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Biorefinery for the co-production of protein, hydrochar and additional co-products from a green seaweed *Ulva* sp. with subcritical water hydrolysis

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ABSTRACT

Marine seaweeds are promising biomass feedstock for the co-production of food, energy and chemicals in a biorefinery. In this study, subcritical water hydrolysis (SWH) was applied to the biomass of green seaweed *Ulva* sp., fast-growing cosmopolitan seaweed. The SWH was done with seawater at 180 °C and 10.5 bar during 40 min with 8% w/w solid load. This treatment resulted in 211 ± 7 mg of hydrochar g^{-1} dry weight (DW) of *Ulva* sp. with higher heating value (HHV) double that of the initial biomass. The liquid fraction content per gram of *Ulva* DW included 5.2 ± 1.15 mg of 5-HMF; 24.1 ± 2.84 mg total monosaccharides (composed of 14.3 ± 1.78 mg glucose, 5.1 ± 0.41 mg rhamnose, 2.3 ± 0.41 mg fructose, 1 ± 0.06 mg xylose, 0.9 ± 0.08 mg galactose and 0.6 ± 0.11 mg glucuronic acid); 58 ± 11.78 mg protein (corresponding to 84.9 ± 13.2% of the total protein); and free amino acids (3.64 ± 0.07 mg leucine, 2.08 ± 0.13 mg arginine, 1.54 ± 0.01 mg isoleucine and 1.06 ± 0.03 mg alanine). Two-step fermentation optimization was done with *Saccharomyces cerevisiae* and *Escherichia coli* in *Ulva* hydrolysate following the SWH, with ethanol yield of 4.67 ± 0.76 mg g^{-1} DW *Ulva*. Based on these process results, some economics and sustainability indicators were estimated for large-scale macroalgae-based biorefinery. The required offshore areas for *Ulva* cultivation to satisfy the entire national demand in Israel for plant-based protein, char for electricity production (20% blend co-firing with coal), or ethanol for transportation (10% blend in gasoline), are 0.8%, 3.1% and 34.3%, respectively of the Israeli exclusive economic zone in the Mediterranean sea. The total revenue expected for the co-products derived from *Ulva* sp. via SWH varies between $0.26 and $1.06 kg^{-1} DW.

1. Introduction

Marine macroalgae can provide a source of biomass for sustainable production of food, fuel, and chemicals [1,2]. A key step in the energy-efficient conversion of macroalgae to chemicals and biofuels via fermentation is the deconstruction of complex carbohydrates [3]. Currently, such a deconstruction relies mostly on multistep acid hydrolysis, diluted-acid and alkaline hydrolysis [3] and enzymatic decomposition [4]. These processes either require handling of large volumes of chemical waste [5], or are unaffordable if the final products of fermentation are low-cost commodities such as food or fuels [6,7]. In addition, only a few enzymes have been reported up to date that can hydrolyze complex macroalgal fibers [4]. An alternative solution for the complex macroalgae carbohydrates deconstruction is subcritical water hydrolysis (SWH) [8–10]. The products of subcritical water (SW) treatment or subcritical water extraction (SWE) include solids such as hydrochar, which is the product of hydrothermal carbonization (HTC) process [11], liquids (products of hydrolysis and liquefaction and intracellular compounds release [12,13]), and gases [14,15].

Hydrolysis of the biomass polysaccharides is expected to produce fragments with molecules of different lengths, including monosaccharides [12,13] that can be used as carbon sources for fermentation [16]. Previous works on the macroalgae hydrothermal treatment investigated the impact of process parameters on the yield of sugars release, hydrochar (biochar) [17] and biocrude formation [18]. The biorefinery processes are designed for simultaneous co-production [2,7,19]. For instance co-production of hydrochar and fermentable monosaccharides has been reported [8]. This paper describes the extension of this approach to a process for co-production of hydrochar, ethanol, proteins and additional hydrolyzed monomers such as monosaccharides and amino acids. Including sugar chemically converted

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Nomenclature

A	area (km^2)
b	blend ratio
h_{fg}	liquid/vapor enthalpy difference (kJ/kg)
M	annual dry mass (kg, t)
p	market sale price ($/kg).
r	ratio of a value (e.g LHV) for a convention source to that of the algae-derived alternative
Y	yield (kg product per kg of algae dry weight)

Subscripts

algae	related to algae
conv	related to conventional (non-algae) sources
H_2O	related to water

Acronyms

AA	amino acids
AGR	annual growth rate per unit area
CFU	colony forming units
DDW	double-distilled water
DW	dry weight
EEZ	exclusive economic zone
GlcA	glucuronic acid
HHV	higher heating value
HTC	hydrothermal carbonization
LHV	lower heating value
SW	subcritical water
SEM	scanning electron microscope
SW	subcritical water
SWH	subcritical water hydrolysis
TR	total revenue
TS	total monosaccharides
WW	wet weight

spices as 5-Hydroxymethylfurfural (5-HMF). Although the production of the mentioned products from *Ulva* has been demonstrated [1,8,11,20], it was done previously in separated processes. This work investigates hydrothermal pretreatment aimed to release simultaneously multiple products including 5-HMF, proteins, fermentable monosaccharides with amino acids, and energy-dense hydrochar. The green macroalgae *Ulva* sp., a promising biorefinery feedstock, is used in this work. The macroalgae from *Ulva* sp. is of particular interest as a feedstock due to its global distribution, fast growth rates and high carbohydrate and proteins contents [21].

SWH was shown to improve the protein extraction yields from plants [22] and microalgae [23]; to the best of our knowledge, it was not previously used to extract proteins from seaweeds [24]. Previous SWH work with seaweed reports the extraction of polyphenols, phlorotannins, neo-antioxidants, amino acids, polysaccharides, and minerals, reviewed by Ciko et al. [25]. Previously several other methods for protein extraction from different *Ulva* species were used [20,26]. Those methods such as pulsed electric field (PEF), osmotic shock, sonication, higher shear homogenization, chemical and enzymatic treatments were applied. The maximal protein extraction yield was reported by Postma et al. as ~39% from total protein [26]. The relatively low protein yield extracted from seaweeds [27] might be because all of the mentioned extraction methods rely on the release of water-soluble proteins due to the algae macrostructure, and the proteins' solubilization properties in water. The seaweeds contain phenols and polysaccharides which can interact with the proteins and hinder their extraction and purification [28]. However, SW rich in H^+ and OH^- is a good polar solvent and a catalyst with self-neutralizing acid-base properties [29]. Therefore, SWH should be an efficient method for solubilizing water-insoluble protein of plant biomass [30]. SWH of plant biomass demonstrated high protein extraction yield, for instance about 50% in soybean and up to 84% in de-oiled rice bran [31].

For the generation of the ethanol end-product, a two-step liquid hydrolysate fermentation was experimentally investigated, using *Saccharomyces cerevisiae*, which mostly consumes glucose [32,33], and *Escherichia coli*, an organism that is able to ferment a wider variety sugars to ethanol [34]. In previously published works, the bioethanol potential production under complete biomass hydrolysis [35] was assessed. This is usually achieved by a combination of thermochemical following enzymatic hydrolysis [4,36]. In this work, the optimal microbial formation sequence was implemented for both the thermochemical [37] and enzymatic process [36] hydrolysate, and the SW hydrolysate.

One of the most important part in the developments of a program for biorefinery is the area allocation of the biomass cultivation, particularly in case of offshore multi-use platforms [38]. For demonstration purposes, as a test case, the experimental results of this study were used for estimating the offshore area required within the Israeli Exclusive Economic Zone in the Mediterranean sea for supplying the national demand for plant based protein, for replacing coal by char in power generation, and for replacing gasoline by ethanol as a fuel for transportation [39]. In addition, the experimental data allows estimating the potential revenue of an *Ulva* based biorefinery.

2. Materials and methods

2.1. Ulva sp. biomass cultivation

Green macroalgae *Ulva* sp. was cultivated at the Israel Oceanographic and Limnological Research (IOLR Ltd., Haifa, Israel), under controlled conditions using 40 L outdoor tanks. The exact taxonomic composition of the *Ulva* sp. used in this study suggests a mix of two morphological and genetically similar types, *Ulva rigida* and *Ulva fascia* [40]. After two weeks of cultivation, the *Ulva* sp. biomass was transferred to larger outdoor tanks having volume of a 1000 L. In both tanks, the biomass was constantly aerated and supplied with running natural Mediterranean seawater pumped from the nearby seashore. During the cultivation in the 40 L tanks, the seaweeds were fertilized once a week, with 0.06 mM NaH_2PO_4 and 0.59 mM NH_4Cl (both chemicals from Haifa Chemicals Ltd., Israel). After 4 weeks of cultivation (from October to November 2018), the biomass was harvested, washed with distilled water and drained using a spinner. This biomass is defined as wet weight (WW). For SWH and HTC the biomass was dried at 40 °C to constant weight, denoted as dry weight (DW).

2.2. Elementary and caloric value analyses

Elemental analysis was done at the Technion (Israel Institute of Technology, Haifa, Israel), using Thermo Scientific CHNS Analyzer (Flash2000). The oxygen atom content in the biomass or in the biochar was determined by a mass balance (see data in Table 1):

$$\%O = 100\% - (\%C + \%H + \%N + \%S + \%Ash) \quad (1)$$

For the caloric value and ash analyses, two gram of untreated algae (DW) and residual carbonized material were analyzed for High Heating Value terms (HHV) according to ASTM D5865 − 13 (Standard Test Method for Gross Calorific Value of Coal and Coke) and for ash according to D5142 standard by a certified laboratory of Israel Electric

Table 1
Properties of *Ulva* sp. biomass before and after SWH. Composition is % wt relative to the original algae DW. The atomic content of CHNS was determined from the elemental analysis. Then, the oxygen content was quantified via equation num. 1 [41]. N ≥ 2. Numbers are represent mean ± SD.

