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Ulva-bacteria interactions and biomass processing for the production of new energy and food sources

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Preface

The thesis is composed of the following scientific papers:

1. **Polikovsky M**, Califano G, Dunger N, Wichard T, and Golberg A, 2020. Engineering bacteria-seaweed symbioses for modulating the photosynthate content of *Ulva* (Chlorophyta): Significant for the feedstock of bioethanol production. *Algal Research*. 49, 10945.
2. **Polikovsky M**, Fernand F, Sack M, Frey W, Müller G, and Golberg A, 2016. Towards Marine Biorefineries: Energy Efficient Proteins Extractions from Marine Macroalgae *Ulva lactuca* with Pulsed Electric Fields. *Innovative Food Science and Emerging Technologies*. 37, 194-200.
3. **Polikovsky M**, Fernand F, Sack M, Frey W, Müller G, and Golberg A, 2019. *In silico* food allergenic risk evaluation of proteins extracted from macroalgae *Ulva* sp. with pulsed electric fields. *Food chemistry*. (276): 735-744.

Additional publications:

During the current thesis, addition papers were published:

1. Vitkin E., Gillis A., **Polikovsky M**, Bender B, Golberg A, and Yakhin Z, 2020. Distributed flux balance analysis simulations of serial two-organism biomass fermentation, *PLos ONE*. 15 (1).
2. Zollmann M, Robin R, Prabhu M, **Polikovsky M**, Gillis A, Greiserman S, and Golberg A. 2019. Green Technology in Green Macroalgae Biorefinery. *Phycologia*. 58(5): 516-534.
3. Negev E., Yezioro A, **Polikovsky M**, Kribus A, Cory J, Shashua-Bar L, Golberg A, 2019. Algae Window for reducing energy consumption of building structures in the Mediterranean city of Tel-Aviv, Israel. *Energy and Buildings*. 204:109460.
4. Ingle K., **Polikovsky M**, Chemodanov A, and Golberg A, 2018. Marine integrated pest management (MIPM) approach for sustainable seagriculture. *Algal Research*. (29): 223-232.
5. **Polikovsky M**, Sharon A, and Golberg A, 2018. Enhancing energy literacy in children using zn/cu/potato batteries. *F1000Reaserch.* f1000research.com/articles/7-24.

Abstract

The rising requirements for the food, animal feed, and energy sources are major global challenges for the coming decades. Such growing demands require new biomass feedstocks. So far, the most dominant biomass sources come from crops that are cultivated in terrestrial agriculture. The challenges associated with terrestrial agriculture such as limited availability of arable land and potable water, the environmental hazards, and the lack of energy-efficient processing technologies question whether terrestrial crops can truly provide the growing demands for such biomass.

Seawater macroalgae are considered as a perfect alternative feedstock. Macroalgae cultivation does not require arable land or potable water. Among macroalgae, *Ulva* species are particularity interesting due to their rapid growth rates, adaptability to diverse climatic conditions, and high carbohydrate and protein content. In addition, *Ulva* sp. is already known as a relatively well-studied model organism.

Controlling the algal growth rates and chemical composition are the major challenges for efficient *Ulva* biomass feedstock for producing food and energy. Utilizing *Ulva* biomass as a new protein source for food, involves two more challenges: efficient extraction and of allergen risk management. These challenges motivated my research that was published in the three papers that are described in this thesis.

The first paper in the thesis shows how two bacteria associated with *U. mutabilis* modulate the algal growth rate and its chemical composition. *U. mutabilis* that was grown in an engineered consortium (tripartite community) was different in sugar, protein, and other chemical content from an axenic (bacteria-free) culture of that alga. Afterward, the ethanol yields of the axenic and the tripartite community biomasses were estimated using the flux balance analysis model ('BioLego'). This metabolic model uses the fermentative organisms, such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *Clostridium acetobutylicum*, in one-step or two-step fermentation processes. The analyses of the modeling results showed that the algae biomass cultivated with bacteria is a significantly better feedstock for bioethanol production than the axenic biomass.

Following the challenge of efficient protein extraction, the second paper in this thesis aimed to develop a new protein extraction method. That work focused on protocol development of a process for protein extraction from *Ulva* sp. biomass, using new pulsed-electric

field (PEF) processing technology. Importantly, PEF technology facilitates an eco-friendly, non-thermal, and chemical-free extraction process. The results of the second paper showed that electroporation with PEF leads to selective protein extraction.

Following protein extraction, the next step for creating a “new protein” for food, requires risk evaluation, including the potential for allergenic properties. This evaluation must take into account the specific protein extraction method. Thus, the third paper of this thesis evaluated *in-silico* allergenicity risk of the *Ulva* sp. proteins extracted with PEF. The extracted proteins were identified and then annotated using comparisons to known allergens. A control for total protein extraction was done with a thermochemical extraction following enzymatic treatment, which is a common protocol for protein extraction for proteomic analyses. Finally, a list of proteins extracted with PEF and the control were compared. According to this comparison, PEF treatment did not lead to the extraction of peptides that are known food allergens. Based on these experiments, we conclude that PEF treatment leads to selective allergens extraction.

This thesis investigated interactions of *Ulva* and its associated bacteria, protein extraction from this alga and allergenicity assessment of the extracted proteins. These topics are of high priority for establishing a large-scale biorefinery based on *Ulva* sp. biomass as a feedstock for new energy and food sources.

Contents

1. Introduction

1.1 The global demands for food and energy

The global population in 2050 is expected to reach nearly 10 billion [1]. In order to meet the rising demand and feed the whole population in the mid-century, food production must increase more than 50 percent [1], while the animal-based food demand needs to increase by nearly 70 percent [1]. The population growth is also a challenge for world energy resources, with associated concerns for negative environmental impacts [2].

The main current biomass feedstocks for food, animal feed, and renewable biofuels rely on classic terrestrial agriculture [3,4]. However, intensive terrestrial plant agriculture is problematic. It causes soil erosion and requires the usage of insecticides, herbicides, and nitrogen fertilizers [4]. Moreover, the requirements of this type of agriculture contribute to water pollution and air pollution, which lead to natural environment degradation. Another problem with the terrestrial plant agriculture is its dependency on fossil-fuel-based energy [5,6], which increases the cost of food and diverts human food resources to costly and inefficient energy production [3,4,6]. With these challenges, it is imperative to search for new biomass sources.

1.2 Macroalgae as a potential source for food and energy

Macroalgae, or seaweed, are aquatic organisms with a multicellular "plant-like" structure [7] and have high distribution diversity [8]. The macroalgae naturally grow in freshwater or seawater. Currently, about 11,000 different seawater macroalgae species are known, divided into three types: green (> 1800 species), brown (~2000 species), and red (> 7200 species) [9]. Yet, among this diversity, less than 20 species of only 11 genera, are commercialized [10,11].

The macroalgae industrial potential is represented by the rapid increase of cultivation and its emerging global market sizes. In the last 50 years, the global macroalgae cultivation has been exponentially expanding [12]. The global macroalgae production (in cultivation) in 1990 was estimated at 3.7 million tons (Mt) [13], while in 2014 it was increased to almost 27 Mt [14]. However, macroalgae contributed only 0.3% of the total world food production in 2012 [15]. Taking into account the next decades' predictions for growing food demand

[16], with the possibility that the investigated macroalgae-based applications [17–20] will be successfully industrialized for bioenergy and animal feed (also materials, chemicals, and other commodities) can easily explain the prediction of an emerging macroalgae global market. In 2017, the global seaweed market size was valued at about 4 billion USD (United States dollar). By 2024, it is expected to reach about 9 billion USD [21].

1.2.1 *Ulva sp.* as biomass feedstock

The green macroalgae *Ulva* sp. belong to the branch of Chlorophyceae plays an important role in marine coastal ecosystems as primary producers [22]. As of today macroalgae *Ulva* sp. is not yet massively industrialized and has a niche market size [11,23]. Even so, it has great potential to become a sustainable feedstock source for diverse applications such as food and energy [24–26].

Ulva sp. biomass rapid growth rates make this biomass attractive for industry. For example, the growth rates of *Ulva* sp. biomass in optimized cultivation conditions might reach up to 45 tons of dry weight (DW) hectare $^{-1}$ year $^{-1}$ [27]. An additional example of *Ulva* sp. biomass growth rates that was cultivated in the sea-coastal area (Tel Aviv, Israel), reported about 20 tons (DW) hectare $^{-1}$ year $^{-1}$ *Ulva* sp. of biomass [28].

Adaptability of the *Ulva* sp. biomass to diverse climatic conditions makes this biomass relevant for large cultivation locations [29–31]. Additionally, *Ulva* spp. are cosmopolitan macroalgae [32], allowing cultivation without ecological risk as invasive species. In general, most of the green, red and brown algae are rich in carbohydrates (1%–55 % of DW) and in proteins (6%–33% of DW) [19,33–40], Table 1. Specifically *Ulva* spp, carbohydrate content (25-40% of DW) [34–38] and significant protein content (7-33% of DW) [38,41–43]. *Ulva*'s composition is an additional factor makes it to be promising biomass feedstock [27,34,44,45]. In addition to the beneficial properties of *Ulva* sp. biomass to serve as a feedstock, the scientific advantage of choosing *Ulva* sp. for the study is that *Ulva* sp. is already being studied and used as a model organism [46], and its genome has been sequenced and published [47].

Content per dry weight	Green (Chlorophyta)	Red (Rhodophyta)	Brown (Phaeophyceae)	References
Carbohydrates (%)	1-40	9-52	0.9-55	[33–38]
Protein (%)	7-33	10-20	6-12	[19,33,39]
Lipid (%)	0.5-7.2	0.28-6	0.7-20	[40]
Ash (%)	20-36	23-41	11-39	[33]

Table 1- The chemical composition of algae types.

1.2.2 The *Ulva* sp. sugar content and composition.

The major sugar content in *Ulva* sp. biomass is composed of polysaccharides such as ulvans, cellulose, and starch [19,48]. The water-soluble polysaccharide ulvan, also named *ulvacin* or *ulvin*, is cell-wall sulfated polysaccharide. It is located between two cell layers in the intercellular area of *Ulva* sp. thallus [49,50]. In *Ulva* sp. the cellulose is a cell-wall polysaccharide, with the structural and insoluble in water properties [51]. It is mainly composed of glucose, but it could be co-extracted with other monosaccharide residues [52]. The starch is the intercellular storage polysaccharide, surrounded in pyrenoids and granules at the chloroplast [48].

Typically the polysaccharide content of the biomass is about 8–29% ulvan [51,53], 1-15% cellulose [52,54] and 1.6-32% [48,55] starch. The cellulose and starch basic monosaccharide building block is mainly glucose [48,52]. The ulvan is composed mainly of rhamnose (16.8–45.0%), sulfate (16.0–23.2%), glucuronic acid (6.5–19.0%), xylose (2.1–12.0%), and iduronic acid (1.1−9.1%) [51]. Minor residues of galactose, glucose, and mannose have been reported in a co-extraction with *Ulva* water-soluble cell wall sulfated polysaccharides, and were reported as part of ulvan [49,56]. The monomers have appeared mostly in disaccharide-repeating sequences [51]. Lahaye and Robic (2007) have previously described the repeating structures of ulvan in different *Ulva* species [51].

Ulvan has not yet been commercialized on an industrial scale. So far, it has been investigated as relevant for different pharmaceutical applications and has been found to have potential uses as an anticoagulant, and to have immunostimulatory and anticancer functions, as well as being antihyperlipidemic [50,57–61]. Additional information about ulvan applications appears in Polikovsky M and Alexander G. 2019 [24].

Polysaccharides' basic building blocks, the monosaccharides of *Ulva* sp., can be used for fermentation with different microorganisms [27,62,63]. During the fermentation, monosaccharides are transformed into different chemicals such as bioethanol. Microbial fermentative feasibility depends on natural or synthetic metabolic pathways available in the fermentative microbes used [62,64].

1.2.3 The *Ulva sp.* protein content and composition

The most critical component for the human and animal global food value is the protein [65]. Due to the increasing global food demand, the relevance to investigate new protein feedstocks has increased [66]. Following the high potential of *Ulva* sp. to be a new feedstock for novel protein (as mentioned above in section 1.2.1), to study protein production from the *Ulva* sp. biomass and to understand its properties has become significant. The *Ulva* sp. proteins could be consumed from the whole organism itself [67], or the proteins could be extracted and concentrated, and the crude protein extract could later be added to different food or feed products [68,69]. Generally, the quantity and quality of *Ulva* sp. protein is considered to be similar to soy or animal proteins [70]. Recent evidence showed that simulation of gastro-intestinal digestibility of *Ulva* sp. protein via proteolysis yielded 89.4 ± 2.6% of the concentrated proteins [68]. Also, the *Ulva* sp. extracted proteins have a high antioxidant activity, and this characteristic might be influenced by associated phenolic compounds [68]. Among *Ulva lactuca* hydrolyzed and fractionated proteins bioactive peptides were identified. This bioactivity was found to be relevant for lowering human blood pressure [71].

Among nine essential amino acids (AAs) and eleven AAs recommended for daily consumption for human adults [72], *Ulva* sp. protein usually contains all AAs, but sometimes lacks the essential AA tryptophan [68,73]. It is important to mention that the protein characteristic in the *Ulva* sp. biomass depends on different biotic and abiotic parameters. The protein content, composition, properties, and its AAs profile, are different between *Ulva* species [70,73]. Moreover, the AAs characteristics are affected by abiotic cultivation conditions, such as nutrient availability [74,75], stress conditions [76–78], salinity level [78] and other parameters [78,79].

1.2.4 Algae associated bacteria; a potential effect on algae composition

Naturally, macroalgae host a wide range of microbial diversity such as Protista, fungi, microalgae, viruses, and bacteria [80]. Macroalgae influence species-specific microbiota in the water column [81]. The physical and biochemical properties of macroalgae are influenced by the complex interactions with microbial epiphytes [82–84]. These interactions can affect the macroalgae lifestyle either positively or negatively [85]. Among microbial epiphytic communities of macroalgae (especially *Ulvacean*), bacteria are the largest and best studied community [86]. Macroalgae provide perfect conditions for bacterial habitat by supplying a solid substrate for growth, nutrients, oxygen, and a carbon source [85]. Some bacteria may cause macroalgae diseases by producing cell-wall digestive toxins, enzymes, and developmental inhibitors [85]. However, most of the macroalgae associated bacteria are beneficial, or harmless. They play a critical role in macroalgae health by producing protective chemicals such as antisettlement, antibiotics, and antiprotozoal agents [87]. Additionally, macroalgae associated bacteria produce essential beneficial chemicals for macroalgae growth, development, and reproduction by supplying growth hormones (e.g., indole-3-acetic acid)[88], developmental and morphogenic compounds [89], spore settlement inducers, and accumulating algal spores [83]. For instance, a macroalgae-associated bacterium, *Bacillus licheniformis*, produces morphogenic compounds that are essential for *U. fasciata* development and for increasing the zoospore productivity [90]. An additional example of how bacteria shape the macroalgal fitness is *Cytophaga* sp., which produces "thallusin" that is a fundamental component for restoring the morphology of axenic ulvalean macroalgae [91]. Moreover, it was found that the presence of two *U. mutabilis*–associated bacteria, *Roseovarius* sp. strain MS2 and *Maribacter* sp. strain MS6, in the algal culture medium affects the metabolic content and the chemosphere composition [92]. In axenic (bacteria-free) *U. mutabilis* Føyn culture the cell walls developed abnormally, while the addition of some specific bacteria (as well with MS2+MS6 bacteria) to the algae culture recovered the normal cell wall development [93]. This observation indicates that the associated bacteria play a role in macroalgae development, including cell wall development. Usually, the main polysaccharide content is located in the algal cell wall. There is a paucity of information about how macroalgae chemical content and composition are influenced by bacteria. In addition, up to the current thesis, there was no available information about how associated bacteria affects these polysaccharides.

1.3 Biorefinery based on marine macroalgae

The biorefinery process includes cultivating biomass as raw material and converting it to different products simultaneously [24]. Those products might replace oil-based products such as fuels, chemicals, and bioplastic [94]. Biorefinery for biofuel production was initially developed based on terrestrial plant feedstocks as raw materials. Terrestrial plants are used as food, animal feed, and a biomass feedstock for biofuels [3,4]. Getting a significant amount of terrestrial plant biomass for biorefinery encourages biomass production via industrial agriculture [95]. This type of agriculture, increases soil erosion, and requires the usage of insecticides, herbicides, and nitrogen fertilizers. All of these lead to increasing negative environmental impact [4]. In addition, using this agricultural type for products other than food leads to competition on food-growing areas. In return, this could increase the cost of food and divert human food resources to costly and inefficient energy production [3,4,6]. An additional major issue with terrestrial biomass usage for biorefinery is the lignin content, which makes the treatment of the biomass difficult [96,97]. Alternatively, macroalgae are promising biomass feedstock. Macroalgae are versatile biomass, useful as a renewable biomass feedstock for human food, animal feed, and fuels; and do not compete with food crops for arable land or potable water [29]. The attractiveness of macroalgae as a feedstock source might be explained by its relatively easy harvesting procedure (compared to microalgae) [98,99], which could be done manually or mechanically [100]. An additional advantage in macroalgae biomass is a low level of lignin (or not at all), allowing much easier processing, compared to terrestrial plants [101,102].

Even with all the advantages of macroalgae biomass, it was only recently that macroalgae fell under the research radar as additional candidates for a future sustainable feedstock source for food, animal feed, and energy [27,103–105]. The macroalgae biorefinery concept is illustrated in **Fig. 1**.

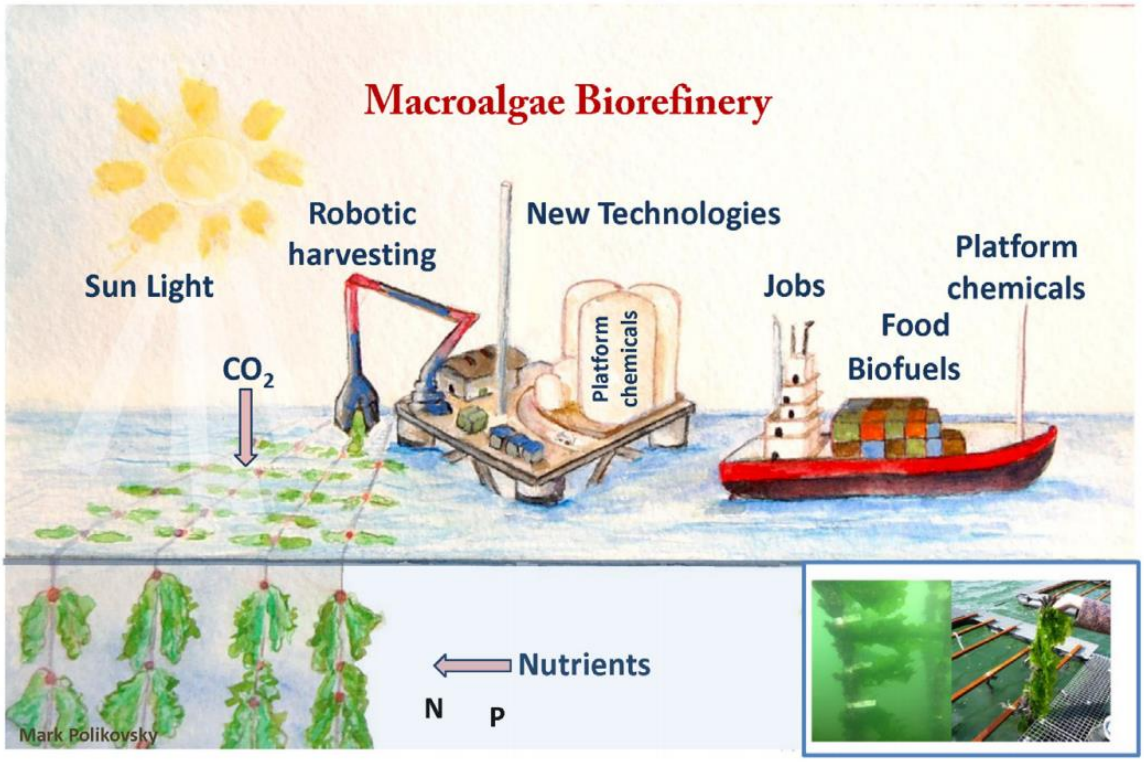

Figure 1 – Illustration of a macroalgae biorefinery [1]. Macroalgae cultivation offshore requires harvesting facilities [36,106]. After harvesting the macroalgae biomass is then shipped to a biorefinery for a conversion of the biomass into feed for animals, food, and biofuels [107–109]. The figure is adopted from the paper of Lehahn Y. 2016 (illustrated by Mark Polikovsky) [29].

1.3.1 Biorefinery based on *Ulva* sp. biomass

So far, the *Ulva* sp.–based biorefinery has not yet become industrialized, though *Ulva* sp. has a rapid growth rate [27], a high carbohydrate [34–38] and high protein content [38,41–43], and additional advantages mentioned above, which make it an attractive biomass feedstock for the marine biorefinery [27,29,110].

For the simultaneous production of different refined products from *Ulva* sp. there are different process design steps that have already been developed and potentially others could be developed in the future. A process design exemplification showed that after the *Ulva lactuca* biomass is treated with aqueous treatment at 150°C, and then enzymatically hydrolyzed, the protein fraction extracted is relevant as feed for animals, and the remaining

[1] "Reprinted from Publication Algal Research, Yoav Lehahn Kapilkumar Nivrutti Ingle Alexander Golberg. *Global potential of offshore and shallow waters macroalgal biorefineries to provide for food, chemicals and energy: feasibility and sustainability*. Vol. 17, Pages 150-160, 2016, with permission from Elsevier. License Number 4862390308176, License date Jul 05, 2020.

hydrolysate contains monosaccharides, which could be fermented anaerobically into acetone, 1,2-propanediol, ethanol, and butanol [111]. An alternative process design showed simultaneous production of salts, starch, lipids, ulvan, proteins, and cellulose from *Ulva ohnoi* biomass with a sustainable fractionation process [112]. However, there are additional process designs that were suggested in the literature, for biorefinery based on *Ulva* sp. [19,48,53,113,114]

Regarding the scalability of *Ulva*-based biorefinery, one of the major challenges is the large-scale biomass cultivation. Thus, a mathematical model was developed for estimating the global *Ulva* sp. biomass cultivation potential for biorefinery, in potential cultivation in shallow-water and offshore [29]. With a given theoretical cultivation density of 4 kg m^{-2} and surface cultivation of ~10^8 km^2, the potential productivity was estimated at 10^{11} ton (DW) $year^{-1}$ of *Ulva* sp. biomass. Considering the near future available technologies for water installation in the depth of 100 m, and a distance from the shore of 400 km, there is potential for 10^9 tons (DW) $year^{-1}$ of *Ulva* sp. biomass. Using this biomass in a biorefinery can lead to simultaneous production of ethanol, butanol, acetone, and proteins. The 10^9 tons (DW) $year^{-1}$ of *Ulva* sp. biomass can potentially replace 20% of the fossil fuel demand for the transportation sector. The same biomass can cover 5–24% of the predicted global plant protein requirement for 2054, and it could supply the entire global demand for butanol and acetone [29].

1.4 Protein extraction from macroalgae biomass

With the polysaccharide content being higher than the protein in *Ulva* sp. biomass and other macroalgae, the protein fraction was considered as a by-product after the extraction of polysaccharides [24,115]. However, the recently understood insight that novel protein is necessary motivated recent studies to find the optimal protocol for protein extraction [68,116]. There are different protocols for protein extraction from green macroalgae biomass. Some of these protocols are for analytical purposes [77,117], while others oriented the protein to animal or human consumption [68,115,116,118–122]. The desired protein extraction method from macroalgae biomass in a biorefinery should have the following parameters: to be cheap, quick, safe (without hazardous chemical remnants), and efficient (reach high extraction yield). However, macroalgae cell wall complexity, combined with viscus and usually charged properties, make the protein extraction a challenging task [123].

Many technologies or different combinations of technologies were used with adjusted protocols for protein extraction (from macroalgae) such as mechanical grinding, ultrasonic treatment, polysaccharide-aided digestion, high shear force, osmotic shock, alkaline or acid treatment [115,118–122].

