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Bioconversion of the Brown Tunisian Seaweed *Halopteris scoparia*: Application to Energy

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Abstract: The brown Tunisian seaweed *Halopteris scoparia* was used as a feedstock for producing renewable bioethanol, biogas, and biodiesel to demonstrate the proof of concept for the North African energy sector. A quantitative and qualitative quantification of *H. scoparia* composition using different colorimetric methods was completed to highlight its bioconversion potential. These substrate inputs were subjected to anaerobic fermentation by *Saccharomyces cerevisiae* to produce bioethanol. The materials were also used to generate bio-hydrogen and volatile fatty acids during dark fermentation by a bacterial consortium and using the oleaginous yeast *Yarrowia lipolytica*. The lipids were extracted and trans-esterified to Fatty Acid Methyl Esters (FAMEs), and their profiles were then analyzed with gas chromatography (GC). A significant ratio of the bioethanol, e.g., 0.35 g ethanol/g DW substrate, was produced without pretreatment, consistent with the theoretical Gay-Lussac yield. The production of the biohydrogen and lipids were up to 1.3 mL H_2/g DW substrate and 0.04 g/g DW substrate, respectively, from the raw biomass. These results were higher than those reported for other well-studied seaweeds such as *L. japonica*. Overall, this work contributes to the current investigations in Tunisia for producing alternative energies from algae and finding new solutions to the current energy situation and environmental challenges in Maghreb.

Keywords: *Halopteris scoparia*; by-product; ethanol; bio-hydrogen; lipid; fermentation

1. Introduction

Macroalgae, commonly referred to as seaweed, are multicellular, macroscopic, eukaryotic, and autotrophic organisms divided into three categories depending on their thallus pigmentations (green, red, and brown), which can be harvested from natural resources but also cultivated [1,2]. Owing to their richness in organic compounds and their ability to generate bioactive primary and secondary metabolites (e.g., pigments, fatty acids, proteins, polyphenols, enzymes, and carbohydrates), seaweeds are beneficial resources widely studied over the past decade [3].

Until recently, several nations have shown a significant interest in the usage and the exploitation of macroalgae for a variety of reasons, such as the production of polysaccharides, which can possess interesting properties for polymers (biocompatibility, non-toxicity, and biodegradability), de facto contributing to their high economic worth, especially in a strong sustainable development context [4]. This is the case for the sulfated polysaccharides, alginate, and laminarin extracted from brown macroalgae, despite their low mass content (up to 5% *w*/*w*) [5–7]. Other active molecules can be extracted from seaweeds, for instance phenolic compounds, which can show significant anti-inflammatory or anti-proliferative characteristics [8]. The macroalgae extracts have many other reported benefits, such as antitumor, antioxidant and antiviral properties [9,10], causing a rise in seaweed derivatives on the markets, owing to these numerous health-promoting characteristics [11,12]. Besides, these products can not only be used in the fields of feed/food, nutraceuticals, and/or cosmetics, but also as fodder fertilizer and for biofuels' production, which thus enhance the blue biotechnologies' economy [13]. Some of the seaweeds contain essential fatty acids, such as linolenic (C18:3) and linoleic acids (C18:2) [14]. Lipids and fatty acids produced by marine macroalgae could be transformed into biodiesel, which has similar qualities to the conventional diesel fuel and may be used either straight in current diesel engines or mixed with diesel from petrochemistry. The production of biofuels from seaweed is a green technology that could minimize environmental damages by limiting the over-exploitation of fossil fuels, expected to rise by 40% between 2010 and 2040 [15]. This is particularly true regarding the political stability in Europe and the strong dependence on fossil fuels and gas in 2022. Many of the studies have reported the contribution of macroalgae as renewable and economical sources to address climate change issues [16]. Shifting to renewable energies and/or new ways for making clean energies is, thus, of utmost interest.

Macroalgae have a higher photosynthetic capability than terrestrial plants, as well as high water and carbohydrate contents [17–20]. Factors such as the productivity, relative ease of growing, harvesting, and scalability are also major considerations for choosing a specific seaweed (raw and/or by-products from seaweed processing) as biofuel feedstock [17–20]. According to Chen et al. [20], macroalgae may produce a net energy of 11,000 MJ/t dry algae [21], in comparison to 9500 MJ/t from microalgae gasification. Brown seaweed contains quantities of fermentable sugars (laminarin, fucoidan, alginate, glucan-like, etc.) without the presence of lignin (as for terrestrial plants). It can thus be used in the macroalgae-based biorefinery technologies for producing biomethane, bioethanol, biodiesel, and bio-oils [20,21]. The red, green, and especially brown macroalgae were subjected to fermentation under anaerobic digestion, using different strains such as *S. cerevisiae* or *P. angophorae* to produce ethanol [22]. For instance, *L. hyperborean*, *J. rubens*, *U. lactuca* or *S. latifolium* from the Egyptian Mediterranean were characterized as bioethanol feedstock after fermentation [22]. Note that the various conversion methods are detailed in recent reviews, such as fermentation, anaerobic digestion, liquefaction, transesterification, or pyrolysis [20,21]. Aboulkas et al. showed that 24.1% and 44% of bio-oil and biochar can be obtained prior to a pyrolysis conversion from the Moroccan *G. sesquipedale* [23]. High quality biodiesel production was reported after some transesterification steps by using *P. padina* from the Abu Qir Bay in North Africa [24].

