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A combined ultrasound + membrane ultrafiltration (USN-UF) process for enhancing saccharides separation from *Spirulina* (*Arthrospira platensis)*

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ABSTRACT

In this study the combination of ultrasound (USN) + membrane ultrafiltration (UF) was used to separate *Spirulina* saccharides (SPS). The results showed that USN significantly ($p < 0.05$) increased the average high-added-value compounds yield (mainly protein 572.8 mg/g, saccharides 133.6 mg/g and polyphenols 33.6 mg/g, dw) when the setting of 400 W/10 min was applied. Scanning electron microscope (SEM) results showed that USN treatment effectively destroyed *Spirulina* microstructure. The extraction efficiency differed according to USN total energy consumption, with higher extraction efficiency observed when a cooling system was not used. Different molecular weight cut-offs (MWCOs) membranes were used to separate SPS. Relative high purity SPS (~70%) were obtained when 4 and 10 kDa membranes were used, while a higher separation efficiency occurred when using 100 and 150 kDa membranes. USN treatment increased the concentration of relatively low molecular weight SPS (4, 10 kDa) in the permeate ($p < 0.05$) but there was no significant ($p > 0.05$) effect on the high molecular weight SPS (300, 500 kDa). Membrane fouling was observed during the saccharides UF process, thus indicating that the process could be further improved.

1. Introduction

As the global population increases so will the demand for food consumption. According to the Food and Agriculture Organization of the United Nations (FAO) it will be difficult to meet the food needs of nearly 10 billion people worldwide by 2050 (FAO, 2021). In addition, the COVID-19 pandemic has disrupted and hampered food industry production, distribution, and consumption, which has triggered a food crisis for humanity (Rivera-Ferre et al., 2021). Furthermore, adverse environmental impacts such as increased greenhouse gas emissions, overuse of pesticides, and intensified use of land and fresh water have affected traditional crop-based food supply systems (Chen, Tang, Shi, Zhou, & Fan, 2022). Within this context, the exploitation of edible marine resources, especially microalgae, is being promoted by the food industry because of their ability to survive in harsh conditions and capture solar energy, water and incorporate inorganic nutrients into biomass, and their assimilation efficiency exceeds that of terrestrial plant (Pramanik, Suja, Zain, & Pramanik, 2019).

Spirulina is a type of cyanobacteria most commonly used in animal and human nutrition due to a high protein content from biomass and the biological activity of components such as saccharides, vitamins, minerals, and fatty acids (Costa et al., 2019). The optimization of the extraction conditions of *Spirulina* bioactive compounds has been widely studied, such as traditional extraction methods, including hot water extraction, Soxhlet extraction, Folch, etc. (Zhou et al., 2021). However, innovative extraction methods need to be developed and optimized, since traditional extraction involves the usage of large amounts of toxic solvents, long extraction times, and high extraction temperatures (Zhou et al., 2022). Compared to traditional extraction techniques, ultrasound (USN) extraction has several advantages (i.e., high efficiency, mild operating conditions, low toxicity, and time efficient), Moreover, it has been used in microalgal biorefinery research (Sivaramakrishnan &

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Incharoensakdi, 2018). USN cell disruption is attributed to acoustic cavitation, where ultrasound waves create molecular motion through a series of rarefaction and compression cycles, creating small gas or vapor-filled bubbles in a liquid (Liu, Liu, Cui, & Yuan, 2022; Liu, Zhu, Sun, & Gao, 2022). When the bubbles reach a size where the USN energy is not sufficient to retain the vapor inside, they collapse violently during the compression cycle, releasing a large amount of energy, which produces mechanical/physical and chemical effects of microalgae cell rupture (Wang, Chen, Zhou, Yuan, & Wang, 2020; Wang, Liu, Chen, Yang, & Cao, 2020).

Spirulina contains a variety of high-added-value compounds (HAVCs) such as proteins, polyphenols, pigments, polysaccharides, etc. (Zhou et al., 2023), the separation of these compounds being of great significance for their potential applications. Among the HAVCs from *Spirulina*, polysaccharides from *Spirulina. platensis* have shown potent immunomodulatory, anti-tumor, antioxidant and anti-fatigue activities (Liu, Liu, et al., 2022, Liu, Zhu, et al., 2022).

The separation of crude saccharides from microalgae extracts is a critical step in polysaccharide purification, as it is related the removal of macromolecular impurities such as proteins, pigments, and lipids. At present, some studies have carried out impurity removal before polysaccharide extraction, including the removal of proteins (Sevag, Trichloroacetic acid (TCA), protease), pigments (active carbon, H_2O_2 oxidation), lipids (ethanol soaking), etc., but each step promotes polysaccharide loss, and has a high economic cost (Kang et al., 2022; Qiu et al., 2022; Tang et al., 2020; Tang, Liu, Yin, & Nie, 2020).

Ultrafiltration (UF) technology is considered a green operation technique, with a high separation efficiency and low cost, making it a good candidate for polysaccharide purification compared to common organic reagents used to remove impurities in multiple steps(Tang, Liu, Liu, et al., 2020, Tang, Liu, Yin, & Nie, 2020). Furthermore, the operating conditions of ultrafiltration are conducted under mild temperatures, thus reducing possible denaturation, inactivation or degradation of biomolecules of interest (Balti, Zayoud, Hubert, Beaulieu, & Massé, 2021).

The separation theory of ultrafiltration is based on the molecular weight cut-offs (MWCOs) of the membrane pore size, that is, under a certain hydrostatic pressure and flow rate, molecules of different sizes and shapes in an aqueous solution are separated through the membrane (Feng, Luan, Ning, Shao, & Sun, 2019). Some previous studies have used ultrafiltration membranes with different MWCOs (1, 5, 10, 100 kDa) to obtain crude saccharides of different molecular weights from *Spirulina*, *Chlorella*, etc., and finally obtained purified microalgae saccharides fraction (Cai et al., 2022; Sheng et al., 2007; Wang, Chen, et al., 2020; Wang, Liu, et al., 2020; Yuan et al., 2020). Based on this, in this study, USN extraction parameters were first selected for the most efficient release of *Spirulina* bioactive compounds into aqueous solutions, and then ultrafiltration technology was further applied using membranes of different MWCOs (4, 10, 50, 100, 150, 300, 500 kDa) to separate the saccharides in the extracts, that is, to obtain the crude saccharides efficiently by ultrasound + membrane ultrafiltration (USN-UF) technology, with the aim to provide a reference for the current microalgae *Spirulina* saccharides (SPS) refining industry.

2. Materials and methods

2.1. Materials

For the *Spirulina* culture conditions, during the experiment, the daytime temperature was 32 °C and temperature decreased to 24 °C at night. The pH of the culture varied from 9.8 to 10.4 controlled by the addition of CO_2. Spirulina biomass was filtered using a tambor filter of 30 micra mesh. Cultivation medium went back to the cultivation pond, while biomass was vacuum-pressed and then frozen in 50 g portions.The sample was freeze-dried (−40 °C for 72 h) to reduce the degradation of poorly resistant before use. The nutrients, microbiology and contaminants information of *Spirulina (Arthrospira platensis)* were as follows on a dry weight basis (dw), protein 652 ± 13 mg/g, saccharides 205 ± 4 mg/g, chlorophyll *a* 12 ± 1 mg/g, carotenoids 6 ± 1 mg/g, polyphenols 38 ± 3 mg/g, Ferric 1497 ± 15 mg/kg, β-carotene 1339 ± 10 mg/kg, mesophilic aerobic flora <2000 UFC/g, *Enterobacteria* < 100 UFC/g, *E.Coli* ND, *Staph aureus* < 10 UFC/g, *Salmonella* ND, Yeasts/molds <100 UFC/g, pesticides ND, cadmium 0.032 ± 0.001 mg/kg, arsenic 0.78 ± 0.03 mg/kg, mercury <0.005 mg/kg, benzopyrene <0.5 μg/kg. Folin-Ciocalteu, gallic acid and phenol were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). D-glucose (98%) and bovine serum albumin (BSA, 98%) standard were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific. Polyether sulfones (PES) membranes with MWCOs of 4, 10, 100, 150, 300 and 500 kDa were purchased from Microdyn-Nadir Company (Germany).