One of the most popular extraction methods is with 1 M sodium hydroxide solution [53]. This type of protein extraction is relatively quick, but typically results in low yield. This method allows the extraction of up to about 15% of the total protein [124]. The same study showed that prolonged aqua-alkaline solution might increase the protein extraction yield, up to 22 % of the total protein.

Even though the above mentioned technologies and protocols might increase the protein extraction yield, they involve either chemical or thermal procedures. These processes could affect the functionality or the safety of the extracted peptides or proteins [104,125]. Alternatively, the non-thermal and chemical-free emerging technologies such as pulsed electric field (PEF) could be used for protein extraction.

1.4.1 Protein extraction using Pulsed Electric Field

PEF technology was first developed between the 1930s and 1950s in the USSR [126,127], after which the development continued in the 1960s elsewhere in Europe [128]. These attempts were mainly focused on phytochemicals, juice extractions [129,130], and microbial inactivation [131].

Appling PEF on a living cell causes additional transmembrane voltage (TMV). This electric voltage is distributed across the cell membrane. To determine analytically how TMV is induced a non-conductive plasma membrane of a single spherical cell, the Laplace equation as a coordinate spherical system is used.

The expression is similar to Schwan's steady-state equation: $\Delta V_m = 1.5\ E\ Rcos\theta$ [132]. The TMV induction depends on: (i) the local electric field amplitude (E), (ii) the cell's radius (R), and (iii) the electric field vector direction relative to the location of the membrane (θ). The induced TMV can be theoretically calculated for spheroid cells shapes, but the

calculation for real cell shapes requires that a numerical determination must be experimentally evaluated [133,134]. Experiments with molecular dynamics showed the membranes' non-homogeneous distribution of the electric field [135]. This phenomenon explains the higher local electric field distribution, which may explain the formation of the pores in the cell membrane, a phenomenon known as electroporation. The pore formation manner is dependent on the bilayer molecular composition and other factors [135,136]. Therefore, based on the cell membrane properties, each type of organism requires a different working range of the electric field for its membrane permeabilisation, usually varying between 0.5 and 20kV cm^{-1} [137,138]. A cell membrane exposed to a high electric field higher than a cell-specific threshold, becomes permeable to ions and molecules [139]. This permeability allows even big molecules to cross the membrane such as plasmid DNA[140]. The cell's survival status (if the cell survived the electroporation after high electric field pulse), categorizes electroporation into two modes called reversible or irreversible electroporation. After reversible electroporation, the cell survives, while irreversible electroporation causes cells to die. Both of these two modes are potentially applicable to a biorefinery.

PEF technology has recently become applicable for multiple fields such as in biotechnology and medicine [138,141]. Regarding PEF relevance for food processing applications, it has already proven to be an efficient technology for protein extraction from diverse biomasses such as bacteria, yeast, microalgae, and plants [142–145].

Interestingly, the PEF technology has advantages in protein extraction as non-thermal and chemical-free technology (as already mentioned in the previous section). This technology has already proven to be scalable and, most importantly, energy-efficient [139,146]. This is evident from the recent PEF large-scale usage for biomass processing in the sugar industry [147]. This technology successfully reduced the energy investment of the downstream process by up to 50% [147].

Experiments in PEF technology for protein extraction from plant and microalgae biomasses have shown encouraging results. For example, PEF pretreatment was done for rapeseed (*Brassica napus* L.) stem, leftover biomass after oil production. The PEF pretreatment significantly increases the total protein extraction yield [148]. Additional

evidence of PEF treatment was with five different microalgae with different cell wall composition: *Haematococcus*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Athrospira platensis,* and *Porphyridium cruentum*. Interestingly, the PEF treatment resulted in high hydro-soluble recovery from total protein 80-25% [149]. Other investigations of phytochemicals extraction from the microalgae *Nannochloropsis* showed a selective protein extraction [142].

All the mentioned advantages of PEF technology encourage the investigation of this technology for protein extraction from macroalgae and especially *Ulva* sp. to design an energy-efficient and sustainable biorefinery.

1.4.2 New protein source; food safety assessment for allergenicity

The fast-growing protein demand for human consumption and for animal feed [16], and the limited per area crop cultivation productivity [150], combined with environmentally harmful effects of the industrial-conventional agriculture [151,152], motivate the search for new environmentally-friendly alternatives for protein sources [153]. So far, the known potential candidates as alternative protein sources are the bean, pea, lentil, and chickpea [154], duckweed [155], lab-grown meat [156], insects [157], and algae [69]. It is forecasted that by 2054, the human protein sources in the global market will change dramatically. The alternative protein is planned to increase from 2.1 to 33 %, while in the future alternative protein market the estimated market share of algae-based protein (among the total) can reach 18% [153].

To make this novel protein available for human or animal consumption, it must be digestible, nutritionally valuable, and most importantly, safe [158]. The food safety issue of the novel proteins is not well studied [159]. The novel protein source can never be ensured of being free of risks. However, proper risk management might evaluate the potential risk involved in the novel food consumption and reduce the uncertainty [158,160]. The novel protein may be considered safe when it is found to be free of biological or chemical toxins and hazardous microbes [161]. Also, the novel protein should be analyzed for its processing in the body by testing absorption, distribution, metabolism, and excretion (ADME) [161]. In addition, the novel protein anti-nutritional factors must be considered [160,161]. Finally,

the potential allergenicity of novel protein must be evaluated [161].

The different allergen intake ways may lead to a hypersensitive immune response, such as: through the gastrointestinal tract (oral intake), through the respiration tract (breath intake), or other ways of exposure, such as through the skin [162]. This response could be IgE mediated, where the antibodies (IgEs) bind to mast cells and basophils and thus activate them. The activated cells then produce inflammatory mediators such as cytokines with and without histamine [162].

The protein allergic effect might happen due to cross-reactivity [158] when a homologs protein with an allergen causes a similar reaction as the original allergen. Hence, the cross-reactivity could be assessed via serological testing or homologs studies, but detecting the allergenicity of new potential allergens is a much more difficult task [158]. The allergenicity evaluation of new protein should include four phases: (a) collecting the information about the protein exposure history or potential future usage; (b) analyzing the taxonomic relationship between the unknown allergen protein sources and the known allergens; (c) comparing the novel protein to the databases, and (d) evaluating the allergenic potential due to matrix change caused by the processing method for the protein preparation [159].

Importantly, the allergenicity risk is increased when extracting and concentrating the protein. Because it can change the protein reactivity in the body and/or by increasing negative dose-response [159]. Therefore, the effect of the extraction method on the potential of allergenicity must be taken into consideration for responsible risk management [159–161].

1.5 Bioethanol from macroalgae

The major fossil fuel alternative for vehicles today is bioethanol. This alternative source is based mainly on corn biomass (sugarcane, sugar beet, and wheat are also used) [163]. The main global producers of bioethanol are located in U.S. and Brazil [164]. The size of the global ethanol fuel market in 2016 was almost 65 billion USD, with the amount increasing every year. The estimation for 2025 is a Compound Annual Growth Rate (CAGR) of 5.8% [165]. Pew Center on Global Climate Change estimates that by 2035, up to 25 percent of

gasoline consumption in the USA will be replaced by bioethanol [166]. In 2017, about 21 million vehicles (flexible-fuel vehicles) in the USA used ethanol [167].

The major problem with ethanol production from corn is environmental degradation increase. Corn production causes irrevocable soil erosion more than any other crop. Besides, corn production uses more insecticides, herbicides, and nitrogen fertilizers than any other crop [96]. All the above factors degrade the agricultural and natural environment and contribute to water pollution and air pollution [96]. Furthermore, it causes an increase in the cost of food and diverts human food resources to costly inefficient production [96]. Alternatively, macroalgae could be used as a renewable biomass feedstock for fuels (as already mentioned in previous sections) [38][29,38,168,169], that does not compete for arable land or potable water and could be massively cultivated offshore [29].

1.5.1 Fermentation of *Ulva* sp. biomass for bioethanol

Among all macroalgae, *Ulva* sp. is a particularly relevant candidate to replace corn for biofuel production, as it has a high sugar content, and this macroalgae type is wildly distributed (as mentioned in section 1.2.1 and 1.2.2).

The use of *Ulva* sp. for bioethanol production involves four main steps: (i) cultivating the alga biomass [28]; (ii) harvesting the algae biomass [170]; (iii) decomposition and hydrolysis of polymers into basic monomers [38]; and (iv) fermentation of the sugars into bioethanol and distillation [38,168].

To improve the yield of the bioethanol per alga biomass, steps iii-iv should be optimized. The *Ulva* sp. biomass hydrolysis leads to the release of multiple monosaccharides (as mentioned in section 1.2.2) in different concentrations [171], and additional chemicals [172]. Every component might affect fermentation productivity [64]. To design the optimal biorefinery, the combination of steps i-iii should be tested at the most optimal fermentation condition. However, experimenting optimization for every protocol steps i-iii with fermentation optimization of multiple fermenting microbes might make this task too complex. For that reason, recently a two-step metabolic model called ‘BioLego’ [62,64] was developed. This model allows the use of the biomass parameters of hydrolyzed

compounds such as monosaccharides, amino acids, fatty acids, and others, for predicting *in-silico* the yield of bioethanol. This model considers *Saccharomyces cerevisiae* (with and without genetic modification), *Escherichia coli* and *Clostridium thermocellum*, in a single fermentation step or combination of two fermentation steps [62,64]. Importantly, this model assists in estimating bioethanol yield from a given *Ulva* sp. biomass, without the experimental part.

2. Statement and motivation

Oil-based refineries are mature technologies that were developed in recent decades that are able to produce a large product diversity including fuels, chemicals, and materials. However, the negative environmental aspects associated with such refineries and its derived products, motivate technology innovators to find new alternative feedstocks accompanied by new processing technologies. Therefore, a biorefinery concept becomes a relevant alternative; this alternative being based on the usage of biomass sources as a feedstock for the production of multiple products. This alternative solution is already developed on a large scale, and allowing to supply fuel and food products simultaneously. Nevertheless, the current biorefineries are relying on terrestrial plants such as maize, sugar cane, and others. All terrestrial crops compete on arable lands for agriculture, and potable water, and involve negative environmental issues. Importantly, processing terrestrial plants is a complex task due to the lignin, a hardly degradable polymer.

The growing demands for proteins and fuels, with the lack of appropriate sustainable feedstock motivated us to develop new macroalgae based feedstocks as sources for sustainable biorefinery. The macroalgae *Ulva* sp. are seawater plant-like organisms, which can be cultivated offshore. Thus, it does not compete on the land territory and does not require potable water. In addition, it has no lignin, which makes this feedstock much easier for processing.

Because the biorefinery process based on macroalgae is a relatively new approach, new efficient methods have to be developed for making such processes applicable to industry. The cultivation of macroalgae in general, and especially *Ulva* sp. are also new processes. These processes require the development of new methods for efficient cultivation.

Improving crop cultivation efficiency can be reached by controlling the biomass growth rates and controlling its composition.

From the markets' perspectives, currently, there are two trends happening simultaneously. The first is the growing demand for "new protein" sources. The second is the need to replace the current feedstock for bioethanol production. The first market trend follows the rising global demand for protein. At the same time, the second market trend follows the rising demand for vehicles powered by bioethanol. While the current bioethanol production feedstock sources are unsustainable.

According to the global requirements for the next decades and the following recent market trends, we aimed to provide basic knowledge and to develop sustainable methods that could supply the increasing worldwide population demand for the simultaneous production of a safe source of protein for food and bioethanol.

Potentially, the biorefinery based on macroalgae could be designed for large-scale production of protein and bioethanol from the same biomass [29]. Therefore, our approach is to close the main scientific gaps for realizing this solution.

3. The study goals, main hypotheses and research approaches

Based on the scientific literature described in the introduction we detected the following gaps in the knowledge that prevent the implementation of *Ulva* based biorefineries:
(i) the ability to control *Ulva* growth rate and its biomass sugar and protein composition; (ii) the ability to control the *Ulva* composition that is relevant for bioethanol production; (iii) the lack of an efficient protein extradition method, that is relevant for the food industry; and (iv) the unknown allergenic effects of *Ulva* proteins. In order to close these gaps, we detailed four main goals.

Goal 1: Identifying the effect of epiphytic bacteria on *Ulva* growth rates and on its chemical composition. According to the literature *Maribacter* sp. and *Roseovarius* sp. bacteria affect *Ulva* development [84,173,174] and its metabolites composition and concentration which are released into the chemosphere [92]. Following that information, our research question was: **if and how do the two algae-associated bacteria affect the sugar and protein concentrations of the algal biomass?**

We hypothesized that *Maribacter* sp. and *Roseovarius* sp. can influence the *Ulva* growth, and its chemical composition. The chemical composition might be affected, including the proportions of amino acids and monosaccharides. Our approach was to compare the growth rate and the chemical composition between the tripartite communities composed of *Ulva* mutabilis with *Maribacter* sp. and *Roseovarius* sp. to axenic (bacteria-free) cultures. We expected that two bacteria added to the algae culture allow a rapid development that leads to fast-growing *Ulva*. In addition, the chemical composition of *U. mutabilis* biomass grown with bacteria will be different from the biomass of an axenic culture.

Goal 2: Defining the relevance of associated bacteria in modulating *Ulva* biomass for bioethanol production. Hydrolyzed algae might be used as feedstock for bioethanol production after microbial fermentation [38,175]. Each microorganism has some favorable carbons sources. Changing this source concentration during the fermentation will result in different ethanol yields [176]. However, there are some other nutrients that may play a fundamental role in the fermenting organism reproduction and the bioethanol generation. The commonly used microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *Clostridium acetobutylicum*, could result in different ethanol yields by using similar biomass feedstock [62,64]. Following this information and the answer from the first research goal, our research questions were: will the fermentation of algae biomass cultivated with bacteria result in different ethanol yields? and what is the optimal fermentation combination for the *U. mutabilis* biomass cultivated in the tripartite community and for axenic culture?

We hypothesized that fermenting *U. mutabilis* biomass grown in a tripartite community or as axenic culture will result in a significantly different ethanol yield. In addition, fermenting *U. mutabilis* biomass in two fermentation steps with *S. cerevisiae*, *E. coli*, or *C. acetobutylicum* will lead to improved ethanol yields than only one fermentation step. Our approach was to compare different microbial fermentation combinations in single or two-step fermentation of two different biomasses: tripartite community biomass and axenic *U. mutabilis* biomass. We expected that biomass with a higher monosaccharide concentration, especially glucose, will lead to higher ethanol production. Moreover, two-step fermentation will lead to more ethanol production than a single fermentation. Also, using *S. cerevisiae* for fermentation *U. mutabilis* hydrolysate will yield higher ethanol than fermentation with other organisms.

Goal 3: Developing an efficient process for protein extraction from *Ulva* sp. using electroporation with Pulsed Electric Field. There are many available methods for extracting proteins. Usually, these methods involve unwanted chemicals, thermal processes which affect the protein value [115,118,120,122], or relatively expensive enzymatic methods [120]. Alternatively, electroporation with PEF increases the tissue permeability [177,178], while in combination with physical pressure it facilitates active biochemical extraction through the cell walls [139,146]. PEF in combination with mechanical press results in a chemical-free, non-thermal, and energy-efficient extraction method that has already been tested in different plant biomasses [139,146]. **Can we efficiently extract proteins from the algae cells with PEF treatment? Thus, we hypothesized that applying PEF treatment with the mechanical press will be an efficient method for protein extraction from *Ulva* sp.** Because macroalgae are salt-water organisms, we thought it would be relevant to integrate osmotic shock as an additional treatment for improving the protein extraction. We assumed that PEF treatment leads to selective protein extraction, due to the different protein biochemical properties. Our approach was to compare the control (osmotic shock and mechanical press) to the same treatment with PEF, to evaluate the energetic efficiency of the treatment, the protein yield, and to do a proteomic analysis of the extracted proteins. We expected efficient extraction and definition of specific proteins after PEF treatment (with the mechanical press and osmotic shock).

Goal 4: Evaluating the allergenicity risk of protein extracted from *Ulva* sp. with PEF. The European regulation for commercial production of novel proteins includes risk assessment [158,160]. One of the major parts in that assessment is the evaluation of potential allergenic risk [161]. This assessment is challenging, but using a new extraction method (for example, PEF) for the new protein production makes this evaluation even more challenging. The extraction method can play a fundamental role in the extracted proteins type and their activity [159–161]. Thus, it is reasonable to link the allergenicity assessment with the specific protocol for protein extraction [159–161].

Thus, our research question was **what is the allergenic risk of the proteins extracted from *Ulva* with PEF? We hypothesized that PEF treatment will affect the allergenicity level of the extracted proteins.** Our approach was first to optimize the extraction process with PEF, then to assess *in-silico* allergenicity for the extracted proteins. We aimed to define an optimal treatment extraction method with PEF, composed of a number of pulses and pulse strengths. We assumed that after a certain threshold of energetic investment further increasing pulse number or higher energetic investment (for the pulses) will not improve the protein extraction yield. In addition, we assumed that optimal extraction would lead to certain protein extraction, with certain allergens. In detail, for the protein extraction optimization from *Ulva* sp., we used PEF treatment; 12 kV or 26 kV in combinations of 0-75 pulses, a treatment that included mechanical press and osmotic shock. Then the proteins were quantified and identified. The controls were the same treatment without PEF and total protein. For total protein extraction, we used a conventional thermochemical method, a universal method for proteomic analysis [179]. After the extraction, the allergenicity assessment was done *in-silico* by annotation of the extracted protein and comparisons to known allergens. In addition, the potential allergenicity was evaluated based on scientific publications and databases. We expected to find the optimal protocol with relevant voltage strength and the pulse amount that will result in optimal protein yield with the minimal energetic requirement. In addition, we expected to detect the specific allergens after extraction with PEF, compared to controls.

4. The study publications

The studies presented in the thesis attempt to answer the research goals, using the research approaches according to our hypotheses. The order of the papers in the thesis is organized following the logic of the biorefinery conceptual structure. For the sake of simplicity, **Fig. 2** presents a graphical illustration of the processes in the biorefinery. Here, in the illustration, the color adjacent to a process is related to the specific research focus of a paper.

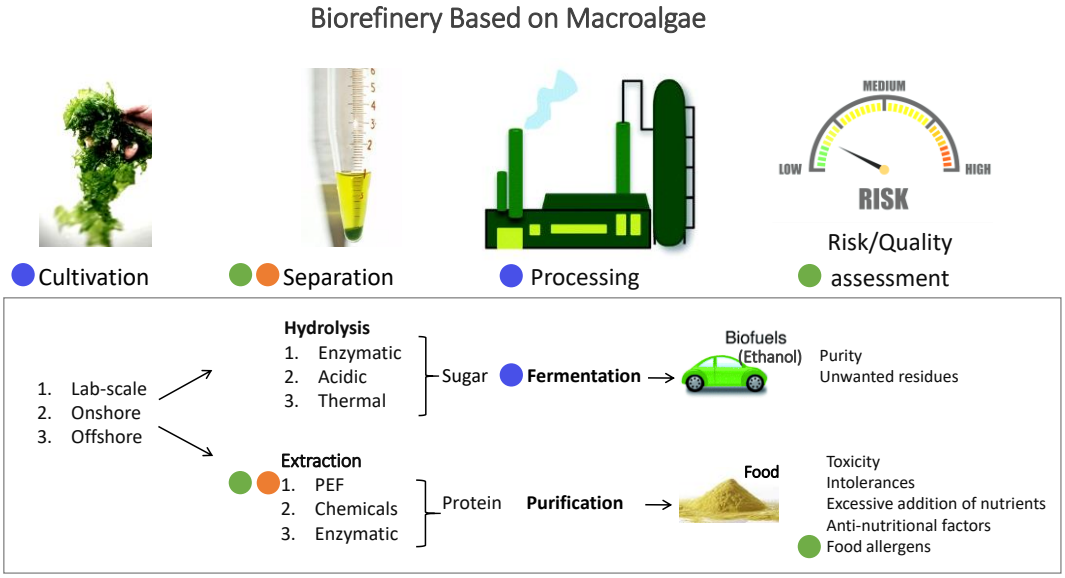

Figure 2 - A graphical illustration of a biorefinery based on macroalgae. Each colored dot is related to different publications: the purple dot is related to paper #1, the green dot is related to paper #2 and the orange dot is related to paper 3#.

4.1 First paper

***Polikovsky M**, Califano G, Dunger N, Wichard T, and Golberg A, 2020. Engineering bacteria-seaweed symbioses for modulating the photosynthate content of Ulva (Chlorophyta): Significant for the feedstock of bioethanol production. Algal Research. 49, 101945.*

In this study, two main results were achieved: the glucose concentration in the macroalgae biomass almost doubled due to bacterial addition to the algal cultivation. Importantly, glucose is the most relevant component in the algae biomass for bioethanol production. Additional results showed how bacteria affect the algal amino acid profile, the basic component for a protein formation in any living organism. **This study presented a new way to control the macroalgal biomass composition using bacteria.** This paper for the first time showed how macroalgae associated bacteria are can modulate macroalgae biomass composition to make it a better feedstock for bioethanol production.

4.2 Second paper

***Polikovsky M**, Fernand F, Sack M, Frey W, Müller G, and Golberg A, 2016. Towards Marine Biorefineries: Energy Efficient Proteins Extractions from Marine Macroalgae Ulva lactuca with Pulsed Electric Fields. Innovative Food Science and Emerging Technologies. 37, 194-200.*

Before this study, many extraction methods were available, but with most of them unwanted chemicals remain, or they are too expensive to be commercialized [115,118–122]. Therefore, we developed a new method for protein extraction from *Ulva* sp. biomass with PEF, which was described in the paper. In this paper, we reported the specific proteins that were extracted from *Ulva* tissue using PEF. An additional part in the paper focused on calculations of the energetic investment for the treatment. **The novelty in this paper was in the development of a new method to extract proteins from macroalgae using PEF**, a scalable, chemicals-free, non-thermal and energy-efficient technology.

4.3 Third Paper

***Polikovsky M**, Fernand F, Sack M, Frey W, Müller G, and Golberg A, 2019. In silico food allergenic risk evaluation of proteins extracted from macroalgae Ulva sp. with pulsed electric fields. Food chemistry. (276): 735-744.*

Following the work presented in the second paper, in the third paper the PEF extraction method from *Ulva* sp. biomass was optimized and proteins were identified. Based on this data, an allergenicity assessment was done. We showed that our new extraction method selectively avoids the extraction of certain allergens, and it can potentially reduce the allergenicity risk compared to the control of total protein extraction. Before this paper was published, an assessment of allergens of the proteins extracted from macroalgae had not been described, although much research was done regarding algae protein (as a "novel protein" source for food). **The novelty in this paper is that it evaluated food allergenicity risk of macroalgae proteins.** This evaluation is obligatory for food risk management of a "novel protein". This fundamental paper is key for the future use of sustainable macroalgae proteins in the human food chain.

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Engineering bacteria-seaweed symbioses for modulating the photosynthate content of *Ulva* (Chlorophyta): Significant for the feedstock of bioethanol production

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ABSTRACT

Seaweed biomass cultivation predicates the quantity and quality of this biorefinery feedstock. Unfortunately, the seaweed growth rate and chemical content are hardly predictable and are affected by environmental factors, including epiphytic bacteria. We hypothesize that microbiome engineering can control the chemical composition of Ulva biomass. We show that the engineered *Maribacter* sp. and *Roseovarius* sp. consortium modulate *Ulva mutabilis* growth rate and photosynthate content of constituents relevant for bioethanol production. Although minimal growth was observed in the axenic cultures (0.04 mm day^{-1}), *Ulva mutabilis* in a tripartite community showed a growth rate of 3.79 mm day^{-1} in the growth phase. Furthermore, the content of glucose and glycerol in *Ulva* of the engineered community increased by 77 ± 19% and 460 ± 207% whereas xylose and glucuronic acid decreased by 37 ± 14% and 46 ± 15% in comparison to axenic culture.