Tunisia, with 1300 km of Mediterranean Sea coastlines, aims to develop the exploitation of macroalgae for creating new operational sectors in energy. *Halopteris scoparia*, belonging to the phaeophycean species, is an abundant brown seaweed on the Tunisian north coast, which is still in the process of having its worth evaluation. To our knowledge, no other works to date have covered the bioconversion potential of *H. scoparia*. Thus, this work aims to provide a proof of concept for using *H. scoparia* biomass (as well as some of the by-products generated after the extraction of its high-value biomolecules) as a material to produce various types of energy vectors [25,26]. Thus, Bioethanol, H_2, and lipids were, respectively, produced by fermentation (*Saccharomyces cerevisiae*) and dark fermentation (microbial consortium and *Yarrowia lipolytica*). The results could contribute to the integration

of *H. scoparia* into a global biorefinery conversion process in Tunisia, and more generally in Maghreb.

2. Materials and Methods

2.1. Algal Biomass

The *H. scoparia* biomass was gathered in February 2020 from Tabarka city, which is in the north-west of Tunisia (36°57′36.223″ N and 8°45′12.018″ E). After harvesting, the thalli were rinsed with distilled water to remove the salts and dried for 20 days at 50 °C [27]. The dried samples were then pulverized into a powder size of 16–40 µm using a blender (Moulinex, France). This item was labeled as the raw seaweed and given the abbreviation "RSF" (Raw Seaweed Fraction). Two further fractions were employed in this study. They were generated after the extraction and purification of the polysaccharides and pigments from the *H. scoparia* dry biomass [27] and were, respectively, termed "BP1-PS" and "BP2-F". The BP1-PS is a by-product obtained from the carbohydrates' (fucoidan/alginate) extraction. Briefly, after depigmenting and delipidating the dry biomass (100 g) using acetone (1.4 L) and methanol (0.6 L) treatment (twice) for 24 h at 25 °C, the obtained biomass (75 g) was treated for 2 h at 60 °C with chlorohydric acid (0.1M, pH = 2) to extract the fucoidans, then filtered and treated with Na_2CO_3 (3%, pH = 11) at 60 °C to extract the alginates, then dried for 24 h at 50 °C [23,24]. The BP2-F is a by-product obtained from the depigmented biomass with successive treatments, using methanol to remove all of the pigments for a 72 h period at 25 °C under stirring (250 rpm). After filtering, the biomass was collected and dried for 24 h at 50 °C [28,29].

2.2. Algal Biomass Characterization and Chemicals Constituents

2.2.1. Carbohydrate Content of the Three Fractions

The nonenzymatic gravimetric technique was used to determine the total polysaccharides of RPS, BP1-PS, and BP2-F (AOAC Official Method 993.21) [30,31]. One hundred mg of RPS and BP1-PS were depigmented with acetone (1400 mL) then delipidated with methanol (600 mL) for 14 h at room temperature with moderate stirring. The obtained suspensions were filtered using a fine mesh strainer with a porosity of 16–40 µm. RPS, BP1-PS and BP2-F previously depigmented and delipidated were macerated in ultra-pure water (100 mL) for 4 h at 80 °C under gentle stirring (400 rpm). Cold ethanol 96% (300 mL, −20 °C) was used to precipitate the polysaccharides fractions. The suspensions were centrifuged at a rate of 20,000× *g* for 20 min at 4 °C, and the pellets were recovered. The following ethanolic precipitation cycle was completed three times. After that, the pellets were suspended in ultra-pure water (50 g/L) and freeze dried. From these fractions, the total carbohydrate contents were determined using the Dubois colorimetric technique, and the reducing sugars using the bicinchoninic acid method, both using glucose as standard [32,33].

