

High-Voltage Pulsed Electric Field Preprocessing Enhances Extraction of Starch, Proteins, and Ash from Marine Macroalgae *Ulva ohnoi*

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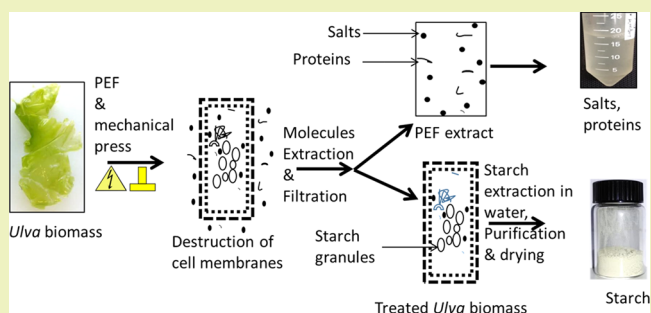
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Supporting Information

ABSTRACT: Marine macroalgae are an attractive source for biorefineries as an alternative to terrestrial crops, and new, sustainable macroalgae biomass fractionation methods are needed. One of the least investigated macroalgae-derived products is starch. In this work, we report on a device and a protocol for pretreatment for starch extraction from a green macroalga *Ulva ohnoi* (*U. ohnoi*) with an emerging, non-thermal, and environmental friendly technology—pulsed electric field (PEF). Using the custom-made insulated gate bipolar transistor-pulsed generator combined with a gravitation press-electrode device, we show that 200 pulses with a field strength of 1 kV cm^{-1} , pulse duration of $50 \mu\text{s}$, and pulse repetition rate of 3 Hz concentrate the starch in the *U. ohnoi* biomass by 59.38% by removing the salts, proteins, and other small molecules. The starch extraction yield from the PEF-pretreated biomass is $59.54 \pm 1.34\%$, compared to $52.31 \pm 3.21\%$ from untreated biomass. In addition, PEF combined with pressing increased the coextracted macroalgae protein by more than 4 times and ash by 1.5 times in comparison with pressing alone. These results indicate the potential of PEF pretreatment for challenging macroalgae biomass fractionation in the sustainable marine biorefinery.

KEYWORDS: pulsed electric fields, macroalgae, seaweeds, *Ulva ohnoi*, starch, biorefinery, marine proteins, deashing



INTRODUCTION

Increasing population and food consumption rate will demand 70–100% more food by 2050.¹ Starch is the main carbohydrate ingredient of human food in addition to the protein. Starches, including modified starches, are important ingredients of various industries and are used for numerous applications including food, fermentation, textile, cosmetics, pharmaceuticals, packaging, synthetic polymer industries, and more.^{2,3} Modified starches and different native starch-based blends are an attractive bio-based alternative to biodegradable synthetic polymers.^{2,4} Starch-based bioplastics represent 80–85% of marketed bioplastic.⁴ In addition, starch from cereals is used for biofuel production for the past 10 years in the United States and has also become prominent in other parts of the world.⁵ These competing applications cause shortage and price increase of cereal-based food starch⁶ and also put pressure on world food supplies.¹ The shortage of arable land for terrestrial agriculture and scarcity of water raise the question on the sustainability of starch supply. To meet these challenges, offshore macroalgae biomass production, known as seagruc-

ture, can provide a sustainable solution for the feedstock required for starch production. Furthermore, starch can provide an additional revenue stream for the emerging macroalgae biorefineries.^{7–10}

Starch is a major storage carbohydrate in various species of seaweeds such as *Cladophora*, *Gracilariopsis*, *Rhodophyta*, *Ulva*, and other species.^{10–16} In *Ulva* sp., starch granules are situated inside the chloroplast of the cell.^{13,15} Starch content for instance in *Ulva rigida* and *Ulva ohnoi* (*U. ohnoi*) fluctuates seasonally and reaches 32 and 21.4% of the dry matter (DM), respectively.^{10,17} Although seaweed starch can serve as a sustainable alternative to conventional starch for food and other industrial applications, it does not require arable land and fresh water for biomass production, and not much attention has yet been given to its extraction. We demonstrated extraction of these starch granules from the macroalga *U. ohnoi*

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and obtained an extraction yield of 51%.¹⁰ Previously, Yu et al. reported extraction and characterization of Floridean starch from red alga *Gracilariopsis lemaneiformis*.¹⁶ These two studies suggest that the starch from marine macroalgae is similar to several terrestrial starches; however, it can be produced offshore.

New and sustainable green methods for ocean-based biomass fractionation are still not mature. The macroalgae biomass structure, electrical conductivity, and chemical composition are very different from crops and other terrestrial plants. Therefore, direct translation of processing technology from land crops to marine biomass is challenging and requires new devices and processes.^{18,19} In this work, we focus on pulsed electric fields (PEF)—an emerging sustainable biomass processing technology.

Pulse electric fields (PEFs) is an emerging nonthermal, nonchemical, energy efficient method, used in the food industry for disinfection, enzyme activity modification, and extraction of various compounds from biomass.^{20–22} PEF treatment is less energy-consuming process compared to the conventional thermal extraction and dehydration operations.²² PEF has been already shown as a potential pretreatment method for potato starch extraction at the industrial scale in Germany.^{23–25} However, compared to the starch extraction process from terrestrial biomass sources such as potato, wheat, maize, and so on, which are rich in starch (around 80%),³ the starch extraction from leaf-like biomass such as *Ulva* is much more difficult to process as the starch granules are present inside the chloroplast. In addition, the starch content inside the seaweed biomass is low,^{13,15} between 2 and 21%,¹⁰ and its concentration could enable more efficient storage and extraction. Starch granules gelatinize and lose their granular and crystalline structure low temperatures between 50 and 70 °C,^{10,26,27} therefore, the extraction or concentration of starch granules in their native form seaweeds requires use of nonthermal processes, such as PEF.^{10,28,29} However, currently used PEF systems at the industrial and laboratory scale do not fit for macroalgae processing because of multiple difficulties related to the application of PEF on seawater and highly conductive biomass.

Indeed, our recent work at the laboratory scale showed that PEF can be used for selective extraction of proteins and ash (minerals) from macroalgae such as *Ulva* sp.^{20,21,30} Extraction of water-soluble protein and carbohydrates from macroalgae using PEF was also demonstrated in refs 19 21 30, and 31 indicating that PEF could be used in the processing of macroalgae biomass. However, to the best of our knowledge, there are no works mentioning the use of PEF as a pretreatment method for starch concentration and extraction from seaweed or even from any green biomass. Such a use can provide an opportunity to the PEF for the simultaneous fractionation and extraction of soluble molecules, such as proteins, and enhancement of starch extraction in the downstream processing, providing a new process for biorefinery.