		Untreated Algae	Hydrochar 180 °C, Solid Load 8%, 40 min, salinity 38 g/L
Ultimate (wt.%)	N (%)	1.46 ± 0.01	3 ± 0.7
	C (%)	24.77 ± 0.13	44.4 ± 0.8
	H (%)	4.88 ± 0.02	5.8 ± 0.0
	S (%)	6.64 ± 0.1	3.1 ± 0.5
	O (%)	34.41 ± 0.26	26.3 ± 1.15
Proximate (wt.%)	Ash	27.85 ± 0.51	17.35 ± 0.15
	Moisture	15.49 ± 1.31	15.6 ± 0.3
Biochemical (wt.%)	Starch	4.53 ± 0.07	–
HHV (MJ kg^{-1})	Boie [42]	10.9	20.9
	Grummel & Davis [43]	9.1	18.2
	Measured value (Calorimeter)	10.04 ± 0.2	20.4 ± 0.8
LHV (MJ kg^{-1})	Calculated **Eq.2**	8.55 ± 0.2	18.9 ± 0.8

company.

The lower heating value (LHV) was calculated using the following equation:

$$LHV = HHV - h_{fg} \cdot M_{H_2O} \quad (2)$$

The HHV calculated as in equation (1). h_{fg} is the latent heat for water condensation: 2257 kJ kg^{-1}. M_{H_2O} is the water produced in combustion reaction: 0.666 kg per kg char. The dry weight (DW) of the biomass and hydrochar was determined after drying at 40 °C.

2.3. Scanning electron microscopy

Images of solid samples of *Ulva* sp. before and after HTC (in Fig. 1), were captured with the scanning electron microscope (SEM) (JCM-6000, JEOL) after mounting them on aluminum stubs and sputter-coating with gold (SC7620, Quorum).

2.4. Ulva sp. biomass thermochemical and enzymatic hydrolysis

Ulva biomass enzymatic and thermochemical hydrolysis experiments were done in order to optimize the fermentations sequence, with *S. cerevisiae* and *E. coli* (Table 2). The *Ulva* sp. biomass was dried at 40 °C until achieving a constant weight and was grounded with an electric grinder (Grinding machine, Henan Gelgoog Machinery GG9FZ-19) to particle sizes of 0.125 > X ≥ 0.063 mm. The ground powder was then sieved using a size-selective metal-mesh (Sieve Sets S3076, Aquatic Ecosystems). The *Ulva* powder was hydrolyzed in two steps. First, a thermochemical hydrolysis was done then enzymes were added to the cold hydrolysate for improving the hydrolysis of polysaccharides (saccharification) into monosaccharides.

For the thermochemical hydrolysis, 4 g of the powder of dry *Ulva* was added to a 100 mL glass bottle with 40 mL of 2% sulfuric acid (v:v) (Sigma-Aldrich), making 10% solid load. Afterwards, the biomass in the

Table 2
Two-step fermentation for ethanol production optimization of *Ulva* sp. biomass hydrolyzed with acid and enzymes. In the column of microbial sequence, the letter "Y" stand for the yeast *S. cerevisiae* and "E" is the bacteria *E. coli*. The fermentation step with *S. cerevisiae* was 24 h while fermentation *E. coli* 48 h. N = 3. Numbers are represent mean ± SD.

Microbial sequence	Fermentation step 1 (mg g^{-1} DW *Ulva*)	Fermentation step 2 (mg g^{-1} DW *Ulva*)	Total Ethanol (mg g^{-1} DW *Ulva*)
S-E	29 ± 3.1	9 ± 1.2	38 ± 3.4
S-S	29 ± 3.1	5.7 ± 0.5	35 ± 3.1
E-S	1.7 ± 1.4	4 ± 1	6 ± 2.1
E-E	1.7 ± 1.4	1.1 ± 0.2	3 ± 1.3
S	29 ± 3.1		29 ± 3.1
E	1.7 ± 1.4		1.7 ± 1.4

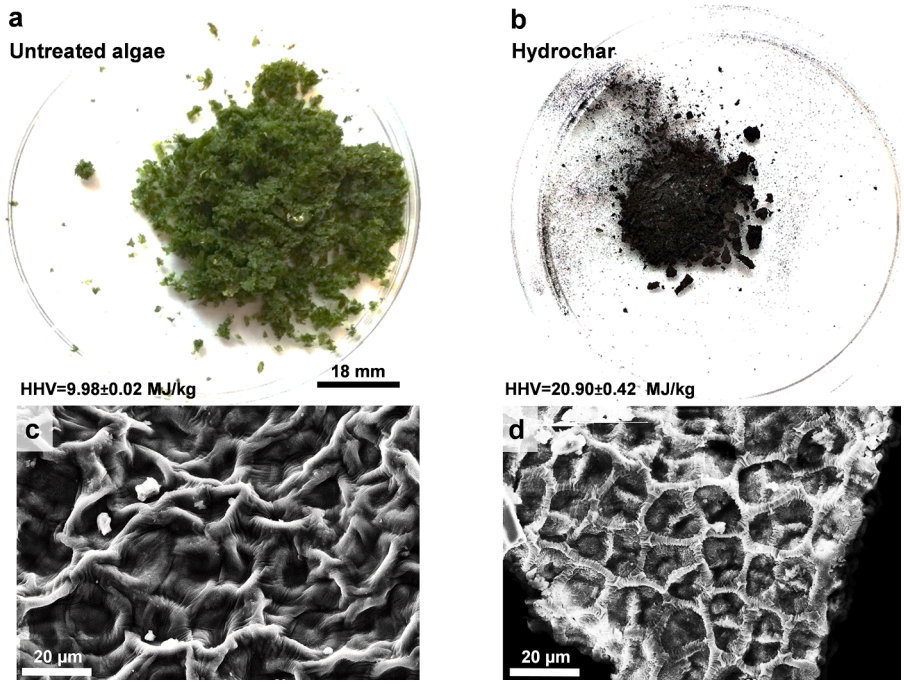

Fig. 1. Chemical and energetic profile of untreated *Ulva* sp. thallus and hydrochar obtained after. SWH treatment - seawater at 180 °C and 10.5 bar during 40 min with 8% w/w algae DW solid load. **a**. Photo of untreated *Ulva* sp. biomass. **b**. Photo of hydrochar after *Ulva* sp. biomass treatment by SWH. In **a** and **b** the HHV is presented. The black scale bare = 18 mm. **c**. SEM photo of untreated *Ulva* biomass. **d**. SEM photo of hydrochar.

acid was autoclaved at 121 °C for 30 min. At the end of the thermochemical hydrolysis, the hydrolysate was prepared for enzymatic hydrolysis by adjusting the pH to 6 with NaOH (Merck, Sodium hydroxide). Subsequently, to the hydrolysate 6.6 mL of 0.5 M phosphate buffer (Phosphate Buffer Powder, Sigma-Aldrich, Israel) [44] was added. A mixture of enzymes that included amyloglucosidase 72 U, α-amylase 38 U and cellulase 66 U in 40 mL of 200 μM sodium acetate buffer (Sigma-Aldrich, Israel) was also added to the hydrolysate. In order to achieve the optimal enzyme activation, the hydrolysate was incubated at 45 °C for 24 h [36]. At the end, samples were taken for monosaccharides measurements in ion chromatography. Additionally, this hydrolysate was used for microbial sequence optimization in two-step fermentation.

2.5. Subcritical water hydrolysis in a batch system

The experimental system [8] consists of a 0.25 L batch reactor (Zhengzhou Keda Machinery and Instrument Equipment, CJF-0.25) heated electrically (Keda Machinery, China). The temperature was controlled and measured with a digital temperature gauge (TM-5005, MRC) using 1/16″ thermocouple type K (Watlow, USA). The pressure was constantly measured with a pressure gauge (PS-9302, MRC) and reading instrument (PS100-50BAR, MRC). A magnetic coupling drive used to mix the slurry inside the pressure reactor with a stirrer at 70 RPM. The magnet coupling was water-cooled from a chiller (Guangzhou Teyu Electromechanical Co., Ltd Cw-5200ai, China) set to 25 °C. The reactor has two gas-sampling ports and one liquid sampling line. The liquid sample passes through a cooler (from the same chiller) and a cold trap (25 °C), before entering the sampling tube. The gas line passes through a condenser and a cold trap before reaching a 1 L gas-sampling bag. Before each test, the air was evacuated from the system with a vacuum pump (MRC, ST-85), down to 0.13 mbar absolute. Hydrothermal treatment was done under process parameters which were previously optimized for *Ulva* sp. biomass monosaccharides SWH [8]: temperature of 180 °C, treatment time 40 min, maximal pressure 10.5 bar, 8% w/DW solid load (8 g *Ulva* sp. DW in 92 mL of seawater) and salinity of 38.2 g L^{-1}. Each test was repeated twice. After the hydrothermal treatment, the treated biomass was transferred into 50 mL sterile tubes. The solids were settled during overnight at 4 °C, then centrifuged at 3000 rpm for 10 min. The supernatant transferred into new sterile 50 mL tubes and stored at −5°C. The solids were dried at 40 °C for 4 h and then stored at room temperature (~25 °C). The supernatant is considered as SW hydrolysate while the solids phase is defined as the biochar.

2.6. Total protein determination

After the subcritical treatment of *Ulva* sp. biomass, the hydrolysate was separated from the solids (as described in previous section) and filtered with 0.22 μm syringe-filter (Millipore, USA), the hydrolysate was diluted 10 times with DDW. Then, total protein content in the hydrolysate was determined applying Lowry method [45].

