude extracts, surfactants impede the protein network formation resulting in an impaired droplet and emulsion stabilization with larger emulsion droplets at equal protein concentrations. Overall, the comparison of emulsion droplet sizes by algae extracts with different degrees of protein purification revealed a clear dependency of droplet size and protein concentration in the emulsion. Furthermore, emulsification using the crude extract was the most efficient regarding initial biomass; however, purification yields protein fractions with enhanced functionality. This should be taken into consideration when stabilizing emulsions with microalgae

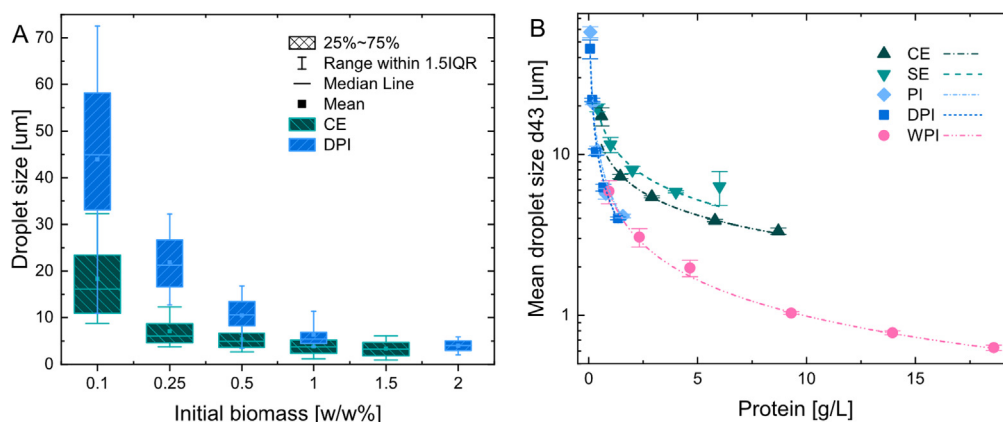


Fig. 5. (A) Volume based droplet size distribution (q_3) in emulsions formed with crude extract (CE) and diafiltrated protein isolate (DPI) for increasing initial algae biomass utilized to form the extracts and emulsions. (B) Mean emulsion droplet sizes from volume based distributions (d_{43}) in a semilogarithmic plot against emulsion protein concentrations for emulsions formed with all algae extracts with different degrees of purification and whey protein isolate (WPI). Dashed lines depict fitted power law functions ($R^2 > 0.98$) whose fitting coefficients are supplied in Table 1. Measurements were performed in triplicates.

Table 1Fitted coefficients of allometric fit ($d_{43} = a \cdot C_{protein}^b$) to describe dependency of mean droplet diameter (d_{43}) from algae extract protein concentration in emulsion ($C_{protein}$).

	CE	SE	PI	DPI	WPI
a	8.94 ± 0.42	11.89 ± 0.31	5.70 ± 0.32	4.98 ± 0.21	5.47 ± 0.30
b	-0.47 ± 0.03	-0.51 ± 0.02	-0.79 ± 0.04	-0.81 ± 0.04	-0.74 ± 0.02
R² [%]	98.2	98.9	98.9	98.6	99.4

extracts. The increase in functionality per protein must outweigh the loss in protein biomass to rationalize the purification process. Otherwise, a crude extract could have an overall superior emulsification function than an isolated protein fraction.

3.4.1. Surface coverage of emulsion droplets by protein differs alongside purification

Depending on the degree of purification, differences in droplet size could be observed as a function of protein concentration in the emulsion. To explain these, attention was drawn to the protein coverage at the oil-water interface. Upon adsorption, emulsifiers lower the interfacial tension and reduce the critical forces that are required to break up droplets. Additionally, the adsorbed protein layer stabilizes droplets from coalescence [5]. Both effects do not depend per se on the protein concentration but are linked to the interfacial surface coverage Γ , which can be derived from Eq. (2) [55,63]:

$$\Gamma = \frac{d_{32} \cdot (C_{protein}^{ini} - C_{protein}^{ser}) \cdot (1 - \Phi)}{6\Phi} \quad (2)$$

Γ denotes the surface coverage in $[\text{mg}/\text{m}^2]$. Φ denotes the oil fraction, which was constant at 0.2. $C_{protein}^{ini}$ is the initial protein concentration in the continuous phase. $C_{protein}^{ser}$ is the protein concentration in the serum, which denotes the continuous phase after emulsification and free from dispersed oil droplets. The difference between the initial protein in the continuous phase and the protein in serum quantifies the fraction of proteins adsorbing to the interface. d_{32} denotes the Sauter mean diameter, which is the surface based mean diameter. Droplet size distributions were measured by laser diffraction particle size analysis. Only strictly monomodal droplet size distributions were utilized for this calculation. In a bimodal distribution, one droplet size is overestimated in comparison to the other one, yielding unrealistic values for d_{32} .