2.2. Ultrasound (USN) extraction

2.2.1. USN extraction process

A UP-400S ultrasound processor (Hielscher GmbH, Germany) with a constant frequency of 24 kHz was used for ultrasound extraction (Zhang, Lebovka, Marchal, Vorobiev, & Grimi, 2020). The ultrasound probe (diameter: 14 mm, length: 100 mm) was submerged in a beaker, containing a 2.0% suspension (Tavakoli et al., 2021a, 2021b). The total treatment time of USN extraction was 5, 10, 15, 20 min, and the amplitude was fixed at 25%, 50%, 75%, 100%, which corresponded to the power of 100, 200, 300, 400 W. The temperature increase was recorded every minute and USN-cooling system (USN-CS) extraction was carried out to evaluate the thermal effects during ultrasound extraction. The control group extraction (2.0%, dw/V) at different temperatures (20/30/40/50/60/70 °C) and time (5/10/15/20 min) using a magnetic stirrer was set to calculate the yield increase after USN treatments. After the extraction process, the extracts were centrifuged using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 ×g for 15 min, then the supernatants were collected for further analysis.

2.2.2. Specific energy analysis

Specific energy consumption, W (kWh/kg suspension), was calculated for ultrasound treatment according to the following formula (Zhang et al., 2020):

$$W(kWh/kg) = P(w)*t(s)/m(kg) \quad (1)$$

where P (W) is the ultrasound power, t (s) is the time of USN extraction process, m is the mass of 2.0% suspension (kg).

2.2.3. Protein quantification

The bicinchoninic acid (BCA) method was used to measure the protein content in the extracts (Balti et al., 2021; Zhou et al., 2022). Protein content was determined using a calibration curve (0–2000 mg/L) using bovine serum albumin (BSA) as a standard. During the measurement, 10 μL of samples or BSA and 200 μL of BCA working solution were added and incubated at 37 °C for 30 min, the absorbance was then measured at 562 nm using an Ultraviolet-visible (UV–Vis) spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA).

2.2.4. Saccharides quantification

The saccharidess content in the extracts was measured with the concentrated sulfuric acid-phenol method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Specifically, 1 mL of sample, 0.5 mL of phenol and 2.5 mL of H_2SO_4 (98%) were sequentially added to a glass reaction flask and reacted for 30 min at room temperature. The absorbance value at 490 nm was measured using a UV–Vis spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). D-glucose was used as a

standard for calculating saccharides content.

2.2.5. Polyphenols quantification

The Folin-Ciocalteu method was used to analyze the polyphenol content in the extracts (Kokkali et al., 2020; Li et al., 2022). That is, 0.2 mL of sample, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, *v*/v) and 0.8 mL of Na_2CO_3 solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 min. Then, the absorbances were measured at 750 nm using a UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). Gallic acid was used as a standard to prepare the calibration curve for quantifying the polyphenol content in the extracts.

2.2.6. Pigments quantification

Pigments analysis was carried out according to the method proposed by (Gustavo, Pedro, Cláudia, Diego, & Celso, 2018) with some minor modifications. Specifically, 200 μL of algae extract was mixed with 1300 μL of pure methanol and incubated in the dark for 1 h at 45 °C. Samples were then centrifuged at 10,000 *xg* for 10 min at 20 °C. The organic phase containing the pigments was recovered and the absorbance was measured using a UV–Vis spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The chlorophyll *a* and carotenoid content were calculated using the following equations (Gustavo et al., 2018), these equations were simplified in view of the absence of chlorophyll b in *A. platensis*, and they were reduced to the following equations:

$$Chlorophyll\ a\ (mg/L) = 12.6{*}A665.2 \tag{2}$$

$$Carotenoids\ (mg/L) = (1000x\,A470 - 1.63x\,Cchlorophyll\ a) \div 221 \tag{3}$$

where A665.2=Absorbance at 665.2 nm; A470=Absorbance at 470 nm.

The final yields of chlorophylls and carotenoids were calculated as mg/g (dw) in microalgae, the equation was as follows:

$$Yield\ (mg/g\ dw) = C_{pigments\ (mg/L)}{*}V_{extracts\ (L)}/W_{microalgae\ (g)} \tag{4}$$

Where C, V and W are the abbreviation of concentration, volume, and weight respectively.

2.2.7. Ultrasound increment analysis

The yield increase after ultrasound treatments was calculated using the following equations:

$$Yield\ increase\ after\ ultrasound\ (mg/g\ dw) = Yields_{USN} - Yields_{E} \tag{5}$$

Where USN is ultrasound extraction, E is control extraction.

2.2.8. Component ratio calculation

$$Dynamic\ proportion\ (\%) = (X/T){*}100\% \tag{6}$$

where X is the content of specific bioactive compounds (protein, polyphenol, chlorophyll *a*, carotenoids, mg/g dw), T is the total amount of the compounds in the extracts (the sum of protein, polyphenol, chlorophyll *a*, carotenoids content, mg/g dw).

2.2.9. Scanning Electron Microscope (SEM) analysis

The precipitate of the extracts after centrifugation was used to analyze the effects of USN on *Spirulina* microscopic morphology (Fang, Xu, Kawashima, Hata, & Kijima, 2021). Scanning electron microscopy (SEM Quanta 250 FEI Company, Eindhoven, The Netherlands) was used to analyze the microstructure of samples after freeze drying (MUT 002A pilot freeze-drier (Cryotec, France)) for 72 h at a cold trap temperature of −65 °C. Freeze-dried samples were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with an SEM microscope at magnifications of 1000×.

2.3. Membrane ultrafiltration (UF) of Spirulina saccharides

2.3.1. UF process

Membranes with different MWCOs were used to separate saccharides from the extracts, following the study of (Tang, Liu, Liu, et al., 2020; Tang, Liu, Yin, & Nie, 2020). Filtration was performed in a stirred cell Amicon 8200 (Millipore, Billaica, USA) with a membrane diameter of 63.5 mm and maximal volume of 200 mL. Compressed air was used to supply a transmembrane pressure (TMP) of 2 bars. The temperature of the extracts was kept at 20 °C during the filtration process. Seven kinds of polyether sulfones (PES) membranes (Microdyn-Nadir Company (Germany)) with MWCOs of 4, 10, 100, 150, 300 and 500 kDa were used to purify the extracts. According to the reference of Microdyn-Nadir Company (Germany), the PES membranes were soaked with deionized water for 30 min prior to use. A magnetic stirrer fixed over the membrane surface provided a constant stirring speed of 500 rpm.

Prior to ultrafiltration, 100 mL of deionized water was ultrafiltered using a soaked membrane, and the volume change of the permeate was recorded. Then, 100 mL of *Spirulina* USN extract was added into the ultrafiltration container and the ultrafiltration process was performed until the permeate volume was 90 mL and the change of the permeate volume was also recorded. The permeate of *Spirulina* extracts was collected for heat concentration (95 °C, 3 h), and the concentrated solution was centrifuged (14,100 *xg* for 15 min) and freeze-dried to obtain crude saccharides powder for subsequent analysis. After the ultrafiltration process of the *Spirulina* extract, deionized water was used to clean the cake layer on the surface of the membrane, and the cleaned membrane was again used for deionized water (100 mL) ultrafiltration with the change in the volume of the permeate was recorded. The membrane ultrafiltration process followed control extraction (E) was abbreviated as *E*-UF.