2.7. Biological assay for determination the S. cerevisiae growth inhibition by SW hydrolysate

The yeast *Saccharomyces cerevisiae* growth was used as a marker for SW hydrolysate toxicity evaluation. SW hydrolysate original pH was 3.41 ± 0.06. Therefore, firstly, the pH was adjusted to 4.5–5 with 3 M NaOH. Secondly, the hydrolysate was diluted in 10 dilutions using autoclaved DDW (double-distilled water). The dilutions were done in 10% steps (100%, 90%, 80% and so on…). The diluted hydrolysate was filtered through 0.22 μm syringe-filter (Millipore, USA) and the salinity was measured with a refractometer (SainSonic, Automatic Temperature Compensating Refractometer). Thirdly, the *S. cerevisiae* was refreshed and one colony was added to the SW hydrolysate. Finely, the level of yeast growth in SW hydrolysate was measured by $OD600_{nm}$ and colony-forming units (CFU). A fresh culture of yeast *Saccharomyces cerevisiae* (Ethanol Red, Batch 62186/2, 'Leaf', France) was prepared for the experiments. Initially, the yeast was stored in glycerol at −80 °C, then refreshed with yeast extract peptone dextrose (YPD) solid medium in a Petri dish and incubated during overnight (O.N) at 32 °C. The petri dish with the fresh culture was stored up to one week in the refrigerator (5 °C). Yeast starter was prepared by transferring one yeast colony from the culture in the petri dish into 2 mL YPD medium and then incubated O.N in 32 °C, with orbital shaking of 180 rpm (IncuShaker Mini, Benchmark Scientific). The yeast starter was concentrated twice and washed three times with 10% SW hydrolysate medium. The washing procedure was done by centrifuge at 7000 rpm for 3 min (centrifuge 5424, Eppendorf). Yeast was cultivated in 2 mL diluted hydrolysate media after 20 μL from the starter (2×10^5 cell/μL) was transferred into the media. The media was sealed in sterile polypropylene (PP) 10 mL tubes (Nalgene™ Oak Ridge High-Speed PPCO Centrifuge Tubes, Thermo-Fisher Scientific, UK), sterilized in an autoclave (IcanClave, STE-23D, Ningbo Ican Machines Co., Ltd). The incubation was done in PP tubes horizontally placed to the shaker incubator, temperature set to 32 °C, shaken with 80 rpm, during 48 h. Each hydrolysate experiment was repeated three times. At the end of the incubation time (48 h), the yeast cells were counted using absorbance and colony-forming units (CFU). The absorbance was measured using O.D optical density 600 $_{nm}$ (Tecan Infinite 200 PRO, TECAN, Switzerland). CFU was done by spread-plating with glass beads on Petri dishes with YPD solid medium (2% agar). The plates were incubated in aerobic conditions at 32 °C during O.N and the colonies were count manually.

2.8. Optimization of sequence of organisms in the two-step fermentation in thermochemical and enzymatic Ulva hydrolysate.

S. cerevisiae (Ethanol Red, Batch 62186/2, 'Leaf from Lesaffre', France) and *Escherichia coli* K-12 MG 1655 wild type (WT) were used, where different fermentation orders were tested to maximize ethanol yield. Six combinations (factorial design) of the sequence in microbial fermentation are described in Table 2. The microorganisms were refreshed on rich media plates from glycerol stock, as described in the previous section. The *E. coli* was cultivated on Lysogeny broth (LB) agar plates [46] and incubated O.N at 37 °C. Microbial starts were prepared for initiating the microbial growth in the thermochemical and enzymatic hydrolysate. One colony from each organism (*S. cerevisiae* or *E. coli*) transferred from rich media plats (YPD or LB) to 2 mL of thermochemical and enzymatic *Ulva* hydrolysate (hydrolase preparation described in section 2.4) in 15 mL sterile test tubes (Culture tubes, PP, 2-stage-cap, Bar-Naor, Israel). The *S. cerevisiae* or *E. coli* were cultivated during 24 or 48 h in 32 or 37 °C, respectively. The two-step fermentation was done in 3 mL of *Ulva* in thermochemical and enzymatic hydrolysate in 10 mL autoclave tubes (Nalgene™ Oak Ridge High-Speed PPCO 185 Centrifuge Tubes, Thermo-Fisher Scientific, CA). The tubes were sealed after adding 75 μL of the microbial starter (with *S. cerevisiae* or *E. coli*). The microbial cultivation was done in tubes, which horizontally incubated in orbital shaker incubator (IncuShaker Mini, Benchmark Scientific) E. coli was incubated at 32 °C for 24 h while *S. cerevisiae* and 37 °C for 48 h, both shaked at 150 RPM. At the end of every fermentation, the ethanol from every sample was evaporated in a water bath at 80 °C for 30 min. For deactivation of the microorganism from the first step, and preparing the hydrolysate for the second step, the samples were autoclaved (121 °C for 30 min). The second fermentation was done similarly to the first with the same volume of the starter, the same temperature, shaking and incubation time, typical for every microorganism.

2.9. Two-step fermentation in SW hydrolysate.

The *Ulva* hydrolyzed in subcritical water was fermented to ethanol in two-step fermentation with yeast *S. cerevisiae* at the first step and *E. coli*

at the second step (same organism as mentioned at previous two sections). The *Ulva* SW hydrolysate was prepared as described in section 2.4. Firstly, the pH of the hydrolysate (3.41 ± 0.06) was adjusted to 4.49 ± 0.01 with 3 M NaOH. Secondly, the hydrolysate was diluted with double-distilled water (DDW), 5 times to a concentration of 20%. After dilution, the media pH was 4.79 ± 0.02 and the conductivity 134.9 ± 0.45 mV measured with electric pH and conductivity meter)SevenExcellence™, Metter-Toledo, Switzerland). The starter of *S. cerevisiae* was prepared (as described in section 2.7) and 20 μL of the concentrated and washed starter (2.8×10^5cell/μL) was inoculated to the hydrolysate. After yeast fermentation in polypropylene (PP) tubes, the samples were transferred to 2 mL Eppendorf tubes and the ethanol was evaporated as described in section 2.7. After the evaporation, the liquid volume was adjusted with DDW to the original volume that was before the evaporation process. An *E. coli* starter was prepared similarly to how the *S. cerevisiae* was prepared with minor changes, the incubation temperature was 37 °C, and the cultivation media was in Lysogeny broth (LB). The starter was similarly washed as a starter with *S. cerevisiae* and 20 μL of the washed starter (2.85×10^6cell/μL) inoculated to the autoclaved media. For the second fermentation step, the yeast was deactivated with autoclave and *E. coli* was added to the hydrolysate and was incubated similarly to as described in Section 2.7. Before and after each fermentation step the CFU, OD 600_{nm}, monosaccharides, GlcA, 5-HMF and ethanol were measured. The AA measured only before each fermentation step.

2.10. Ion chromatography of monosaccharides, glucuronic acid and 5-HMF analysis

Monosaccharides contents in the hydrolysates were determined using high-pressure ion chromatography (HPIC) via Dionex ICS-5000 (Dionex, Thermo Fischer Scientific, MA, USA) using a Dionex™ AminoPac™ PA10 IC analytical column with its corresponding guard column (Thermo Fischer Scientific, UK). An electrochemical detector was used with a gold disposable working electrode and an AgCl reference electrode. The voltage waveform "Carbohydrates (Standard quad)" was used. The autosampler containing the diluted sample and the standards was kept at 5 °C. The analysis started with an isocratic gradient of 4.8 mM KOH generated by the Eluent Generator technology through first pump (Dionex, Thermo Fischer Scientific, MA, USA) to elute the common monosaccharides, then a gradient of three eluents (A: ultrapure water, B: 480 mM NaOH, and C: 100 mM NaOH + 1 M sodium acetate) was applied by a second pump after switching the flow valve to elute sugar acids (such as glucuronic acid) as well as to wash the column. Following the acetate gradient, acetate ions were washed with NaOH (B) for a few minutes. Then a flow of 100 mM KOH generated by the eluent generator (after the flow valve was switched back to pump 1) was applied for a few minutes before the system was re-equilibrated to initial condition for 31 min. The total duration of the analysis including column washing and equilibration was 75.2 min (more details of the eluent gradients are available in Table S1). The mobile phase flow rate was 0.25 mL/min, the injection volume 25 μL and the column temperature was set to 30 °C. The identification and quantification of monosaccharides in the HTC hydrolysates were performed by comparison with reference standards: fructose, xylose, glucose, galactose, rhamnose, GlcA (glucuronic acid, Sigma-Aldrich, Saint-Louis, USA) and 5-HMF (5-Hydroxymethyl-2-furaldehyde, stabilized with Water, TCI America™). The calibration curves were at the range of monosaccharides and GlcA were in the range of 22–0.21 μg/mL and for 5-HMF the range was 95–0.74 μg/mL. Prior to analysis, the hydrolysates of the SW samples were diluted in ultrapure water and filtered with 0.22 μm syringe-filter (Millipore, USA) into HPIC vials (Thermo Fischer Scientific, USA).

2.11. Ion chromatography for amino acids analysis

Analysis of the amino acid (AA) content in the hydrolysates was performed following the product manual "Dionex *AAA-Direct*, Amino Acid Analysis System" [47] from Dionex Inc. (Thermo Fischer Scientific, MA, USA). Total amino acid content was analyzed by HPAEC-PAD (High-Pressure Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection) using a Dionex ICS-5000 platform (Dionex, Thermo Fischer Scientific, MA, USA) with an analytical column (Dionex™ AminoPac™ PA10 IC analytical column) and its corresponding guard column. An electrochemical detector with a working non-disposable gold AAA^{TM} electrode and a pH reference electrode were used for detection. The eluent gradient program (**Table S2**) and the waveform for the electrochemical detector used were the ones used in the product manual (page 18) previously mentioned [47], other conditions were as follow: flow rate; 0.25 mL/min, injection volume; 25 μL, column temperature; 30 °C, autosampler temperature; 5 °C. The program was validated by using a commercial amino acid mix (AAS18, Sigma Aldrich, MO, USA) and four dilutions of the mix (1/50, 1/100, 1/250, 1/500 and 1/1000) which were used to build a calibration with a correlation factor $R^2 > 99\%$.