The objective of this work was to evaluate the use of PEF for nonthermal preprocessing of the extraction of native starch granules from macroalga *U. ohnoi* coupled with the fractionation of biomass into ash and proteins. To attain this task, we developed a PEF system that consists of an insulated gate bipolar transistor (IGBT)-based pulse generator, coupled to a press-electrode device for simultaneous electric field delivery and intracellular juice extraction. We show that PEF

coupled to pressing pretreatment concentrates starch at the residual macroalgae biomass and enhances the starch yield in the downstream processing by extraction of intracellular water, ash, proteins, and other water-soluble molecules. The application of PEF as reported in this paper is innovative and critically important for future production of starch from marine sources—a truly sustainable approach for the production of this important commodity chemical in a marine biorefinery.

EXPERIMENTAL SECTION

***U. ohnoi* Biomass Cultivation.** A culture of *U. ohnoi* was collected from an outdoors seaweed culture cultivation system at Israel Oceanography and Limnological Research, Ltd. (IOLR), Haifa, Israel. *U. ohnoi* originated from random collections conducted over the past years along the Israeli Mediterranean Sea.³² The cultivation units were made up of 1000 L fiberglass tanks equipped with aeration and running seawater pumped from a Mediterranean shore nearby IOLR, Haifa. *U. ohnoi* cultivated in 1000 L tanks got fertilized once a week with sodium dihydrogen phosphate (NaH₂PO₄, added to 0.057 mM in the tank) and ammonium chloride (NH₄Cl, 0.59 mM).

Initial Characterization of *U. ohnoi* Biomass Composition. At the end of a 4 week growth period, *U. ohnoi* was harvested from the cultivation tank and transported to our laboratory in a cool and dark condition within 2 h post-harvesting. The surface water from the biomass was removed at 3200 rpm, using a portable spin dryer (Beswin Electric Co. Ltd., Zhejiang Province, China). This surface dewatered fresh biomass, defined as wet mass (WM), was used for the estimation of moisture content, DM content, and starch concentration.

Determination of Moisture, Dry Mass, and Ash Content. Moisture content and DM were determined by drying *U. ohnoi* at 105 °C to a constant mass using a moisture analyzer (BM-50-5, Biobase Biodustry (Shandong) Co. Ltd., China). Ash content was measured at 550 °C for 3 h based on ISO 5984,³³ and calculations were made using eq 1

$$\text{ash content(\%)} = 100 \times \frac{(M_4 - M_1)}{(M_3 - M_1)} \quad (1)$$

where M_1 is the mass of the empty crucible at 105 °C, M_3 is the mass of the crucible and sample dried at 105 °C, M_4 is the mass of the sample and crucible at 105 °C, after treating at 550 °C.

Determination of Starch Content. Starch concentration was measured by using the total starch assay kit (K-TSTA-100A, Megazyme, Ireland).¹⁰ Briefly, harvested *U. ohnoi* was dried to a constant mass at 40 °C in a conventional oven and ground to a particle size of less than 1 mm using a mortar and pestle with the help of liquid N₂. From this powder, 10 mg of sample ($n = 3$) was weighed in 2 mL tubes and washed twice in 500 μL of 80% (v/v) ethanol to remove any glucose present. Two hundred microliters of 2 M potassium hydroxide (KOH) was then added, and the tubes were shaken horizontally for 30 min at 37 °C and 150 rpm. At the end of 30 min, the mixture was further incubated at 100 °C for 1 min to completely dissolve the starch. Tubes were short spun for 1 min at 23 °C, and sodium acetate buffer (800 μL, 1.2 M, pH 3.8) was added. Immediately, 10 μL of α-amylase and 10 μL of amyloglucosidase were added and mixed using a vortex mixer. The mixture was shaken for 2 h at 50 °C and 150 rpm. The tubes were then centrifuged at 1800g for 10 min (Eppendorf centrifuge 5424, Hamburg). The glucose released was measured at 510 nm by reacting 0.01 mL of supernatant with 0.3 mL of glucose oxidase–peroxidase (GODPOD) enzyme mixture for 20 min against the glucose standard. Starch concentration, as the percentage of the DM, was calculated with a molar mass conversion from glucose to anhydroglucose (the starch monomer unit) of 0.9.

IGBT-Based PEF Generator and Gravitation-Based Electrodes for Biomass Pretreatment and Fractionation. A custom-made pulsed electric field generator was developed. The generator provides at a maximum voltage of 1000 V and a current of 160 A at a

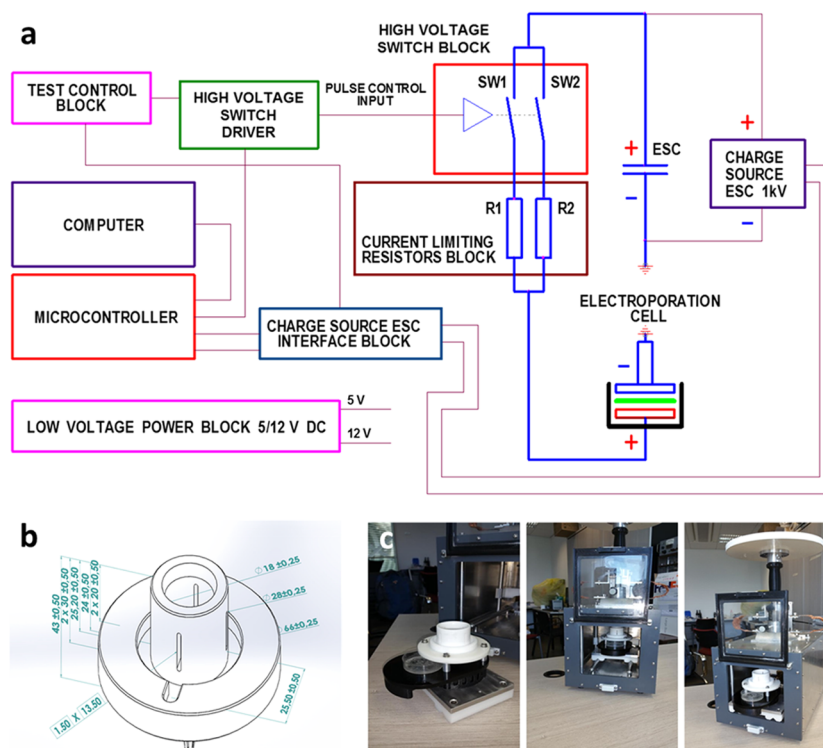


Figure 1. (a) Functional circuit diagram of the electroporation device 1 kV, 160A. (b) Electroporation cell. (c) Mechanical chamber for high-voltage electroporation.

5 ohm load. The maximal pulse duration, the number of pulses, and pulse frequencies are limited by the permissible heating of the IGBT transistors. In our system, as described below, for a 5 ohm load and 1 Hz pulse repetition rate, the maximum pulse duration is 100 μ s.