Interestingly, bacterial addition affected the rhamnose/xylose/glucuronic acid ratio (1.96:1:1: vs 1.34:0.85:1 in xenic vs axenic culture), indicating the impact of bacteria on ulvan synthesis. In addition, tyrosine and histidine increased by 191 ± 61% and 40 ± 26%; however, valine, isoleucine, aspartate, threonine, serine, and phenylalanine decreased by 22 ± 19% - 42 ± 23%. Flux-balance analysis of *Saccharomyces cerevisiae*, *Escherichia coli*, and Clostridium acetobutylicum was used to estimate the bioethanol yield from hydrolyzed *Ulva* biomass, in a one-step or two-step fermentation process. Simulation using *S. cerevisiae* (RN1016) with xylose isomerase resulted in a bioethanol yield of 85.62 for xenic vs. 71.31 mg/g dry weight (DW) axenic cultures of *Ulva*.

The increased growth rate and the relative amounts of photosynthates of *U. mutabilis* are modulated by the engineered microbiome. Moreover, it results in biomass with a higher potential for bioethanol fermentation in comparison to axenic cultures.

1. Introduction

Conventional fossil sources for energy supply have adverse side effects of climate change [1,2]. Terrestrial plants, which are current alternative feedstocks for biofuels, conflict with food production [3]. Moreover, the agriculture for cultivating those plants is contributing to water consumption, greenhouse gas emissions, and the degradation of natural environments [4,5].

Seagriculture emerges as an alternative to agriculture practice to produce seaweed biomass for the sustainable biofuel feedstock supply chain [6–8]. In seagriculture, green, red, and brown seaweed biomass could massively be cultivated in seawater. Thus, seagriculture does not compete for arable land or potable water [9,10].

From all green macroalgae species, *Ulva* spp. are particularly attractive as a potential biomass feedstock for biorefinery [7,11] due to its rapid growth rate [6] and adaptation to varied habitats with different abiotic conditions [12,13]. *Ulva*'s carbohydrates are composed mainly of C5 and C6 monosaccharides, iduronic acid, and glucuronic acid [14–16]. The monosaccharides derived from Ulva biomass could be fermented into bioethanol, a versatile chemical, and biofuel [11,15].

However, the chemical content and the composition in *Ulva* sp. varies between the species and is influenced by seasonality and other environmental abiotic and biotic conditions [17–20]. This fluctuation in the chemical composition of the biomass, challenge the optimization of efficient fermentation processes [21]. Therefore, control of the macroalgae biomass chemical composition is required. This control

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could improve the yield of the downstream biomass conversion to biofuels. Even though the power of abiotic environmental parameters is commonly investigated for seaweed aquaculture [22–24], the understanding of the biological microenvironment [25] is still understudied.

Indeed, the natural microbiome of seaweeds plays an intricate role in the algal physiology [26], nutrition, metabolism [27], and immune function [28]. The seaweeds have a proper surface and chemosphere, which may serve as an attractive environment for the bacterial existence [26,27,29]. This environment includes beneficial compounds for bacterial growth such as oxygen [30], carbon source, nutrients [29,31], and metabolites [28]. Recent studies explicitly investigated the cross-kingdom interactions between *Ulva* spp. and its associated bacteria [26]. Metabolomics research compared the chemosphere of axenic *U. mutabilis* culture with a tripartite community of *U. mutabilis* and its two naturally associated bacteria, *Roseovarius* sp. strain MS2 and *Maribacter* sp. strain MS6. Bacteria can recognize *Ulva* as a reliable food source through chemoattractants [32]. In turn, bacteria induce algal growth and morphogenesis settling around the algal holdfast [32]. Bacteria of the *Rosoebacter* clade often promote algal growth to develop their own (bacterial) benefits [33].

The photosynthate secreted by *Ulva* spp. includes carbon sources such as glycerol [31]. Notably, the glycerol is the backbone of triacylglycerols (TAG) and the primary form of energy storage in plants [34]. These storage lipids are essential to plant development, being used, for example, in seedling growth during germination [35]. As glycerol is essential for both algal and bacterial growth, we hypothesize that algal growth- and morphogenesis promoting bacteria trigger the sugar and glycerol production of *Ulva mutabilis* in standardized algal aquacultures.

Moreover, we also tested, whether bacteria modulate the amino acids (AAs) pattern of *Ulva*, due to their potential role in the *Ulva*'s morphogenesis. Such modulation of photosynthate is essential not only for *Ulva* growth and development but also for the utilization of *Ulva* and its downstream processing, such as bioethanol fermentation. For testing these hypotheses, we applied a targeted analysis of organic compounds critical for bioethanol production in *Ulva* tissue such as monosaccharides, glucuronic acid, glycerol, and AAs. The engineered tripartite community composed of *U. mutabilis* with *Roseovarius* sp. and *Maribacter* sp. was compared with the *U. mutabilis* axenic culture. The chemical content of *U. mutabilis* from these two cultures types served as feedstocks for a flux balance analysis of bioethanol fermentation in BioLego. This specially designed software that uses flux balance analysis (FBA) to predict bioethanol yield from biomass with various fermenting microorganisms [36]. Our study demonstrates that engineering of *Ulva*'s microbiome leads to a better understanding of the bacterial role in the macroalgal biomass production, critical for developing an efficient seaweed-based biorefinery.

2. Materials and methods

2.1. Induction of U. mutabilis gametes

The cultivation was carried out as previously described by Alsufyani et al. (2017) for 63 days [31]. The fast-growing natural developmental mutant of *U. mutabilis* Føyn (mating type mt+; morphotype "slender" (sl)) was cultivated [37,38]. The *Ulva* cultivation was started from haploid gametes to achieve reproducibility and synchronization of the algae. For preparing the *Ulva* seed stock, gametogenesis was induced in mature thalli (a four weeks old culture started from gametes). Gametogenesis was induced by fragmentation with a herb chopper (Zyliss, Zurich, Switzerland) into smaller fragments (1–3 mm size) (Fig. S1A). Sporulation inhibitors were removed from the *Ulva* tissues with the immersion of the fragments three times in 50% artificial seawater [39]. After three days of cultivation, the *Ulva* culture medium (UCM) was changed for removing the swarming inhibitor that led to the gametes discharge.

2.2. Preparation axenic gametes

The feedstock of U. mutabilis axenic germlings was prepared by separating gametes from the associated bacteria in Pasteur pipettes under strictly sterile conditions using the phototactic properties of the gametes. This method is a standard operational procedure (Fig. S1B) [40]. The axenicity of the gametes was tested by plating 10 μL of the gametes seed stock on marine broth (Roth, Germany) agar plates (1.5%; w/v) (Sigma-Aldrich, Germany) and by performing polymerase chain reactions (PCR) of the 16S rRNA gene [41]. Gametes were counted by flow cytometry. About 6×10^3 axenic gametes were inoculated as seed stock in 250 mL sterile UCM in polycarbonate tissue culture flasks (V = 650 mL, BD Falcon, Franklin Lake, NJ, USA). The feedstock was incubated for 24 h in the dark for settlement of the gametes [31,40].

2.3. Engineering U. mutabilis and bacteria symbioses

Algae were cultured under light: dark (17:7 h) regime and the illumination of a photon flux of 60–120 (μmol $m^{-2} \cdot s^{-2}$) (50% GroLux, 50% daylight fluorescent tubes; OSRAM, München, Germany) at 18 °C. Sock cultures of the two bacterial strains, *Roseovarius* sp. (MS2) (Genbank EU359909) and *Maribacter* sp. strain (MS6) (Genbank EU359911) [41], were grown on the orbital shaker at 20 °C in liquid marine broth medium (Roth, Germany). For preparing the tripartite community of *U. mutabilis* and two associated bacteria (i.e., the xenic cultivation), the exponentially growing bacterial cultures were harvested by centrifugation (3000 × g) for 5 min. The cell pellet was resuspended and washed three times with sterile UCM. Finally, the two bacterial strains were added to the axenic gametes of *Ulva* [31] (Fig. S1C). The bacterial suspension was diluted to a final optical density (OD) of 0.001 in the cell tissue flask.

After 14 days of cultivation of *Ulva* in tissue flasks, propagules of the xenic and axenic cultures were transferred to the 25-l polycarbonate bottles (i.e., bioreactors) filled with 15 L UCM. The experiment was started with 5×10^3 germlings for both treatments, while for the tripartite community preparation, each bacterial inoculum was added (OD_{620nm} = 0.0001, OD in the bioreactor after inoculation). Half of the culture medium was renewed after 4 weeks. *Ulva* was collected from the tripartite community and axenic cultures equivalent to 100–350 mg dry weight (DW) after 8 weeks of cultivation . Each culture, tripartite community or axenic culture, was cultivated in three independent replicates.

The growth rate (mm day^{-1}) was calculated by a length with the following equation:

$$Growth\ Rate\ (mm\ day^{-1}) = \frac{L_t - L_0}{T_t - T_0}$$

L = length (in mm), T = time (in days). t = time of the cultivation. The parameters of the growth rate during the growth phase (T_t = 35 and T_0= 7 days after inoculation in the bioreactor) of *Ulva* in tripartite community and axenic culture were; L_t = 117.11 mm, L_0 = 10.9 mm, and L_t= 1.06 mm, L_0= 0.01 mm.

2.4. Biomass hydrolysis for the quantification of monosaccharides, glucuronic acid, and glycerol

Biomass was dried (16 h) at 50 °C in an oven (Fig. S1D) and subsequently grounded into powder using mortar and pestle. The powder was then stored at −28 °C. For every biological replicate, a duplicate of hydrolysis treatment was performed. Thermochemical hydrolysis [42] was conducted with 2% sulfuric acid (v/v) for 30 min at 121 °C in a ratio of 1:250 (solid: solvent) using 10 mL autoclavable centrifuge tubes (Nalgene™ Oak Ridge High-Speed PPCO Centrifuge Tubes, ThermoFisher Scientific, CA) in the autoclave (Tuttnauer 2540MLV, Netherlands). Each batch, 4 ± 0.5 mg of dried biomass was weighed (Mettler Toledo, Switzerland). Sulfuric acid (Sigma-Aldrich, Israel) was

diluted to 2% (v/v) and was added to the tube. Hydrolysates were stored at 4 °C. Triplicate of algae samples were hydrolyzed in duplicate before being analyzed (Fig. S1E).

2.5. Monosaccharides quantification

The monosaccharides were determined (Figs. S1F, 2) by high-pressure ion chromatography (HPIC) according to a protocol of Robin et al. (2017) with small adaptations [43]. In brief, aliquots of the hydrolysates were taken and diluted in ultrapure water before being filtered through a 0.22 μm syringe-filter (Millipore, USA) in HPIC vials (Thermo Fischer Scientific, MA, USA). Monosaccharide content in the hydrolysates were measured by high-performance anion exchanged chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS-5000 platform (Dionex, Thermo Fischer Scientific, MA, USA) equipped with an analytical column (Dionex™ AminoPac™ PA10 IC) and a guard column (Thermo Fischer Scientific, UK). An electrochemical detector with an AgCl reference electrode was used for measuring the compounds. The analysis was performed using an isocratic flow gradient of 100–4.8 mM KOH generated with an eluent generator (Dionex, Thermo Fischer Scientific, MA, USA) (for details see Table S1). 100 mM KOH, for 20 min was used for rinsing the analytical column between each run. Before the analysis of the samples, the system was reequilibrated with 4.8 mM KOH. During the analysis of the samples, the flow rate was 0.25 mL/min, the temperature of the column was set to 30 °C, and the autosampler temperature set to 5 °C. Calibration curves for monosaccharide standards such as rhamnose, galactose, glucose, xylose, and fructose (Sigma-Aldrich, Saint-Louis, Missouri, USA), were produced independently in triplicates. The appearance of monosaccharides in the biological samples was verified in comparison to reference standards (Fig. 1A).

2.6. Quantification of glucuronic acid (GlcA) and glycerol

GlcA was determined following the same workflow as described above (Fig. S1F) but with a different gradient and eluents for chromatographic separations (Table S2). Glycerol was measured with HPIC using a program involving two eluents, namely NaOH and ultrapure water. The analytical column, Dionex™ CarboPac™ MA1 IC, and its corresponding guard column were from Thermo Fischer Scientific. The flow rate was set at 0.4 mL/min, and the column temperature was kept at 30 °C.

2.7. Bacterial cultivation on various carbon sources

Roseovarius sp. (MS2) were grown aerobically in 50 mL UCM at 20 °C for 18 days and enriched with 1% (w/v) of various carbon sources: glycerol [31,32], glucose, rhamnose, galactose, xylose, and fructose. The bacterium was cultivated with each carbon source separately. The bacterial cultures grew in an orbital shaker in 250 mL polystyrene tissue culture flask (Flask T75, Sarstedt, Germany). Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) in a 1cm polypropylene cell on a UV/Vis spectrophotometer (Genesys, ThermoFisher, Germany). The bacterial growth rate of *Roseovarius* sp. in UCM with 1% glycerol (w/v) as carbon source, was calculated using the following equation; $F(x) = x_{(0)}e^{(\mu * xt)}$, where $F(x) = OD_{600,}$ $x_{(0)}$ is the initial time point of the logarithmic phase, $x_{(t)}$ = the last time point of the logarithmic growth phase and μ = growth rate (change of OD day−1).

2.8. Biomass hydrolysis for amino acids quantification

The biomasses of axenic *U. mutabilis* or tripartite community were hydrolyzed according to the manual “Dionex AAA-Direct, Amino Acid Analysis System” (Thermo Fischer Scientific, MA, USA) and Kazir's protocol with some modifications [44]. The biomass was dried, grounded, and stored, as described in Section 2.4. The biomass powder (4 ± 0.23 mg) was transferred into 3 mL micro-reaction vials (Sigma Aldrich, MO, USA). The headspaces of the vials were rinsed with N_2 during 10 s. The biomass was added to the vials and incubated in 1 mL of 6 M HCl (Sigma Aldrich, MO, USA) for 16 h at 112 °C (Fig. S1E) with continuous headspace N_2 gas flushing. During the incubation, the vials with the biomasses were in a dry bath with a set of needles for gas flushing (Bio-Base, China). After the incubation, the vials were cooled down to room temperature, and the acid (HCl) was evaporated. The evaporation process was done with N_2 (99%); the gas was purged into open vials through the needles for 3.5 h (flow rate of 4 ± 1 L/min). After complete evaporation of acid, the dry samples were reconstituted with 1 mL of ultrapure water. All samples were diluted with ultrapure water and were filtered with 0.22 μm syringe-filter (Millipore, USA), before the HPIC analysis.

2.9. Amino acids quantification

Analysis of AAs content was performed (Fig. S1F) according to the Kazir's protocol and the manual of Thermo Scientific [44,45]. Total AA content was analyzed with the same equipment and the set up as described in Section 2.5, but with a non-disposable gold AAA™ electrode. The eluent gradient was run, as described in Table S3. The waveform for the electrochemical detector was adopted from the Application Note 163 [46]. The AAs peak areas were compared to commercial AA standard mix (AAS18, Sigma Aldrich, MO, USA). The program was validated with the commercial AA mix (AAS18, Sigma Aldrich, MO, USA). The commercial mix was diluted (1:50, 1:100, 1:250, and 1:1000). Calibration curves were built for 17 AAs: alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine (Fig. 1B). DL-norleucine (Sigma Aldrich, MO, USA) was added to all the samples and standards as an internal standard. The internal standard was used for normalizing the system's sensitivity variations between the samples. The correlation factor for each of them was $R^2 > 99\%$. Cysteine and methionine are probably underestimated [46] because of their sensitivity to the hydrolysis procedure.

2.10. Modeling bioethanol production using flux balance analysis

‘BioLego’, a software for flux balance analysis [36] was used for the prediction of bioethanol yield. This model relies on the complete metabolic models of the microorganisms with the ability to produce bioethanol. The tested organisms were *Saccharomyces cerevisiae* [47], *Escherichia coli* [48] and *Clostridium acetobutylicum* [49]. Online website was used (http://wassist.cs.technion.ac.il/~edwardv/BioLego/html/ BioLego.html) [50] for running the model; the products of *U. mutabilis* axenic and tripartite community biomasses were used as the input. “Other particles” of the model were defined as compounds of the biomass, which do not appear in the default medium. The calculation of the “other particles” was carried out as follows: total chemical components measured in this study was removed from total chemical components in the simulation of default medium (of U. lactuca). This difference corresponds to the “other particles” in the default medium.

2.11. Statistical analysis

Tripartite community and axenic culture were carried out in three independent biological replicates. Six individual analytes from each biological replicate were collected for length measurements. The quantification of the monosaccharides, GlcA, glycerol, and AAs for every biological replicate were performed in two technical replicates. Statistical differences between the replicates were measured via a twotailed Student's t-test using Excel software (Microsoft Office 2013). P – values < 0.05 were considered as significant difference.

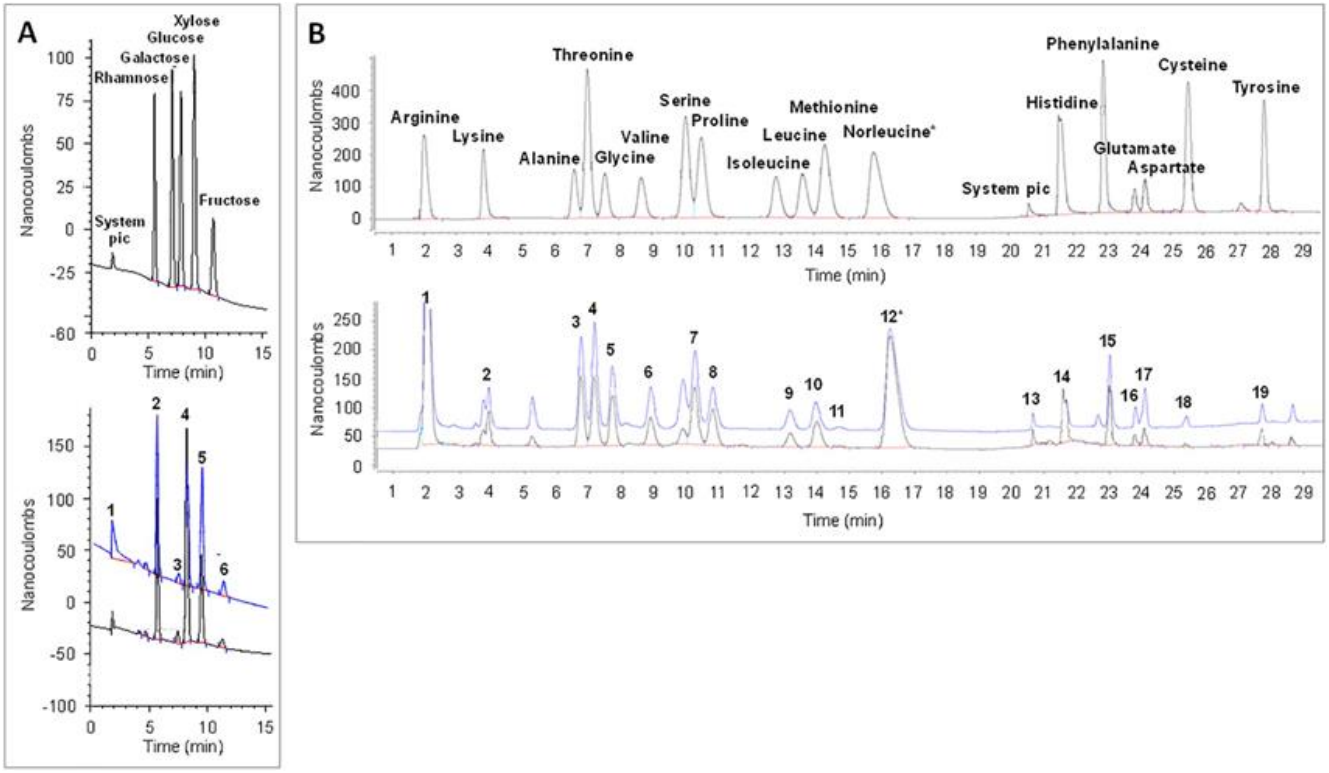

Fig. 1. Ion chromatography for the separation of monosaccharides and amino acids (AAs). Intensity in electric charge (nanocoulombs) over time (in min). A. Monosaccharides separation. The numbers in the chromatogram means: 1 = System peak, 2 = Rhamnose, 3 = Galactose, 4 = Glucose, 5 = Xylose, 6 = Fructose. B. AAs separation. The numbers in the chromatogram means: 1 = Arginine, 2 = Lysine, 3 = Alanine, 4 = Threonine, 5 = Glycine, 6 = Valine, 7 = Serine, 8 = Proline, 9 = Isoleucine, 10 = Leucine, 11 = Methionine, 12* = Norleucine (internal standard), 13 = System peak, 14 = Histidine, 15 = Phenylalanine, 16 = Glutamate, 17 = Aspartate, 18 = Cysteine, 19 = Tyrosine. In A and B the chromatogram on the top, are the separations of monosaccharides or AAs standard mixtures. The chromatograms in the bottom are showing the separation of monosaccharides or AAs in the samples. The blue chromatogram shows the monomers separation in sample of axenic culture. In black are the monomers separation in the sample of tripartite community. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. U. mutabilis growth rate increased under xenic conditions

The growth phase of axenic and xenic *U. mutabilis* culture was determined between 7 and 35 days after the inoculation of axenic gametes with bacteria (Fig. 2). The growth rate of *U. mutabilis* cultivated in the tripartite community was 3.79 mm day $^{-1}$ compared to 0.04 mm day $^{-1}$ in the axenic culture. The maximal length of the thallus reached to 117 ± 19 mm after 35 days within the tripartite community. The average diameter of the callus was only 1.7 ± 0.3 mm in the axenic culture at the end of cultivation (day 63). The effects of bacteria species on *U. mutabilis* growth and morphogenesis corroborated with previous observations, which showed that *Maribacter* sp. and *Roseovarius* sp. (i.e., xenic) modulates the *U. mutabilis* growth and development [26,31,41].

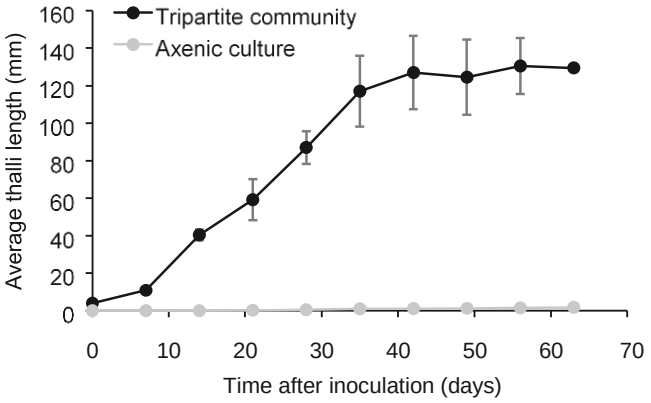

Fig. 2. Growth curve of *Ulva mutabilis*. Average thallus length was measured during growth. *Ulva* biomass was collected from the onset of the algal culture in bioreactors (Day 0) until the mature specimen reached the steady-state growing phase. Error bars represent averages ± standard deviation (n = 18, collected from three biological replications). In axenic cultures, error bars are smaller than the symbol size.

a. Xenic conditions affect the monosaccharide and sugar acid profiles of U. mutabilis biomass

The monosaccharides, namely, rhamnose, glucose, xylose, fructose, and galactose were identified by comparison to reference standards using HPIC (Fig. 1A, Table S1) and subsequently quantified. After summing up the total amount of monosaccharides, no significant difference was observed in the content, while comparing the axenic and the engineered tripartite community (21.3 ± 0.99 23.2 5± 1.00% of DW respectively). Importantly, the percentage of monosaccharides per dry weight (DW) were in the expected range compared to other studies of *Ulva* spp. [7,42,43].

Specific tissue-derived sugars have already been described in *Ulva* spp. several times [11,43,51]. In the current study, the content of rhamnose and fructose did not change either in the xenic or axenic biomasses. Galactose was below the limit of quantitation (LOQ) in all of the samples.