2.12. Ethanol measurement

Ethanol was measured using (K-ETOH, Magazme, Ireland) with a spectrophotometer (Tecan, infinite M200 PRO) with 340 nm wavelength. Before using the kit, the samples were frozen at −20 °C and centrifuged at 14,000 RPM for 5 min. The ethanol was measured in the supernatant.

2.13. Calculation for needed area to cultivate Ulva

The Exclusive Economic Zone (EEZ) of Israel in the Mediterranean sea, about 26,000 km^2, might be used for macroalgae cultivation [48]. Three co-products of an algae biorefinery are considered for covering the Israeli national demand in the following applications: the char can displace coal in power plants; ethanol can displace gasoline as a vehicle fuel; and the algae protein can displace conventional sources of alimentary protein. The annual consumption rates in Israel for these three applications in 2017 were: $8.3 \cdot 10^9$ kg of bituminous coal (providing about 33% of the national demand for electricity). This demand was based on the annual energy derived from coal which was 4968 ktoe [49] ($2.08 \cdot 10^{11}$ MJ) with LHV of 25 MJ kg^{-1} [49]; $3.2 \cdot 10^9$ kg of gasoline for land vehicles [50] (excluding diesel vehicles); and $1.5 \cdot 10^8$ kg of plant-based protein (average protein requirement per person is 47 g per day [51], population 8.7 million [52]). The annual dry mass of algae (M_{algae}) needed to displace the conventional sources can be calculated separately for each of these applications:

$$M_{algae} = \frac{M_{conv} br}{Y} \tag{3}$$

M_{conv} is the annual mass consumed from the conventional source, as given above. b is the blend ratio, i.e., fraction of the conventional mass to be displaced by the algae-derived product. Generally, the hydrochar created after SWH could be used for co-firing blended with coal, at different proportions. However, according technical parameters of char firing (e.g., high ash content) and environmental factors, previous LCA [53] and techno-economic analysis [54] have shown that 10–20% is the optimal char proportion for co-firing [53]. So far the largest industrial-scale pilot for coal co-firing with biomass (not with biochar) uses 15% biomass [54]. It is much cheaper to burn the biomass directly without processing into biochar. However, when the char production leads to multiple co-products with high economic value, it can be beneficial to consider co-firing biochar instead of the raw biomass. The ethanol product is best used as E10 blend (10% ethanol in gasoline) that is commonly used in regular cars with no need for engine modifications

[39], and can lead to easier commercialization of bioethanol. We select values of $b = 0.2$ for coal, $b = 0.1$ for ethanol; and $b = 1$ for protein (assuming that the composition of algal proteins is adequate for all nutritional needs that are currently served by conventional sources of protein). r is the ratio of conventional fuel lower heating value to the substitute algae-derived fuel LHV for the char and ethanol cases, and $r = 1$ for the protein. Y is the yield (kg product per kg of algae DW) for each of the algae-derived products.

The area required in the Mediterranean Sea for producing these annual amounts of algal mass is:

$$A_{algae} = \frac{M_{algae}}{AGR} \quad (4)$$

AGR is the annual growth rate per unit area, with value of 12.3 kg m^{-2} in the case of intensified cultivation nearshore [55]. For extensive cultivation that can be carried out in deeper water, the value of the AGR is lower by a factor of 5.8 [48] ($AGR = 2.1$ kg m^{-2}) and the required area will be larger by the same factor.

2.14. Market value analysis of the products from Ulva

The specific Total Revenue (TR, US$ kg^{-1}) per unit mass of algae DW from the three co-products is calculated by the following equation:

$$TR = \sum_{i=1}^{3} Y_i p_i \quad (5)$$

p_i is the market sale price for product i. Y_i is the yield of product i per unit mass of algae DW, based on data from the current study, as described in the result section.

2.15. Statistical analysis

All samples were hydrolyzed in duplicates and each hydrolysate was injected twice for HPIC analysis. All data were reported as the mean weight fraction of the AA per mg of biomass (μg of AA mg^{-1} DW biomass ± standard deviation. Statistical analysis was performed with Excel (ver. 13, Microsoft, WA) Data analysis package and R-studio (R-Studio: an Integrated development environment for R (Version 1.1.383) [Windows]. Boston, MA).

3. Results and discussion

3.1. Ulva sp. biomass hydrothermal carbonization (HTC)

The ultimate, proximate, starch and the HHV (measured and calculated) of the untreated algae and the carbonized biomass (hydrochar) are shown in Table 1, Fig. 1, **and Fig. S1**. The SWH was done as mentioned 'method' section, *Ulva* sp. 8% w/w solid load in seawater (salinity 38.2 g L^{-1}) treated with temperature of 180 °C for 40 min with maximal pressure of 10.5 bar (Fig. 1**a, c**). We chose to use this specific SWH protocol because of a previous optimization work where a similar protocol led to optimal sugar hydrolysis of *Ulva* sp. biomass [8]. After the SWH treatment, the solid biomass/hydrochar (Fig. 1**b, d**) was separated from the hydrolysate. The hydrochar yield was 211 ± 7 mg per gram *Ulva* (DW) biomass. The hydrochar's carbon content increased and oxygen and ash content were decreased compared to the untreated biomass due to the carbonization process. This leads to an upgrading in the HHV by 9–11 MJ (energy densification of 1.9–2.2) compared to the untreated biomass (Fig. 1**a, b**).

The energy yield (the densification of energy multiplied by hydrochar yield) is 35–39%, comparable to other carbonization studies on macroalgae which show 40–67% energy yield [11,17,56]. Arable biomass such as rice and corn have a higher reported energy yield of 79%, 69%, respectively (HTC at 178 °C and 30 min residence time) [57]. Additional energy densification requires operation at higher temperatures and longer times, which, however, leads to the degradation of the released fermentable monosaccharides [58]. Indeed, the maximization of sugar recovery requires operation at lower temperatures, typically below 220 °C [58]. In contrast, maximizing energy densification of the HTC char requires higher temperatures [58]. This could suggest a two-step process, for realizing high sugar recovery at the first step and maximizing char densification at the second step. The carbonization led to a change in surface morphology of the biomass (Fig. 1**c, d**). Initially the surface was comparatively smooth. However, after the SWH the porosity of the surface was increased. The pores origin are probably in the *Ulva* cell walls, and its extracellular matrix. It has already was proven that carbonization increases the porosity of the biomass, relevant for the combustion reaction activation factors (temperature, precursor, degree of heat treatment, and activation agent) [59].

3.2. Proteins extraction with SW

In this work, it was found that SW extracted 58.4 ± 11.8 mg protein from one gram of *Ulva* (DW) biomass. The total nitrogen content of this biomass was 1.38 ± 0.006%, thus after the multiplication by 5 [60], the total protein content of the *Ulva* was 6.9 ± 0.3% or 69.3 ± 3.3 mg protein g^{-1} *Ulva* (DW). This means that SW extracted about 84.9 ± 13.2% of the total protein, a recovery level much higher than other currently reported methods for protein extraction from seaweeds.

The nitrogen to protein ratio used here is a viable method for protein estimation in seaweed biomass [60]. This ratio could not be applied to the liquid extract, therefore the Lowry assay was used, which is a common protein determination method [61], including for total protein extracted from *Ulva* [20,26]. However, it is important to mention is that Lowry assay has sometimes interference issues with other molecules such as phenol, lipids, sugars and polysaccharides, and various reducing agents [61]. This interference might cause protein overestimation or underestimation [61]. Therefor we tested interference of SW hydrolysate with BSA, and the Lowry reagent (used for protein quantification method) reactivity with SW hydrolysate in high concentrations (data not shown). Both tests showed that the protein content in hydrolysate is potentially underestimated by about of 20%. More work is then needed to estimate more precisely the protein content and the level of interference of the extract, e.g., by using different assays, isolating the protein and/or the interfering compounds in order to better assess the efficiency of protein extraction from green seaweed with SWH. In addition, the impact of the SW process parameters on the textural, functional [62] and bioavailability [63] of extracted seaweed proteins should be further investigated in the following studies.

3.3. Determination the growth inhibition of S. cerevisiae by Ulva SW hydrolysate

Growth inhibition or chemical sensitivity of *S. cerevisiae* is traditionally measured by agar-based plating methods [64,65]. Additional turbidity measurements are usually used as complementary tests [65]. Multiple previous studies showed that biomass hydrolysates could be toxic to fermenting microorganisms both by inhibiting their growth and/or by inhibiting the metabolism [66–68]. This inhibition takes place due to the formation of inhibitors such as acetic acid, levulinic acid and 5-HMF during the hydrolysis [66–68]. In the present case, the salinity (**Fig. S1**) might also affect the growth of the inhibition [69]. Thus, it is common to dilute the hydrolysate to detect the growth inhibitory hydrolysate's concentration range [70].