The functional circuit diagram of the pulsed generator developed is shown in Figure 1a. The main functional nodes of the system include (1) an energy storage capacitor (ESC) with a capacity of 50 μ F for a voltage of 1.25 kV, (2) a high-voltage source of charge of energy storage capacitors (CCM1KW, Spellman, NY), (3) parallel-connected high-voltage switches for pulsed discharge of ESCs (IXYN120N120C3 (IXYS, CA) with parameters of 1200 volts, 120 A), (4) a driver of high-voltage switch with electrical circuits of control of transistor gates and own power supply (Gate Driver Optocoupler FOD3184, Fairchild, CA), (5) high-power current-limiting resistors (RR02–3 OHM-2 W), (6) a circuit node for manual control of high-voltage switch and high-voltage power supply in the testing mode, (7) a microcontroller for controlling PEF treatment, calculating the current through the treated biomass, and transferring the results to the computer recorder, and (8) a low-voltage power supply for control circuits and fans of the device. The device is connected by a USB interface to a computer for inputting the experiment parameters in the microcontroller, displaying the current state of the process and recording the received data in the experiment file. Currents were measured using a PicoScope 4224 oscilloscope with a pico current clamp (60A AC/DC). The voltage was measured with a PicoScope TA044 70 MHz 7000 V differential oscilloscope probe 100:1/1000:1. Voltages and currents were analyzed with Pico Scope 6 software (Pico Technology Inc., U.K.).

The electroporation extraction cell holds the treated biomass between the electrodes during the application of PEF. It also allows separation of the seaweed biomass into liquid and solid phases simultaneously with the application of electric fields and mechanical pressure. The electroporation cell consists of a cylindrical Teflon chamber (2.5 cm diameter), and the positive electrode is located at the bottom of the chamber. In the lateral part of the electroporation cell, there are narrow slit-like openings for the outlet of the liquid fraction during the electroporation of the biomass. The extracted liquid is collected and discharged through a groove at the base of the

cell. The gravitational press-electrode device is shown in Figure 1b. The load-receiving platform is fixed at the upper end of the rod, and at its lower end, there is a negative electrode, which can freely slide inside the electroporation cell. The complete developed experimental setup is shown in Figure 1c. A load weighing up to 10 kg can be placed on the load-receiving platform to exert the necessary interelectrode pressure on the biomass (Figure 1c). A displacement sensor (optoNCDT, Micro-Epsilon, Germany) is installed on the sliding rod to monitor the volume change of the biomass during electroporation (Figure 1b).

PEF Treatment Coupled with Mechanical Press. A custom-made PEF generator and gravitation-based electrodes were used for the electroporation of the *U. ohnoi* thalli. Ten grams (WM) of *U. ohnoi* was suspended in 100 mL of distilled water. The biomass (10 g fresh, corresponding to 2.61 ± 0.08 g dry) was then loaded in the electroporation cell in batches of 2 g, and PEF was applied with the following conditions: electric field strength of 1 kV cm^{-1} , pulse repetition rate of 3 Hz, number of pulses, 200 with 50 μ s pulse width duration.

These parameters (field strengths $E \geq 1 \text{ kV cm}^{-1}$) were reported to be sufficient to result in permanent pore formation of plant cell membranes and were recommended in refs 20 and 36. Thus, parameters were chosen to have conditions that enable the breaking of covalent bonds within the cell wall and allow the investigation of the PEF effect on *Ulva* biomass fractionation and starch extraction. The distance between the fixed electrode inside the electroporation cell (positive electrode) and the free slide gravitational press electrode (negative electrode) was adjusted before applying the first pulse. The distance between the two electrodes was measured continuously with the displacement sensor (opto NCDT 1302, Micro-Epsilon, Germany). An additional pressure of $107,682 \text{ N/m}^2$ was applied during the PEF. Internal resistance, R_i , for the device with 1000 V and 160 A was 0.75 ohm. The initial (I_0) and residual (I) currents were determined from the oscillogram. V_{ESC} is the voltage applied on the energy storage capacitor (ESC), C_{ESC} is the capacitance of the energy storage capacitor (1×10^{-6}), and t_i is the width of the pulse in microseconds. The electroporation cell (EPC) resistance was calculated using eq 2

$$R_{\text{EPC}} = \left(\frac{V_{\text{ESC}}}{I_0} \right) - R_i \quad (2)$$

The electroporation cell initial voltage (V) and residual voltage (V_0) were calculated as in eqs 3 and 4, respectively,

$$V_0 = V_{\text{ESC}} - I_0 \times R_i \quad (3)$$

$$V = I \times R_{\text{EPC}} \quad (4)$$

The energy of the single pulse of electroporation (A_{imp}) was calculated using eq 5

$$A_{\text{imp}} = \left(\frac{V^2 \times t_i}{R_{\text{EPC}}} \right) + 0.5C_{\text{ESC}} \times (V_0^2 - V^2) \quad (5)$$

After the treatment, the thalli were resuspended in 100 mL of distilled water into which they were suspended before applying PEF. The biomass was shaken at 32 °C and 150 rpm to allow the intracellular products to diffuse out into the aqueous medium (extraction). The thalli were removed from the water after 1 h, and the water medium was filtered through a 50 μm nylon filter using a glass vacuum filtration system. The thalli at the end of filtration were referred to as "PEF-treated biomass", and the aqueous juice thus obtained was referred to as "PEF-extract". For control experiments, the whole process was repeated but no pulses were applied in the chamber. The biomass thus obtained was called as "control-biomass", and the liquid thus obtained was called as "control-extract". In total, six replicates of 10 g of fresh *U. ohnoi* were treated each for PEF and control treatment.

Measurements of Conductivity, Starch, Protein, Total Solids, and Ash Content in the PEF-Extract. The conductivity of the control-extract and PEF-extract was measured using an InLab 731 ISM conductivity sensor mounted on the pH meter (Mettler-Toledo, Melbourne, Australia). All four components, control-extract, control-biomass, PEF-extract, and PEF-treated biomass, were analyzed for their ash, protein, and starch contents. The liquid extract was dried at 105 °C using a conventional oven to determine the total solids (TS). Ash content was measured based on ISO 5984.³³ Biomass fractions were dried at 40 °C until constant masses were reached. The presence of starch was analyzed by using the Megazyme's total starch assay kit as described above (in the [Determination of Starch Content](#) section).

Total protein was analyzed using a modified version of the Lowry method.³⁵ The filtered liquid extract was centrifuged at 1800g for 10 min. Solid biomass (~15 mg) was accurately weighed in 2 mL tubes and filled to one-third with beads (zirconia, 2 mm, Sarstedt) and 1.5 mL of 2 M sodium hydroxide (NaOH) solution. The tubes were run through three sessions of bead beating of 60 s each in a bead beater (BioSpec, OK, USA) with intermittent cooling for 10 min at 23 °C. The tubes were then centrifuged at 14,000 rpm for 20 min. The supernatants from all the tubes were appropriately diluted with ultrapure water. Diluted samples were analyzed by adding 100 μL in a well of a 96-well plate. Biuret reagent was prepared by mixing 0.5 mL of 1% cupric sulfate with 0.5 mL of 2% sodium potassium tartrate, followed by the addition of 50 mL of 2% sodium carbonate in 0.1 N NaOH. Two hundred microliters of biuret reagent was added to each well and mixed thoroughly using a micropipet. The mixture was then equilibrated at 23 °C for 10 min prior to the addition of 20 μL per well of 1.0 N Folin-Ciocalteu reagent. Samples were mixed immediately by repeated pipetting following each addition. The color was allowed to develop for 30 min at 23 °C, following which absorbance was measured at 750 nm using a spectrophotometer (Infinite 200 Pro, TECAN, Switzerland). A standard curve was produced using bovine serum albumin (BSA) at different concentrations (0–500 $\mu\text{g}/\text{mL}$). As a blank, water was used in place of the sample. Analyses were done in triplicate, and the results were expressed as BSA equivalent in mg/L.