Interestingly, the comparison of the monosaccharides content in the

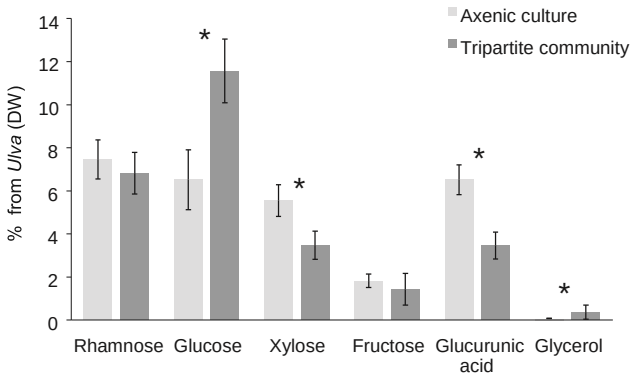

Fig. 3. Monosaccharide, glucuronic acid, and glycerol content in *U. mutabilis* in axenic culture and the tripartite community. *U. mutabilis* axenic culture and the tripartite community biomasses were hydrolyzed after the algae reached the steady-state growth phase, after 8 weeks of cultivation. The monosaccharides were quantified by using HPIC and by comparison to standards. An asterisk indicates the significant difference between the two cultures (two-tailed Student's t-test, $P < 0.05$). Error bars represent averages ± standard deviation for n = 3 (biological replicates), n = 2 (technical replicates).

hydrosylates derived from axenic and tripartite communities of *U. mutabilis* revealed significant monosaccharide type-specific differences (two-tailed Student's t-test, $P < 0.05$) in glucose (Fig. 3, Table S4). In detail, when *U. mutabilis* was cultured with the bacteria, its glucose content increased by 77.42 ± 18.6% from 6.51 ± 0.44% to 11.55 ± 0.61% per dry weight (DW). The higher amount of glucose per DW in the tripartite community compared to the axenic samples could be explained by the nitrogen source limited availability (Fig. 2). In a previous study with *U. mutabilis* and the same cultivation conditions, nitrate was entirely utilized by the tripartite community after 20–30 days [31], which might result in a nitrogen limitation. It is known that under nitrogen starvation conditions, *Ulva* accumulates starch [52]. Starch is a polymeric carbohydrate consisting of a large number of glucose units joined by glycosidic bonds, which were hydrolyzed in this study. Interestingly, microbe-algae interactions might trigger starch production, as demonstrated for the green green microalgae Chlorella spp., which accumulated starch and carbohydrates in the presence of the heterotrophic bacterium Azospirillum brasilense [53].

At the same time, the xylose content decreased by 37.37 ± 14.5% from 5.45 ± 0.49 to 3.47 ± 0.18% per DW in the presence of the bacteria. GlcA content per *Ulva* DW in the axenic culture was higher (two-tailed Student's t-test, $P < 0.05$) compared to the tripartite community (Fig. 3, S4). The GlcA content in tripartite community biomass decreased by 46.15% from 6.5 ± 0.38 to 3.5 ± 0.46% per DW, respectively. Like glucose, GlcA can be a carbon source for a bioethanol fermentation [54].

Rhamnose xylose and GlcA, are the building blocks of the cell wall polymer ulvan [16,18], which contribute to 8–29% of DW [16,18,55]. In the ratio of xenic culture rhamnose to xylose to GlcA is 1.96:1:1, while in axenic culture 1.34:0.85:1. Previously, the ratio between rhamnose, xylose, and GlcA was detected in *Ulva* wild-type was 3.51:0.92:1 [43]. Therefore, rhamnose to xylose to GlcA ratio in xenic cultures culture is closer to the biomass composition of *Ulva* sp. wildtype that was grown with the natural microbiome. Importantly axenic algae possess malformed cell walls forming protrusions without any further cell differentiation compared to xenic conditions (i.e., with bacteria) [26,41,56]. Therefore, further studies will show how the Maribacter-mediated cell wall formation [57] might interfere with the biosynthesis of ulvan and its composition.

These results indicate again that the reduced microbiome of only two bacterial strains is sufficient enough to mimic the natural microbiome and can be used for land-based algal aquacultures under standardized conditions. It is important to note that the bacterial effect on the Ulvan-building block ratio also leads potentially to changes in the Ulvan structure and its functional properties. This evidence could be a key finding for further Ulvan manipulations by using different engineered bacterial consortiums, for controlling the Ulvan properties.

3.3. Xenic growth increases the glycerol content of U. mutabilis biomass

The essential role of glycerol in the cross-kingdom interactions between *U. mutabilis* and its associated bacteria [31,32] motivated the analysis of glycerol. The glycerol content increased by 4.6 times (twotailed Student's t-test, $P < 0.05$) in the tripartite community (0.38 ± 0.11% DW) in comparison to the axenic culture (0.069 ± $6.46 \cdot 10^{-3}$% DW) (Figs. 4, S4). The potential amount of glycerol thus increased, which can be secreted into the chemosphere of *U. mutabilis*, providing a carbon source for heterotrophic growth of *Roseovarius* sp. [31].

3.4. Xenic conditions affect the amino acid profile in U. mutabilis biomass

Considering the significant differences in algae development under xenic and axenic conditions [41], we assumed changes in the AA profile

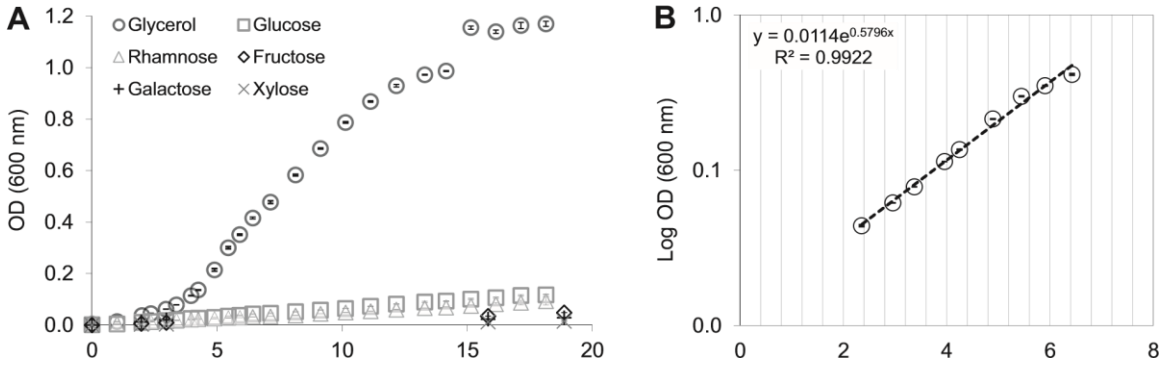

Fig. 4. *Roseovarius* sp. growth with different carbon sources. (A) *Roseovarius* sp. growth in UCM with 1% (w/v) various carbon sources; glucose, rhamnose, galactose, fructose, xylose and glycerol during 18 days of cultivation. The bacterial growth was monitored by the OD_{600} and reached the highest optical densities when supplemented with glycerol. With any other tested carbon source, *Roseovarius* sp. grew up to 10% of the final OD_{600} achieved with glycerol as a carbon source. (B) The growth phase of *Roseovarius* sp., which was grown in UCM supplemented with glycerol as a carbon source, from day 2 to 6. Data represent the mean ± standard deviation for n = 3 (biological replicates). Error bars are smaller than the symbol size.

Table 1
Amino acid (AA) content comparison between axenic and the tripartite community (mg/g of biomass). Data represent average ± standard deviation for n = 3 (biological replicates), n = 2 (technical replicates). Hashtag (#) indicates underestimated AA content due to its sensitivity to the hydrolysis treatment. An asterisk (*) indicates the significant difference between the two cultures (twotailed Student's t-test, P < 0.05). Dicarboxylic acid (when in nonionic form).

Group of AA		Tripartite community	Axenic culture
Monocarboxylic	Glycine	4.36 ± 0.87	4.86 ± 0.37
	Alanine	7.34 ± 1.65	9.22 ± 0.4
	Valine*	3.81 ± 0.90	5.38 ± 0.44
	Leucine	4.50 ± 0.78	4.40 ± 0.33
	Isoleucine*	2.28 ± 0.49	3.13 ± 0.30
	Total	22.28 ± 4.58	26.99 ± 1.57
Dicarboxylic	Aspartate*	4.37 ± 1.62	7.48 ± 0.78
	Glutamate	4.23 ± 1.73	5.82 ± 0.64
	Total*	8.59 ± 3.34	13.30 ± 1.42
Hydroxy	Threonine*	0.29 ± 0.04	0.48 ± 0.04
	Serine*	3.03 ± 0.38	4.52 ± 0.40
	Total*	3.31 ± 0.42	5.00 ± 0.43
Diamino	Arginine	40.43 ± 5.83	34.70 ± 2.29
	Lysine	2.37 ± 0.54	2.87 ± 0.39
	Total	42.80 ± 6.17	37.57 ± 2.47
Aromatic	Tyrosine*	0.95 ± 0.15	0.33 ± 0.078
	Phenylalanine*	3.34 ± 0.56	4.29 ± 0.40
	Total	4.29 ± 0.70	4.62 ± 0.39
Heterocyclic	Histidine*	0.70 ± 0.09	0.50 ± 0.05
	Proline	2.31 ± 0.34	2.61 ± 0.28
	Total	3.02 ± 0.42	3.11 ± 0.32
Sulfur-containing	Cysteine#	0.30 ± 0.12	0.34 ± 0.05
	Methionine#	0.40 ± 0.11	0.47 ± 0.12
	Total	0.70 ± 0.18	0.81 ± 0.15
	All AA	79.77 ± 6.40	85.24 ± 3.94

as well. The AA profile might change according to the conditions synthesis and activity of some enzymes, gene expression, and redoxhomeostasis [58]. In this study, 17 AAs were quantified (Table 1, Fig. 1B). The total AA (sum of 17 AAs) content of the tripartite community and axenic culture did not differ significantly (two-tailed, Student's t-test, P > 0.05). Tyrosine (aromatic) and histidine (heterocyclic) significantly increased (two-tailed Student's t-test, P < 0.05) by 191 ± 61% (from 0.33 ± 0.078 to 0.95 ± 0.15 mg/g of *Ulva* DW) and by 40 ± 26% (from 0. 5 ± 0.05 to 0.7 ± 0.09 mg/g of *Ulva* DW respectively in xenic culture). The content of six AAs significantly decreased (by about one-third) in the stationary phase (two-tailed Student's t-test, P < 0.05). The valine content decreased by 29 ± 24%, isoleucine, decreased by 27 ± 27%, aspartate, decreased by 42 ± 23%, threonine, decreased by 40 ± 11%, serine, decreased by 33 ± 16%, and phenylalanine, decreased by 22 ± 19% in the tripartite community. The content of arginine, lysine, alanine, glycine, proline, leucine, and glutamate did not depend on bacterial treatment. Clustering the AAs into groups (Table 1) showed that the total dicarboxylic- and the total hydroxy-AAs were significantly higher (twotailed, Student's t-test, P < 0.05) in the axenic culture. The total content of the groups, including of monocarboxylic-, diamino-, aromatic-, heterocyclic-, and sulfur-AAs, were not significantly different between xenic and axenic cultures. In a previous study, a dramatic difference was observed in the intercellular content of AA in diatoms upon bacterial addition [59]. The profile of intercellular dissolved AAs in diatoms considerably changed after co-cultivating the diatoms with bacteria [59]. The intercellular content of histidine was significantly higher in axenic culture, and the content of isoleucine was much higher in the consortium of diatoms and bacteria [59]. In our study, an opposite pattern was observed for both AAs.

3.5. Change of profile in Ulva photosynthates indicates the need for bacterial growth

Algae provide photosynthate for heterotrophic bacteria in symbiosis. Besides, algal compounds are utilized by the bacteria during the algal decomposition [60]. Therefore, we studied whether the bacterium-induced change in *Ulva*'s chemical profile of the photosynthate could be correlated to the bacterial eco-physiological function in the cross-kingdom interaction [31,32]. In other words, if the bacterium induces the changes in the algae biomass for its benefits, it could be the explanation for bacterial influence on the algal monosaccharides composition during the algal growth (Fig. 3). After testing the growth of *Roseovarius* sp. in *Ulva* culture medium (UCM) with different major *Ulva*'s monosaccharides as a carbon source (Fig. 4A), only weak bacterial growth was measured. Only glucose contributed slightly to the growth of *Roseovarius* sp. as reported by Spoerner et al. (2012) [41], but the optical density (OD_{600}) did not reach values higher than 0.15. The inability to grow sufficiently on glucose was also found for *Roseovarius mucosus* [61]. We thus argue that *Roseovarius* sp. (MS2) did not gain benefit from the algal monosaccharides. However, after 18 days of cultivation with 1% (w/v) glycerol in UCM, the OD_{600} reached 1.17 (± 8.16 E−03), showing the typical growth curve (Fig. 4A). The growth

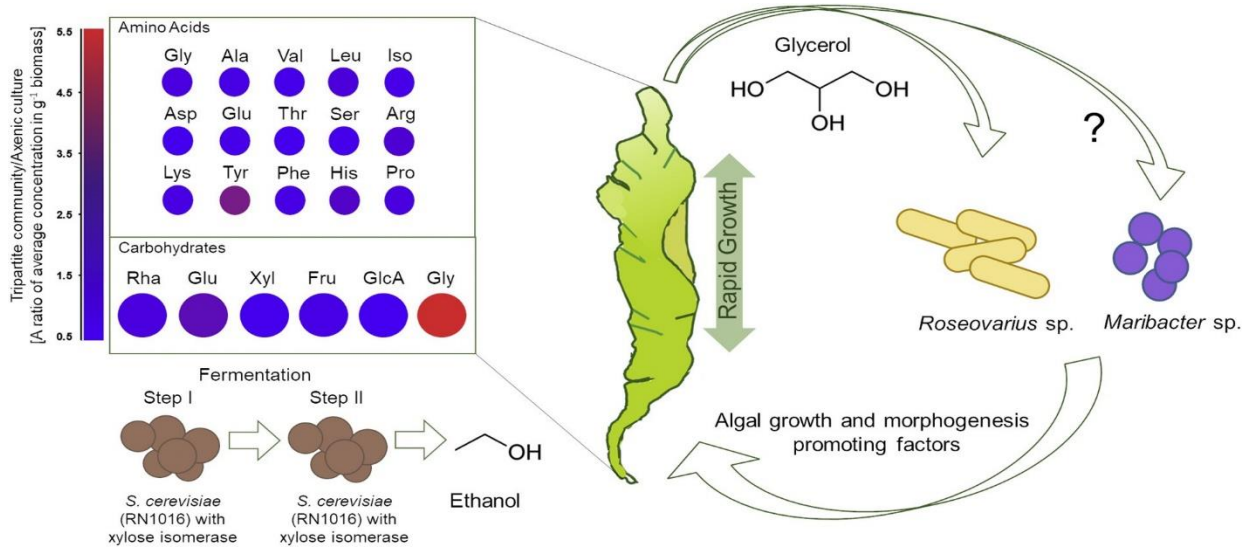

Fig. 5. Schematic diagram summarizing the *U. mutabilis* and it's associated bacteria interactions and the metabolic model analysis.

growth rate was 0.58 (change of OD_{600} per day) (Fig. 4B). It means that *Roseovarius* sp. (MS2) efficiently utilized and grew on glycerol as the only carbon source (Figs. 4, and 5) [31,32]. The current data support the previous report, where bacteria promote algal growth and morphogenesis [31,41,62,63]. In return, *Ulva* can provide the bacterial carbon source, as indicated by the elevated amounts of glycerol in the tissue. It supports the observation that algae growth-promoting bacteria are enriched in intensive land-based algal aquacultures compared to the seawater supplied to the aquaculture system [64].

At the same time, *Maribacter* sp. (MS6) did not grow on UCM supplemented with different monosaccharides or glycerol and needs complex media such as marine broth [32,Wichard and Weiss pers. observation]. The *Maribacter polisphoniae* might be an interesting exception because it grows on glycerol [65].

Overall, the insights in the algal-bacterial interaction pave the way to improved culture conditions, which might yield higher amounts of glycerol. Importantly, glycerol is an efficient carbon source for fermentation and biofuels production, such as bioethanol, under standardized conditions [66]. Our study paves the way for microbiome engineering to develop *Ulva* as a cash crop. *Ulva* affects its microbiome in intensive algal aquaculture, which promotes beneficial bacteria for the alga [67]. Inoculates of those bacteria need to be applied in order to test their effect on growth and the production of specific constituents. Indeed, our results support that the presence of bacteria is associated with changes in the content of photosynthates. Improved plant breeding has already been performed with plant probiotic bacteria successfully [68]. Also, bacterial-based biofertilizers have been considered as a promising application for increasing the yield of terrestrial crops in an environmentally-friendly manner, improving the plant's nutrient availability, and making the plant biomass to be more efficient for human needs [69]. We believe that the current data are a first step towards the development of algae promoting probiotics.

3.6. Simulation of bioethanol fermentation from Ulva biomass using metabolic flux balance analysis

The bioethanol yields from xenic and axenic cultured were estimated in-silico using 'BioLego' [36,50] (Table S5). In all simulations, we used the fermentation broth composition based on the measured values of monosaccharides, GlcA, and glycerol, and AAs in both biomasses. The fermentation was simulated for *S. cerevisiae* wild type (WT) [47] and recombinant strain with xyole-isomerase from *Piromyces* sp. [70], *E. coli* [48] and *C. acetobutylicum* [49].

Tripartite community derived *U. mutabilis* was a preferred feedstock for bioethanol production in most simulations. In those simulations, the majority of the bioethanol yield relayed on glucose and glycerol metabolism. Those two components were higher in *U. mutabilis* from the tripartite community (Table S4). Among all combinations, the two-step fermentation with the same organisms (Table S5, simulation no. 9, and Fig. 5) *S. cerevisiae* RN1016 (+xylose isomerase) resulted in the highest bioethanol yield, of 85.62 g/kg (using tripartite community). It is probably due to the additional pathways leading to bioethanol production in the presence of xylose isomerase [36,70]. The highest difference in bioethanol yields between approaches using the *Ulva* biomass from the tripartite community or axenic culture was detected in single step or two-step fermentation with *C. acetobutylicum* (Table S5, simulations No. 16 and 20).

Only in simulations where *C. acetobutylicum* was used in the first step and *S. cerevisiae* RN1016 (+xylose isomerase) or *E. coli* used in the second step, the axenic biomass led to larger bioethanol yield (although the total yields are low: 16.95–30.45 g/kg). Those exceptional simulations results might be explained by available carbon source during the fermentation. The first-step, contributed to higher bioethanol yield, using the tripartite community (3.85 g/kg) than from axenic culture (2.25 g/kg). At the second-step, more bioethanol was produced using axenic culture (with *S. cerevisiae* RN1016 (+xylose isomerase) 28.2 g/ kg or 14.7 g/kg with *E. coli*) than from tripartite community (with *S. cerevisiae* 19.5 g/kg or 11.54 g/kg with *E. coli*). On the first-step, *C. acetobutylicum* consumed all carbon sources except the xylose, which produced a relatively low bioethanol yield. *C. acetobutylicum* was thus the weakest bioethanol producer among all tested organisms so far [49,71–73]. In the second fermentation step, the bioethanol production was based on xylose metabolism. The larger the xylose content, the higher the yield of bioethanol in this fermentation step. Therefore, the usage of axenic biomass was probably more efficient for bioethanol production. In those two exceptional simulations, numbers 18 and 19 (Table S5), the bioethanol yield consuming axenic culture was higher in 30.4 and 10.1%, respectively, than consuming tripartite community biomass.

Conclusion

The current study demonstrates that macroalgae *U. mutabilis* associated bacteria modulate *Ulva* growth rate and the major photosynthate components. The studied constituents were monosaccharides, glycerol, glucuronic acid, and amino acids content after cultivation during the algal stationary phase before the occurrence of the next sporulation event. The quantity of the compounds was normalized to the dry weight of the harvested biomass. The tissue of *U. mutabilis* cultivated with the bacteria *Maribacter* sp. and *Roseovarius* sp. was enriched with glucose, glycerol, histidine, and tyrosine but decreased in the content of xylose, GlcA, valine, isoleucine, aspartate, threonine, serine, and phenylalanine compared to the axenic culture. The addition of two bacteria to *U. mutabilis* cultivation changed the ratio of rhamnose/xylose/GlcA, which became closer to the ratio found in *Ulva* with its natural microbiome. Although the factors are unknown, which are required to understand the complicated cause-effect relationship of bacteria-algae interactions, our observations linked the presence of the bacteria in the environment of *Ulva* with the formation of an essential constituent of the algal cell wall, development, and growth. Glycerol was the most affected component in the algal photosynthate by the bacteria. As glycerol is the preferred component for the growth of *Roseovarius* sp. (MS2), it is an additional insight into the glycerol function in the cross-kingdom interactions. The metabolic model simulations of *U. mutabilis* fermentation with *S. cerevisiae*, *E. coli*, and *C. acetobutylicum*, suggested the higher bioethanol yield after fermenting in xenic than axenic culture biomass. The highest yields were estimated from a two-step fermentation with S. cerevisiae (RN1016) that included the xylose isomerase.

In summary, our results are a valuable example of how the understanding of chemical ecology can help us to use associated macroalgal bacterial interactions to adjust the biomass feedstock for bioethanol production. Overall, this type of modulation opens new pathways for developing an efficient biorefinery based on macroalgae.

Authors' contributions

M.P. conceived the project, performed the chemical analysis and model calculations. G.C. carried out the cultivation and harvested the algae for further sample preparation. N.D. carried out the bacterial cultivation and their growth measurements. T.W. supervised the project in Jena during the Short Term COST Scientific Mission. A.G. conceived and supervised the project. All authors have analyzed the data and drafted the paper

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.101945.

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Towards marine biorefineries: Selective proteins extractions from marine macroalgae *Ulva* with pulsed electric fields

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ABSTRACT

Macroalgae are potential feedstock for biorefineries. However, integration of macroalgae into biorefinery network requires new processing technologies that will lead to energy efficient and zero waste conversion of macroalgae biomass into food, chemicals and fuels. Here we report on the selective extraction of proteins from green macroalgae from *Ulva* genus by electroporation with energy efficient pulsed electric field (PEF) process. We show that application of 75 pulses with an average electric field strength of 2.964 ± 0.007 kV cm^{-1}, and pulse duration 5.70 ± 0.30 μs, delivered at approximately 0.5 Hz, combined with hydraulic pressing of the treated samples for 5 min with force of 45 daN cm^{-2} led to the total protein concentration of 59.13 ± 3.82 μg mL^{-1} in the extracted juice. The final temperature of the extracted juice was 35.50 ± 2.02 °C. The energy consumption of the process is 251±3 kWh kg^{-1} of protein. We show that PEF process is selective and its extraction efficiency and damage are protein specific.

1. Introduction

Global population growth combined with the increase in quality of life in the era of changing climate will increase the demand for food, chemicals and fuels. The global demand for plant proteins is expected to grow from 4.73 in 2014 to 9.44 · 10^8 ton protein in 2054 (Stice, 2014). This growth in protein demand is expected to require additional 100 · 10^6 arable land hectares (Stice, 2014). Previous studies clearly show the positive impact of plant proteins consumption on sustainability and reduction of land, water, fertilizers and energy consumption (Pimentel & Pimentel, 2003). There is a consciously growing interest in exploring different plant sources for direct proteins use in the diet, either directly as entire plant or combined in the processed food products (Tuso, Ismail, Ha, & Bartolotto, 2013). In the last 5 decades microalgae biomass gained a lot of interest as a feedstock for proteins production (Becker, 2007). More recently food protein production is considered as a valuable co-product with biofuels in the algal biorefineries (Vanthoor-Koopmans, Wijffels, Barbosa, & Eppink, 2013). The production of microalgae biomass, however, is still cost prohibitive and further advances in the cultivation and harvesting technologies are required. Macroalgae, large multicellular organisms, have been mostly overlooked as a feedstock for protein production for many years (Golberg et al., 2014; Harnedy & FitzGerald, 2011; Lehahn, Ingle and Golberg, accepted for publication). However, many of the marine red and green macroalgae species have shown significantly higher content of proteins in comparison to the terrestrial plant proteins sources such as soy, nuts, and cereals (Fleurence, 2004; Harnedy & FitzGerald, 2011). In addition to their high yields and nutritional properties, marine macroalgae derived proteins and peptides have shown additional value because of their nutraceutical, pharmaceutical and cosmeceutical properties such as antioxidant, antihypertensive, immune-modulatory, anticoagulant and hepeto-protective substances (Fleurence, 2004; Harnedy & FitzGerald, 2011).