The impact of various concentrations of the SW *Ulva* hydrolysate on the growth of *S. cerevisiae* was investigated. Growing the *S. cerevisiae* on different hydrolysate concentrations was found to have a significant difference in growth (Fig. 2, ANOVA p-value $< 2.21 \cdot 10^{-10}$). It is relevant to assume that the salinity (**Fig. S1**) might cause *S. cerevisiae* growth inhibition. But the acute growth inhibition in the range of

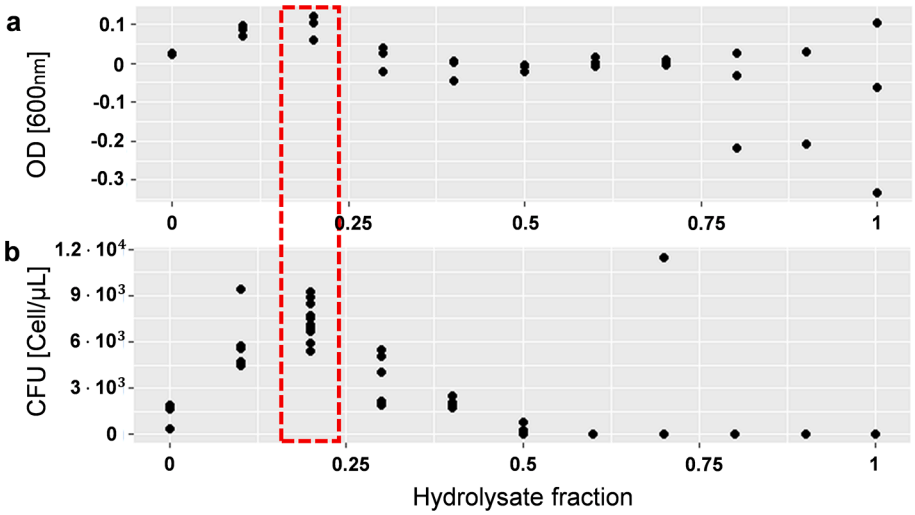

Fig. 2. *S. cerevisiae* viability in SW hydrolysate dilutions. **a** and **b**, *S. cerevisiae* viability in different hydrolysates fractions measured with OD 600 nm or with CFU, respectively.

50–100% hydrolysate (Fig. 2), where the salinity range is 4–8% w:v, is not compatible with the literature [69,71]. Usually, the acute *S. cerevisiae* growth inhibition by salinity is in the range of > 15% w:v [69]. Therefore, the observed growth inhibition might be due to other factors formed during hydrolysis. The growth inhibition might be due to the limited carbon source availability; however, slow growth on high concentrations (30% and higher, Fig. 2 and **Table S3** for Tukey analysis) is probably due to high concentrations of inhibitors, including 5-HMF (355–555 mg L^{-1}) [72]. There is a large scatter and negative results in OD in > 70% of hydrolase (Fig. 2**a**) that might be explained by the way of calculating the results (The OD 600 $_{nm}$ of the *S. cerevisiae* culture presented by the yeast growth minus the background of a sterile hydrolysate). In the concentrated samples, the hydrolysate was more turbid. This turbidity might cover the signal of the culture. Therefore, to understand the *S. cerevisiae* growth efficiency in the different concentrations of the *Ulva* hydrolysate after subcritical water hydrolysis, especially in the highly concentrated hydrolysates, a CFU measurement was performed (Fig. 2**b**). It was found that 20% hydrolysate medium led to the highest growth of the yeast (Fig. 2**a,b** and **Table S3)** and this concentration was used in the following fermentation steps. Previously, the haloarchaea *Haloferax mediterranei* was cultivated in similar *Ulva* hydrolysate after subercritical water hydrolysis with the same solid load during the hydrolysis [73]. The growth of *H. mediterranei* was tested in different hydrolysate concentrations. The optimal microbial growth was in the range of 25–50% hydrolase. 75–100% hydrolysate significantly inhibits microbial growth. That might indicate that this hydrolysate contains growth-inhibitory environment that affecting microorganisms in general.

3.4. Optimization of sequence of organisms in the two-step fermentation in thermochemical and enzymatic Ulva hydrolysate

Here, the sequence of microbial fermentation was optimized in two steps (Table 2). For the optimization, *Ulva* biomass was hydrolyzed with thermochemical treatment and then enzymatically. The released TS yield in the hydrolysate was 227.76 mg sugar per g *Ulva* DW. This TS yield composed of glucose 101.74, rhamnose 44.12, galactose 3.17 xylose 28.27 fructose 12.1 and glucuronic acid 38.4 mg g^{-1} *Ulva* DW.

In order to verify if the second fermentation step improves the ethanol yield compared to a single fermentation step, all ethanol yields after two fermentation steps were compared to a single formation step (**Table S4**). The results show that every additional fermentation increased significantly ($p > 0.05$, one-tailed Student's *t*-test) the ethanol yield compared the single fermentation step with *S. cerevisiae* or *E. coli*, except in the cases of comparing two-step fermentations with same organism to a single fermentation with the similar organism. For instance, the ethanol yield after S-S fermentation was not significantly higher ($p > 0.05$, one-tailed Student's *t*-test) than just fermenting with S (*S. cerevisiae*). The best ethanol yield was produced after the first fermentation with *S. cerevisiae* and the second fermentation with *S. cerevisiae* or with *E. coli.* Comparing the yields after S-S fermentation to the yield, S-E was not significantly different ($p > 0.05$, one-tailed Student's *t*-test). Because of yields after S-E fermentation was significantly higher than the yield of just S it was considered that S-E fermentation is preferable.

Fermentation with *S. cerevisiae* was followed with *E. coli* (S-E) produced after the first step 29.5 ± 3.1 mg ethanol g^{-1} *Ulva* DW (Table 2). At this fermentation, only glucose and fructose were consumed. The conversion ratio to ethanol was 0.3 mg ethanol mg^{-1} glucose. The conversion ratio to ethanol is similar compared to other studies on fermented *Ulva* sp. with *S. cerevisiae* [36]. However, unlike other studies, no yeast extract was added, that might contain additional sugars, to the hydrolysate [74–76]. In the second step after *E. coli* fermentation, an additional 8.8 ± 1.2 mg ethanol g^{-1} *Ulva* DW was achieved. In total, the fermentation S-E yielded 38.3 ± 3.4 mg ethanol g^{-1} *Ulva* DW with average 16.8 ± 1.4% mg ethanol g^{-1} TS. It is reasonable to considering the glucose as the potentially dominant carbon source for ethanol production by *S. cerevisiae* [77,78] and the maximal theoretical ethanol production yield which is 0.51 g ethanol g^{-1} glucose [79]. The yield of conversion from glucose to ethanol was 73.81 ± 6.5% of the maximal theoretical production yield (0.51 g ethanol g^{-1} glucose). The two-step S-E fermentation produced 23% more ethanol than the single-step fermentation with *S. cerevisiae*. An additional 74% of the sugar decomposed during S-E fermentation and about 23.22% of that sugar fermented to ethanol. This experimental finding supports previous results from metabolic flux balance analysis [35], which suggested that a combination of *S. cerevisiae* and *E. coli* would increase the utilization of macroalgae *Ulva* sp. biomass-derived monosaccharides to ethanol. On the other hand, fermentations that started with *E. coli* produced low ethanol yields, probably because of the *E. coli* lacks a base ingredient that prevents it from fermenting and/or just because of it a weaker ethanol producer then *S. cerevisiae* [80,81].

3.5. Two-step fermentation of hydrolysate after SWH for bioethanol production with S. cerevisiae and E. coli

The fermentation by *S. cerevisiae* resulted in 4.7 ± 0.76 mg Ethanol

g^{-1} DW *Ulva* and reduction of total monosaccharides (TS) in the medium from 24 ± 2.84 mg TS g^{-1} DW *Ulva* to 6.6 ± 0.3 mg TS g^{-1} DW *Ulva* (Fig. 3**a,b**). This corresponds to 66 ± 18.7% of the theoretical maximum yield from the given amount of TS, similar to the fraction of the theoretical maximum yield achieved with the hydrolysate produced by the thermochemical and enzymatic process, as described in previous section.

Detailed monosaccharide and sugar acid analysis showed that released glucose, xylose and fructose were mostly fermented by *S. cerevisiae* (at the first fermentation step, $p < 0.05$), Fig. 4**d,e,f**. However, rhamnose, galactose, and GlcA were not metabolized and their concentrations almost did not change during the first fermentation step ($p > 0.05$), see Fig. 4**b,c,g**. In addition, almost all produced 5-HMF was metabolized by *S. cerevisiae* during 48 h of fermentation (Fig. 3**a**, $p < 10^{-4}$). Adaption of 'Ethanol Red' strain used in this study to 5-HMF in media was previously reported by Wallace-Salinas et al. [82].