The fraction of ash and protein in TS of the extract was calculated using eq 6

$$\text{ash or protein}(\% \text{ of TS}) = \left(\frac{P_f}{\text{TS}} \right) \times 100 \quad (6)$$

where P_f is the amount of ash or protein in the extract (in g) and TS is the amount of total solids in the extract (in g).

Total ash (TA) and total protein (TP) extracted in the liquid (in the PEF-extract) from the initial biomass were calculated using eq 7

$$\text{TA or TP}(\%) = \left(\frac{P_f}{P_i} \right) \times 100 \quad (7)$$

where P_f is the amount of ash or protein (DM) present in PEF-extract and P_i is the amount of ash or protein present in initial *Ulva* biomass (DM).

Starch Extraction from *U. ohnoi* and Characterization.

Starch was extracted from control-biomass and PEF-treated biomass samples (10 g each, $n = 5$) of *U. ohnoi* using the homogenization and filtration method as explained in our previous paper.¹⁰ Briefly, *U. ohnoi* was mixed with cold distilled water (1:20 (w/v)) and homogenized into fine particulate suspension using a homogenizer (HG-300, Hasigai Machinery Industry Co., Ltd., Taiwan). The rotation speed of the homogenizer blades (step 9) and duration (3 min) were kept constant during the homogenization of each sample. The slurry thus obtained was filtered by sequentially passing through nylon filter bags of 50 and 10 μm pore size with the help of the centrifuge. Starch granules from the filtrate were collected using a centrifuge at 4500 rpm and 15 min (Rotanta 46 RSC, Hettich Instruments, LP, Germany). The pellet obtained was purified by washing with 30 mL of 80% ethanol and dried at 40 °C for 24 h. The starch-rich fractions extracted from PEF-treated biomass and from control-biomass were called as "US-PEF" and "US-C", respectively. The quantity of extracted starch-rich fraction was measured gravimetrically. The percentage of extracted starch-rich fraction (SE) was calculated using eq 8

$$\text{SE}(\%) = \left(\frac{S_w}{S_i} \right) \times 100 \quad (8)$$

where S_w is the mass (in g) of the extracted starch-rich fraction and S_i is the initial mass (DM) of the *U. ohnoi* PEF-treated biomass.

The starch content in the extracted starch-rich fraction was measured using the total starch assay kit (as mentioned above in this section under [Determination of Starch Content](#)), and the starch extraction yield (SEY) was obtained using eq 9

$$\text{SEY}(\%) = \left(\frac{S_f}{S_i} \right) \times 100 \quad (9)$$

where S_f is the amount of starch (g) present in the extracted starch-rich fraction and S_i is the amount of starch (g) present in the initial DM of harvested *U. ohnoi* biomass used in the experiment. The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrum was recorded for extracted dry *Ulva* starch, US-PEF, and US-C using a Bruker Platinum ATR-FTIR spectrometer from 400 to 4000 cm^{-1} . The scanning electron microscopy (SEM) image of *Ulva* starch was obtained using a Quanta 200 FEG ESEM (Oregon, USA) after fixing the sample on a silicon tape and then coating with gold using a sputter coater. AFM scans were obtained using NanoWizard II Bio-AFM (JPK Instruments, Germany) at 25 °C. Samples were imaged in air in an intermittent contact mode using NSC21/NoAl silicon tips (NT-MDT, spring constant between 8.5 and 33 N/m).

Protein Extraction from *U. ohnoi*. Following PEF treatment, protein was extracted from PEF-treated biomass and control-biomass of *U. ohnoi* (1 g fresh, corresponding to 0.261 g dry). Total protein was extracted using the thermochemical (TC) method (0.25 N NaOH solution at 60 °C for 1 h) as described by Mhatre et al.³⁶ The protein-rich extract thus obtained was analyzed for protein content using the Lowry method,³⁵ and the protein extraction yield (PEY) was obtained using eq 10

$$\text{SEY}(\%) = \left(\frac{P_f}{P_i} \right) \times 100 \quad (10)$$

where P_f is the amount of protein (g) present in the extracted starch-rich fraction and P_i is the amount of starch (g) present in the initial DM of harvested *U. ohnoi* biomass used in the experiment.

Statistical Analysis. For statistical analysis, a data analysis package in Excel (ver. 13, Microsoft, WA) was used. All samples and controls were prepared and measured, at least in triplicates, if not mentioned differently. The error bars are the standard error of the mean (SEM). To compare the extracted starch yield from PEF-treated biomass to the controls, a two-tailed Student's *t*-test was performed.

RESULTS AND DISCUSSION

Dry Mass, Moisture, Ash, Starch, and Protein Content in *U. ohnoi*.

U. ohnoi, a species of light-green colored seaweed in the family Ulvaceae, was selected for this study as the representative species. *Ulva* sp. show a high growth rate and have unique biochemical composition including attractive polysaccharide composition, which in addition to the common cellulose and starch, including large amounts of sulfated polysaccharide, Ulvan.³⁷ In addition, *Ulva* sp. are distributed globally from polar to tropical regions³⁸ and have a relative advantage in warmer climate zones, thus being less sensitive to climate changes.^{39,40} Several authors have studied *Ulva* sp. as a potential candidate for seaweed biorefinery.^{9,10,41} Dewatered, fresh (WM) *U. ohnoi* was characterized for dry mass and moisture and ash contents. The DM and moisture content were 26.12 ± 0.94 and $73.88 \pm 1.88\%$, respectively. The ash content was $27.8 \pm 0.54\%$ of the DM. Starch concentration was $7.35 \pm 0.25\%$, and protein content was $8.41 \pm 0.11\%$ of the DM. The ash content in *Ulva* sp. can vary between 11 and 57% of DM.⁴² The ash content in the present biomass was slightly less than the values reported for laboratory grown *Ulva* sp., which showed 36.02%.⁴³ The protein in *Ulva* is required to maintain the nitrogen balance inside the cells. Its concentration in natural environments can vary from 2.9 to 29% of DM.^{42,44} The protein value in this study was slightly less than other *Ulva* grown in laboratory conditions.⁴⁴ Starch is a storage polysaccharide in *Ulva*, and its concentration in natural environment can vary between 0.45 and 9.3% of DM depending on nutrient conditions, season, and life cycle and can be significantly increased upon nutrient starvation.¹⁰ In this study, the starch concentration in *U. ohnoi*, grown in nutrient-rich conditions, was higher than observed in our previous study, which could be due to variation in abiotic growth conditions such as temperature and light.¹⁰