2.3.2. pH and conductivity analysis

The electrical conductivity (μS/cm) and pH of *Spirulina* membrane permeates were measured with a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany). The volume reduction ratio (VRR) was defined as follows (Zhu et al., 2015):

$$VRR(mL) = \frac{V_{initial}}{V_{concentrate}} \tag{7}$$

where $V_{initial}$ is the initial extracts volume (mL) and $V_{concentrate}$ is the concentrate volume (mL).

2.3.3. Permeation flux analysis

The permeate flux ($mL.min^{-1}.m^{-2}$) was calculated according to the change of permeate volume over time, which was used to evaluate the filtration efficiency of the PES membrane, including the water flux, permeate flux of extracts during ultrafiltration and water flux after extracts ultrafiltration. The calculation formula is as follows (Zhu et al., 2015):

$$J\left(mL.min^{-1}.m^{-2}\right) = \frac{dv}{dt}{*}A^{-1} \tag{8}$$

Where *dv* is the derivative of volume (mL) and *dt* is the derivative of time (min), A is membrane area (m^2).

2.4. Statistical analysis

The results of bioactive compounds yield at different extraction parameters (100/200/300/400 W, 5/10/15/20 min) were analyzed using an ANOVA test followed by Tukey post-test. The results were expressed as mean ± standard deviation, and results were considered significant when the *p*-value was lower than 0.05. Different lowercase letters represent significant differences ($p < 0.05$), and ns represent no significant differences ($p > 0.05$). Those parameters of interest were subjected

to principal component analysis (PCA) using the Graph 9 software to measure the correlation matrix. The inputted variables among different samples were mapped as a biplot where the loadings and scores were distributed in two-dimensional section determined by the first two principal components (PCs) with eigenvalues >1.0.

3. Results and discussion

3.1. Yield of high-added-value compounds and specific energy consumption

Fig. 1 showed the yield and specific energy consumption of *Spirulina* USN extraction of HAVCs and evaluation of the impact of thermal effect on USN extraction efficiency. From Fig. 1, it could be seen that the temperature of USN-no cooling system (USN-NCS) increased from 18 °C to 40–80 °C (100–400 W) from 5 to 20 min, and the median temperature of USN-CS was below 30 °C (100–400 W), which indicates that USN produced a strong thermal effect. Based on this, the extraction efficiency and energy consumption of USN-NCS and USN-CS was compared. Overall, it was found that the combination of USN-NCS is beneficial to recover *Spirulina* HAVCs. Specifically, USN-NCS with prolonged extraction time (up to 20 min) allowed for obtaining around 550–600 mg/g protein, 35–40 mg/g polyphenols, 130–140 mg/g saccharides, 1.1–1.3 mg/g of chlorophyll *a*, and 0.25–0.28 mg/g of carotenoids. Correspondingly, USN-CS could yield 500–550 mg/g protein, 35–40 mg/g polyphenols, 115–125 mg/g saccharides, 1.1–1.3 mg/g mg/g of chlorophyll *a*, and 0.25–0.27 mg/g of carotenoids. From the perspective of energy consumption, the biomass extraction yield, upon reaching a plateau, will no longer depend on power intensity, but on total energy consumption. The energy requirements (kWh/kg) for USN-NCS extraction to reach the maximum recovery yields of HAVCs were lower than those for USN-CS, i.e., protein (380 vs 450 kWh/kg), polyphenols (350 vs 450 kWh/kg), saccharides (460 vs 470 kWh/kg), chlorophyll *a* (350 vs 450 kWh/kg), carotenoids (360 vs 460 kWh/kg) and total compounds (350 vs 450 kWh/kg) suggesting that ultrasound extraction without a cooling system is beneficial for improved extraction yields and energy savings.

The increased yields of the bioactive compounds were due to the disruption effects of USN on microalgae. Acoustic cavitation was an accepted theory of ultrasonic cell disruption (Liu, Liu, et al., 2022; Liu, Zhu, et al., 2022; Wani & Uppaluri, 2022), and low ultrasonic frequencies in the 20–40 kHz range was the most commonly used frequencies in the extraction process of microalgae (González-Balderas, Velásquez-Orta, Valdez-Vazquez, & Orta Ledesma, 2020; Greenly & Tester, 2015), especially those with thin cell walls. When the intensity of ultrasonic waves was high enough, it generated severe shear forces, high pressures and temperatures, thus promoting microalgal cell disruption and the release of HAVCs (Al Khawli, Martí-Quijal, Pallarés, Barba, & Ferrer, 2021), resulting in a higher yield of USN-NCS than US-CS (Chen, Li, Zhang, Tyagi, & Dong, 2018). The high temperature generated by USN-NCS was detrimental to the properties of proteins and some pigments. Since one of the purposes of this study was to separate the crude saccharides from *Spirulina* (stable at 80 °C), considering the time efficiency, the USN-NCS extraction method (400 W, 10 min) was selected for further analysis.

3.2. USN-NCS extraction yields increase

The thermal effect in the USN-NCS extraction process can promote the extraction of the HAVCs of *Spirulina*, and it is necessary to evaluate the increase attributed to ultrasonic extraction in addition to the thermal effect. The results in Fig. 2 showed that compared with the hot water extraction method, the USN-NCS greatly increased the extraction yield of *Spirulina* biomass, especially when the ultrasonic power was 200 W, 300 W and 400 W. When the ultrasonic power was 400 W and the extraction time was 20 min, the USN-NCS increases were 354.6 ± 18.2 mg/g dw for protein, 15.8 ± 2.6 mg/g dw for polyphenols, 59.8 ± 3.8 mg/g dw for saccharides, 1.2 ± 0.1 mg/g dw for chlorophyll *a*, and 0.25 ± 0.02 mg/g dw for carotenoids. Some related studies have demonstrated results similar to this study. For instance, Vernès et al. (2019) obtained an increased protein recovery up to 28.42 ± 1.15 g/100 g dw after using ultrasonic isothermal extraction (20 kHz, 1000 W, 20 min) (Vernès et al., 2019), while Tavakoli et al. (2021a, 2021b) significantly increased ($p < 0.05$) the yields of chlorophyll *a*, and total carotenoids of ultrasound obtained *Spirulina* extracts (30 kHz, 100 W, 5–30 min) (Tavakoli et al., 2021a, 2021b).