The free AA that were released after SWH, and were available for fermentation include arginine, isoleucine, leucine and alanine. These are the only free AA that could be successfully detected using the HPIC in the SW hydrolysate. Therefore, only those AA were quantified by comparing them to commercial standards. The concentrations of arginine ($0.17 \pm 6.3 \cdot 10^{-3}$ μg mL^{-1}), isoleucine ($0.09 \pm 3.5 \cdot 10^{-4}$ μg mL^{-1}) and leucine ($0.35 \pm 6.1 \cdot 10^{-3}$ μg mL^{-1}) in the SW hydrolysate did not change significantly after the first fermentation step followed by yeast hydrolysis, see Fig. 5. However, alanine concentration significantly dropped from 1.06 mg ± 0.03 g^{-1} DW *Ulva* at S1TO ($0.1 \pm 5.1 \cdot 10^{-3}$ μg/ mL) to 0.01 ± 0.004 g^{-1} DW *Ulva* ($8.7 \cdot 10^{-4} \pm 3.5 \cdot 10^{-4}$ μg mL^{-1}) at S2T0, see Fig. 5. The second fermentation step by *E. coli* resulted in 0.12 ± 0.04 mg Ethanol g DW $Ulva^{-1}$ and reduction of TS in medium from 6.74 ± 0.21 mg TS g^{-1} DW *Ulva* to 1.99 ± 0.72 mg TS gDW $Ulva^{-1}$ (Fig. 3**a,b**). Different from *S. cerevisiae,* a significant reduction in rhamnose and galactose ($p < 0.05$) concentrations were observed from 48 fermentation by *E. coli* (Fig. 4**b,c**). No significant reduction in GlcA content was observed (Fig. 4**g**). Finally, a relatively low ethanol amount was produced ($p > 0.05$, two-tailed Student's *t*-test) (Fig. 3**c**).The final CFU was $1.1 \cdot 10^4 \pm 8.4 \cdot 10^2$ cells μL^{-1}. Significant cell growth could potentially be used for additional applications where direct biomass production is required. The total ethanol yield from the two-step fermentation process was 4.79 ± 0.77 mg Ethanol per g DW *Ulva*, which is the highest obtained yield per TS, but it is in the lower range of bioethanol yields from *Ulva* as reported previously [36], because of the low saccharification efficiency. However, in this study, the biomass SWE required only seawater without any additional chemicals. At the first fermentation step, the monosaccharides (glucose, xylose, and fructose) were consumed by *S. cerevisiae* and converted by 32.2 ± 1.5%. The theoretical conversion yield of those sugars to ethanol is 0.51 g ethanol/ g sugar [79,83,84]. This means that sugars conversion efficiency was 76.4 ± 3% of the theoretical yield. While at the second fermentation step with *E. coli* additionally to *S. cerevisiae* during the fermentation, also rhamnose and galactose were consumed. However, during the second step only 1.9 ± 0.12% from the sugar leftovers were converted to ethanol.

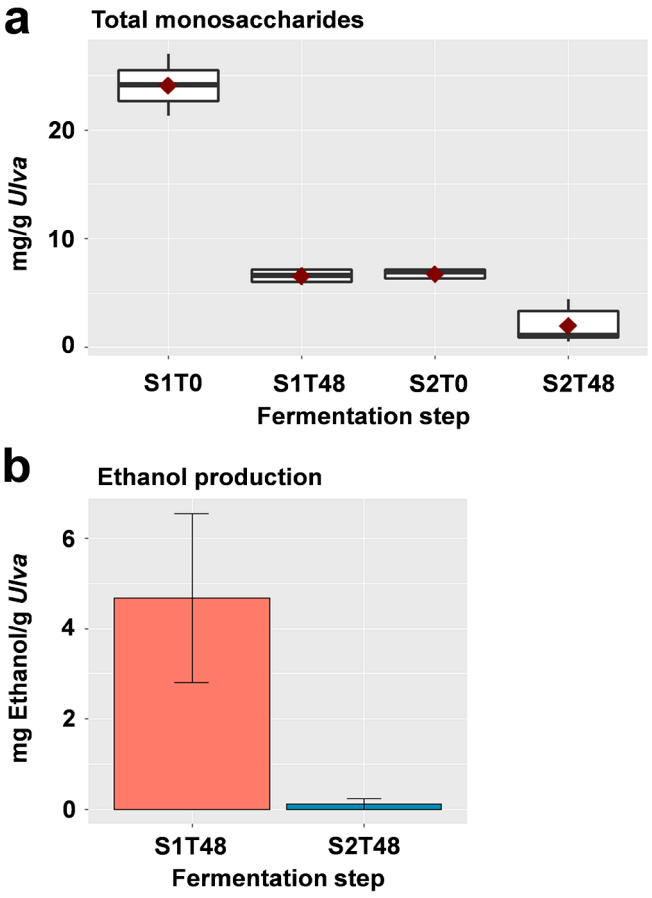

Fig. 3. Total monosaccharides and ethanol yield in SW hydrolysate after every fermentation step. **a**. In axis X the labeling S1 = first fermentation step (with *S. cerevisiae)* and S2 = second fermentation step (with *E. coli*). T0 = initial time before fermentation, or T48 = 48 h after fermentation **b**. Ethanol production yield ethanol production yield (mg ethanol/ g *Ulva*) after each fermentation step.

The glucose to ethanol conversion efficiency in thermochemical with enzymatic hydrolysate was similar to the SW hydrolysate. 20% SW hydrolysate was the favorable cultivation environment for *S. cerevisiae*. According to the efficient conversion of glucose to ethanol in SW hydrolysate, it is reasonable to assume that the carbon source in 20% SW hydrolysate is the major growth-limiting factor. Further study is conceived on modifying the fermenting organisms, to genetically design recombinants with metabolic pathways for consuming all the carbon sources including GlcA [85] and also with improved stress tolerance to fermentation inhibitory conditions [86]. Another approach for improving the process is by detoxifying the SW hydrolysate [68]. In addition, the decomposition of *S. cerevisiae* biomass in the SWH before the second step of the fermentation should be optimized.

3.6. Required area for algae cultivation to meet demand in Israel

The annual mass of algae and the needed area for cultivation in the Israeli EEZ in the Mediterranean Sea are presented in Table 3. Production of char to displace 20% of the coal demand in power plants (2017 data) requires about 3% of the EEZ area. Displacing 10% of the gasoline for transportation with low-yield fermentation following SWH will require 34% of the EEZ, an impractically high requirement that results from the low yield of ethanol from the fermentation of the SW hydrolysate. For production of the annual demand for protein, less than 1% of the EEZ area is needed. If a larger area is used, e.g. 3% of the EEZ to address the char demand, then a large surplus of protein will be produced and it will have to be exported. It is not likely that protein from a single plant species will replace the entire national protein demand, due to the need for variety in dietary intake. But, this calculation shows the potential of algae-based protein production, which is very high compared to other protein extraction methods [20,26]. If the thermochemical and enzymatic process with higher yield is used instead of the SWH process (for ethanol production), the required area is reduced on 4.3% of the EEZ. The ethanol yield in both hydrolysis options was estimated after two-step fermentation. At the first step, the fermentation was with *S. cerevisiae,* at the second step with *E. coli*. All results are for algae growth rate that corresponds to intensive cultivation; using extensive cultivation will increase the area requirements by a factor of 5.8 as explained above.

Previous work has estimated that displacing 10% of Israeli demand

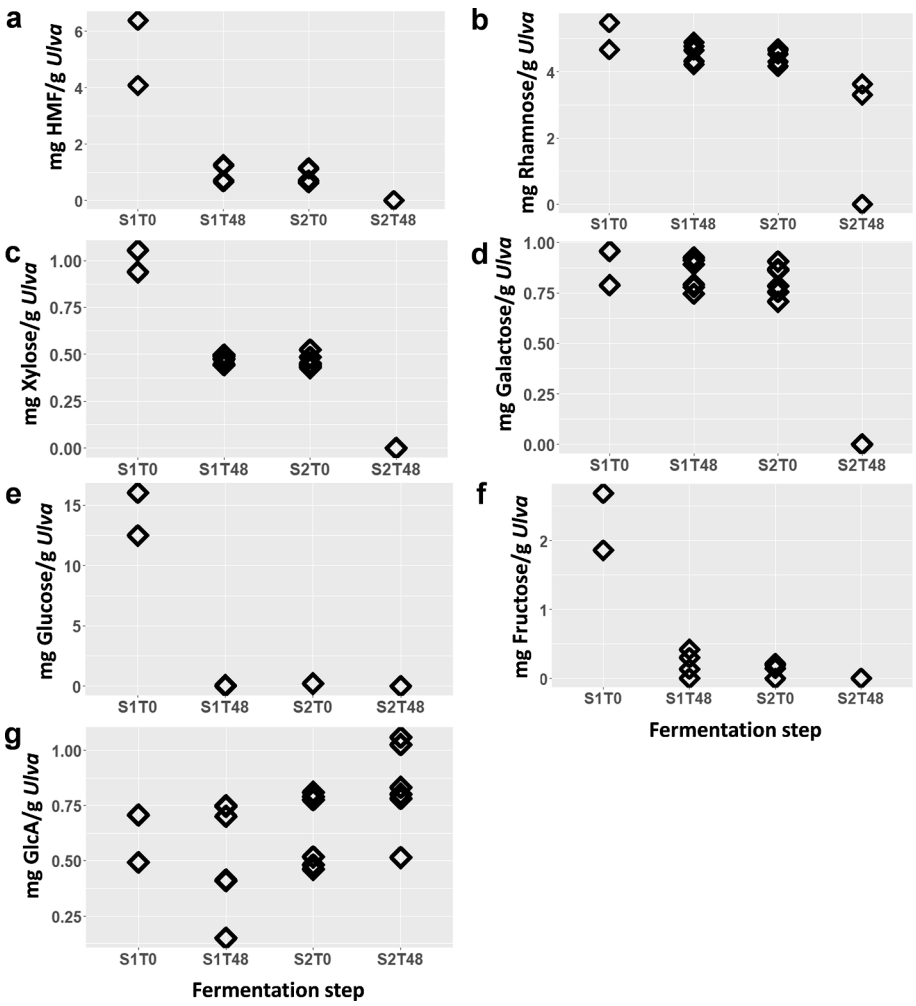

Fig. 4. Detailed TS dynamics in each fermentation step. **a**. 5-HMF, **b-f** monosaccharides, **g**. glucuronic acid. The data is presented by the monomer as mg/g *Ulva* in every fermentation step in time 0 or after 48 h of fermentation. In axis X the labeling S1 = first fermentation step (with *S. cerevisiae)* and S2 = second fermentation step (with *E. coli*). T0 = initial time before fermentation, or T48 = 48 h after fermentation.