PEF Treatment. *U. ohnoi* biomass, post-harvesting and dewatering (WM), was subjected to PEF treatment to extract ash and protein, followed by starch extraction from the residual PEF-treated biomass. The whole flow process is shown in Figure 2. During the PEF treatment, resistivity of the biomass decreased from 112.75 ± 0.35 ohm·cm during the first pulse of the treatment to 35.71 ± 0.87 ohm·cm at the end of the last pulse. Similarly, the current increased while the voltage decreased during the first pulse and the last pulse of the treatment (current 7 ± 0.90 A and voltage 990 ± 5 V and current 24.5 ± 2.3 A and voltage 965 ± 9.5 V during the first and the last pulse of the treatment, respectively) (Figure 3), indicating that the biomass was electroporated. Such phenomena were observed in earlier studies^{19,21,45,46} and occur due to altered resistance and capacitance. The distance between the electrodes at the start of the first pulse was $10.5 \pm$

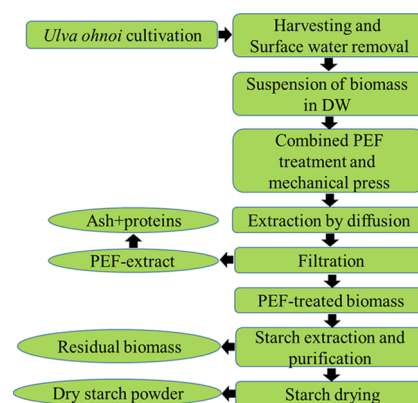


Figure 2. Flow diagram of PEF-assisted process for the recovery of starch, salt, and protein from *U. ohnoi*. (DW = distilled water; PEF = pulsed electric fields).

2.76 mm. The electric field strength at the first pulse was 98.5 ± 0.34 V mm⁻¹, and the invested energy was 24.67 ± 3.43 J pulse⁻¹. The distance between the electrodes at the end of the last pulse was 9.51 ± 2.37 mm. The electric field strength at the last pulse was 98.5 ± 0.34 V mm⁻¹, and the invested energy was 35.919 ± 2.33 J pulse⁻¹. The distance between the electrodes is adjusted only during the first pulse. It was observed that during the application of the pulse, ionic matter from the cytoplasm of the cell starts oozing out as indicated by the increase in conductivity. Furthermore, due to the gravitational pressing during the application of the pulses, the distance between the electrodes spontaneously decreases. With the highest energy consumption of 35.919 J pulse⁻¹, the total energy invested for pretreatment will be 1375 kJ/kg DW of *Ulva* biomass. Previous work on potato starch extraction showed energy saving on cutting probably because of tissue softening and loss of turgor pressure. As the water content of the intracellular content of the biomass decreases due to extraction, this wilting could decrease the energy required for mechanical breaking of thalli in the following steps. Our previous work²² has shown that PEF treatment is nonthermal and less energy-consuming compared to the conventional thermal extraction and dehydration operations and enables to better valorize biomass.

Conductivity and Contents of Protein, Starch, Total Solids, and Ash of the PEF-Extract. *Conductivity.* The conductivity of the control-extract was 2988.33 ± 73.39 μS/cm, and that of the PEF-extract was 5075.60 ± 396.66 μS/cm, which was 69.84% higher than in the control-extract (Figure 4a). The applied PEF affects the membrane permeability of the *Ulva* cells, allowing free, nonselective passage of ions and salts from the cellular cytosol. These salts are responsible for the higher conductivity in the PEF-extract.¹⁹

Starch. Starch granules were expectedly absent in the control-extract as well as in the PEF-extract, which is similar to the results of the process when applied to potatoes.²⁴ By applying PEF, it was possible to pierce the walls of the potato cells.²⁴ It was observed in the literature that the tissue is altered during PEF treatment as the cell wall is affected by PEF treatment.⁴⁷ Whether cell wall components are changed directly due to the PEF treatment or due to cell membrane disintegration and the release of cytoplasm is still not clear. However, the content of the cell wall biopolymer lignin decreases during the PEF application, which may occur due to the effective break of intermolecular and intramolecular bonds