The value of the macroalgae as a protein source depends on the yields and functional properties. Previous work on the microalgae food proteins has shown that the economic viability of the algae proteins critically depends on the extracted protein yields (VanthoorKoopmans et al., 2013). In addition to yield, to achieve nutraceutical, pharmaceutical and cosmeceutical properties it is vitally important to preserve native proteins function. The complex, viscous and often charged macroalgae cell wall and extracellular matrix make the extraction process challenging (Joubert & Fleurence, 2007). Osmotic shock, mechanical grinding, high shear force, ultrasonic treatment, acid and alkaline pretreatment and polysaccharidase aided digestion and their combinations have been used to increase the extraction yields (Barbarino & Lourenço, 2005; Fleurence, Le Coeur, Mabeau, Maurice, & Landrein, 1995; Galland-Irmouli et al., 2000; Harnedy & FitzGerald, 2013; Rouxel, Daniel, Jérôme, Etienne, & Fleurence, 2001; Wong & Chikeung Cheung, 2001). Although the mentioned methods were shown to increase the extraction yields, they involve either thermal or

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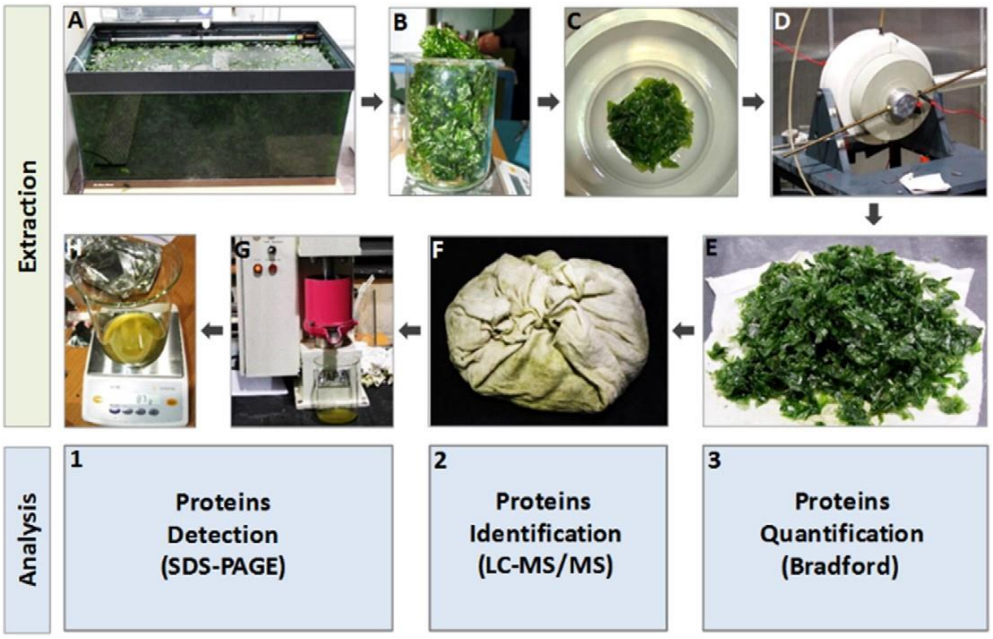

Fig. 1. Water soluble proteins extraction from macroalgae *Ulva* with pulsed electric field system. Extraction. A. Macroalgae storage system. B. External water removal. C. Biomass loading in the PEF treatment chamber. D. Application of pulsed electric fields for cell membrane electroporation. E. Treated biomass F. PEF treated biomass packing for mechanical extraction. G. Extraction of macroalgae intracellular liquid with mechanical press. H. Weighing of the extracted juice. Analysis. 1. Proteins detection with SDS-PAGE 2. Protein identification. 3. Total Protein identification.

chemical procedures that could affect the functionality of the extracted proteins and peptides. In this work, for the first time, we report on the chemical-free, non-thermal pulsed electric field (PEF) aided extraction of macroalgae water soluble proteins.

PEF is an emerging, non-thermal food processing technology already used to the energy-efficient extraction of proteins from microalgae (Goettel, Eing, Gusbeth, Straessner, & Frey, 2013; Parniakov et al., 2015), yeast (Ganeva & Galutzov, 1999; Ganeva, Galutzov, & Teissié, 2003), bacteria (Haberl Meglic, Marolt, & Miklavcic, 2015) and plants (Bluhm & Sack, 2008; Doevenspeck, 1961; Martin Sack & Bluhm, 2008; Vorobiev & Lebovka, 2010; Zagorulko, 1958). Although the exact mechanism of biological tissue permeabilisation by PEF is not fully understood, PEF technology is currently used in multiple applications in medicine and biotechnology (Kotnik et al., 2015; Rubinsky, 2007; Yarmush, Golberg, Serša, Kotnik, & Miklavčič, 2014). The current theory suggests that the membrane permeabilisation is achieved through the formation of aqueous pores on the cell membrane, a phenomenon known as electroporation (Weaver & Chizmadzhev, 1996). In the recent years significant advances in the industrial scale PEF system enabled the large scale use of the PEF process for biomass processing the sugar industry, saving up to 50% of the downstream energy investment in the process (Bluhm & Sack, 2008; Sack et al., 2009, 2010a,2010b;Sack & Bluhm, 2008; Sack et al., 2010a,2010b; Sack, Schultheiss, & Bluhm, 2005). Encouraged by this non-thermal, chemical-free, scalability and energy efficiency properties of PEF processes, we set out to test the working hypothesis that PEF will enable selective protein extraction from green macroalgae from Ulva genus, which has a potential to become a feedstock for marine biorefineries (Korzen, Abelson, & Israel, 2015b; Korzen, Peled, et al., 2015a).

2. Experimental

2.1. Biomass material

Ulva biomass was obtained from Alga Plus Company, Portugal (cultivated in the certified integrated aquaculture facility). Macroalgae were then stored for 2 days in a 400 L aquarium with a salt concentration of 3.5%.

2.2. PEF treatment

The fresh biomass was centrifuged at about 840 rpm for 3 runs of 1 min each to remove the external water, so that b5 g of water has been removed during the third run. 140 g of Ulva biomass in a 2 L becher was weighted with scale of type KERN 440-49N. This amount of macroalgae was then poured and pushed into the PEF treatment chamber with a volume of 232 cm^3 for the application of a homogeneously distributed pulsed electric field. Water was added to the macroalgae to fill the chamber completely. The chamber was closed to begin the PEF treatment. After the PEF treatment was applied, the macroalgae were collected and returned to the becher. The electroporated macroalgae were weighted again. The treatment parameters were:

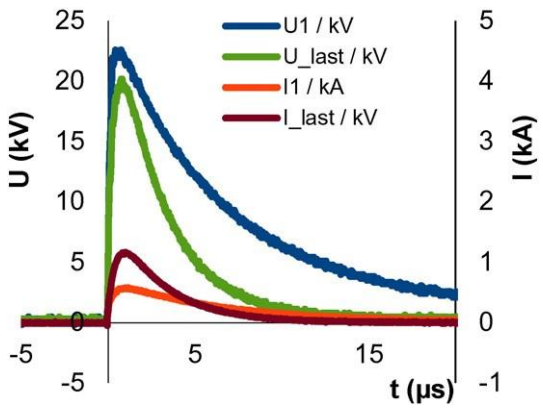

Fig. 2. Shape and magnitude of the single electric pulse delivered for macroalgae biomass electroporation. The shape for actually delivered voltage and measured current is shown for the first and last pulse in the series of 75 pulses applied on the biomass.

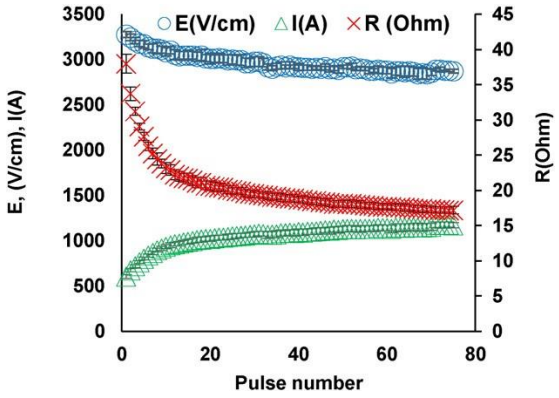

Fig. 3. Pulsed electric field and sample resistance behavior during macroalgae biomass treatment. Critical process parameters such as electric field (E), current (A) and biomass resistance (R) were monitored during each pulse. The experiment was done in triplicate. Error bars show ± SEM.

average field strength (average was taken between all pulses at all repeats, 225 measurements) 2.964 ± 0.007 kV cm^{-1}, and pulse duration 5.70 ± 0.30 μs, delivered at 0.5 Hz. These parameters were chosen based on the previous studies with PEF dehydration of various types of biomass with this system (Sack et al., 2009,2010a,2010b; Sack et al., 2008). Temperature was measured with a digital thermometer (TFA Type 30.1018). Current and voltage across the electrodes of the treatment chamber during each pulse were measured with a current probe (PEARSON 110 A) and a voltage divider (HILO-Test HVT 240 RCR), both connected to an oscilloscope (Tektronix TDS 640A). The impedance of the treated sample was derived from the current and voltage measurements.

2.3. Energy consumption

The total energy consumed for the PEF treatment was calculated based on the energy stored in the pulse capacitor with the following Eq.1:

$$E_t = 0.5 \cdot C \cdot 10^{-9}(V \cdot 10^3)^2 \cdot N \quad (1)$$

where E_t (J) is the total energy consumed for the treatment of one treatment chamber, C is the discharging capacitor capacitance (nF), V (kV) is the applied voltage and N is the total number of pulses. Additional losses of the capacitor charger have not been considered. The energy consumed with PEF for protein extraction was then calculated with Eq.2:

$$E_p = \frac{E_t}{m} / (C_p \cdot m_{PEF}) \quad (2)$$

where E_p (kWh kg^{-1}) is the PEF energy required to extract 1 g of protein, E_t (kWh kg^{-1}) is the total energy consumed to treat the PEF chamber, m (kg) is the raw mass of treated macroalgae, C_p (kg mL^{-1}) is the concentration of the proteins in the extracted juice and m_{PEF} (mL) is the volume of the extracted juice.

2.4. Mechanical juice extraction

The electroporated algae were placed in a cloth material that was folded so that the algae could not escape during pressing. The algae wrapped up in the fabric were placed in the mechanical press (HAPA type SPM 2.5S). A force of 45 daN cm^{-2} was applied for a determined time of 5 min using the automatic mode of the press that keeps the pressure applied to the piston constant. Extracted juice from pressing was collected in a 2 L becher and weighted at the end of the pressing. The pressed material was taken out of the press, weighted, reorganized

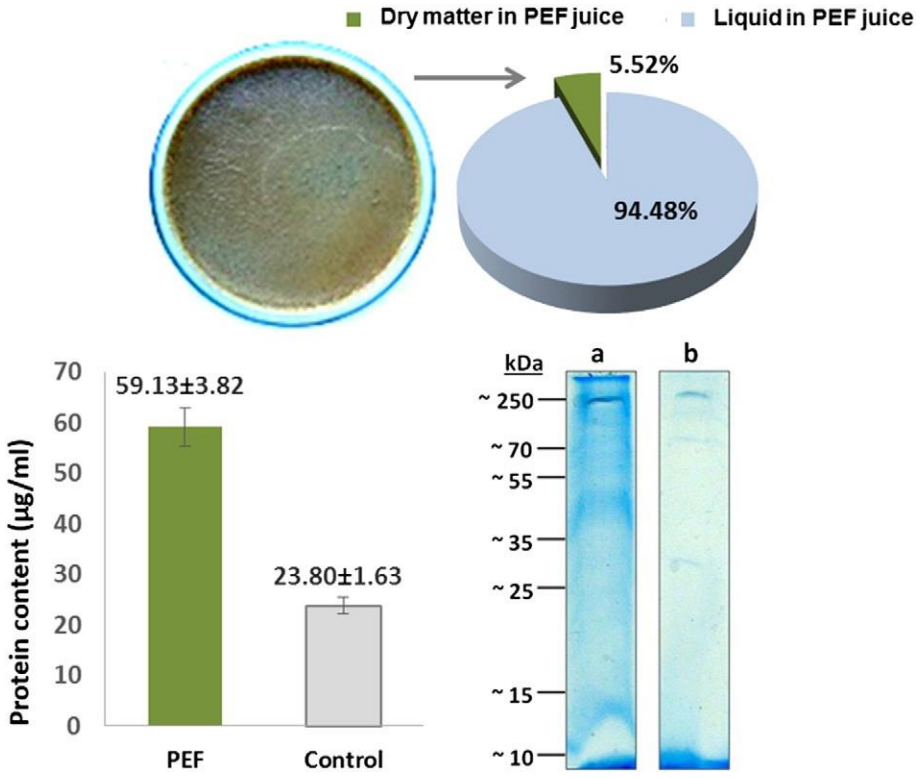

Fig. 4. Proteins extraction from Ulva. SDS gel electrophoreses (right) and total protein quantification with Bradford (left) Process parameters and total yields for 24 kV, 75 pulses applied. Error bars show ± SEM. Insert in the top row shows the total water soluble solids extracted from Ulva with PEF. The complete composition of the extract is still to be determined.

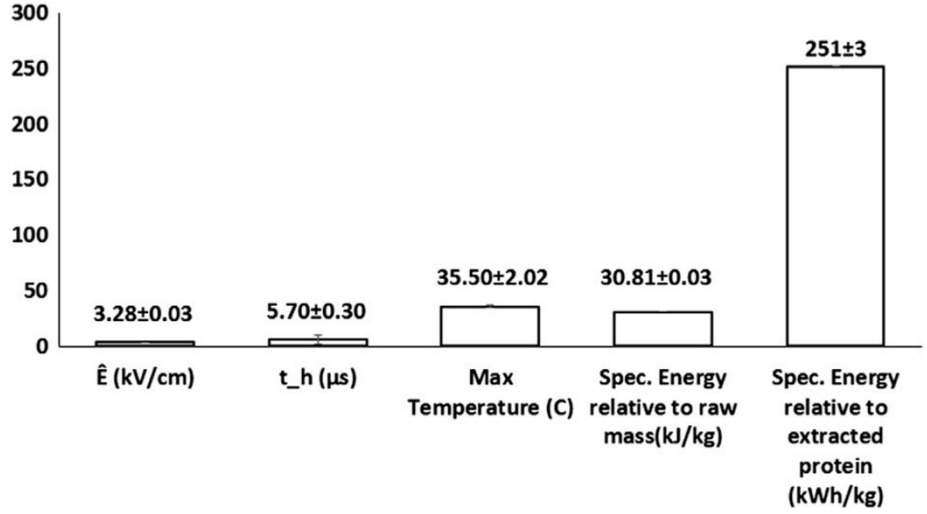

Fig. 5. Process parameters and energy requirement for proteins extraction from *Ulva* thalli.

and put back into the press for a second pressing step. The extracted juice and the pressed algae were again weighted.

2.5. Crude protein quantification

Bovine serum albumin (BSA) in DDW calibration curve was done in following concentrations:

1 mg mL^{-1}, 500 μg mL^{-1}, 250 μg mL^{-1}, 200 μg mL^{-1}, 150 μg mL^{-1}, 150 μg mL^{-1}, 100 mL^{-1}, 50 μg mL^{-1}, 25 μg mL^{-1}, 0 μg mL^{-1}. Every 10 μl BSA concentration mixed with 115 μl Bradford buffer. Extracted juice was filtered with 0.2 μm filter, 10 μL of samples mixed with 115 μL Bradford buffer. The BSA concentrations and the extracted juice samples with Bradford buffer were measured at optical density (OD) 450 nm and 590 nm. The numbers of OD 450 parts 590, were the basis for linear calibration curve. OD detection was done with an EL808, BioTek spectrophotometer (Winooski, VT, USA).

2.6. Gel electrophoresis

Extracted juice from pressing with/without PEF treatment was filtered with a 0.2 μm filter. Protein precipitation was made: 1 volume of Trichloroacetic acid (TCA) 100% (w/v) added to 4 volumes extracted juice samples. Then moved to 1.5 mL tubes. The samples incubated in 10 min at 4 °C. The tube spin in microcentrifuge at 14 K rpm, 5 min. Supernatant was removed, protein left in the pellet intact. Pellet was washed with 200 μL cold acetone. Spin in microfuge at 14 K rpm, 5 min. We made total of 2 acetone washes. Pellet was dried by placing tube in 95 °C heat block for 5–10 min. SDS-PAGE, 4X sample buffer (with βME) and sample boiled for 10 min in 95 °C heat block. The samples were run on SDS gel 12% agarose 200 V, 30 min.

2.7. Extracted proteins identification quantification with LS–MS/MS

2.7.1. Proteolysis

200 μL of the samples was brought to 8M Urea. The protein in 8M Urea, was reduced with 2.8 mM DTT (60 °C for 30 min), modified with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 2 M Urea, 25 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37 °C. One microgram from each sample was injected into a LC–MS/MS device.

2.7.2. Mass spectrometry analysis

The tryptic peptides were desalted using C18 tips (Homemade stage tips) dried and re-suspended in 0.1% formic acid. The peptides were resolved by reverse-phase chromatography on 0.075× 180-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 60 minute gradient of 5 to 28% 15 minute gradient of 28 to 95% and 15 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 μL/min. Mass spectrometry was performed by a Q Exactive plus mass spectrometer (Thermo) in a positive mode using repetitively full MS scan followed by collision induces dissociation (HCD) of the 10 most dominant ions selected from the first MS scan.

2.7.3. Computational analysis

The mass spectrometry data was analyzed using either the MaxQuant software 1.5.1.2 (Mathias Mann's group) or Peaks 7 software (Bioinformatic Solutions). The analyses were done vs. the green algae section of the NCBI-nr database with 1% FDR. The data was quantified by label free analysis using the same software. Intensity parameter mean: Summed up eXtracted Ion Current (XIC) of all isotopic

Table 1
Proteins detected only after PEF treatment.

Protein identification	NCBI accession number (GI)	Protein found in the species	MW (kDa)	Average normalized intensity (N = 3)
Calreticulin	255089467	*Micromonas sp. RCC299*	48	2.57E + 07
Ferredoxin-NADP+ reductase	545356935	*Coccomyxa subellipsoidea C-169*	38	6.26E + 07
Fructose-1,6-bisphosphatase	145345160	*Ostreococcus lucimarinus*	35	3.53E + 07
Fructose-bisphosphate aldolase 1	302831241	*Volvox carteri f. nagariensis*	41	3.42E + 07
Phosphoglycerate kinase	654120603	*Tetraselmis sp. GSL018*	45	2.30E + 07
Ribosomal protein L12 (chloroplast)	11467764	*Nephroselmis olivacea*	18	4.70E + 07
Predicted protein	145346523	*Ostreococcus*	32	3.41E + 07
Predicted protein	612389598	*Bathycoccus prasinos*	78	4.32E + 06

Table 2
Proteins extracted with water without PEF treatment. Not detected in the PEF treated samples.

Protein identification	NCBI accession number (GI)	Protein found in the species	MW (kDa)	Average normalized intensity (N = 2)
Amidohydrolase 2	693500897	*Ostreococcus tauri*	40	2.14E + 08
Calmodulin	654126732	*Tetraselmis sp. GSL018*	16	2.66E + 07
Cytosolic 80S ribosome and 40S small subunit	302839477	*Volvox carteri f. nagariensis*	16	3.45E + 07
Histone H4	761973387	*Monoraphidium neglectum*	11	1.33E + 07
Photosystem I iron–sulfur center, partial (plastid	269925003	*Volvox carteri f. nagariensis*	3	2.56E + 07
Hypothetical protein	612393855	*Bathycoccus prasinos*	36	3.28E + 07
Predicted protein	158274897	*Chlamydomonas reinhardtii*	58	1.33E + 07
Predicted protein	303280339	*Micromonas pusilla CCMP1545*	40	1.97E + 07

clusters associated with the identified AA sequence. For specific protein quantification, the intensity measured for each protein was normalized to the total intensity of all proteins from the same sample.

2.8. Statistical analysis

Statistical analysis was performed with Excel (ver. 13, Microsoft,WA) Data analysis package. All experiments and controls were done in triplicates unless stayed differently. Standard error of the mean (SEM) is shown in error bars. One side Student's t-test was performed for compare the total protein extraction yield to controls. MS statistical analysis for proteins identification was done as described in2.7.3.Thecriterion for inclusion was that the same protein was identified in at least two repeats from three.

3. Results and discussion

3.1. Process of proteins extraction from macroalgae Ulva with PEF

The process of protein extraction from macroalgae with PEF system for cell membrane disruption is shown in Fig. 1. First we analyzed the shape of the individually delivered electric pulse. Because of the cell membrane electroporation, the resistance of the treated macroalgae biomass reduces. Therefore we expected mild changes in the shape and pick values of each individual pulse. Fig. 2 shows the shape of delivered voltage and current at the first (U1, I1) and last (U_last, I_last) pulse in the delivered series of 75 pulses. The pulse source has been designed such, that a series of pulses of equal energy are applied. A pulse circuit based on a capacitor discharge has been employed. For a series of pulses the charging voltage of the capacitor has been kept constant. Pulses with an aperiodically damped shape are applied to the load. The peak current of the pulse is influenced by the resistance of the electrode system inside the treatment chamber and the stray inductance of the pulse circuit. The resistance of the treatment chamber decreases with the number of applied pulses, as discussed later. As a consequence, the pulse shape changes with the decreasing resistance, and the voltage across the electrode system decreases slightly with increasing number of pulses.

3.2. PEF parameters and changes in the macroalgae biomass during extraction

Next, we analyzed the changes in the peak electric field and current during the whole treatment (Fig. 3). We observed the decrease of the actual delivered electric field strength per pulse and increase of the current per pulse (Fig. 3). These changes are expected because of treated media conductivity increase (Fig. 3 shows the decrease of the resistance), which follows cell membrane electroporation and release of intracellular cell content. In the application of the 75 pulses on the biomass, the actual peak electric field decreased from 3.215 ± 0.033 kV cm^{-1} at the beginning of the treatment (first five pulses) to 2.864 ± 0.040 kV cm^{-1} at the end of the treatment (last five pulses in the series). The current increased from 713.6 ± 16.8 A at the beginning of the pulse series (first five pulses) to 1173.86 ± 30.8 A. These changes in the actual electric field and current are explained by the 46% drop of the sample resistance during the application of pulsed electric fields. Interestingly, we observed that the major decrease in the resistance (35%) and increase in the current from 713.6 ± 16.8 A to 1024 ± 26.7 A took place during the first twenty pulses. This is probably the number of pulse required to electroporation the majority of cells in the treated Ulva thalli. Previously, we have observed similar pattern of rapid resistance decrease and current increase in the skin tissue, where electric fields also electroporated cells inside the complex extracellular matrix (Golberg et al., 2013) and also in sugar beet tissue (Bluhm & Sack, 2008).

3.3. Extracted proteins yield

With the treatment parameters used in this study the average electric field strength applied on the macroalgae thalli was 2.964 ± 0.007 kV cm^{-1}, the pulse duration was 5.70 ± 0.30 μs (Fig. 5). The total dry matter (Fig. 4) consisted 5.52 ± 0.20% of the total extracted juice weight (Fig. 4). PEF increased the total protein extraction yields (p-val b 10^{-6}) (Fig. 4). The total protein concentration in the extracted

Table 3
Proteins detected in samples with PEF and without PEF treatment

Protein identification	NCBI accession number (GI)	Protein found in the species	MW	Averagenormalized intensity	
				(−PEF) (N = 2)	(+PEF) (N = 3)
Actin	116222105	*Pterosperma cristatum*	38	8.96E + 07	3.40E + 07
Heat shock protein 70	304555563	*Ulva pertusa*	73	2.80E + 07	4.81E + 06
Iron-superoxide dismutase 1	149275667	*Ulva fasciata*	25	3.04E + 07	9.46E + 06
Plastocyanin	3024399	*Ulva pertusa*	11	1.59E + 09	1.27E + 09
Plastocyanin precursor	48526878	*Ulva pertusa*	15	8.24E + 08	.14E + 09
Sedoheptulose-1,7-bisphosphatase	545353814	*Coccomyxa subellipsoidea C-169*	37	1.19E + 07	1.02E + 07
Ubiquitin	552821086	*Chlorella variabilis*	8	3.82E + 07	2.57E + 07
Uroporphyrin-III C-methyltransferase	303285200	*Micromonas pusilla CCMP1545*	32	1.66E + 08	1.01E + 07
Hypothetical protein	761971964	*Monoraphidium neglectum*	113	1.82E + 08	1.03E + 07
Predicted protein	145348138	*Ostreococcus lucimarinus CCE9901*	39	2.10E + 09	3.78E + 08

juice was 59.13 ± 3.82 μg mL^{-1} (Fig. 4). This is in contrast to 23.80 ± 1.33 μg mL^{-1} observed in the control samples. Importantly, the developed process has almost no thermal effects on the produced proteins as the maximum observed temperature was 35.50 ± 2.02 ^{0}C.