The surface coverage revealed clear trends with increasing purification of the algae extracts. The surface coverage by WPI with $\Gamma = 2.7 \pm 0.3 \text{ mg}/\text{m}^2$ was slightly above a dense monolayer of whey protein, which was identified by Tcholakova et al. [63] at $2.0 \pm 0.2 \text{ mg}/\text{m}^2$. For the isolated proteins, surface coverage ranged even lower, at 1.2 ± 0.3 and $1.2 \pm 0.2 \text{ mg}/\text{m}^2$ for PI and DPI, respectively. These values could be influenced by low adsorbed protein fractions of only 40 to 50 %. However, the comparable surface coverage data suggest a monolayer of microalgae proteins adsorbed at the interface similar as for WPI. For CE and SE, the algae extracts with a lower degree of purification, the surface coverage increased to 5.1 ± 1.5 and $4.9 \pm 2.3 \text{ mg}/\text{m}^2$, respectively. These are more than twice the amounts calculated for PI and DPI. Consequently, multilayers and/or agglomerates were likely to be formed at the droplets' surface by algae extracts with a low degree of purification.

This matches with the results obtained in interfacial shear rheology and drawn from the stabilized emulsion droplet sizes. A reduction in emulsion droplet size requires more interfacial area to be stabilized. Consequently, smaller emulsion droplets can be stabilized with increasing interfacial stabiliser. In case of the protein isolates, the protein concentration seems to pose the limitation on the emulsion droplet's size. According to surface coverage calculation, they cover newly generated interface with a protein monolayer maximizing the interfacial area per protein and thus minimizing the emulsion droplets. The crude extracts draw a slightly different picture. Their proteins were calculated to cover the interfacial area with multilayers and/or agglomerates. Hence, the stabilized droplets were larger per employed protein than in case of isolated proteins or WPI. Likewise in interfacial shear rheology, the soluble extract showed to contain surfactants, which impeded the establishment of a viscoelastic network. The crude extracts contain interfacial active substances that limit the potential of the protein fraction isolated during the purification. This could be either surfactants, insoluble particles or proteins, which

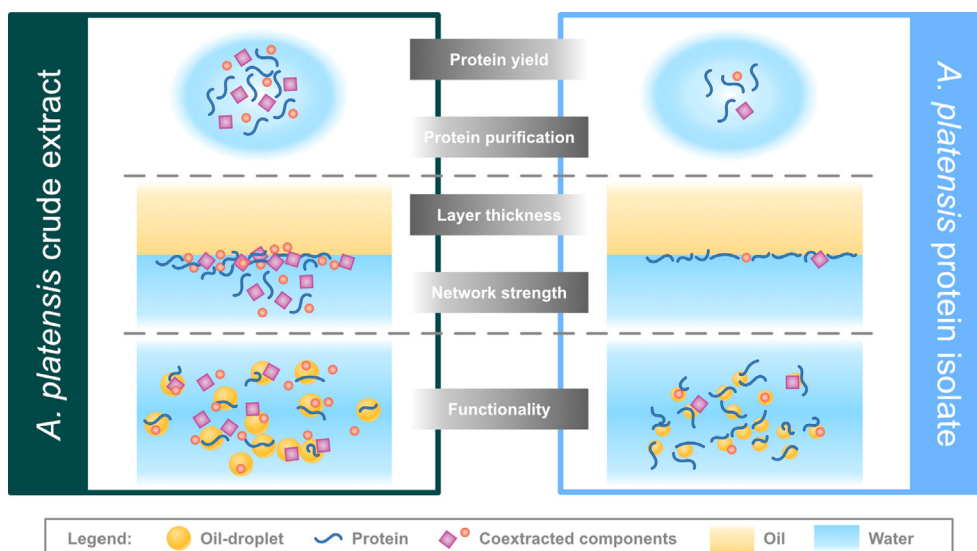


Fig. 6. Schematic figure comparing qualitatively the composition, interfacial adsorption and emulsification potential of crude extracts and protein isolate from *A. platensis*.

are lost during the purification. Fig. 6 attempts to visualize these findings.

4. Conclusions

We demonstrated that extracts from the microalgae *A. platensis* stabilize emulsions independently of protein purification. The crude and water-soluble protein extracts were more efficient with respect to used biomass; however, purification yielded isolated proteins with enhanced functionality yielding smaller emulsion droplets and faster established stronger viscoelastic networks at the oil-water interface.

The study combined the emulsion formation by different microalgae extracts with its network formation at the oil-water interface studied by interfacial shear rheology. Thereby, it could be reasoned that crude extracts of *A. platensis* contain surfactants, which impede the network formation by proteins and thus limit its emulsification potential. Using interfacial shear rheology for evaluating emulsification potential is unique compared to previous literature on microalgae protein extracts which restricted to interfacial tension measurements [64,30] and focused on the performance of protein isolates [28,27].

The insights on emulsion droplet sizes per applied biomass strengthen that purification efficiencies and product requirements have both to be taken into account when designing an alternative protein emulsifier. By isolating proteins, the proteins' specific emulsification potential was enriched but purification efficiencies hampered the yield. This principle should be extended to other single cell protein alternatives such as yeast, fungi, bacteria and other microalgae. Moreover, interfacial shear rheology should be used for future work on the adsorption behaviour and network formation also under varying pH and ionic strength conditions or for differently isolated proteins from microalgae biomass e.g. by filtration approaches.

CRediT authorship contribution statement

Lukas Böcker: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration. **Pascal Bertsch:** Conceptualization, Methodology, Writing - review & editing. **David Wenner:** Formal analysis, Investigation, Writing - original draft. **Stephanie Teixeira:** Formal analysis, Investigation, Writing - original draft. **Jotam Bergfreund:** Methodology, Visualization, Writing - review & editing. **Severin Eder:** Methodology, Writing - review & editing. **Peter Fischer:** Conceptualization, Writing - review & editing. **Alexander Mathys:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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.

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