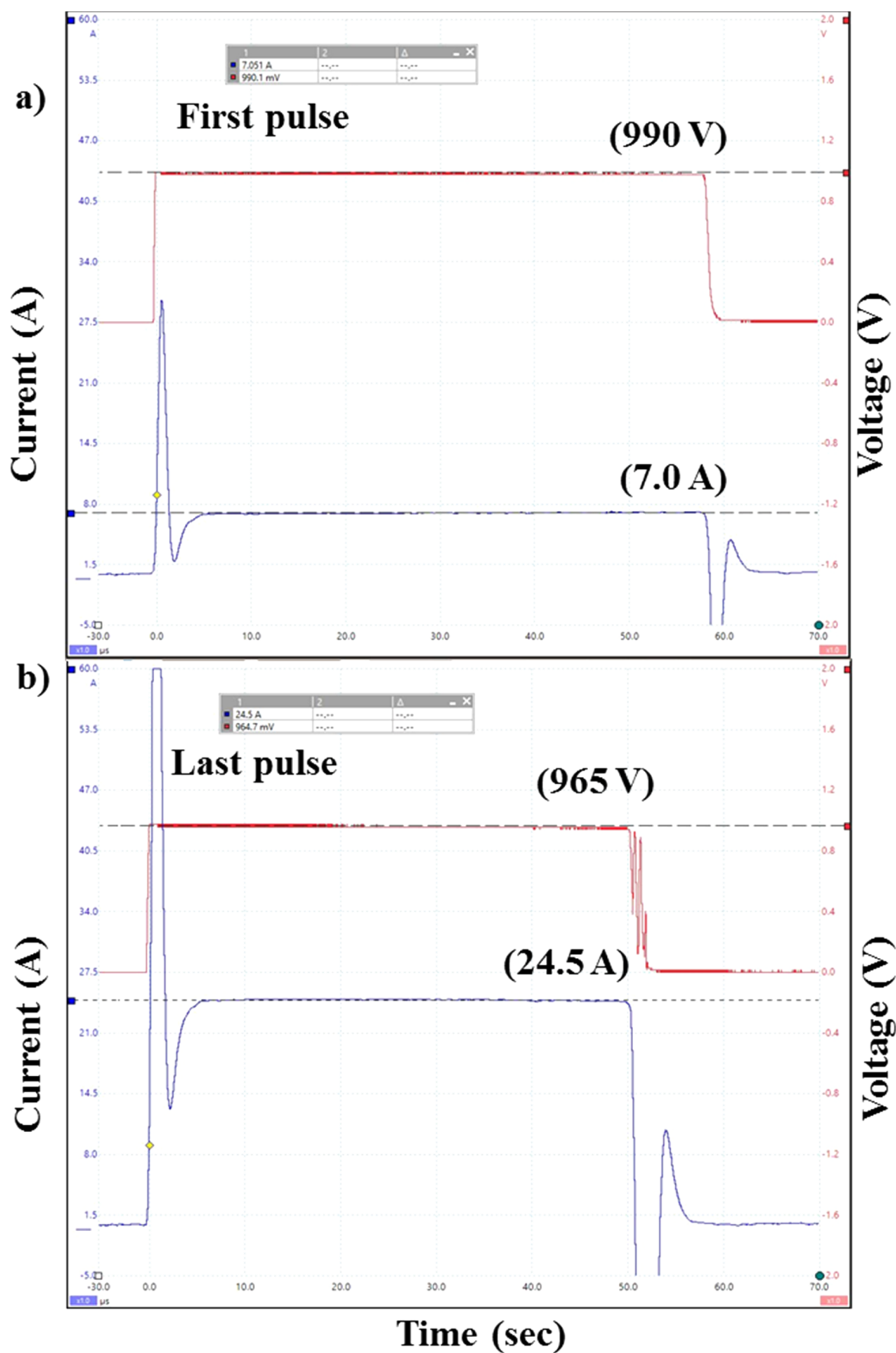


Figure 3. Electrical voltage and current shape for single pulse: (a) first pulse and (b) last pulse. Pulse strength, 1 kV cm^{-1} ; pulse number, 200; pulse duration, $50 \mu\text{s}$; pulse frequency, 3 Hz; biomass load, 2 g.

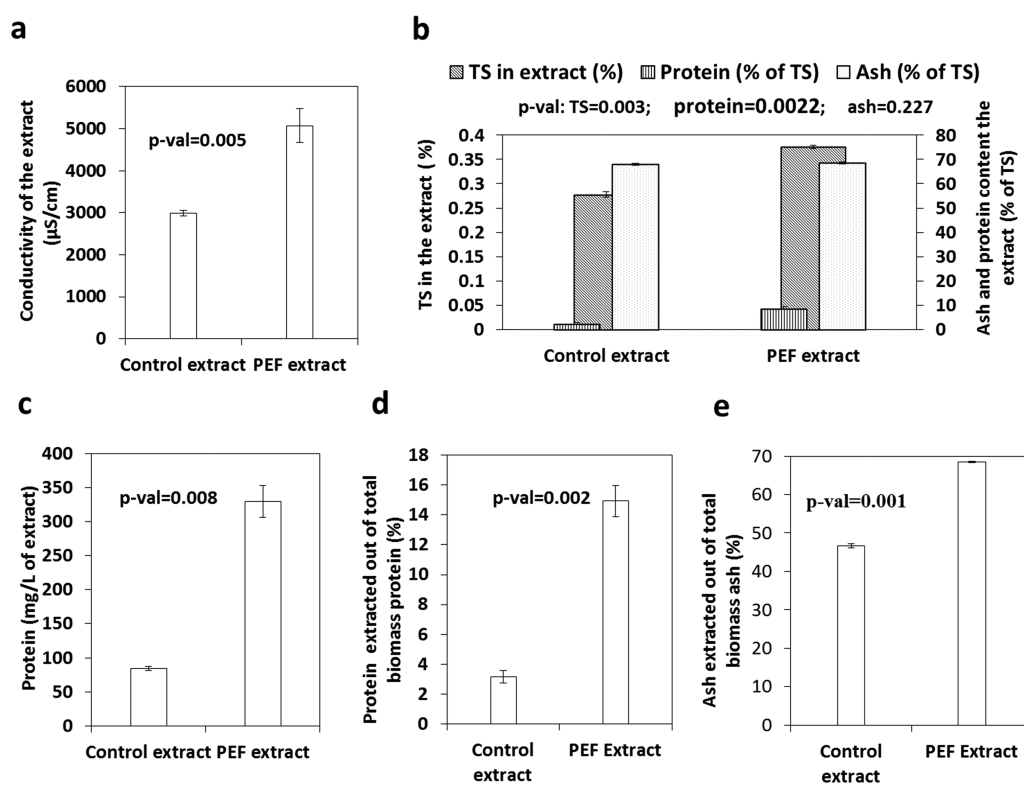


Figure 4. (a) Conductivity, (b) total solids (TS), (c, d) proteins concentration, and (e) ash concentration in control-extract and PEF-extract, $n = 3$, which is the number of repeated PEF treatments.

between or within the cellulose, hemicellulose, and lignin.⁴⁷ Starch extraction from leafy biomass and macroalgae having a similar leaf-like structure is challenging due to distribution of starch granules inside the chloroplast and their relative lower concentration and smaller size of granules as compared to other common starch from seeds and tuber plant sources.^{3,10} Above changes in the cell wall created by PEF could facilitate the extraction of starch during a later starch extraction step.²⁴

Protein. The protein content as % of TS was higher in PEF-extract (8.79%) compared to the control-extract (2.51%) (Figure 4b). The protein concentration in control-extract was 84.58 ± 3.11 mg/L, whereas in PEF-extract, it was 329.62 ± 23.10 mg/L (Figure 4c). This corresponds to the protein concentration in the PEF-extract, 289.7% higher than that in the control-extract. The protein extracted out of the total biomass protein was 3.16 and 14.94% in the control-extract and in the PEF-extract, respectively (Figure 4d). The protein yield in this study was higher than the protein yield reported earlier by Robin et al.,¹⁹ who got only 2.93% extracted out of total biomass protein. This could be due the diffusion step carried out after the PEF treatment. However, the concentration of proteins in the PEF-extract was lesser than reported by Robin et al.¹⁹ This could be due to the lower voltage and lesser number of pulses used for the PEF treatment in our experiments. Robin et al.¹⁹ showed that the protein extracted from *Ulva* can be significantly increased by increasing the applied voltage from 20 to 50 kV, as well as the number of pulses from 10 to 50. However, in our study, we could not go beyond 1 kV due to current restrictions from the custom-made PEF device and also due to doubt of damaging the native structure of the starch granule at higher voltages.

Notably, although PEF treatments seem to be effective for ash removal (>68% of as extracted out of total biomass ash,

Figure 4e), the protein extracted out of total biomass protein (%) is still low (14.94%, Figure 4d). Therefore, this new method may not be sufficient to meet economical requirements when it is compared with current methods of protein extraction, due to the lower protein removal. However, it should be also noted that protein content of raw *Ulva* biomass in this study was low ($8.41 \pm 0.11\%$ of the DM), and thus, the efficiency may be higher when the protein content in the biomass is high. In addition, although PEF extracted only 14.94% protein out of initial total protein, it selectively extracted a protein fraction that has proteins within a certain molecular weight range and specific functional properties (e.g., high water solubility) as observed from our previous work on protein extraction using PEF.^{19,20,30} Further, such protein fractions are known to have the phenolic and antioxidant compound and fractions of sulfated polysaccharides that can be useful as an additional benefit during its use as a food supplement.^{19,30} Thus, the PEF method is still a useful method for protein extraction for various specific applications, which may require such a protein fraction. More importantly, the notable feature of PEF extraction is that the tissue structure of the *Ulva* thallus is left intact after the PEF treatment, making the separation of protein and salts in liquid fraction from the solid biomass much easier.¹⁹ To further increase the efficiency of protein recovered in the PEF-extract, extraction conditions such as different polarity solvents, pH values, and diffusion times can be optimized in the future work. This may also further facilitate starch extraction.