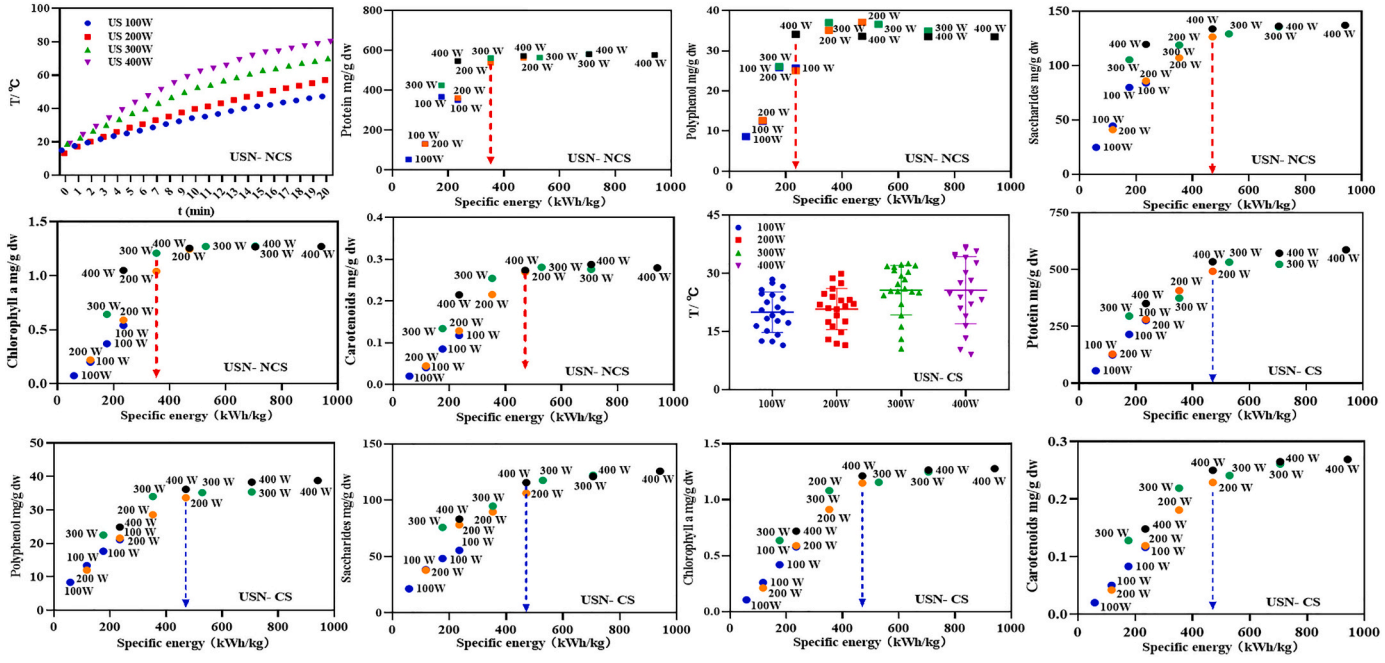

Fig. 1. The thermal effects and specific energy consumption (kWh/kg) in terms of various bioactive compounds yields (mg/g dw) during ultrasound (USN) extraction. USN-NCS, Ultrasound-Not using cooling system. USN-CS, Ultrasound-Using cooling system.

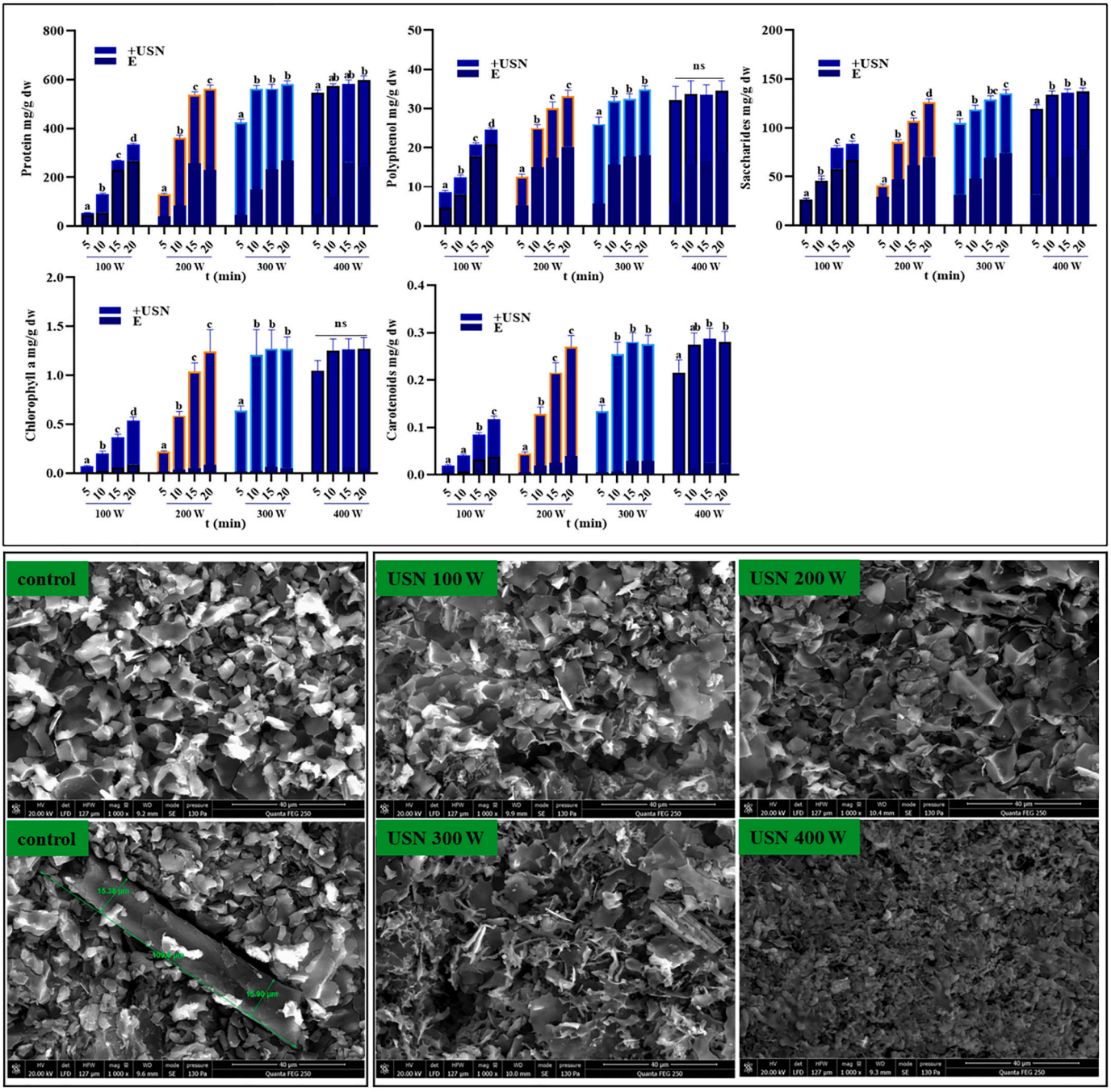

Fig. 2. Increment of specific bioactive compounds yields by ultrasound extraction under different extraction parameters (100/200/300/400 W, 5/10/15/20 min) and Scanning electron microscope (SEM) analysis of *Spirulina* extraction residue microstructure (control /USN100 W/ USN 200 W/ USN 300 W/ USN 400 W, 10 min). USN-ultrasound, *E*-aqueous extraction. Different lowercase letters represent significant differences ($p < 0.05$), and ns represent no significant differences ($p > 0.05$).

When evaluating the ultrasonic extraction yields at different extraction times, the results showed that the extraction yields increased significantly ($p < 0.05$) with the elapse of time when the extraction powers were 100 W and 200 W. However, when the extraction powers were 300 W and 400 W, no significant increase ($p > 0.05$) were found with the elapse of extraction time, especially when the extraction power was 400 W. This result was attributed to the working principle of USN. When the USN intensity was large enough (300–400 W), the attractive forces between the molecules in the liquid phase were exceeded by the expelling force, and the cavities were generated into the liquid (Chemat et al., 2017). The high energy intensity generated severe shearing forces, high pressures, and high temperatures (Chen et al., 2018), which results in the extraction process being carried out efficiently, thus reaching the extraction target in a relatively short time.

3.3. Effects of USN process on Spirulina morphology

The effect of different USN conditions (100/200/300/400 W, 10 min) on the microscopic morphology of *Spirulina* was shown in Fig. 2. The results showed that the morphology of *Spirulina* in the control group was complete, and a complete rod-like structure could be observed, with a diameter of 15.38–15.90 μm, the length being 109.8 μm. When using USN extraction, the morphology of *Spirulina* became fragmented with increasing USN power. Relatively intact structures remained after

ultrasonic extraction with low power (100 and 200 W), while the *Spirulina* morphology became more fragmented when high-power ultrasonic extraction (300 and 400 W) was used, especially at 400 W. There are three mechanisms for ultrasonic-induced *Spirulina* cell rupture. First, the acoustic cavitation generated by the mechanical effect can generate enough shear force to directly rupture the microalgal cells and lead to cell fragmentation (Yao, Mettu, Law, Ashokkumar, & Martin, 2018). Secondly, if the size of the oscillating bubbles in the ultrasonic system is comparable to that of microalgal cells, mechanical resonance can be excited effectively, thereby inducing cell rupture (Kurokawa et al., 2016). Finally, the cavitation bubbles explode rapidly during sonication, which can lead to local temperature and pressure increases as high as 5000 K and 100 MPa, respectively (Peng et al., 2020). When using 24 kHz with 400 W USN power, the *Spirulina* cells are disrupted by both mechanical and thermal effects, resulting in a rapid release of intracellular bioactive compounds, which accelerates the extraction process.