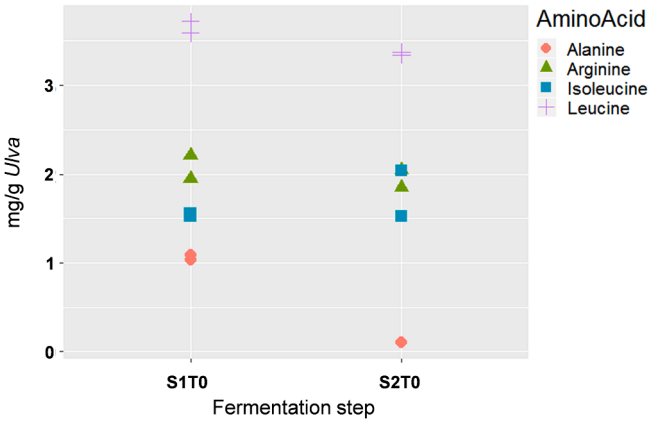

Fig. 5. AA profile at the SW hydrolysate before each step of fermentation. AA are expressed as mg g^{-1} *Ulva* DW. S1T0 = the initial time before fermentation of the SWH hydrolysate of *Ulva* sp. by *S. cerevisiae*. S2T0 = the initial content before fermentation with *E. coli* of the residue after the first step of fermentation of the SWH of *Ulva* sp.

for transportation fuel by *Ulva* based ethanol should require less than 300 km^2 using intensified cultivation [48]. This was based, however, on a higher algae annual growth rate (16 kg m^{-2}, achieved with intensification under laboratory conditions), and a higher ethanol yield (0.1 kg kg^{-1} DW) [48]. The current results reporting higher required *Ulva* cultivation area for ethanol production should be more realistic.

Since all three products are co-produced simultaneously, a selection of a specific cultivation area will create a set of different contributions towards the national demand. For example, if an area of 1000 km^2 is chosen, the corresponding char production will displace 24% of the coal demand instead of the 20% defined above. The ethanol production will displace only 1.1% of the gasoline demand. The protein production will be higher than the national demand by a factor of 4.8, possibly allowing export if the economics are suitable for this product. The reference process of ethanol production with thermochemical with enzymatic process from the same cultivation area will displace 9% of the national gasoline demand but without the co-products of char and protein. Allocating the large-scale area required for a significant contribution to the national energy balance (Table 3) is challenging. There are some technological developments for offshore multidimensional seaweed farming [87], which could produce much more seaweed mass per unit area compared to costal cultivation in shallow water (the basis for calculation in the current study). Unfortunately, those technologies not yet available for large-scale seaweed production. It is interesting to note that China, now producing about two thirds of the global seaweed production [88], uses coastal seaweed farming with low AGR of 1604 t DW km^{-2}, occupying about 1250 km^2 of the coastal area [88] (approximately 0.3% of the Chinese territorial water [88]. Such a

Table 3
Required annual mass of algae and sea area for cultivation in order to provide the hypothetical annual demand in Israel according to 2017 data for char [49] (20% blend in coal power plants), ethanol (E10 blend in gasoline cars) [50], and plant-based protein [51,52] using SWH process, and for ethanol only with thermochemical and enzymatic treatment. The area requirement was calculated based on intensive cultivation *Ulva* biomass (*AGR* = 12.3).

Product	**Yield** (kg kg^{-1}DW)	Annual mass of product (kt a^{-1})	Annual mass of algae (kt a^{-1})	Area intense cultivation (km^2)	% of EEZ
Hydrochar (SWH)	0.211	2139	10 1138	824	3.1
Protein (SWH)	0.058	149	2 570	209	0.8
Ethanol (SWH)	0.0048	526	10 970	8 920	34.3
Ethanol (thermochemical + enzymatic)	0.0383	526	1 370	1 115	4.3

solution is clearly not acceptable for Israel that has a relatively short coastline and its EEZ has many additional uses such as shipping, fishing, natural gas fields etc.

3.7. The revenue from Ulva biomass co-products

In order to illustrate the economic viability of this conceptual biorefinery assessment of the potential revenues for all products from *Ulva* sp. biomass with three alternative treatments: SWH, SWH with one-step fermentation (since the second step was shown to be ineffective as shown in Fig. 3**b**), or thermochemical and enzymatic treatment with two-step fermentation. It is important to mention that calculating revenues is only one side of the economic issue: expenses are different for each scenario, so the complete economic competitiveness is not defined only by the current results, and requires further study. Fig. 6 **and Table S5** show the yields of the different products as presented here, together with a range of yields and market prices for each product as found in the literature, and the contribution of each product to the TR of the biorefinery. The total revenue (TR) for all products from *Ulva* sp. biomass treated by SWH without fermentation (i.e., sugars are considered as end products) is \$0.66 kg^{-1} DW *Ulva*, based on the yields obtained in this study. When considering the range of yields and product prices found in the literature (**Table S5**), the TR can vary over a wide range between \$0.26 and \$1.06 kg^{-1} DW *Ulva* (Fig. 6 **and Table S5**). The TR from *Ulva* sp. biomass treated by SWH with one-step fermentation is \$0.56 kg^{-1} DW *Ulva* according to this study, and varies between \$0.23 and \$0.88 kg^{-1} DW *Ulva* for the range found in the literature. The addition of the fermentation step is then not beneficial in terms of

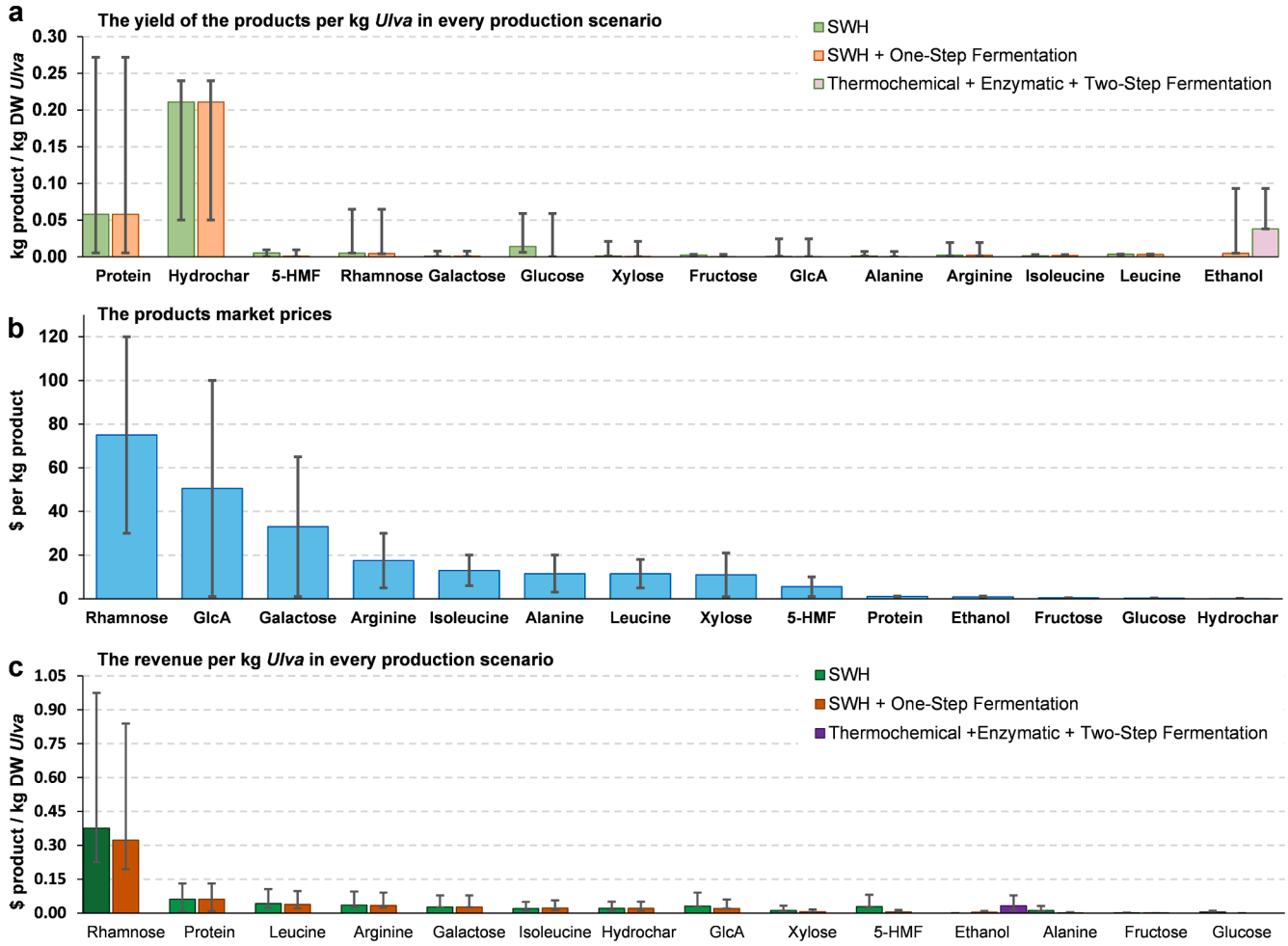

Fig. 6. All products yield kg^{-1} *Ulva* in every production scenario. **a**. The production scenario are: SWH, SWH following single fermentation step with *S. cerevisiae*, and two-step formation (*S. cerevisiae* then *E. coli*) in thermochemical following enzymatic hydrolysate. **b**. The products market prices, USD kg^{-1} product. From left to right, is the order of the products are from the most expensive to most cheap. **c**. Every product revenue, USD product per kg product produced from *Ulva* in every production scenario. The similar production scenarios as in 'a'. From left to right, is the order of the highest to the lowest revenue, based on the scenario of SWH following a single fermentation step.

revenue: the conversion of monosaccharides (high-value products) to ethanol (a relatively cheap product) leads to a reduction in TR. Inclusion of the fermentation process will also involve additional expenses that may lead to a disadvantage on the cost side (which is not analyzed here). However, producing ethanol could be considered as a national strategic priority for energy independence, and this may influence the economic considerations. The reference process of thermochemical and enzymatic treatment (Fig. 6**c**) produces only one product (ethanol), and its revenue was 0.03$ kg^{-1} *Ulva* for the current results, and between 0.02 and 0.04 $ kg^{-1} *Ulva* based on the literature (**Table S5**). Even though the thermochemical and enzymatic process yields significantly more ethanol than the fermentation of SWH hydrolysate, the TR of all products after SWH is much higher because of the multiple products, some of which have much higher prices compared to ethanol.