Total Solids and Ash Content. Total solids present in the control-extract and the PEF-extract was 0.27 and 0.37%, respectively (Figure 4b). These values of TS were significantly lower compared to 2 and 3.8% in the control and PEF-treated extract obtained in the study carried out by Robin et al.²¹ Ash

contents were 67.97 ± 0.48 and $68.53 \pm 0.44\%$ out of the total extracted solids in the control-extract and the PEF-extract, respectively (Figure 4b). This shows that the ash was a major component of the TS. The difference in the ash content in both extracts was not significant, and the results are comparable to those of Robin et al.²¹ However, there was a higher amount of protein in the PEF-extract indicating the higher fraction of organic content in the TS of the PEF-extract. The ash contents extracted out of the total ash in the initial biomass in the control-extract and PEF-extract were 46.67 and 68.52%, respectively (Figure 4e).

The relative percentage content of ash and protein to the dry raw material reflecting the yield is reported in Table 1. The

Table 1. Relative Percentage Content of Ash and Protein to Dry Raw Material

(% dry biomass)	fraction extracted		
	raw biomass	PEF-extract	control-extract
ash	27.8 ± 0.54	19.05 ± 0.06	12.98 ± 0.15
protein	8.41 ± 0.11	1.26 ± 0.29	0.27 ± 0.06

extraction yield of ash (68.5%) and protein (14.94%) observed in our study using PEF coupled with the mechanical press followed by extraction into distilled water was much higher than that by PEF followed by the mechanical press alone (46% ash and 2.9% protein) as observed by Robin et al.¹⁹ The higher yield could be due to a more effective extraction by distilled water by washing the electroporated tissue during 1 h shaking compared to mechanical pressing for a short time. Previously, it has been shown that PEF treatment on *Ulva* biomass releases ash (salts) and small proteins out of the cells.^{19–21} Our results of higher values for conductivity, ash, TS, and protein in the PEF-extract compared to the control-extract further support these findings. Furthermore, the salts and proteins from the PEF-extract can be separated using a suitable method, for instance, ultrafiltration.¹⁹ The salts can be used for various purposes such as fertilizer or table salt, and the protein can be used in human and animal nutritional supplements.^{48–50}

The mechanism behind the effect of PEF on the cell wall and the cell membrane is complex and still only partially understood.⁵¹ Current theory explains that when PEF is applied, small pores are created in the cell wall and membranes. Through these pores, most of the salts, water-soluble proteins, and soluble carbohydrates exit the *Ulva* cells^{20,21,31} and bigger molecules are left inside the cells. The reason behind the absence of starch in PEF-extract could be that the size of the pores, created in the cell envelop by the PEF treatment in this work, was significantly smaller than the size of the starch granules ($5\text{--}7\ \mu\text{m}$, see ref 10). Moreover, the starch granules are located inside the chloroplast,¹³ thus making it difficult for them to become extracted. Another reason could be lack of the chemical potential gradient for diffusion and spontaneous release of the starch granules, as large molecules have very small molar concentration, leading to small motive force to diffuse out of the cells during extraction in distilled water.³¹ More radical PEF conditions for extraction or release of starch granules in the PEF-extract were not tested, as it might cause heating. Furthermore, higher PEF treatment ($30\text{--}50\ \text{kV/cm}$) could cause dissociation, denaturation, and also damage the starch granules, as observed in the case of pure corn and potato starch granules in earlier studies.^{52–54}

The starch concentration in the control-biomass and PEF-treated biomass of *U. ohnoi* was measured and compared with the starch concentration in freshly harvested biomass. As ash content in the biomass decreases with PEF treatment, protein and starch contents in the biomass increase significantly (Figure 5).^{9,19} The starch concentrations in the harvested

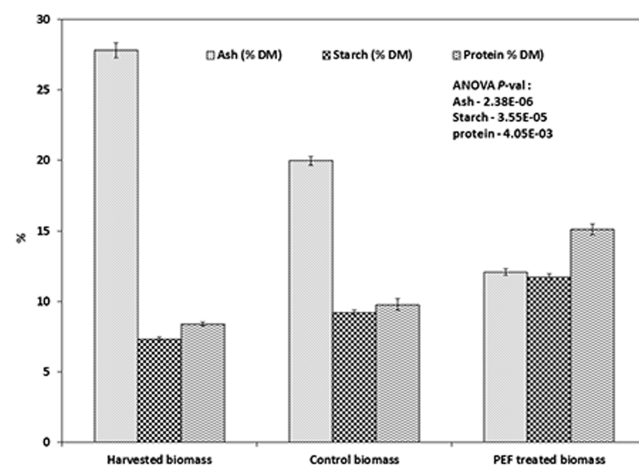


Figure 5. Ash, starch, and protein concentrations in the biomass (% DM) of *U. ohnoi* at various treatment steps. (Harvested biomass = fresh biomass (FM); control biomass = PEF treatment process only with mechanical press + diffusion + filtration; PEF-treated biomass = PEF treatment with electric pulses + mechanical press + diffusion + filtration). $n = 3$ (number of experimental repeats with applied PEF for the analysis).

biomass, control-biomass, and PEF-treated biomass were 7.35 ± 0.15 , 9.21 ± 0.18 , and $11.71 \pm 0.23\%$ of the DM, respectively. The increases in the starch concentration in control-biomass and PEF-treated biomass compared to harvested biomass were by 25.4 and 59.38%, respectively. This was further true for the protein concentration also, which increased from 8.41% in harvested biomass to 9.78 and 15.11% in control-biomass and PEF-treated biomass (an increase of 16.28% and 79.68%), respectively (see Figure 5).

Protein and Starch Extraction and Characterization.