3.4. PEF process energy consumption

An important parameter in the production of proteins for food and feed application is the energy consumptions. Non-thermal pulsed electric field has been shown previously to reduce the total energy consumption of the sugar extraction by 30–50% at the industrial scale (Bluhm & Sack, 2008; Sack et al., 2010a,2010b;). Here we analyzed the energetic consumption of the pulsed electric field process for water soluble solids, including proteins, extraction from macroalgae Ulva. The specific energy consumed relative to raw mass of macroalgae was 8.56 ± 0.01 Wh kg^{-1} (30.81 ± 0.03 kJ kg^{-1}) and the specific energy relative to the extracted proteins was 251 ± 3 kWh kg^{-1}(Fig. 5).

3.5. PEF extracted proteins identification

Previous works have shown the use of PEF to extract proteins from microalgae (Goettel et al., 2013; Parniakov et al., 2015), yeast (Ganeva & Galutzov, 1999; Ganeva et al., 2003), bacteria (Haberl Meglic et al., 2015) and plants (Bluhm & Sack, 2008; Doevenspeck, 1961; Sack & Bluhm, 2008; Vorobiev & Lebovka, 2010; Zagorulko, 1958). However, to the best of our knowledge these were reported as crude protein extraction. In this work we identified and quantified specific proteins extracted from Ulva genus with PEF using LC/MS/MS (Supplementary information Table 1S shows all identified proteins in at least one sample). Proteins that have been uniquely identified in the PEF treated sample extracts that appear in Table 1 (criteria for inclusion werethe same protein that has been identified in at least two repeats from three). Proteins detected only in samples from Ulva biomass treated only with water, that appear in Table 2 and criteria for inclusion were the same protein that has been identified in at least two repeats from two). These proteins were not observed in the PEF treated samples and probably have been damaged by electric fields. In Table 3 we report on the proteins that have been detected in both PEF treated and nontreated samples. Interestingly, we observed that besides Plastocyanin precursor, PEF reduced the quantities of proteins extracted from Ulva biomass with tap water (based on intensity parameter). Our previous work with DNA showed that specific PEF parameters lead to DNA nicking (Golberg & Rubinsky, 2010). Additional studies showed that specific PEF parameters could lead to either activation or inactivation of enzymes, depending on enzyme type (Ohshima, Tamura, & Sato, 2007). However, most of the studies until today showed that PEF increases the extraction yields. Indeed, in our study PEF increased ~3 times the extracted proteins yields if measured in a bulk. However, the detailed identification and quantification of individual proteins, reported here for the first time, reveals a more complex scenario. Some proteins are extracted with PEF (Table 1 and Table 3), but some, which can be extracted with tap water are partially (Table 3) or completely (Table 2) degraded by the treatment. To the best of our knowledge this is the first report that identified proteins extracted from biomass with PEF; previous studies used bulk proteins characterization (Coustets et al., 2015; Ganeva et al., 2003). These findings are new and important, as they open a possibility to optimize PEF parameters for the extraction of specific proteins from macroalgae and other biomass. An important future application could be the inactivation of the ingenious proteases during protein extraction processes. Additional future studies will address the effect of PEF extraction on the functional properties of the extracted proteins. PEF provides a potentially unique non-thermal, chemicals-free proteins extraction method that could preserve the functional properties of the proteins, important for food and pharmaceutical applications.

4. Conclusions

Macroalgae are promising, but challenging sustainable feedstock for biorefineries. Complete zero waste conversion of macroalgae into food, chemicals and fuels will reduce the burden of the agriculture from arable land. Here we report on a new technology to extract green macroalgae Ulva proteins with electroporation by PEF. PEF is an emerging, energy efficient technology for biomass processing. We showed that PEF increases ~ by 3 times the total protein extraction, and is selective, as it increases the extraction yields of some specific proteins but damages others. This study demonstrates the scalable, energy efficient technology for extraction essential for food supply chemicals-proteins.

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In silico food allergenic risk evaluation of proteins extracted from macroalgae *Ulva* sp. with pulsed electric fields

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ABSTRACT

Extraction of protein from macroalgae, currently defined as "novel food", is challenging and limited information about the health impacts of these proteins is available. Here, we report on a non-thermal, chemical-free green macroalgae *Ulva* sp. protein extraction by osmotic shock combined with pulsed electric fields (PEF) followed by hydraulic pressure. The extracted proteins were identified and annotated to allergens using sequence similarity. The allergenicity potential of PEF extracted proteins was compared to osmotic shock extracts and complete *Ulva* sp. proteome, extracted with the thermochemical method. The PEF extracts contained 'superoxide dismutase' (SOD), a known food allergen, osmotic shock extract contained 'troponin C', and thermochemical extract contained two additional potential food allergens 'aldolase A' and 'thioredoxin h'. This study shows an importance and the need for deep investigation of algal proteins and protein extraction technology health impacts prior to large-scale release to the market of "novel food" derived proteins.

1. Introduction

The world population is growing and as a result, the need for food that doesn't require arable land and fresh water is increasing too (Subasinghe, Soto, & Jia, 2009). Indeed, the food supply will have to be increased by 70% until 2050 (Godfray et al., 2010), in order to answer the whole population demand. Although the 2014 global protein consumption was approximately 473 million metric ton (MMT), the 2054 protein consumption is currently forecasted to reach 943 MMT (Stice, 2014). The current worldwide challenge is to meet this demand sustainably. This challenge is tougher than a few decades ago when agriculture intensification with synthetic fertilizers, herbicides, and pesticides was the solution for the growing food demand (Alston, Beddow, & Pardey, 2009).

However, these forms of intensifications will no longer be an option due to its severe environmental impacts, such as reducing biodiversity, increasing greenhouse gas emissions and the pollution of the terrestrial ecosystems, freshwater, and marine habitats as a result of the nutrient run-off from the fertilizers (Tilman, 1999). The increasing protein demand is expected to require an additional $100{\cdot}10^6$ ha of arable land (Stice, 2014). If the source of the required protein supply remains the terrestrial agriculture, it will magnify the negative environmental impact and cause more ecological shifts (Tilman, 1999). The world protein demand for human diet and animal feed emphasizes the importance of finding new sustainable and environmentally friendly sources (Tilman, 1999; Van Krimpen, Bikker, Van der Meer, Van der Peet-Schwering, & Vereijken, 2013).

To accommodate this growing protein demand, alternative protein sources recently have been investigated (Bleakley & Hayes, 2017; Stice & Basu, 2015). The considered alternative protein sources for human diet come from well-known plants such as pulses (pea, chickpea, lentil, and bean) (Boye, Zare, & Pletch, 2010) and more exotic options: algae, insects, and lab-grown meat. The predicted protein market share of alternative proteins is expected to increase from 2.1% to 33% of the global protein market by 2054 (Stice & Basu, 2015). Among the alternative protein, the algae market share is predicted to be 18% (Stice & Basu, 2015). The algae consist of two main groups: plant-like organism-macroalgae (seaweed) and unicellular organismsmicroalgae. Both groups are considered in the recent years as feedstock for protein supply (Becker, 2007; Bleakley & Hayes, 2017). Macroalgae and microalgae could provide higher protein yield per unit area than terrestrial plants used as protein

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sources such as wheat, soybean and pulse legumes (Bleakley & Hayes, 2017; Van Krimpen et al., 2013). However, to make algal protein available for human and animal consumption, it should be extractable, digestible, and, most importantly, to be safe. Food allergy is one of the main concerns for the food safety in novel foods (Thomas et al., 2007). However, to the best of our knowledge, the question of potential food allergy has not been addressed for extracted proteins from macroalgae. This question must be addressed due to the fact that previously was found evidence for clinical sensitivity to green algae (Bernstein & Safferman, 1973).

The goal of this work is to address the two challenges for the development of new sustainable sources of macroalgal proteins: new technologies for extraction and preliminary assessment of allergenic potential. Our model species is a green macroalga from *Ulva* sp., a promising feedstock for biorefinery (Bikker et al., 2016). In *Ulva*, the protein varies between 9 and 33 % of the dry weight, depending on the growth location, the season of the harvesting, the specific species, and the pre and post-processing procedures that were done with the algae biomass (Fleurence, 2004). In controlled, cultivation condition, yields up to 45 tons (DW) per hectare per year were reported in Denmark (Bruhn et al., 2011), suggesting a theoretical yield of 4–14.8 tons per hectare per year of protein. At the same time, the richest proteins source that comes from terrestrial plants, forage legumes, could provide only 1–2 tons per hectare per year of protein (Van Krimpen et al., 2013).

Ulva sp. biomass could be used as a protein source as the entire organism (Fujiwara-Arasaki, Mino, & Kuroda, 1984) or the protein could be extracted or concentrated in the cake after extraction of other components (Bleakley & Hayes, 2017). Different methods to increase the extraction of the protein yield for macroalgae were described: aqueous, acidic, alkaline, enzymatic, mechanical grinding, high shear force etc. (Bleakley & Hayes, 2017). Recently have been investigated new cell disruption approaches which assisting in the protein extraction such as ultrasound or microwave-assisted, high-pressure homogenization extractions (Barba, Grimi, & Vorobiev, 2015). Each method or their combinations could be used (Bleakley & Hayes, 2017; Parniakov, Apicella et al., 2015; Parniakov, Barba et al., 2015). The concentrated protein extraction potentially could be added to different food products as an ingredient (Fleurence, 1999). An extraction of water-soluble protein from *Ulva* shows efficient digestibility by human intestinal juice (Fleurence, 1999).
However, current methods used for protein extraction often involve thermal or chemical procedures that could affect the nutritional value of the extracted proteins and peptides, and unwanted chemicals also could remain. Moreover, these methods may alter the allergenic properties of the food proteins (Thomas et al., 2007). To address these problems non-thermal, chemical-free protein extraction methods from macroalgae are needed. Pulsed electric field (PEF) is an emerging method for that is already used as an energy-efficient extraction of proteins from microalgae and plants (Bluhm & Sack, 2009; Parniakov, Apicella et al., 2015; Parniakov, Barba et al., 2015). We recently described a water-soluble proteins extraction from *Ulva* using PEF (Polikovsky et al., 2016). We also showed that PEF enables selective protein extraction (Polikovsky et al., 2016).

In the current work, we investigated the impact of various PEF regimes on crude protein extraction. In addition, we analyzed *in silico* the potential allergenic effect of extracted *Ulva* proteins. For this analysis, *Ulva* sp. protein extractions were done with osmotic shock and mechanical press with or without PEF or thermochemically. This study will support further the integration of sustainably produced macroalgae derived proteins into the global food and feed supply chain.

2. Materials and methods

2.1. Source of Ulva sp. biomass

Biomass of macroalgae *Ulva* sp. was supplied by AlGAplus (Aveiro, Portugal). The cultivation was done in a certified facility for aquaculture. After obtaining the macroalgae, it was stored for two days in an aquarium with a volume of 400 Liter, in seawater with a salinity of about 3.5%.

2.2. Proteins extraction using pulsed electric fields, osmotic shock, and mechanical press

In order to remove the external water from the *Ulva* sp. biomass, the biomass was centrifuged three times for 1 min each, at 840 RPM. After the centrifugation, 140*g* of *Ulva* sp. biomass were weighted in a 2 Liter Becher (by using KERN balance, model 440-49N). The *Ulva* sp. biomass was loaded into the PEF treatment chamber (working volume 232 cm^3). Freshwater was added to fill the chamber completely. This fresh water created an osmotic shock. The chamber was closed and PEF were applied. After the PEF treatment, the biomass was collected and weighed again. The treatment parameters were: 0–75 pulses, 12 or 26 kV of applied voltage (1.56 or 7.26 kV cm^{-1} field strength), and pulse duration 2.2–7.2 μs, delivered at 0.5 Hz. For each pulse, voltage and current the data were collected using a high-voltage divider (Hilo-Test Company, HVT 240 RCR). The current was measured with a probe from Pearson electronics (110 A). For collecting data about the voltage and the current, the high-voltage divider and the current probe were connected to an oscilloscope (Tektronix TDS 640A). For the temperature measurements, TFA digital thermometer (30.1018) was used. In total 74 samples were treated with at least three repeats per experimental condition with at least triplicates per experimental condition. The invested energy (E_t) was calculated using Eq. (1):

$$E_t = 0.5 \cdot \cdot (C\,V\,N) \cdot^2 \quad (1)$$

where C is the capacitance of the discharging capacitor (Farad); the applied voltage is *V* (Volt), the number of pulses is *N*. Any additional losses in the capacitor charger were neglected.
The specific energy that was invested for the protein extraction (e_p) was calculated by using Eq. (2):

$$e_p = E\,Yield_t / \quad (2)$$

where *Yield* (gram) is the extracted protein yield.

During the mechanical extraction with pressing, the algae biomass was wrapped in a folded cloth, for preventing the biomass escape during the process. The pressing with 45 decanewtons per square centimeter (daN cm^{-2}) was done with the mechanical press (HAPA Company (SPM 2.5S). The pressing was applied for 5 min in the automatic mode. During the extraction with the press, a juice was collected into a two Liter Becher and was weighed after the pressing process. The pressing matter that was left in the press was weighted, then after reorganizing the pressed biomass, the biomass was loaded back into the press for another pressing step. Finally, the extracted juice was frozen on a dry ice. As a control, we repeated the procedure exactly, excluding, however, the application of pulsed fields. During the control experiments, cells were broken partially by an osmotic shock.

2.3. Extracted proteins identification with LC-MS/MS

2.3.1. Thermochemical, PEF with osmotic shock and mechanical press proteins extraction for proteomic analysis

Proteins extracted by three methods were used for proteomic analysis: thermochemical extraction, PEF with osmotic shock and mechanical press and osmotic shock and mechanical press.

The thermochemical protein extraction method was done with urea buffer. 9 M urea, 400 mM Ammonium bicarbonate, 10 mM DTT were added to 50 mg (dry weight) of a sample, vortexed, and sonicated (5′, 90%, 10-10). Then, the protein reduction was done at 60 °C for 30 min.

Proteins extracted with PEF, osmotic shock, and mechanical press as described in Section 2.2 with the following specific PEF parameters:75 pulses, 24 kV (total capacitance 200 (nF)), average applied field strength of 2.964 ± 0.007 kV cm^{-1}, and pulse duration
5.70 ± 0.30 μs, delivered at 0.5 Hz.
Osmotic shock and mechanical press extraction were done as described in Section 2.2.

2.3.2. Proteolysis for proteomic analysis

The 200 µL samples algae after the osmotic shock and with PEF or without PEF was added to 8 M urea. Then the protein was in 8 M urea, and reduced by using 2.8 mM DTT (at a temperature of 60 °C, for 30 min), the modification done with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in room temperature for 30 min, in the dark conditions) and digested in 2 M urea, 25 mM ammonium bicarbonate.

After the protein extracted with the thermochemical method, samples modified with 37.5 mM iodoacetamide (in the dark, room temperature for 30 min) and the digestion is done in 1 M urea, 60 mM ammonium bicarbonate. An additional second digestion was done for 4 h. Modification with trypsin (Promega) at a 1:50 enzyme-to-substrate ratio done to all samples during an overnight at 37 °C. Finally, from each sample, one microgram was injected into an LC-MS/MS device.

2.3.3. Mass spectrometry analysis

The desalting of tryptic peptides was done by using C18 tips (UltraMicro, Harvard) then dried. The re-suspension has done in 0.1% Formic acid. The peptides resolved in reverse-phase chromatography on 0.075 × 180-mm fused silica capillaries (J&W), the capillaries were packed with 'Reprosil', a reversed phase material (Dr. Maisch GmbH, Germany). The elution of the peptides was done with Linear A gradient of 5–28% during 60 min, the gradient of 28–95% during 15 min and finally 15 min at 95% acetonitrile with 0.1% formic acid with a water flow rates of 0.15 µL/min. Mass spectrometry done with the positive mode of the 10 most dominant ions which selected from the first MS scan by using repetitively full MS scan with collision induces dissociation (HCD), in a Q Exactive plus mass spectrometer (Thermo Fischer Scientific, CA).

2.3.4. Computational analysis

The mass spectrometry data from the biological samples were analyzed using the MaxQuant software 1.5.2.8 (Mathias Mann's group) vs. the green algae section in the NCBI-nr database using 1% FDR. Data quantification was done by label-free analysis with the same software.

2.4. Extracted protein quantification

After protein extractions were done with osmotic shock, and mechanical press with or without PEF, or thermochemical extraction method all samples are filtrated with 0.22 µm pore size filter, and the protein was quantified using Bradford buffer (Sigma-Aldrich, Israel) using EL808, BioTek spectrophotometer (Winooski, VT, USA) with an optical density (OD) of 450 nm and 590 nm. Bovine serum albumin (BSA, Amresco) was used for a standard curve.

2.5. In silico allergenic risk evaluation of macroalgal proteins

All identified proteins were evaluated for potential allergenicity using two databases: AllergenOnline database (allergenonline.org) and SDAP-Structural Database of Allergenic Proteins (fermi.utmb.edu) (Ivanciuc, Schein, & Braun, 2003). Each protein was checked for allergenicity potential using the cutoff E-scores, which indicate homology with allergens detected in other organisms, of 10^{-7} for AllergenOnline and 0.01 for SDAP (Ivanciuc et al., 2003). The complete protocol for *Ulva* proteins extraction and allergenicity determination is shown in Fig. 1 and Table S8.

2.6. Statistical analysis

For statistical analysis, a Data analysis package in Excel program (ver. 13, Microsoft, WA) was used. All samples and controls were prepared and measured, at least in triplicates, if not mentioned differently.

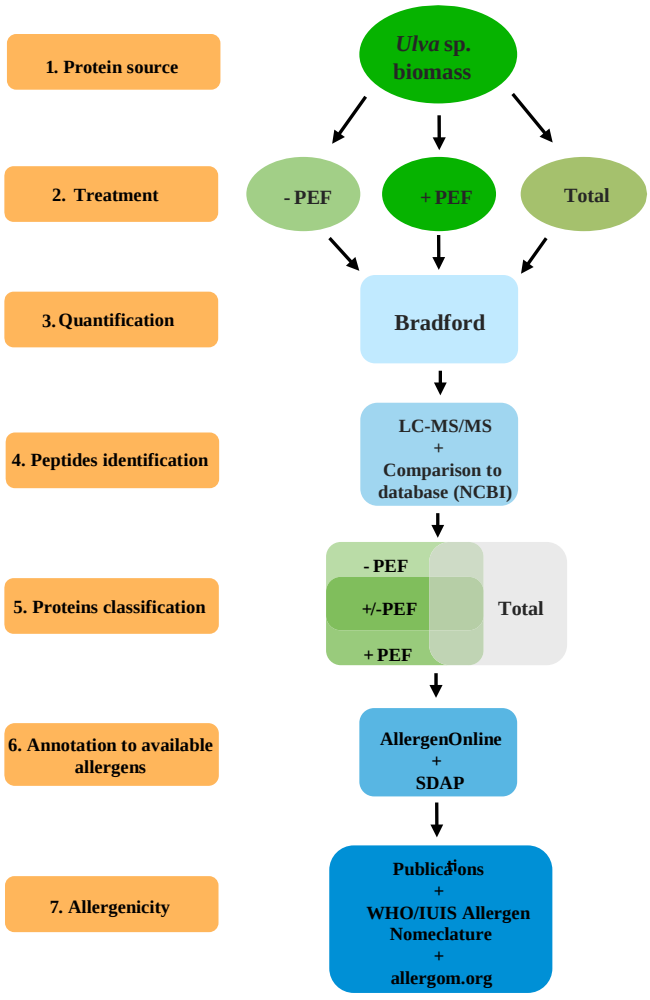

Fig. 1. Protein extraction and allergenicity determination method. The flowchart describes the allergenic identification procedure from extraction the proteins step up the functional analysis of the annotated allergens. The allergen annotation and allergenicity demined by following steps: 1. Protein source- was the *Ulva* sp. 2. Treatment- (−) PEF means treatment only with osmotic shock and mechanical press, (+) PEF means only with osmotic shock and mechanical press with PEF treatment. Method for total protein extraction that includes urea (9 M), sonication and high temperature (60 °C), as described in the methods section. 3. The quantification was done with Bradford. 4. Proteins identified after proteomic analysis. LC-MS/MS used for identifying peptides in the samples and the peptides analyzed with MaxQuant program, then the identified peptides compared vs. the green algae section in the NCBI-nr database for the proteins identification. 5. Proteins classified into groups appeared in Polikovsky et al. (2016) and in Table S8 (under the title 'Treatment') the classification done by the presence of the proteins after different proteins extraction methods. The proteins classification was to: (i) osmotic shock with the mechanical press = (−) PEF (ii) osmotic shock with the mechanical press and PEF = (+PEF). Osmotic shock with the mechanical press with or without PEF = (+/−) PEF. Total protein extraction includes urea, sonication, and heat = Total. 6. The identified proteins were annotated to allergens in two databases AllergenOnline and SDAP = Structural Database of Allergenic Proteins. 7. The identified allergens were discovered for its allergenicity effect using scientific publications, WHO/IUIS Allergen Nomenclature and allergom. org websites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The error bars are the standard error of the mean (SEM). To compare the extracted total protein yield to the controls, a two-tailed Student's *t*test was performed. Spearman correlation (r_s) was performed using RStudio (*RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/*). One-way

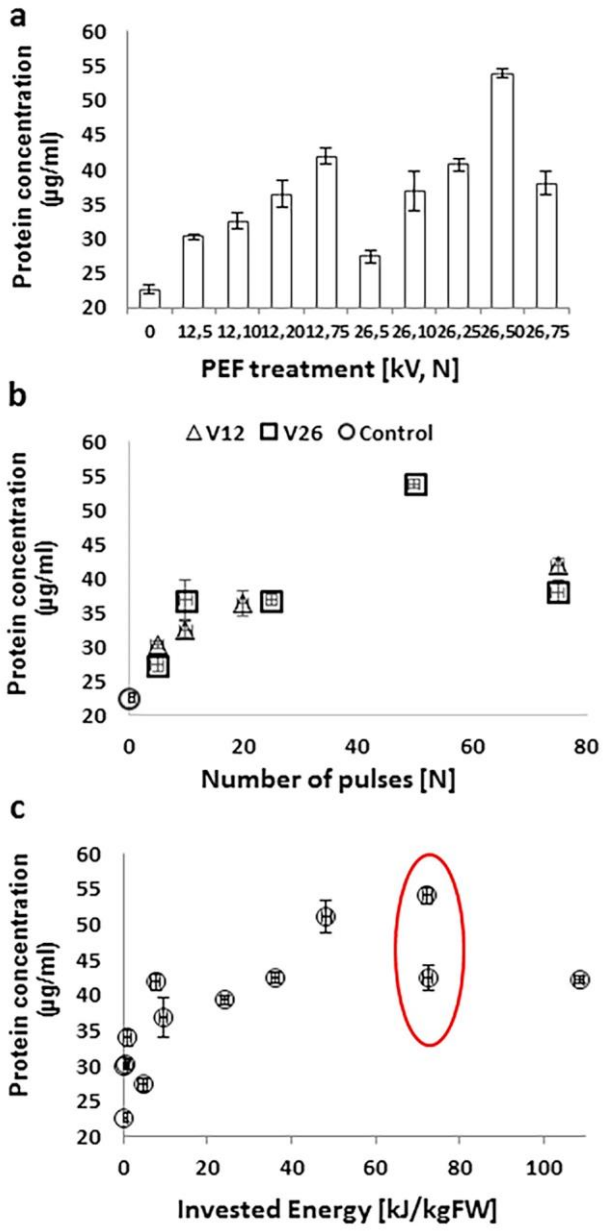

Fig. 2. The protein PEF extraction optimization from *Ulva* sp. a. Protein extraction (µg ml^{-1}) depend on PEF treatment (voltage and number of pulses). xaxis = first number (from left) is the charging voltage per stage [kV], the second number is the number of pulses. b. The protein concentration (µg/ml) dependence on a number of pulses. Triangles (V12) = PEF treatments with a voltage of 12 kV (kilovolts), squares (V26) = PEF treatments with a voltage of 26 kV. Circle = control, a protein extraction with an osmotic shock and press (without PEF). c. A protein concentration (µg ml^{-1}) dependence on the energy invested to extract the protein kJ per kg of fresh algae biomass. Detailed treatment protocols are described in Table S1, the x-axis values describe the numbers shown in the column 'spec. Energy relative to raw mass (kJ/kg)'. The dots in the figure are the averages of the PEF treatment with a difference in the range of ± ~1% in the invested energy. The y-axis of the chart displays the averages numbers of the extracted proteins for every invested energy. a–c: The protein extraction included PEF, osmotic shock, and pressure. The control was only osmotic shock and pressure without PEF. Protein quantified with Bradford assay. The columns and dotes represent averages of the biological replicates, respectively. Arrow bars = ± standard error, $n \geq 3$. Averages included at least of 95% of the biological replicas ($\mu \pm 2\sigma$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Analysis of variance (ANOVA) was done for group comparison with the significance level set up on 0.05. Identified protein is taken into consideration in the analysis, based on a peptide that appeared more than once in each sample and it detected in at least two biological replicates out of three.