3.4. Membrane ultrafiltration process (UF)

3.4.1. Membrane permeability analysis

All membrane ultrafiltration finished with the collection of 90 mL of permeate (total volume of the feed: 100 mL), with the membrane separation fluxes (J, mL.min^{-1}.m^{-2}) of different MWCOs calculated (Fig. 3). Figs. 3A, B, C correspond to the ultrafiltration permeate fluxes of deionized water, ultrafiltration permeates fluxes of *Spirulina* extract, and permeate fluxes of deionized water of the cleaned membrane after ultrafiltration process of *Spirulina* extract. The results in Fig. 3A showed that the permeate flux of membrane (deionized water) differed according to membrane MWCOs, that was, 500 > 300 > 150 > 100 > 10 > 4 kDa, and the permeation fluxes were 0.955, 0.786, 0.658, 0.547, 0.537 and 0.377 mL.min^{-1}.m^{-2}, respectively.

Figs. 3B showed the ultrafiltration flux curves of *Spirulina* USN extracts. The results showed that it took 170–280 min to concentrate 100 mL of USN extract to 10 mL, and the order of ultrafiltration was 500, 300, 150, 100, 10 and 4 kDa, which was related to the size of the membrane MWCOs. With the elapse of ultrafiltration time, the permeate flux value gradually decreased. The apparent decrease in membrane ultrafiltration flux with the elapse of ultrafiltration time was attributed to the occurrence of membrane fouling (Xu et al., 2022). In general, the fouling on ultrafiltration membranes was mostly affected by the filter 'cake layer' or aggregates (Tanudjaja, Ng, & Chew, 2022), and the pH value of the ultrafiltration liquid and the concentration of HAVCs, as well as the membrane material and operating variables (Marson et al., 2021).

Fig. 3B also showed that the membrane permeation flux of *Spirulina* extracts did not always depends on the size of the membrane MWCOs. For example, in the first 70 min of ultrafiltration, the permeate flux of 150 kDa membrane was higher than 300 kDa, and after 120 min, the permeate flux of 10 kDa membrane was higher than 100 kDa and 150 kDa. Similar results were reported by Chaiklahan, Chirasuwan, Loha, Tia, and Bunnag (2011), who used a membrane with cut offs of 50 kDa, 70 kDa and 100 kDa to isolate *Spirulina* phycocyanin, with permeation flux of 70 kDa being <50 kDa at 85 min (Chaiklahan et al., 2011).

Membrane cleaning and surface 'cake layer' removal was performed after the ultrafiltration of *Spirulina* extracts, and the membrane permeation flux was tested again with deionized water.

The results in Fig. 3C showed that the membrane permeation flux was not completely dependent on the MWCOs of the membrane, which was, 150 > 100 > 500 > 300 > 10 > 4 kDa, and the final stable permeation fluxes corresponded to 0.324, 0.209, 0.123, 0.122, 0.050 and 0.035 mL.min^{-1}.m^{-2}, respectively. The difference between Fig. 3A and Fig. 3C indicated that an irreversible membrane fouling occurred during the *Spirulina* extracts UF process. Related previous reports showed that if the fouling on the membrane surface was dominant, the

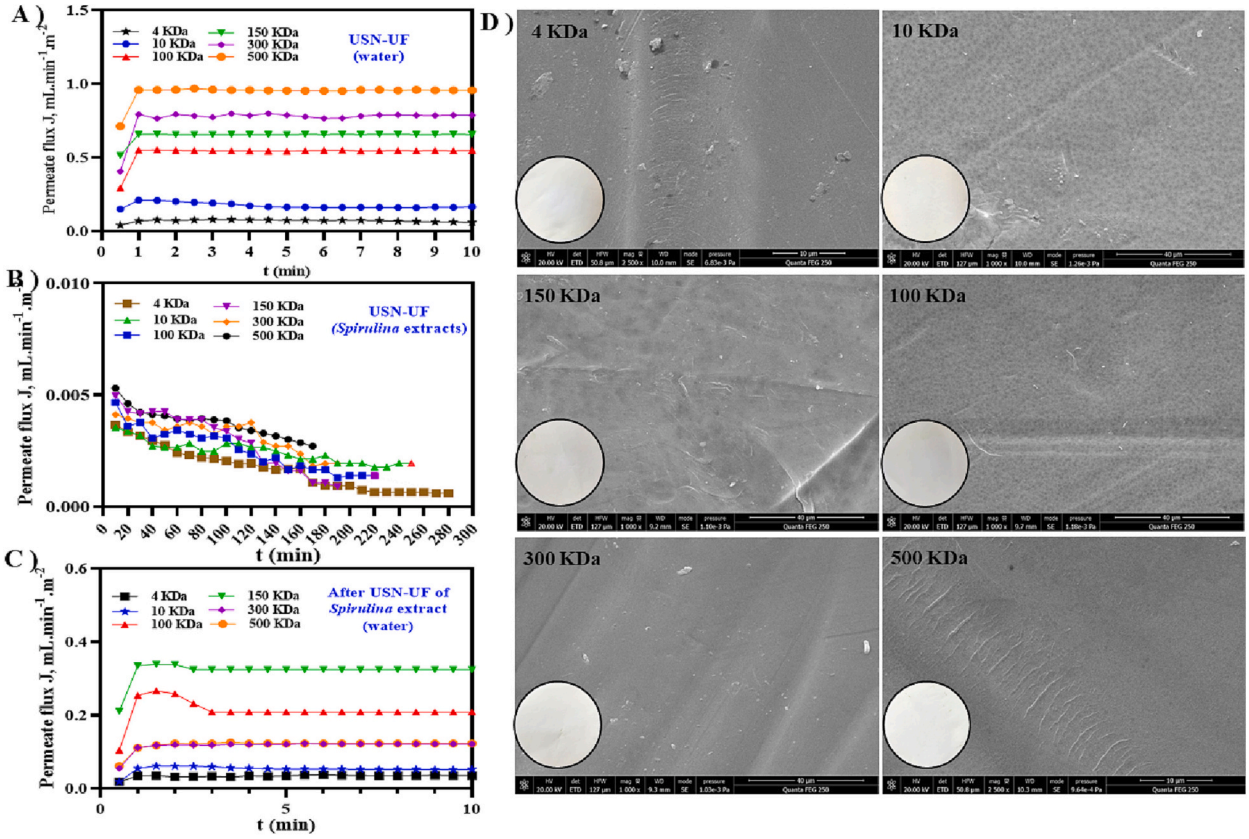

Fig. 3. Membrane ultrafiltration (4/10/100/150/300/500 kDa) permeate flux. A) USN-MUF of water, B) USN-UF of *Spirulina* extracts, C) After USN-UF of *Spirulina* extracts (water). D) is the micrograph of membrane surface after ultrafiltration.

increase in reversible fouling led to a decrease in membrane permeate flux (Marshall, Munro, & Trägårdh, 1993). When the internal fouling increases, the opposite result is found, that is, the membrane permeability is irreversible (Tanudjaja et al., 2022). The results of this study showed that the HAVCs entered the membrane during the membrane separation process, resulting in an irreversible membrane fouling. Fig. 3D shows a microscopic view of the surface of the ultrafiltration membrane after cleaning the 'cake layer'. It is obvious that there are 'granular' black spots on the 10 kDa, 100 kDa and 150 kDa membranes, these being HAVCs that penetrated into the membrane layer, thus confirming that an irreversible fouling occurred in the UF process.