The amount of protein extracted and protein extraction yield (PEY), similarly, the amount of starch extracted, that is, the actual starch content in extracted starch fraction and starch extraction yield (SEY) are shown in Table 2. Protein extracted from *Ulva* (1 g fresh, corresponding to 0.261 ± 0.008 g dry) control-biomass was 4.04 ± 0.05 mg and from PEF-treated biomass was 10.80 ± 0.24 mg. This corresponds to protein extraction yields of 18.40 ± 0.21 and $49.15 \pm 1.11\%$ from control-biomass and PEF-treated biomass, respectively. Total protein extraction yields (combined from PEF and TC extract protein) in this study were 21.56 ± 0.6 and $64.09 \pm 1.6\%$ from control-biomass and PEF-treated biomass, respectively. A result of protein extraction indicates that PEF treatment and mechanical pressing, followed by diffusion and TC extraction, significantly increased protein extraction by 197.26% over control extraction with only mechanical pressing followed by diffusion and TC extraction. The protein extraction yields from *Ulva* observed in this study using PEF, followed by aqueous diffusion and alkaline TC extraction, were higher than only chemical extraction those reported by ref 38 or ultrasonic treatment followed by chemical extraction reported by Kazir et al.⁵⁵

Table 2. Comparison of Starch Extraction Parameters and Protein from Control and PEF-Treated *U. ohnoi* Biomass^a

parameters/sample	control-biomass	PEF-treated biomass	<i>p</i> -value(<i>n</i> = 5, <i>x</i> = 0.05)
protein extracted (in mg) from 1 g of fresh biomass	4.04 ± 0.05	10.80 ± 0.24	<0.001
protein yield (%) from initial biomass total protein	18.40 ± 0.21	49.15 ± 1.11	0.0007
total protein yield (PEF extract + TC extract)	21.56 ± 0.6	64.09 ± 1.6	<0.001
starch content in initial biomass (% DM)	7.35 ± 0.25	7.35 ± 0.25	NA
starch content in initial DM (g)	0.20 ± 0.01	0.20 ± 0.01	NA
extracted starch-rich fraction (g/2.61 ± 0.08 g <i>Ulva</i> DM)	0.175 ± 0.003	0.224 ± 0.01	2.43 × 10 ⁻⁶
extracted starch-rich fraction (% DM)	6.71 ± 0.12	8.64 ± 0.21	0.0015
purity of the extracted starch-rich fraction (%)	59.40 ± 2.47	53.05 ± 0.49	0.077
starch extraction yield (SEY) (%)	52.31 ± 3.21	59.54 ± 1.34	0.029

^a*n* = number of experimental repeats with PEF; NA = not applicable

The amounts of extracted starch-rich fractions were 6.71 ± 0.11% (0.175 ± 0.03 g/2.61 ± 0.08 g *Ulva* DM) of DM from the control-biomass and 8.64 ± 0.21% of DM (or 0.22 ± 0.01 g/2.61 ± 0.08 g *Ulva* DM) from the PEF-treated biomass. The amount of extracted starch-rich fraction (as % DM) from the PEF-treated biomass was 28.85% higher (*p*-value of 0.0015) compared to the control-biomass (8.64% vs 6.71%).

The purity of extracted starch-rich fraction from control-biomass was 59.40 ± 2.47% and from PEF-treated biomass was 53.05 ± 0.49% corresponding to 0.104 ± 0.008 and 0.12 ± 0.006 g of actual starch, respectively. The purity of extracted starch-rich fraction from PEF-treated biomass (53.05 ± 0.49%) compared to extracted starch-rich fraction from control-biomass (59.40 ± 2.47%) was less by 6.35 ± 1.48% of starch DM but less significantly (*p*-value of 0.077). However, the SEY in PEF-treated biomass increased to 59.54 ± 1.34%, compared to 52.31 ± 3.21% from the control biomass on DM basis. The difference in the SEY between PEF-treated biomass and control-biomass was 7.23 ± 2.28%. This corresponds to a relative increase of 13.85% in SEY from PEF-treated biomass. Although the purity of starch was lower, the SEY was significantly higher from PEF-treated biomass (*p*-value of 0.029, Table 2). This could be due to the PEF-induced damages to the cell wall cellulose and cytoskeleton,⁵⁶ which further favored the cell lysis in a more effective manner during the homogenization. Additionally, the increase in starch concentration in the PEF-treated biomass, due to a higher release of salts, ions, and small protein molecules, may have favored the higher starch extraction yield. ATR-FTIR absorbance data showed that the extracted starch-rich fractions from “PEF-treated biomass and “control-biomass” were similar and did not show a difference in the absorbance pattern (see Figure S1 in the Supporting Information). SEM images also indicated similar spherical and ovoid morphology of granules with size ranging from 5 to 7 μm in both the starch samples (see Figure S2 in the Supporting Information).

PEF is known to modify the tissue and the cell and to disintegrate the cell wall by delignification by leakage of lignin-cellulose bonds.^{34,47,54} The increase in starch and protein extraction yields may be due to one of the reasons such as weakening and irreversible damage to the cell wall and membrane,^{34,47,54} osmotic dehydration, and loss of turgor pressure, followed by an increased osmotic flow of salts, ions, and small sized proteins and by moisture redistribution inside the *U. ohnoi* cell. Additionally, increased protein and starch concentration per DM of PEF-treated biomass (see Figure 5) could have influenced protein and starch extraction yields due to increased availability, as observed in the literature for proteins, where pretreatment with cell-disruption techniques

showed higher protein yields during the later extraction process by increasing the availability of proteins.⁵⁷ However, this hypothesis requires further investigation. Our results suggest that the PEF process can be effectively used as a pretreatment to increase the protein and starch concentration in the *U. Ohnoi* biomass before further processing for the extraction. Several native starches and modified starches are used for commercial applications in various industries including food, fermentation, textile, cosmetics, pharmaceuticals, packaging, and synthetic polymer industries.³ Certain modifications are done on starch to enhance its specific properties for unique applications.² PEF-assisted modification of starch has been achieved for several starches;^{58,59} however, it was not studied in this work.

CONCLUSIONS

Extracting multiple products from the same biomass is a crucial step in a marine biorefinery. Harvesting starch from seaweeds will reduce the pressure on terrestrial agriculture and has the potential to feed the growing global population. However, macroalgae biomass fractionation is challenging, and new sustainable technologies and processes are needed. In the present study, the use of PEF for nonthermal fractionation of marine biomass and increasing the starch extraction yield from *U. Ohnoi* was studied for the first time. PEF concentrated the protein by 79.68% and starch by 59.38% in the pretreated biomass, compared to their initial concentrations. PEF pretreatment allowed higher yields of starch extraction from *U. ohnoi*. Starch and protein extraction yields from PEF-treated biomass were 13.85 and 197.26% higher relative to the yield from control biomass (percentage increase), respectively. Furthermore, using PEF coupled to mechanical pressing, 15% protein and 69% salt (from initial biomass protein and ash) were extracted from *U. ohnoi* compared to mechanical pressing alone. This novel study provides missing information and suggests that the PEF can be used as a chemical-free, nonthermal method for the macroalgae fractionation into salt, protein, and starch-rich residue. At the same time, it can be used as a pretreatment method for a more efficient extraction of protein and starch from a strenuous macroalgae biomass. Future work will focus on increasing the yields of products by optimizing various conditions involved in the process. The developed devices and pretreatment approach provide a new process for marine biomass fractionation important for marine biorefinery.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acssuschemeng.9b04669](https://doi.org/10.1021/acssuschemeng.9b04669).

ATR-FTIR absorbance pattern and SEM image of extracted starch-rich fractions ([PDF](#))

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Notes

The authors declare no competing financial interest.

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