3. Results and discussion

3.1. Protein extraction with pulsed electric fields from Ulva sp. biomass

The pulse shape of the voltage delivered in the first and last pulse of the 75 pulses (delivered in series) is shown in Fig. S2. Various combinations of PEF protocols for protein extraction were tested (Fig. 2 and Table S1) and showed significant differences between treated groups (ANOVA: df = between groups = 9, within groups = 41, $P = 9.17 \cdot 10^{-16}$, n = 51). We found that increasing the number of pulses from 5 to 75 at 12 kV led to the increase of the protein in the extract from 22.5 ± 0.64 to 41.9 ± 1.09 µg ml^{-1} monotonically (Fig. 2a, b). In addition, increasing the number of pulses from 5 to 75 at 26 kV led to the increasing of protein content in the extract until 50 pulses (from 27.3 ± 0.96 to 53.8 ± 0.69 µg ml^{-1}), further increasing of the number of pulses to 75 led to the total extracted protein yield reduction to 38 ± 1.67 µg ml^{-1} (Fig. 2a, b). This extracted protein yield reduction is in agreement with previous work that showed the effects of PEF processing on egg protein content and aggregation (Wu, Zhao, Yang, & Chen, 2014), which could prevent extraction.

The influence of the energy investment in PEF treatment for the protein extraction was calculated using Eq. (1) (Fig. 2c). After any energy investment, the extracted protein was higher than in control (df = 7, $P < 5.4 \cdot 10^{-5}$, two-tailed Student's t-test, n = 4), even after investing the lowest amount of energy (0.26 kJ kg^{-1} (Fresh Weight, FW, of *Ulva*), Fig. 2c, red circle). The extracted protein yield tended to increase with the increase of the invested energy (r_s = 0.77). However, the investment of 108 kJ kg^{-1} FW decreased the extracted proteins yield in comparison with 72 kJ kg^{-1} FW (Fig. 2c, Table S3, df = 4, $P < 0.01$, two-tailed Student's t-test, n = 3).

Interestingly, energy investment alone, could not explain the differences between extracted protein yields (Tables S3 and S4). For example, in samples where invested energy was 7.71 and 36.15 kJ kg^{-1} FW, no significant difference was observed in the extracted proteins yields. However, the investment of 72.29 kJ kg^{-1} FW led to 27.4% higher extracted protein yield than 72.43 kJ kg^{-1} FW (Fig. 2c red circle). In these two samples, the applied voltage was the same (26 kV) but the pulses amount and the capacitance were different, 50 or 75 pulses and the capacitance of 600 or 400 nF respectively for 72.29 kJ kg^{-1} FW and 72.43 kJ kg^{-1} respectively. These results show that the form of energy investment is critical in PEF process development.

The highest extraction yield of 53.8 ± 0.69 µg ml^{-1} was obtained with 50 pulses with 2.3 µs duration, applied at 26 kV, 7.26 kV cm^{-1} field strength. The final temperature after extraction with these parameters was 26.9 ± 0.4 °C. The energy investment was 72.29 kJ kg^{-1} FW or 1.5 ± 0.5 kJ $mg_{extracted\_protein}^{-1}$. It is important to emphasize that Bradford assay with the BSA standard curve, done in this work for protein quantification has limitations. When quantifying algae proteins and other stains reported in the literature, it showed significantly higher protein yields on the same samples probably because of the variation in the amino acid composition (Barbarino & Lourenço, 2005).

3.2. Protein quantification for proteomic analysis

The PEF method allowed to extract proteins but not all of them, for the comparison a method for total protein extraction including urea (9 M), sonication and heat (60 °C) was used. This method used before for proteomic analysis (Levitan et al., 2015). By using that method 738.1 ± 51.5 µg protein was extracted out of 50 mg dry weight (DW)

Table 1
Estimation of the potential allergenicity of proteins extracted from *Ulva* sp. biomass with osmotic shock and mechanical press (Bauermeister et al., 2011; Chen, Yang, Wei, & Tao, 2014; De Coaña et al., 2010; Hindley et al., 2006; Jeong et al., 2010; Ledesma, Villalba, & Rodrıguez, 2000; Tinghino et al., 1998).

Allergen name	Organism	Sequence Link in SwissProt /NCBI/PIR	GI source	Database	Identity %	*E* score (full FASTA)	Allergenicity
Tyr p 24.0101	*Tyrophagus putrescentiae*	ACL36923	219815476	AO	45.6	2.30E-21	Sera from 5 of the 47 subjects displayed positive IgE responses to
Tyr p 24.0101	*Tyrophagus putrescentiae*	ACL36923	219815476	SD	44.97	2.50E-26	the recombinant troponin C (Jeong et al., 2010).
Bla g 6.0101	*Blattella germanica*	ABB89296	82704032	AO	42.9	5.70E-19	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be
Bla g 6.0101	*Blattella germanica*	ABB89296	82704032	SD	42.28	1.90E-23	involved in muscle contraction (Hindley et al., 2006).
Per a 6	*Periplaneta americana*	Q1M0Y3	60678791	AO	41.5	6.40E-19	**Not food allergen** Per a 6 allergen was predicted to have nine strongly binding nonamer core epitope
Per a 6	*Periplaneta americana*	Q1M0Y3	60678791	SD	40.94	2.20E-23	sequences and 28 weakly binding sequences (Chen, Yang, Wei, & Tao, 2014).
Bla g 6.0301	*Blattella germanica*	ABB89298	82704036	AO	42.1	1.40E-18	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be
Bla g 6.0301	*Blattella germanica*	ABB89298	82704036	SD	42.95	5.30E-23	involved in muscle contraction (Hindley et al., 2006).
Bla g 6.0201	*Blattella germanica*	ABB89297	82704034	AO	41.5	1.90E-18	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be
Bla g 6.0201	*Blattella germanica*	ABB89297	82704034	SD	40.94	8.20E-23	involved in muscle contraction (Hindley et al., 2006).
MLC-1	*Gallus gallus*		55584149	AO	46.6	2.60E-18	
Cra c 6.0101	*Crangon crangon*		238477333	AO	40.8	2.00E-17	**Food allergen** 6/25 (24%) of shrimpallergic patients had IgE that reacted with Cra c 6 in IgE immunoblotting (Bauermeister et al., 2011).
Hom a 6.0101	*Homarus americanus*	P29291		SD	39.6	1.50E-22	**Food allergen** 6/25 (24%) of shrimpallergic patients had IgE
							that reacted with Hom a 6 in IgE*
Pen m 6	*Penaeus*	ADV17344	317383200	SD	39.6	8.50E-22	**Food allergen*** *monodon*
Jun o 4	*Juniperus oxycedrus*		5391446	AO	44.4	1.3E-16	**Not food allergen** Of 41 human sera from subjects allergic to Cupressaceae, 6 displayed IgE binding to
Jun o 4	*Juniperus oxycedrus*	O64943	5391446	SD	42.28	1.30E-20	run o 4 on immnublot (Tinghino et al., 1998)
Ole e 8	*Olea Europea*		6901654	AO	37	2.00E-15	**Not food allergen** The recombinant protein binds IgE antibodies from patients allergic to olive pollen (Ledesma,
Ole e 8	*Olea europea*	AAF31151	6901654	SD	36.24	3.40E-19	Villalba, & Rodríguez, 2000).
Amb a 10.0101	*Ambrosia artemisiifolia*	Q2KN25	AY894659**	SD	35.57	5.00E-19	**Not food allergen***
Cup a 4	*Cupressus arizonica*		261865475	AO	42.2	2.00E-15	Sera from 9.6% *Cupressus arizonica* allergic patients contain specific IgE antibodies against recombinant Cup a 4 (De Coaña et al., 2010)
Cup a 4.0101	*Cupressus arizonica*	ABP87672	145581052	SD	39.6	5.40E-19	

Database "AO" = AllergenOnline; "SD" = SDAP, *E* score < 10^{-7} indicates significant homology for AllergenOnline. *E*-score < 10^{-2} indicates significant homology for SDAP (Ivanciuc et al., 2003). Description of evidence for allergenicity is shown. Allergens annotated in both databases are highlighted in grey. In the allergenicity description all allergens that describe as 'not food allergen' or a food allergen, that information was taken from allergen.org site. In case of this information is not described this means that it was not described at allergen.org site. Asterisk = ALLERGEN NOMENCLATURE WHO/IUIS Allergen Nomenclature Sub-Committee – www.allergen.org. Two asterisks = No GI number. GenBank nucleotide number (NCBI).

Table 2
Estimation of the potential allergenicity of proteins extracted from *Ulva* sp. biomass with either only osmotic shock or PEF and mechanical press with osmotic shock (Achatz et al., 1995; Aki et al., 1994; An et al., 2013; Andersson et al., 2004; Crameri, 1998; Cui et al., 2016; De Vouge et al., 1998; Gruehn, Suphioglu, O'Hehir, & Volkmann, 2003; Miao & Gaynor, 1993; Postigo et al., 2011; Rihs, Chen, Rozynek, & Cremer, 2001; Shen et al., 1997; Wagner et al., 2001).

	Organism	Sequence Link in SwissProt/ NCBI/PIR	GI source	Database	Identity %	*E* score (full FASTA)	Allergenicity
Tyr p 28	*Tyrophagus putrescentiae*		105536584 2	AO	73.2	2.60E-179	**Not food allergen** 8 of 17 dust mite allergic subjects with IgE binding to *E. coli* synthesized recombinant protein (Cui et al., 2016).
48(	*Cladosporium herbarium*		729764	AO	70.70	1.30E-168	**Not food allergen** Of 62 *C. herbarium* sensitized patients (positive immunoblot of C.herbarum extract), 22% showed IgE binding to Cla h 4 on immunoblot (Achatz et al., 1995).
Cla h 5	*Cladosporium herbarum*	P40918, P42039		SD	70.44	5.10E-170	
Der f 28	*Dermatophagoi des farinae*		685432788	AO	69.50	7.40E-166	**Not food allergen** Serum IgE binding in 28 of 41house dust mites allergic subjects reacted to Der f 28. In Skin Prick Testing detected 7 of 10 (70%) dust mite allergic patients showed a positive reaction to Der f 28 (An et al., 2013).
Der f 28	*Dermatophagoi des farinae*		442565876	AO	67.30	8.50E-136	
Pen c 19	*Penicillium citrinum*	Q92260.1	14423733	AO	74.70	1.30E-131	**Not food allergen** Sera from 14 (41%) of 34 *Penicillium*- allergic patients showed IgE-binding to the recombinant Pen c 19 in dot immunoassay (Shen et al., 1997).
Pen c 19	*Penicillium citrinum*	Q92260		SD	54.75	9.40E-133	
Mala s 10	*Malassezia sympodialis ATCC 42132*		465797105	AO	29.80	2.40E-46	**Not food allergen** 69% of 28 atopic dermatitis patients had IgE to rMala s 10 by ELISA. ~35% inhibition of IgE binding to recombinant heat shock protein when rMala s 10 (heat shock protein) was in a concentration of 0.25 mg/ml and ~86 inhibition in the concentration of 3mg/ml of rMala s 10 (Andersson et al., 2004).
Putative heat shock protein	*Malassezia sympodialis*		28564467	AO	29.60	9.20E-46	
Mala s 10	*Malassezia sympodialis*	CAD20981	28564467	SD	29.11	3.20E-46	
Alt a 3	*Alternaria alternata*		14423730	AO	62.70	2.10E-30	**Not food allergen** rAlt a 3 recognized on immunoblot by 5% of the sera from *Alternaria alternata* sensitive patients (De Vouge et al., 1998).
Alt a 3	*Alternaria alternata*	P78983		SD	15.54	7.10E-31	
Cor a 10	*Corylus avellana*	CAC14168		SD	59.43	3.60E-145	**Not food allergen** Five of seven allergic patient sera demonstrated IgE binding to the Cor a 10 in the purified protein fraction as well as in the crude hazel pollen extract (Gruehn, Suphioglu, O'Hehir, & Volkmann, 2003).

(*continued on next page*)

Table 2 (*continued*)

Der f mag29	*Dermatophagoides farinae*	BAA04556		SD	12.37	7.40E-26	**Not food allergen** A 9.8% binding frequency of IgE in mite-allergic sera (41 cases) to a blot of Mag29 (Aki et al., 1994).
Amb a 4.0101	*Ambrosia artemisiifolia*	CBK52317		SD	4.51	1.8e-03	
Superoxide dismutase	*Hevea brasiliensis*		348137	AO	37.30	7.80E-28	
Hev b 10.0101(Mia o & Gaynor, 1993)	*Hevea brasiliensis*	AAA16792	348137	SD	37.66	5.80E-28	
IgE-binding protein MnSOD	*Hevea brasiliensis*		10862818	AO	39.80	1.40E-27	**Not food allergen** rHev b 10 able to abrogate 93% of IgE binding to rHev b 10 at a concentration of 1 µg/ml, and almost 100% of binding at concentrations greater than 50 µg/ml (Wagner et al., 2001).
Hev b 10.0103	*Hevea brasiliensis*	CAC13961	10862818	SD	34.2	1.00E-27	
MnSOD	*Hevea brasiliensis*		5777414	AO	39.30	3.80E-27	**Not food allergen** Two sera (1.27 kU/l and 0.43 kU/l) from 20 SB patients tested showed specific IgE antibodies to recombinant latex MnSOD (Rihs, Chen, Rozynek, & Cremer, 2001).
Hev b 10.0102	*Hevea brasiliensis*	CAB53458	5777414	SD	33.77	2.80E-27	
Manganese superoxide dismutase-like protein	*Pistaci*		149786150	AO	35.30	1.90E-26	**Food allergen***
Pis v 4.0101	*Pistacia vera*	EF470980	149786150	SD	35.06	1.40E-26	
Manganese superoxide dismutase	*Aspergillus fumigatus*		1648970	AO	34.70	1.90E-25	**Not food allergen** Of 54 patients with allergic bronchopulmonary aspergillosis (ABPA), 30 (56%) showed IgE binding to r Asp f 6 in ELISA. Of 35 A.fumigatus-sensitized patients without ABPA, 0 (0%) showed IgE binding to rAsp f 6 (Crameri, 1998). 1 µg of Asp f 6 inhibit ~ 8% of the IgE and 100 µg of Asp f 6 inhibit ~ 35% of IgE (Wagner et al., 2001).
Asp f 6	*Aspergillus fumigatus*	Q92450, AAB60779	1648970	SD	31.6	7.20E-25	
Asp f 6	*Aspergillus fumigatus*		83305645	AO	36.70	1.40E-24	
Manganese superoxide dismutase	*Malassezia sympodialis*		28569698	AO	33.50	2.50E-24	**Not food allergen** 75% of 28 atopic dermatitis patients had IgE to rMala s 11 by ELISA (Andersson et al., 2004)
Mala s 11	*Malassezia sympodialis*	CAD68071	28569698	SD	31.17	1.90E-24	
Allergen	*Malassezia sympodialis ATCC 42132*		465795607	AO	34.20	7.30E-24	
Alt a 14.0101	*Alternaria*		529279957	AO	34.20	5.20E-20	**Not food allergen** 28 from 30 patients with the previous diagnosis of respiratory allergies caused by *Alternaria* showed positive IgE (Postigo et al., 2011)

Database "AO" = AllergenOnline; "SD" = SDAP, *E* score < 10^{-7} indicates significant homology for AllergenOnline. *E*-score < 10^{-2} indicates significant homology for SDAP (Ivanciuc et al., 2003). Description of evidence for allergenicity is shown. Allergens annotated in both databases are highlighted in grey. In the allergenicity description all allergens that describe as 'not food allergen' or a food allergen, that information was taken from allergen.org site. In case of this not mentions meaning that not described at allergen.org site. Asterisk = ALLERGEN NOMENCLATURE WHO/IUIS Allergen Nomenclature Sub-Committee – www.allergen.org. Two asterisks = No GI number. GenBank nucleotide number (NCBI). The table derived 'Amb a 4.0101' allergen with a black line, means all the allergens above (includes this allergen) are annotated to Heat shock protein 70 and all allergens below are annotated to superoxide dismutase. kU/L = measurement of total IgEs, > 0.35 kU/L were considered positive (Inc, 2012).

algae. After PEF treatment with osmotic shock and the mechanical press 39. 04 ± 1.19 mg was extracted out of 140 mg dry weight (DW) algae while without PEF only with osmotic shock and the mechanical press was extracted 22.5 ± 0.64 mg out of 140 mg dry weight (DW) algae.

3.3. In silico estimation of the potential allergenicity of proteins extracted from Ulva sp. biomass

Extracted proteins, after identification (Table S7), were annotated to allergens from two databases (Fig. 1): AllergenOnline database (allergenonline.org) and SDAP-Structural Database of Allergenic Proteins (fermi.utmb.edu) (Ivanciuc et al., 2003). *In silico* identified potential allergens are summarized in Tables 1, 2, and S8. The allergens in Tables 1 and 2 are with significant similarity to the proteins found in samples after specific treatment, more details on those proteins described in Tables S5 and S6. All allergens found in two databases (AllergenOnline and SDAP) after comparing the sequence of calmodulin found in samples after the osmotic shock and mechanical press or with thermochemical method displayed in Table 1. The allergens presented in Table 2 are identified after sequence comparison to two databases, the proteins superoxide dismutase (SOD) and heat shock protein (HSP) found in samples either after osmotic shock with the mechanical press and osmotic shock with mechanical press including PEF treatment or after thermochemical method. All allergens identified in all treatments presented in Table S8.

The potential allergens and proteins, which were extracted only with osmotic shock and mechanical press treatments are shown in Tables 1 and S5. The protein detected only after osmotic shock or thermochemical treatment is calmodulin, annotated to 13 allergens, most of which are described as troponin C. Troponin C – is a calciumbinding domain. Troponin, actin, and tropomyosin are proteins that compose striated muscle (skeletal and cardiac). Troponin is a complex of three proteins: troponin C, troponin I and troponin T. This complex is a calcium receptive protein at the calcium regulation of muscle contraction (Grabarek, Tao, & Gergely, 1992). Troponin C protein is a calcium binding protein which is one of the most important families of allergens (Radauer, Bublin, Wagner, Mari, & Breiteneder, 2008). It has a helix–loop–helix structural motif with four EF-hand motifs (Grabarek et al., 1992). Troponin C is a parvalbumin (Grabarek et al., 1992), which is the major allergens coming from fish, ubiquitous pollen allergens (Radauer et al., 2008), mold mite (Tyr p 24.0101) (Jeong et al., 2010) and cockroach (Bla g) (Hindley et al., 2006). Bla g is troponin C mite allergen that recently reported to be a calcium-dependent (Hindley et al., 2006). Previous studies showed that 10.6% from the study group observed IgE binding to *Tyrophagus putrescentiae* recombinant troponin C. After addition of $CaCl_2$, the sera from some patients showed strong IgE responses and the effect increased approximately two-fold (Jeong et al., 2010).

The potential allergens and proteins which were extracted with either with PEF and osmotic shock and mechanical press or osmotic shock and mechanical press without PEF treatments are shown in Tables 2 and S6. The two proteins annotated to allergens are ‘Heat shock protein 70’ and ‘Iron-superoxide dismutase 1’. These proteins were annotated mainly to the allergens described as ‘Heat shock protein’ and ‘Superoxide dismutase’ respectively.

Heat shock proteins (HSP’s) are a family of proteins produced in the cells as a response to stressful conditions. Hsp-70 is recognized by antigen presenting cells (APCs) and can activate these cells (Nishikawa, Takemoto, & Takakura, 2008). All the annotated Hsp-70 allergens (Table 2) coming from dust mite (Tyr p 28, Der f 28 and Der f mag29) (Aki et al., 1994; An et al., 2013; Cui et al., 2016) and fungi (Cla h IV, Pen c 19, Mala s 10 and Alt a 3) (Achatz et al., 1995; Andersson et al., 2004; De Vouge et al., 1998; Shen et al., 1997). Previous studies described a large range of allergenic effects from approximately 5% (De Vouge et al., 1998) to 70% (An et al., 2013) (Table 2). For instance: the results of Skin Prick Test on dust mite allergic patients were that 7 of 10 (70%) showed a positive reaction to Der f 28 (An et al., 2013). The allergen.org site labels all the allergens that annotated to Hsp in Table 2 (and Table S6), as not food allergens.

Superoxide dismutase (SOD) is an enzyme that converts ion of superoxide (O_2^-) and hydrogen into peroxide (H_2O_2). This function is a defense mechanism against highly reactive oxygen spices at the cell. SODs are divided by its metal molecule in the active site Cu, Zn, Fe or Mn (Candas & Li, 2014).

The SOD type annotated in the current study is MnSOD (Table 2), which is a mitochondrial antioxidant encoded by genomic DNA and it’s gene upregulated by oxidative stress (Candas & Li, 2014). Previous studies show that SOD activity increased by a salinity stress in *Ulva fasciata* (Lu, Sung, & Lee, 2006). MnSOD was described as an allergen in *Aspergillus fumigatus* (Asp f 6) with cross-reactivity with the human MnSOD (Crameri, 1998). The allergen sources of all the annotated MnSOD allergens (Table 2) coming from the rubber tree (Hev b) (Wagner et al., 2001), pistachio (Pis v 4.0101 from allergen.org) and fungi (Asp f 6 and Mala s 11) (Andersson et al., 2004; Crameri, 1998; Wagner et al., 2001). Previous studies describe that recombinant Mala s 10 (HSP) and Mala s 11 (MnSOD), could play role in atopic eczema/ dermatitis syndrome (AEDS) (Andersson et al., 2004), both allergens were annotated at the study protein in either osmotic shock or PEF and mechanical press samples (Table 2).

The potential proteins and allergens which were extracted with thermochemical method appear in Tables S7 and S8. With thermochemical extraction, we successfully identified 98 proteins, which included 13 identified proteins extracted with PEF. Based on the correlation between the two databases for allergen identification, 13 proteins (extracted thermochemically) were identified with allergic potency, while only four of them are potential food allergens (Table S8, the details about calmodulin allergenicity displayed in Tables 1 and S5). In addition to previously found potential food allergens in PEF and osmotic shock extracts, thermochemically extracted proteins contained fructose-bisphosphate aldolase and thioredoxin. Those were annotated to allergens, aldolase A, and thioredoxin h, respectively (Table S8).

Aldolase A – known as fructose-bisphosphate aldolase – is a glycolytic enzyme that catalyzes the reversible conversion of fructose – 1,6 bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Previous studies showed that 50% of the patients with a clinical history of reaction to fish extract were found with IgE to aldolase A (Kuehn et al., 2013). The authors mentioned the importance of IgE to aldolase when IgE to parvalbumin (Kuehn et al., 2013).