3.4.2. pH and conductivity (μS/cm)

Permeate was collected every 10 mL during each ultrafiltration process, and the pH, conductivity (μS/cm) and resistivity (Ω/cm) of the permeate were monitored. The results in Fig. 4 showed that the pH of the ultrafiltration permeate in the USN-UF and *E*-UF groups remained stable at around, ~7.0. With the ultrafiltration process, the conductivity of the permeate exhibited an upward trend. The initial conductivities of USN-UF and *E*-UF were 1055–1197 μS/cm and 1008–1145 μS/cm, respectively, and the final conductivities were 1366–1578 μS/cm and 1310–1499 μS/cm, respectively. Balti et al. (2021) reported similar results, showing a decrease in the conductivity of the retentate as diafiltration progressed while the pH remained relatively constant between 7.1 and 7.4 during diafiltration (Balti et al., 2021). The changes in conductivity should theoretically be related to the type and concentration of molecules contained in the permeate, which could indirectly reflect the content of bioactive components in the permeate.

3.4.3. Analysis of permeate components

The original extract of microalgae typically contains a complex mixture of HAVCs, such as proteins, polyphenols, saccharides, pigments, etc. (Zhou et al., 2023). Therefore, removal of impurities in a clean and efficient way to obtain the targeted HAVCs is an extremely important step. The results in Fig. 5 showed that the removal rates of both proteins and polyphenols increased with decreasing membrane MWCOs, and the removal rates of pigments were close to 100%. After the application of USN-UF, the removal rates of proteins and polyphenols were 88.35% (4 kDa) ~ 79.88% (500 kDa) and 87.66% (4 kDa) ~ 82.14% (500 kDa), respectively. For *E*-UF, the removal rates of proteins and polyphenols were 70.41% (4 kDa) ~ 60.70% (500 kDa) and 80.56% (4 kDa) ~ 73.77% (500 kDa), respectively. The results showed that the protein, polyphenol, and pigment removal efficiency of USN-UF was higher than that of *E*-UF.

On the other hand, the effects of protein and polyphenol removal by 75% ethanol precipitation and 90 °C heating concentration were also evaluated. The results showed that compared with UF process in this study, 75% ethanol precipitation and 90 °C heating concentration was less effective in removing proteins, polyphenols, and pigments.

Fig. 6 showed the results of the concentration of *Spirulina* saccharides in different MWCOs permeates (Fig. 6A, B, C) as well as the appearance (Fig. 6F, G) and purity (Fig. 6I) of the final crude saccharide products. From the results obtained, whether it was USN-UF or E-UF, the saccharide concentration in the permeate increased as membrane MWCOs increased. The saccharide concentration in the USN-UF group was similar to that in the E-UF group when 100 kDa and 150 kDa membranes were used. A slightly higher saccharide concentration was obtained in the USN-UF permeate than the E-UF permeate when using 300 kDa and 500 kDa membranes, while the saccharide concentration was significantly higher in USN-UF permeate when the 4 kDa and 10 kDa membranes were used in the UF process. This indicates that USN extraction promoted the separation of *Spirulina* saccharides with molecular weight <4 or 10 kDa, and slightly promoted the separation of *Spirulina* saccharides <300 or 500 kDa, whereas the saccharides with molecular weight <100 or 150 kDa were not significantly affected. The crude saccharide permeate from *Spirulina* was further concentrated at a high temperature (95 °C, 3 h), centrifuged (14,100 ×*g*, 15 min), and finally freeze-dried to obtain the light-yellow powder Fig. 6G. The saccharides ratio in the initial extract (Fig. 6E) was <20% (Fig. 6H), while the purity of the final crude saccharides powder was close to 70% (Fig. 6I). Furthermore, the results in Fig. 6I showed that the purity of the final crude saccharides gradually decreased with the increase of

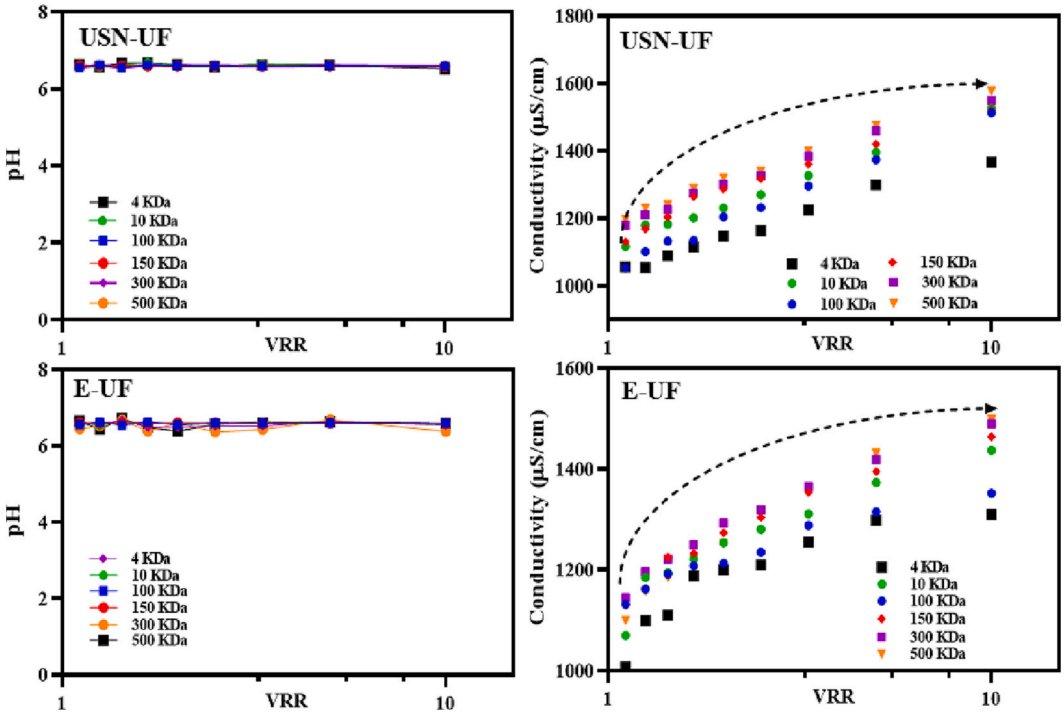

Fig. 4. Evaluation of pH and conductivity (μS/cm) change of permeates with volume reduction rate (VRR). USN-UF, ultrasound extraction + ultrafiltration. E-UF, aqueous extraction + ultrafiltration.