Many of the biorefinery products have a very small contribution to the TR, as shown in Fig. 6**c**. It would then make sense to save the expenses on separation and purification processes for these low-revenue products, with only a modest reduction in TR. For example, the four leading products derived from SWH without fermentation (rhamnose, protein, leucine, and arginine), produce a TR of $0.49 kg^{-1} DW *Ulva*, which is 73% of the total TR of all products. The same four leading products of the SWH with one-step fermentation produce a TR of $0.43 kg^{-1} DW *Ulva*, which is 78.4% from the TR of all products. It is interesting to note that hydrochar is the leading product in terms of mass produced in both cases (Fig. 6**a and** Fig. 7), but it is lagging far behind in terms of contribution to TR. However, it is likely that the hydrochar will be used in any case due to the simplicity of separating the solid phase from the rest of the products.

The separation feasibility of products that are produced by SWH might be critical for the realization the commercial-scale production. Separating the hydrochar in the solid phase from the liquid phase containing all other products is a relatively an easy process using the difference in density, either by natural settling or by centrifugation (as described in section 2.5). In order to separate the total protein, separation with an industrially relevant method could be done, such as: using alkaline solution treatment, neutralizing, and finally purifying with dialysis [89]. The separation of all monosaccharides from the liquid phase may use ion exchange technologies [90]. This type of technology is already used on an industrial scale for extracting monosaccharides from hydrolyzed cellulosic biomass [91]. AA may also be separated with appropriate ion-exchange based technology [92]. The commercial-scale of ethanol separation after fermentation is commonly used with a series of distillation columns [93]. There are different methods to separate GlcA for analytical purposes for quantification and identification using ion-exchange chromatography [94] including the ion chromatography method that has been used in the current research (as described in section 2.10). However, the GlcA separation is not yet available on a commercial scale. The low yield of HMF achieved in the current study is not suitable for separation with known methods such as a catalyst and organic solvents [95]. However, it might be reasonable to convert monosaccharides after purification (especially fructose) to HMF using a suitable catalyst. It is important to mention that we calculated the TR assuming a prefect separation. Obviously, in reality there are always losses in the process, and the final product amounts after the separation are specific to the production design and to the separation methods. These losses related to separation need to be further estimated. Currently, large-scale demand exists for hydrochar [48,49], glucose [96], fructose [95,96], ethanol [39], protein and 5-HMF [95]. The demand for the other products is relevant for applications in smaller size market, usually sold in smaller amounts and at higher purity (additional information are in references cited in table S5). Those products with a niche market are more expensive, but, so far, their demand is much lower. Among those products such as GlcA, galactose and rhamnose, which are the building blocks of *Ulva* polysaccharide called Ulvan. The SW hydrolysis process opens a new path for production of these niche products, since so far the only industrial-scale option to hydrolyze the unique *Ulva* polysaccharides (for instance Ulvan) into the mentioned monomers is the thermochemical method with a catalyst [37]. SWH, on the other hand, does not require the addition of a chemical catalyst. The algae export prices depend on different countries. For instance, the export prices of red seaweeds for producing carrageenan in Indonesia, Philippines and Tanzanian in 2011 ranged between ~0.3 and ~2 USD per DW kg^{-1} [97]. Only the low end of this range may be relevant for a biorefinery where the total revenue is around 0.6 USD per DW kg^{-1} as described above, and cost reduction of the raw biomass is required. This may be done either with economies of scale, or by increasing yield with advanced intensive cultivation methods. In the study mentioned above, the price of biomass was acceptable because the carrageenan product price was about 8 USD kg^{-1} algae DW. An additional comparison can be done vs. *Ulva ohnoi* biomass revenue, based on the separation of the *Ulva* into different fractions [89]. The revenue was fluctuating between 1.56 and 3.93 USD kg^{-1} (DW), the estimated revenue was 2.21 USD kg^{-1}. While the most contributing fraction to the revenue was the Ulvan, which is relatively expensive and considered as a niche market [89]. In the current study, the total revenue was lower than these two cases, but the SWH addresses large-scale markets with high national demand such as hydrochar and protein, rather than only niche products. At the same time, SWH enables also production of exclusive high-value monomers for niche markets or for fermentation, in addition to the large-scale products. However, some limitations have to be noted. Using SWH is challenging for control precisely the operational conditions for selective production of desired products. The additional challenge is to separate

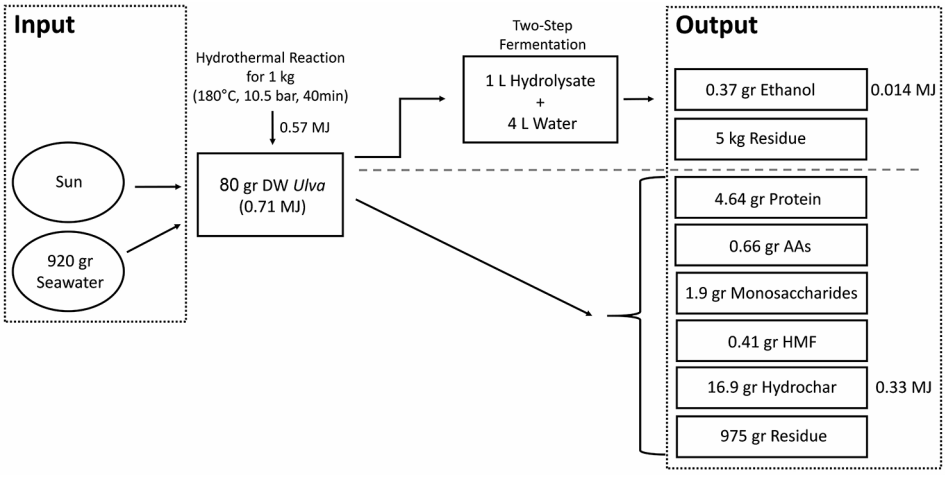

Fig. 7. Mass and energy balance analysis of the *Ulva* sp. biomass SWH process. The gray dashed line is separating two potential processes directions. Above the dashed line is ethanol production via fermentation and the phytochemicals consumption. Below the dashed line, the phytochemicals without the fermentation process. The energy-required processes such: algae cultivation, harvesting, fermentation, and separation of chemicals, have to be further investigated and properly estimated. The heat in the process can be recycled and solar energy can be used as an energy source for the process.

and purify the value-added products in an economic manner. This challenge even bigger in the case of simultaneous production. The presented revenues based on the cost of the different products are a contribution to the assessment of economic feasibility. However, a full techno-economic study is needed with an account of the expected revenue vs. process costs including separation into the final products. This analysis will allow realizing the exact economic feasibility and the processes scalability, based on the data in the current work.

4. Conclusions

SWH of *Ulva* sp. biomass in HTC process and formation of 21.1 ± 0.7% hydrochar on dry basis. The HHV of the hydrochar was almost double than in untreated algae. Usually, the energy densification in HTC processing requires longer time at higher temperatures than was done in the current study. Moreover, SWH resulted in very efficient protein extraction yield compared to other methods that were used in previous studies on protein extraction from *Ulva*. Optimal two-step fermentation was done in *Ulva* sp. hydrolysate produced with the thermochemical following enzymatic method. Which included *S. cerevisiae* following *E. coli* fermentation, led for a significant increase of the ethanol yield in the second step. However, applying the same microbial fermentation sequence in SW hydrolysate from *Ulva*, the second fermentation step did not contribute significantly to increase the ethanol yield, and can be eliminated.

The needed area for cultivating *Ulva* biomass in the Israeli EEZ of the Mediterranean offshore territories was assessed based on the products derived after SWH or thermochemical following enzymatic treatment. The algae cultivation area to produce char with the SWH process, sufficient for replacing 20% of national coal demand, requires 3.1% of the EEZ. In protein production with SWH, the area required to satisfy the national demand is less than 1% of the EEZ. The area required for ethanol production to provide 10% of the transportation fuel is over 30% of the EEZ. The multiple product production with SW hydrolysate led to higher potential revenue (0.66 $) of the products per kg of algae, compared to the fermented SW hydrolysate and thermochemical following enzymatic processes, which led to an increase in ethanol production but reduced the TR.

SWH treatment is relatively quick and cheap [98], does not require hazardous materials and might be efficiently integrated into potential marine biorefinery based on *Ulva* biomass. Thus, using SW treatment for seaweed biomass can accelerate achieving the biorefinery approaches for simultaneous multiple products production. In the Israeli exclusive economic zone in the Mediterranean Sea a platform for *Ulva* cultivation can be conceived in order to provide the national demand for plant-based protein, replacing part of the coal for electricity and blending ethanol for transportation fuel. The findings described in this study can contribute to the design of biorefinery processes. Producing multiple products is essential for energy, cost and environmental efficiency of biorefineries [54].

CRediT authorship contribution statement

Mark Polikovsky: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Amichai Gillis:** Formal analysis. **Efraim Steinbruch:** Formal analysis. **Arthur Robin:** Methodology. **Michael Epstein:** Investigation, Validation, Data curation. **Abraham Kribus:** Investigation, Validation. **Alexander Golberg:** Conceptualization, Investigation, Validation, Visualization, Writing - original draft, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enconman.2020.113380.

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