The thioredoxin, a small redox protein, plays a role in many biological processes such as redox signaling. In human, this protein is a protein involving in indirect reactive oxygen species (ROS)-mediated response (Adler, Yin, Tew, & Ronai, 1999). This protein is annotated to three thioredoxin allergens Plo i 2.0101, Tri a 25.0101 and Mala s 13. Only Tri a 25 considered as a food allergen (Weichel, Glaser, BallmerWeber, Schmid-Grendelmeier, & Crameri, 2006). Tri a 25 is a sequence encoding to wheat thioredoxin. In a previous study, a recombinant protein was created for immunological studies (Weichel et al., 2006). Sera of bakers with occupational asthma for IgE-binding structures were tested. The recombinant protein cause for sensitization rate of 47% among bakers. Tri a 25 is sharing 74% identity to Zea m 25 a maize allergen which previously exhibited distinct IgE cross-reactivity (Weichel et al., 2006).

The information provided in this study is the first sign of the potential existence of allergens in the proteins extracted from *Ulva* sp. Our study also shows that the extraction method affects the extraction of potentially allergenic proteins. These results are

intriguing, as they suggest that a method for protein extraction with fewer allergens could be developed if the mass transport of allergens from the seaweed tissue to the solvent is understood. Future studies should provide more detailed information about the identified proteins as allergenic if are active or not. This understanding is critical before *Ulva* sp. derived proteins could be considered as a protein source for humans.

5. Conclusions

Macroalgae *Ulva* is a promising protein source. However, to be one of the sustainable alternative proteins it should have an optimal extraction process and most importantly, to be safe for a human consumption. One of the main risk assessments for human protein consumption is the allergenicity. Here we report an optimization of a water-soluble extraction method by using combinations of an osmotic shock, a mechanical press and an electroporation with PEF. The highest extraction yield of 53.8 ± 0.69 μg ml^{-1} was obtained with 50 pulses with 2.3 μs duration, applied at 26 kV, 7.26 kV cm^{-1} field strength. The final temperature after extraction with these parameters was 26.9 ± 0.4 °C. The energy investment was 72.29 kJ kg^{-1} FW or 1.5 ± 0.5 kJ $mg_{extracted\_protein}^{-1}$. The proteins that were released by using PEF, without PEF or thermochemical method for protein extraction were identified. The identified proteins sequences were annotated to allergens. A PEF treatment selectively avoids releasing of calmodulin protein compared to the control without PEF. This protein annotated to allergen type troponin C, which is a calcium-binding protein and one of the most important families of allergens, includes the food allergens. From the proteins that were released none selectively; with PEF treatment but also without treatment, two proteins were detected: 'Heat shock protein 70' (HSP) and Superoxide dismutase (SOD). Only SOD was annotated to food allergens. In the proteomic analysis of the proteins extracted with a thermochemical method, four potential food allergens were detected. These included SOD, calmodulin fructose-bisphosphate aldolase and thioredoxin, annotated to SOD (Hev b), troponin C, aldolase A and thioredoxin h (Tri a 25), respectively. To the best of our knowledge, the first evidence for macroalgae proteins to be a potential cause an allergic reaction done *in silico*. Nevertheless, more research on this topic should be conducted to get more practical information about the human immune system allergic reaction to the proteins extracted from macroalgae.

6. Author's contributions

MP quantified the protein content and did bioinformatics work on proteins identification and allergy estimation, and drafted the paper. FF, MS, FF, and GM did the PEF extraction experiments. AG conceived the study, analyzed the data and drafted the paper. All authors read and approved the paper.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.foodchem.2018.09.134.

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5. Discussion and synthesis

To close the scientific gaps for realizing marine biorefinery based on *Ulva* spp. as a feedstock, we defined four most relevant goals (section 3). The first paper [180] aimed to reach the two first goals. Indeed, we successfully identified how epiphytic bacteria modulate the *Ulva* growth rates and their photosynthate components (chemical composition), including sugar and protein. In addition, we defined the relevance of associated bacteria in their ability to efficiently modulate the *U. mutabilis* biomass for bioethanol production, rather than the axenic culture. These results fit our expectation for the first goal. The addition of *Maribacter* sp. and *Roseovarius* sp. to *U. mutabilis* culture significantly improved the *U. mutabilis* growth rate (in the growth phase) from 0.04 mm day $^{-1}$ in axenic culture to 3.79 mm day^{-1} in tripartite community. Furthermore, the concentration of monosaccharides, glycerol, glucuronic acid, and AAs were changed in the *U. mutabilis* tissue. In detail, after bacteria were added to *U. mutabilis* culture, the glucose and glycerol yields were increased (by 77 ± 19% and 460 ± 207%), while xylose and glucuronic acids yields were decreased (37 ± 14% and 46 ± 15%). A prediction of the bioethanol production via metabolic flux balance analysis ('BioLego') using the tripartite community and axenic culture as biomass feedstocks, met our expectations of the second goal. In fact, the biomass with a higher concentration of easily fermentable monosaccharides such as glucose and glycerol produced a higher ethanol yield. The metabolic analysis simulations evaluated two biomasses feedstocks: the axenic culture and the tripartite community, fermenting with *S. cerevisiae* (wild type or with xylose isomerase), or *Escherichia coli* or *Clostridium acetobutylicum*, in single fermentation or in two-step fermentation. In our case, the tripartite community biomass in a two-step sequential fermentation using (twice) *Saccharomyces cerevisiae* (RN1016) with additional xylose isomerase resulted in the highest bioethanol yield (85.62 mg g^{-1} DW) among all possible simulated combinations.

It is important to mention that as a preceding analysis for using the 'BioLego' model, we did an experimental work to validate the original *in silico* model [64]. Following this validation work, an updated and more advanced model was established, 'BioLego' 2.0 [62]. Naturally, the *Ulva* sp. biomass composition and growth rate are fluctuating due to abiotic and probably biotic environmental conditions [48,171,181,182]. This fluctuation challenges the biotechnological applications, which usually rely on robust feedstock, with a high yield of certain compounds [183]. For example, higher glucose amounts in the macroalgae

biomass resulted in a higher bioethanol production [184]. Thus, this chemical fluctuation challenge motivated the study of different methods for regulating the macroalgae biomass composition. These methods aimed to reduce uncertainties and increase the concentration of certain compounds [48,184]. Most of these methods are focused on the regulation of environmental abiotic conditions during the macroalgae cultivation, such as salinity [185], light [185], temperature [186,187], pH [188], oxygen [188], nutrients [48,185], and others [189]. For example, nitrogen starvation of *Ulva* sp, usually causes starch accumulation, by which means the glucose concentration in the biomass is increased [48].

The first paper in this thesis presented a valuable example of a potentially new biotic method using associated bacteria for controlling the biomass composition. The proven fact that bacteria affect the content of the biomass phytochemicals opens a door for new types of studies. These studies' orientation could be biorefinery development. For example, using the macroalgae-associated bacteria for controlling the macroalgae biomass composition and regulating its metabolic pathways, engendering the natural microbiome, and enriching certain bacteria by using them as 'probiotics'.

The methodological limitation in the first paper is the tested xenic conditions, which composed of addition of MS2 and MS6 bacteria with *U. mutabilis*. The *U. mutabilis* growth in the tripartite community resulted in dramatic difference in the algae growth rate compared to axenic conditions. According to the different growth rates and the small portion of biomass (~ 4 mg per sample), the analysis of the axenic culture become challenging. We could hypothesize that the algal growth rate is the dominant factor that correlated with the glucose yield. The rapid consumption of nitrogen can cause a nitrogen starvation that lead to a starch accumulation (high sugar yield) for the algae. For testing this hypothesis, future work can use an additional control by adding the nitrogen periodically during the experiment. However, to gain a better understanding of the bacterial role in the algae metabolites formation, the scope of tested bacteria should be extended and wider metabolic analyses should be done. Future studies can extend the control conditions by testing the metabolic effect on *U. mutabilis* of only MS2 or MS6 and *Ulva* natural microbiome. In addition, it is relevant to test additional bacteria types isolated from *U. mutabilis*, and their influences on the phytochemical profile of the algae. The biochemical mechanism under the bacterial effect of the described phenomena can be tested using further analyses such as untargeted metabolomics, transcriptomics and proteomics, in the chemosphere, and in the

algae tissue or in bacterial cells.

After the macroalgae was cultivated (and harvested), the next step in the biorefinery process is the extraction process. Following our motivation to extract protein efficiently we developed a new method using electroporation with PEF, as described in the second paper [20]. Adding PEF to the treatment significantly increased the protein extraction (59 ± 3.8 μg mL^{-1}) compared to the control (23.8 ± 1.6 μg mL^{-1}). The control included only mechanical press and osmotic shock (without PEF). The PEF treatment protocol was included by washing the *Ulva* sp. biomass with freshwater then applying 75 pulses, with an average electric field strength of 2.964 ± 0.007 kV (kilovolts) cm^{-1}, the intervals between pulses 5.7 ± 0.3 μs, along with hydraulic pressing of 45 daN cm^{-2} during 5 min. The invested energy for the treatment was 251 ± 3 kWh kg^{-1}extracted protein. It is important to note that this study was the first in the research field preforming protein extraction with PEF from *Ulva* sp. Since then, the amount of energy required for extracting protein from *Ulva* sp. with PEF has been improved by other researchers [116]. These researchers' protocol included an electric field of 7.5 kV cm^{-1} with a pulse duration of 0.05 ms, and their energy investment was only 6.6 kWh kg^{-1}extracted protein. Apart from to the PEF extraction method, in the second paper we listed the specific proteins that were extracted from *Ulva* sp. with PEF treatment. The properties of protein extracted with PEF were further investigated by others [190]. Interestingly, 20% of protein extracted with PEF performed antioxidant activity in 10-20 higher than other well-known proteins (protein from potato, β-Lactoglobulin, and bovine serum albumin). The high antioxidant activity might be due to non-protein extracted molecules [190].

Since we applied PEF treatment for extracting proteins from the macroalgae *Ulva* sp., this treatment method became a method for extraction or pre-treatment relevant to additional phytochemicals (for *Ulva* sp. biomass), such as starch extraction [18], desalting [18], de-ashing [191], and dewatering [192].

The final step in macroalgae-based biorefinery producing proteins is the risk assessment. After the proteins were synthesized in the alga cells, extracted, and purified, they must be evaluated for different food safety parameters (as detailed in section 1.4.2), to predict the risk of potential consumption by animal or human. Since the allergenic effect has a high chance to become potent when proteins are concentrated [193], we aimed to evaluate the

allergenic effect in case of consuming *Ulva* sp. protein (Goal 4). This evaluation is much more reliable when the optimal extraction protocol is considered [159–161], hence we evaluated the protein allergenicity extracted with PEF. In addition, we compared the results to two controls: the same treatment without PEF (osmotic shock and mechanical press) and total protein extracts. The results of this work were published in the third paper [194]. First, the PEF treatment was optimized using the pulse strength of 0,12 and 26 kV, with a number of pulses 0-75. Pulse duration, osmotic shock condition, and mechanical press were identical to those we had already used in the second paper (in the thesis). The optimal energetic treatment was performed in the field, with a strength of 26 kV ($7.26\ kV\ cm^{-1}$), 50 pulses with duration 2.3 µs, the energy investment was $72.29\ kJ\ kg^{-1}_{extracted\ protein}$, yielded $53.8 \pm 0.69\ \mu g\ ml^{-1}$ extracted protein. The proteins sequences were identified using a proteomic analyses, and compared *in silico* to known allergens. The PEF treatment exhibited a selective extraction of food allergens. Using PEF, the known food allergen superoxide dismutase' (SOD) was extracted. The extraction control with only osmotic shock and the mechanical press released the food allergen 'troponin C'. The thermochemical method extracted two mentioned allergens, with an extra two food allergens; 'thioredoxin h' and 'aldolase A'. These results show that we met our expectations of goal 4, by defining the optimal PEF treatment in a given range of parameters and by successful definition of selective allergen extraction.

One limitation of the described studies in the second and third papers is the *Ulva* type, which was not genetically identified. This study was done with biomass from AlgaPlus Company hence the main *Ulva* sp. was probably *Ulva rigida* [195]. However, future study could include genetic identification of the *Ulva* sp. Future analysis could check if different species of *Ulva* are affected differently by a PEF treatment that might influence on the extracted proteins and other phytochemicals yield and on the extraction of different allergens. An additional limitation in the third paper is the database-based approach of the study, which restricts the analysis to the limited scope of the database and to the known information about *Ulva* spp.. This study approach provides only theoretical conclusions.

The raising of scientific studies can extend the data that will be enquired in such studies. Although the *Ulva* genome has been recently sequenced, a further annotation via dipper transcriptomic and proteomic analyses might assist to provide more relabel allergenic analyses. Future studies can use the concision of this work as a base for developing more

practical approach, for instance by concentrating the allergens and testing their allergenic potential in the conventional immunological methods. Finally, the theoretical work accompanied by laboratory tests could be a basis for future clinical trials.

Since the third paper was published, it attracted the attention of researchers focused on the macroalgae food safety issue [196,197]. Interestingly, the study of Garcia-Vaquero [71] used a similar approach (as in the third paper) for protein extraction and *in silico* peptides identification from macroalga *U. lactuca*, but with additional bioactivity tests. In this study, the authors detected peptides with the bioactive properties of lowering human blood pressure. Finally and importantly, after the second and third papers were published, the PEF treatment became an additional standard option for protein extraction from macroalgae [18,116,190,198].

Marine biorefinery based on *Ulva* sp. biomass could become efficient and sustainable once the challenge of co-production at a high yield of different products will be solved. The additional challenge in the biorefinery approach is to design the production of the products in a low environmental footprint and still keep the safety of the products for humans. Therefor we tried to develop a new and creative industrial approaches for address these challenges. Those approaches dealt with the challenge of improvements the biomass from the upstream cultivation step up to the downstream processing steps and involved safety tests. According to this work, bacteria can influence the biorefinery in the upstream step of algae growth and increase the sugar yield and protein composition (based on AA profile) of *U. mutabilis* biomass. This finding can be relevant for the downstream processing step of co-extraction proteins and also sugars using PFF [18]. Even though in the second and third papers the PEF treatment was focused on the protein extraction, a further work followed these papers described how PEF treatment is separating the *Ulva ohnoi* protein fraction from the sugar fraction [18]. The PEF treatment solubilized proteins and release them into the liquid phase while the sugars remained at the solid fraction [18]. The two fractions are shown in Fig. 4, Paper 2.

As it was already mentioned, the PEF treatment does not require any additional chemicals during the processing hence this method could be definitely considered safe for the environment [199]. Moreover, PEF treatment is not only safe to the environment but the non-chemically treated extracted sugars potentially can be easily fermented by yeast (for

instance to bioethanol), since there are low chances that yeast inhibitors will appear after PEF treatment as although it should be verified in future studies. However, it was already shown that PEF treatment can remove the de-ashing of the solid fraction (mostly carbohydrates) in *Ulva* sp. [191]. Importantly, deashing is already proven to be an efficient step for enhancing bioethanol production [200]. Interestingly, it will be relevant to test the fermentation of bioethanol sugars from the *Ulva* biomass after it was cultivated in an engineered microbiome and extracted with PEF. The sugar yield can be increased in the biomass due to the bacteria, the sugar extraction can be done efficiently with PEF and the fermentation can be improved due to the de-ashing process. In addition to the chemical environmental safety issue, minimalizing the algae cultivation site could certainly reduce the biorefinery ecological footprint [201]. Therefore, increasing dramatically the sugar yield in the algae, for instance by two times (Paper 1), might lead to the reduction of the algae cultivation site by two times for providing the same demand.

So far the most common approaches to control the biomass are usually via chemicals or genic engineering, using these approaches for macroalgae cultivation might cause environmental and humans safety issues, as it is described in case of terrestrial agriculture [202–204]. Therefore, our motivation was to exclude external chemicals or genetic manipulations but still improve the algae growth and control its metabolites content. Thus, we chose to test the algae-associated bacteria. We found how the algae-associated bacteria could improve the algae biomass, yet this biomass was not yet tested for food safety. Anyway, future work should test how manipulation of algae-associated bacteria affects the environment and food safety including the allergenicity potential of the algae.

Successful industrial implementation of the findings in this thesis might be done after testing the investigated new approaches and experimenting with a wider range of algae and bacteria. In addition, further works could experimentally validate the *in silico* works of ethanol yield and the allergenicity predictions. Finally, increasing the working scale from a lab-scale to a semi-industrial pilot scale and then to an industrial marine biorefinery scale.

6. Conclusions

The marine biorefinery based on macroalgae feedstock is a promising but challenging solution for the production of energy, food, and chemicals. The green macroalgae *Ulva* sp. has high potential to become the optimal biomass feedstock for marine biorefinery, due to its worldwide distribution, rapid growth rate, and high content of sugar and protein. Therefore, it was targeted as the most relevant biomass feedstock for marine biorefinery. Finding attractive new approaches for improving biomass growth, extraction yield, and energy efficiency upstream and downstream of biomass processing will allow realizing large-scale marine biorefinery.

Here we report three approaches relevant for every biorefinery step. In the first step that includes biomass growth and development, we showed how the associated bacteria of *Ulva* affected the algal growth of the photosynthate. The epiphytic bacteria affected the AAs, monosaccharides, glucuronic acid, and glycerol concentration in the alga biomass. Importantly, the affected biomass becomes more relevant for bioethanol production. After the biomass growth, the second step in the biorefinery is the extraction process. Following the need for a new protein extraction method, which should be production-efficient, energy-efficient, and chemical-free, we developed an extraction method for *Ulva* sp. biomass, using electroporation via PEF. We performed a protein extraction optimization and a proteomic analysis for extracts and included a control proteomic analysis of total protein (as possible). This work was the first attempt of using this emerging technology application in *Ulva* sp..

The final step in the biorefinery is the product safety assessment. The extraction of proteins concentrates them; increasing the hazard of an allergenic effect. Because we intended to do a responsible hazard assessment, we took into consideration the extraction method. Therefore, the *Ulva* sp. protein food allergenic risk was evaluated *in-silico* after it was extracted with optimal PEF protocol. Interestingly, PEF treatment led to selective extraction of potential allergens.

These novel approaches will maximize potential of the *Ulva* sp. biomass to be a new feedstock for large-scale biorefinery. The energy-efficient and feasible transformation of *Ulva* sp. biomass into protein and biofuels could provide a viable alternative to the current use of agricultural crops.

7. References

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תקציר

קצב גידול האוכלוסין המהיר והצורך בייצור בר-קיימא של למזון ומקורות אנרגיה הם בין האתגרים הגדולים של האנושות בעשורים הקרובים. על מנת לענות על צרכי האנושות באופן בר-קיימא נדרשים מקורות ביומסה חדשים. שטחי היבשה המוגבלים, המשאב הנדיר שמהווים מי השתייה, יחד עם מפגעים סביבתיים והיעדר טכנולוגיות עיבוד חסכוניות באנרגיה מעמידים בספק את יכולתם של גידולים היבשתיים לספק את הדרישות ההולכות וגדלות של מזון ומקורות אנרגיה.

לחלופין, אצות ימיות רב-תאיות יכולות להוות ביומסה אלטרנטיבית, משום שמקור זה אינו מתחרה על מי השתייה או שטחים יבשתיים שמשמשים לגידולי מזון. האצה מסוג *Ulva* (חסנית הים) היא מקור אטרקטיבי במיוחד לביומסה, בשל קצב הגידול המהיר, יכולת ההסתגלות שלה לתנאי אקלים מגוונים, קלות הקצירה ותכולת הפחמימות והחלבון הגבוהים הקיימים בה. למרות הפוטנציאל הגלום לשימוש באצות ים כמשאב, בקרת ההרכב הכימי של האצות בייחוד הסוכרים וחלבון, הוא אחד האתגרים העיקריים להשמשת *Ulva* כביומסה לצרכי מזון, הזנת בעלי חיים ומקורות אנרגיה. אתגרים נוספים לייצור מקורות חלבון חדש מבוסס על *Ulva*, כוללים מיצוי יעיל של החלבון יחד עם ניהול סיכונים של צריכה פוטנציאלית של אותו חלבון כמזון.

עבודות המחקר המוצגות בתזה בוצעו על מנת לספק פתרון לאתגרים שהוזכרו. עבודה ראשונה המוצגת בתזה מעלה כי שני חיידקים החיים באופן טבעי על האצה, והיו חלק מקונסורציום מהונדס של *Ulva mutabilis* השפיעו על קצב גידול האצות ובנוסף על תכולת הסוכר, החלבון וכימיקלים אחרים. בנוסף נעשה שימוש במודל מטבולי ממוחשב לצורך הערכת תפוקת האתנול לאחר תסיסה תיאורטית של אצות אקסניות (ללא חיידקים) לעומת הקהילה המשולשת (אצות עם שני חיידקים). המודל לקח בחשבון אורגניזמים בעלי יכולת התססה כמו *Saccharomyces cerevisiae, Escherichia coli* ו-*Clostridium acetobutylicum*. המודל בדק אפשרויות שונות של בתהליכי תסיסה חד-שלבית או דו-שלבית. לבסוף, מודל זה הראה כי הוספת החיידקים במהלך הגידול של האצה השפיעו על הרכב ה- *Ulva* באופן כזה שביומסת האצות הפכה ליותר יעילה ורלוונטית לצורך ייצור ביו-אתנול.

בעקבות האתגר של מיצוי חלבונים יעיל לצורך שילוב ביומסת *Ulva* בבתי זיקוק ביולוגיים, עבודה נוספת בתזה התמקדה בפיתוח פרוטוקול להפקת חלבון באמצעות טכנולוגיה המערבת פולסים חשמליים (PEF). בעבודה זו הוצג מיצוי של חלבונים מביומסה של האצה *Ulva* שהתאפשר באמצעות אלקטרופורציה באמצעות תהליך (PEF); טכנולוגיה ידידותית לסביבה, חסכונית באנרגיה ולא מערבת חימום, בנוסף לכך שתהליך המיצוי לא דורש הוספת חומרים כימיים. כמו כן, דגימות החלבון שהופקו מהאצות עברו ניתוח פרוטאומי, בו התגלה כי שימוש בתהליך PEF גורם להפקה סלקטיבית של חלבונים ספציפיים.

על מנת ליצור "מקורות מזון חדשים" או מקורות "חלבון חדשים", נדרשות הערכות סיכון בעקבות חשיפה פוטנציאלית לאותו חלבון, כולל בדיקת אלרגניות. כחלקת מהערכת הסיכונים יש חשיבות לכך ששיטת

מיצוי החלבון תילקח בחשבון. הפרק הבא בעבודה התמקד בהערכת *in-silico* של סיכוני האלרגניות לחלבונים המופקים באמצעות PEF. החלבונים שהופקו באמצעות PEF מ- *Ulva* עברו ניתוח נתונים, זוהו והושוו לאלרגנים ידועים. לצורך בקרה, רצפי החלבונים שהופקו באמצעות PEF הושוו לפרוטוקול ידוע לניתוח פרוטאומי של סה"כ החלבונים. השוואה זו הראתה שטיפול עם-PEF מנע שחרור של מספר חלבונים אשר ידועים כאלרגנים במזון. עובדה זו הובילה למסקנה כי הטיפול ב- PEF משחרר מתאי האצות אלרגנים באופן סלקטיבי.

ההבנה הבסיסית של אופי האינטראקציות בין החיידקים לבין *Ulva*, יחד עם הצורך במחקר בסיסי של חלבוני אצות בהקשר של שיטת המיצוי, נחוצה כדי להעריך טוב יותר את ההשפעות של "חלבון חדש" פוטנציאלי בעתיד על בריאות הצרכנים. המחקרים שהוזכרו הם עונים על הפערים המדעיים החשובים ביותר על מנת לאפשר הקמת בית-זיקוק ביולוגי בקנה מידה גדול על בסיס ביומסה של האצות הרב תאיות מהסוג *Ulva*, לצורך יצירת מוצרי מזון ומקורות אנרגיה חדשים.

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בית הספר לסביבה
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על שם פורטר

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הפקולטה למדעים מדויקים
ע"ש ריימונד ובברלי סאקלר
אוניברסיטת תל אביב

יחסי הגומלין בין אצות אולוה לחיידקים ועיבוד ביומסה לצורך ייצור מקורות אנרגיה ומזון

מארק פוליקובסקי

עבודה זאת נעשתה במסגרת

בית הספר לסביבה ולמדעי כדור הארץ על שם פורטר

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אוגוסט 2020