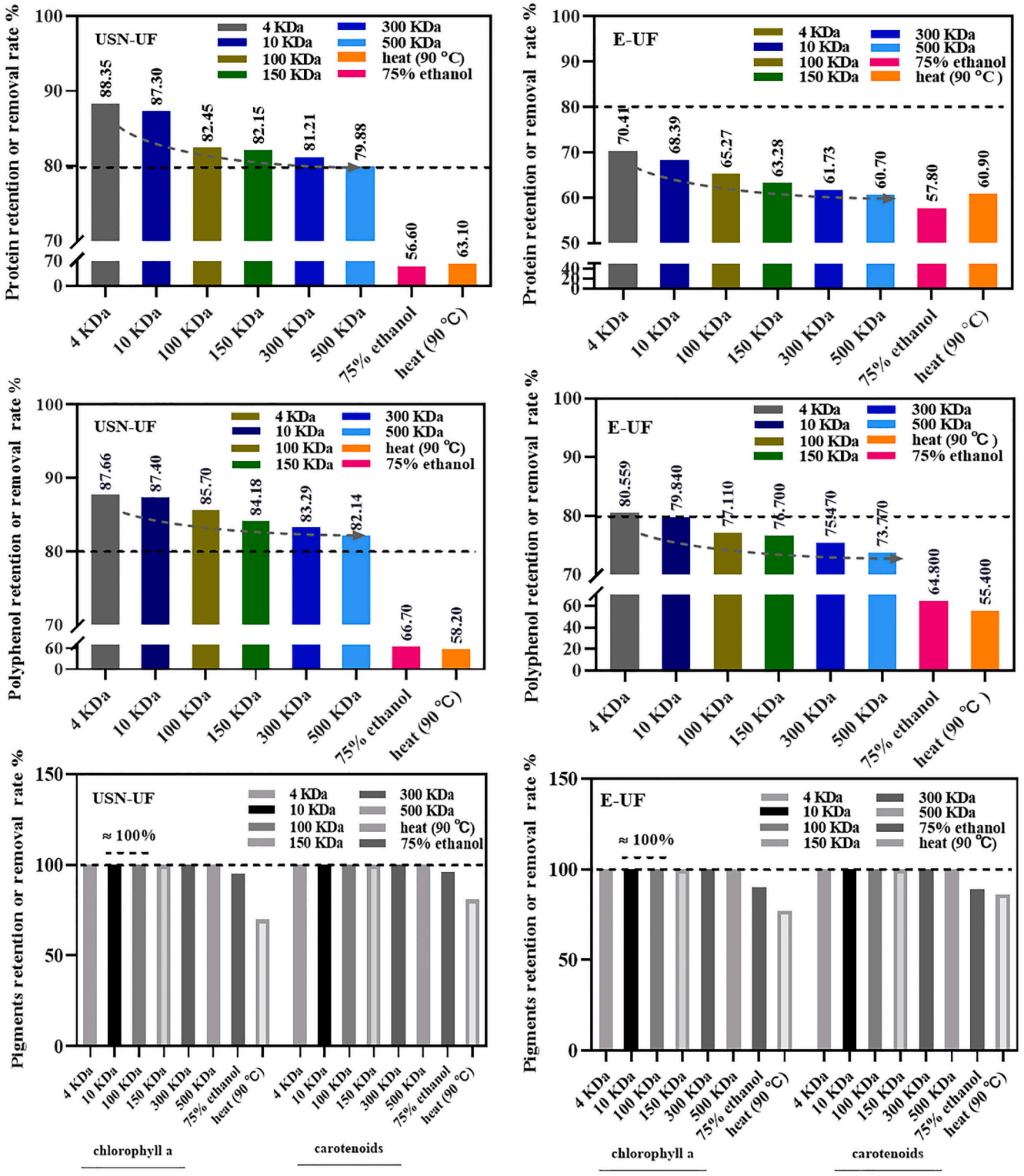

Fig. 5. Retention rate of proteins, polyphenols, and pigments through different cut-off membranes during polysaccharides separation process. USN-UF, ultrasound extraction + ultrafiltration. E-UF, aqueous extraction + ultrafiltration.

membrane MWCOs.

Based on all the results, we analyzed the yields of USN-, E-, UF- on bioactive compounds using multifactorial analysis (principal component analysis, PCA). The two principal components explained the 97.5% and 1.4% of variability, respectively, in total about 98.9% (Fig. 7). The results showed that the energy of 352.9–942.1 kWh/kg was close to the distribution of bioactive compounds, indicating that this energy range was suitable for USN extraction of *Spirulina* nutrients. However, due to the loss of bioactive substances caused by the ultrafiltration process, the ultrafiltration conditions, and the yield of bioactive substances in the permeate are distributed in different quadrants.

In previous related reports, the separation of crude SPS from microalgae requires step-by-step removal of pigments, proteins, polyphenols, etc., while involving a variety of chemical and biological

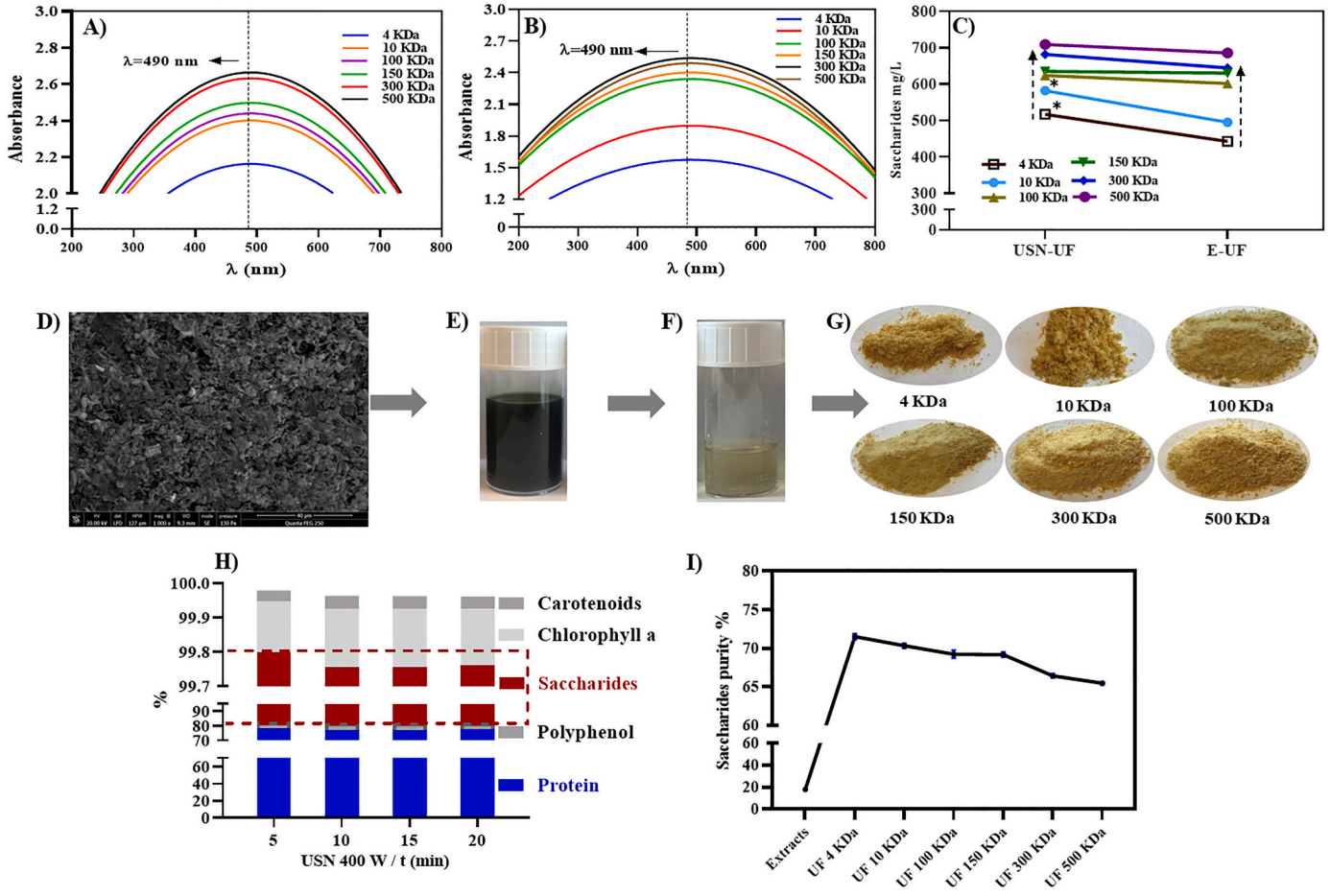

Fig. 6. Saccharides concentration of membrane ultrafiltration permeates and appearance and purity of final crude saccharide. Fig. 6A) and 6B) corresponds to the UV–Vis (490 nm) absorbance of USN-UF and E-UF permeates during sulfuric acid-phenol analysis. Fig. 6C) is the saccharides concentration in USN-UF and E-UF permeates. Fig. 6D), E), F), G) corresponds to the *Spirulina* microscopic morphology after USN, *Spirulina*-USN extracts, *Spirulina*-USN-UF permeates and final saccharides powder. Fig. 6H) is the initial ratio of bioactive compounds in the *Spirulina*-USN extracts, Fig. 6I) is the final saccharides purity.

reagents, such as chloroform, n-butanol, TCA, protease, activated carbon, H_2O_2, ethanol, etc., which not only led to the gradual loss of SPS but also increased cost and pollution (Kang et al., 2022; Qiu et al., 2022; Tang, Liu, Liu, et al., 2020; Tang, Liu, Yin, & Nie, 2020). In contrast, no complicated steps or contaminating reagents were used during the UF process to separate crude saccharides in this study, which show the green and high-efficiency advantages of UF process.

Membrane separation techniques have been used to separate saccharides from different plant tissues. For example, Tang, Liu, Liu, et al. (2020), Tang, Liu, Yin, & Nie, 2020 isolated crude saccharides from *Lentinus edodes* using UF with MWCOs of 2.5 kDa (GH2540F30, GE, USA), 5 kDa (PE5, SEPRO, USA) and 10 kDa (PE10HR, SEPRO, USA). The final saccharide purity ranged from 22.4% to 86.1%, which contained some amino acid components derived from polypeptide or protein moieties (Tang, Liu, Liu, et al., 2020, Tang, Liu, Yin, & Nie, 2020).

Wang, Chen, et al. (2020), Wang, Liu, et al. (2020) used Sevag, Sephadex G-200 column chromatography combined with ultrafiltration membrane technology to separate SPS from *Spirulina*, and fobtained total saccharides with a purity of 92.60% ± 2.01%, which contained 1.67% ± 0.35% of protein (Wang, Chen, et al., 2020; Wang, Liu, et al., 2020). Therefore, these studies demonstrated that membrane technology could be used for the separation of HAVCs due to its advantages of high product yield and separation efficiency, simple scale-up and equipment cleaning (Marson et al., 2021). Similar to our study, these related studies failed to obtain pure saccharides, due to the presence of protein-saccharide conjugates (naturally occurring hydrogen bonds and interactions between saccharides and proteins) in the *Spirulina* extract (Jackson, 1977), which was extremely difficult for saccharides separation.

Combined with the membrane permeate flux and the final saccharide purity, under the USN extraction conditions (400 W, 10 min), choosing a 4 or 10 kDa membrane could obtain *Spirulina* saccharide with a relatively higher purity, while choosing 100 or 150 kDa membranes was more efficient in UF process. In general, this study obtained saccharides with over 70% purity (50% ↑) from *Spirulina* original extract (~ 20%) by USN-UF technology, which provided the basis for further obtaining higher purity SPS. Furthermore, we are working on the structural and compositional analysis of polysaccharides isolated from different MWCOs membranes, as well as their effects on human gut health, which will be reported in the future.

4. Conclusions

Ultrasound is an efficient extraction technique for obtaining high-added-value compounds from *Spirulina*. During the ultrasound extraction process, not using the cooling system can increase the extraction efficiency and decrease the energy consumption. This study suggests that the ultrasound extraction parameters can be set to 400 W/10 min to achieve the ideal extraction target with a 2.0% *Spirulina* suspension. When water is used as the extraction solvent, ultrasound combined with membrane separation technology can be used as a solvent-free and simple-technology process to separate crude saccharides from *Spirulina*

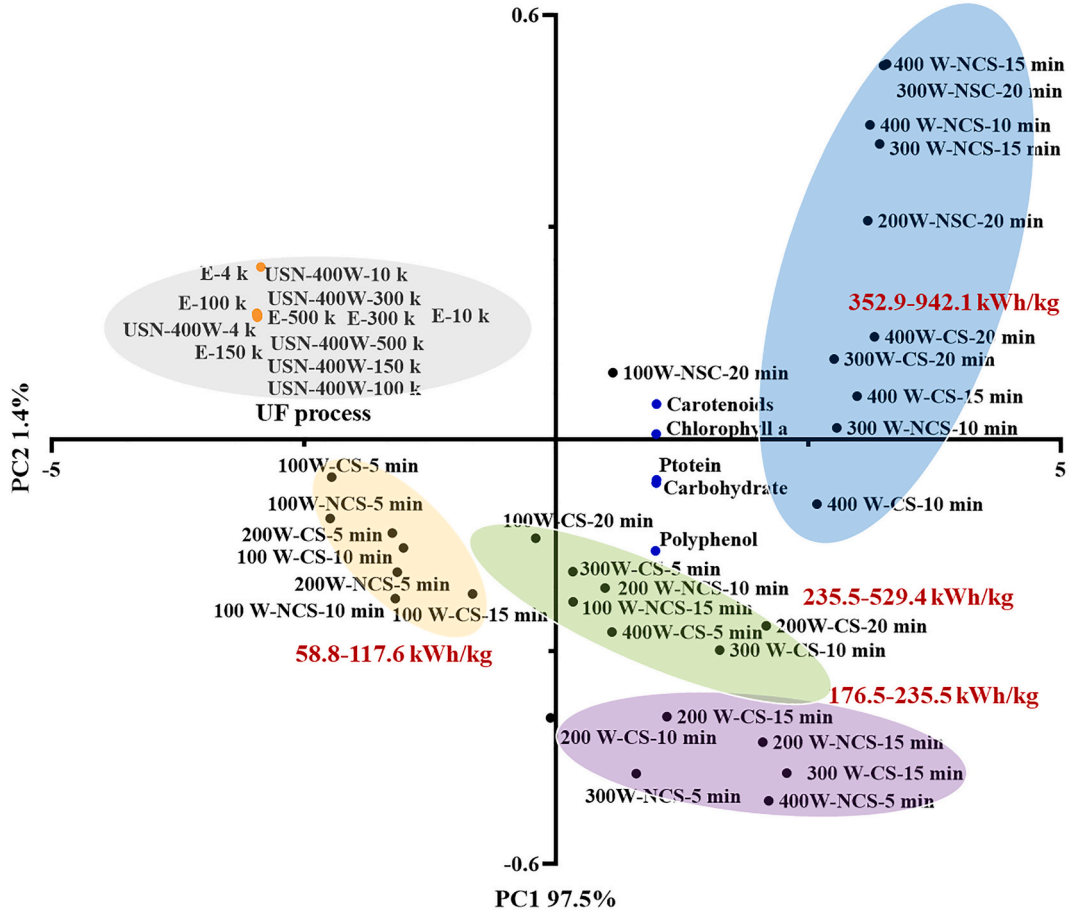

Fig. 7. Principal Component Analysis (PCA) based on the extraction parameters, energy consumption, bioactive compounds yield and membrane ultrafiltration process. USN-NCS, Ultrasound-Not using cooling system. USN-CS, Ultrasound-Using cooling system. UF- ultrafiltration process.

extract. When using a 4 kDa membrane, crude saccharides with relatively high purity can be obtained, which can be used as a reference for the purification process of algae polysaccharide. Membrane fouling occurs during membrane separation process, which reduces permeate flux. Therefore, in industrial applications, many factors such as the concentration of the extract, the pressure value, and the speed of magnetic stirring should be considered to mitigate the occurrence of membrane fouling.

CRediT authorship contribution statement

Jianjun Zhou: Conceptualization, Methodology, Software, Data curation, Formal analysis, Investigation, Validation, Writing - original draft. **Min Wang:** Conceptualization, Methodology, Software, Visualization, Investigation, Formal analysis, Writing - original draft. **Francisco J. Barba:** Conceptualization, Visualization, Supervision, Funding acquisition, Investigation, Methodology, Project administration, Writing - review & editing. **Zhenzhou Zhu:** Supervision, Investigation, Visualization, Writing - review & editing. **Nabil Grimi:** Conceptualization, Visualization, Methodology, Software, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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