2.2.2. Phenolic Content

Ultrasound was used to quantify the phenolic chemicals RPS, BP1-PS, and BP2-F (main fractions from Section 2.1). Ten g of the dried macroalgae were mixed with 200 mL of acetone (80%) and incubated for 45 min at 4 °C, 120 W, and 40 KHz. The suspension was then centrifuged (5000× *g* for 10 min at 4 °C), and the supernatant was collected and dried, using a rotary evaporator (Stuart RE300B, Stone, UK) at room temperature [34]. The total phenolic content was determined using the Folin–Ciocalteu technique, established by Singleton (1965) and using Gallic acid as the standard [35].

2.2.3. Composition in Chemical Elements

This composition was determined after heating the RPS, BP1-PS, and BP2-F at 550 °C for 6 h. Prior to an acidic treatment with 5 mL chlorohydric acid (37%) and 5 mL nitric acid (2M), the ions concentrations for the fractions were assessed, using an Atomic Absorption Spectroscopy (iCETM3300, Thermo Scientific, Waltham, MA, USA) [36].

2.2.4. Lipid Extraction

The Soxhlet technique was used to determine the total lipids of the RPS, BP1-PS, and BP2-F. In a nutshell, 500 mg of each of the fractions were inserted in a thimble in a Soxhlet extractor, along with 170 mL of the hexane in a round-bottomed flask for 4 h at 60 °C, followed by 40 min of solvent distillation. After 30 min of hexane evaporation using a rotary evaporator at 55 °C (Stuart RE300B, Bibby Scientifics, Stone, UK), the round-bottom flask was placed in an oven to be weighted, and the percentage of lipids that it contained was determined [37].

2.2.5. Ash, Moisture, and Protein Contents

The dry weight (DW) of the RPS, BP1-PS, and BP2-F was measured, using the AOAC technique, by drying 0.5 g of each sample in an air oven at 105 °C for 24 h. The ash content was calculated after drying 50 mg of RPS, BP1-PS, and BP2-F in a furnace at 550 °C for 4 h. The protein content in the RPS, BP1-PS, and BP2-F was quantified, using the Kjeldahl test and an AOAC International nitrogen conversion factor of 6.25 (method 981.10) [38].

2.3. Fermenting Microorganism, Medium Culture, and Production Process

2.3.1. Ethanol Production

Saccharomyces cerevisiae ATCC 7754 was used to produce bioethanol [39]. The experiment was conducted in 500 mL Erlenmeyer flasks containing 200 mL of culture medium, described by Kristiansen (1994), and consisting of glucose (50 g/L); yeast extract (3 g/L); monobasic potassium phosphate (KH_2PO_4) (5 g/L); ammonium sulfate $(NH_4)_2SO_4$ (2 g/L); KCl (120 mg/L); $CaCl_2$, $2H_2O$ (60 mg/L); $MgCl_2$, $2H_2O$ (520 mg/L); $FeSO_4$, 6 H_2O (35 mg/L); $MnSO_4$, H_2O (3.8 mg/L); $CuSO_4$, 5 H_2O (0.5 mg/L); thiamine-chlorohydric acid (5 mg/L); pyridoxine-chlorohydric acid (6.25 mg/L); nicotinic acid (5 mg/L); D-biotin (0.125 mg/L); pantothenic acid (6.25 mg/L); myo-inositol (125 mg/L) and traces of $ZnSO_4$, 7 H_2O (2.3 µg/L); $CoSO_4$, 4 H_2O (2.3 µg/L); $NaMoO_4$, 2 H_2O (3.3 µg/L); H_3BO_3 (7.3 µg/L); KI (1.7 µg/L); $NiSO_4$, 6 H_2O (2.5 µg/L) [40].

The *S. cerevisiae* was inoculated in snugly closed flasks and fermentation was carried out for 48 h at 30 °C under gentle stirring of 200 rpm (INFORS HT, Bottmingen, Switzerland). The biomass growth was measured at 550 nm. To estimate the ethanol production, 5 mL were taken and centrifuged at 10,000× *g* for 5 min at 25 °C every 6 h. Before further investigation, 1 mL of each supernatant was deproteinized with 125 mL of barium hydroxide solution (0.3 M) combined with 125 mL of zinc sulfate solution (5% *w*/*v*) and centrifuged at 10,000× *g* for 5 min at 25 °C. The ethanol was quantified using a high-performance liquid chromatography (HPLC) apparatus (1260 Infinity Quaternary LC system, Agilent Technology, Palo Alto, CA, USA) outfitted with two ionic exclusion columns in series (Rezex ROA 300 7.8 mm, Phenomenex, Torrance, CA, USA). The samples were filtered using a cellulose acetate filter (0.2 µm), injected (10 µL), and eluted in an aqueous sulfuric acid solution (5 mM) mobile phase at a flow rate of 0.7 mL/min and 50 °C. The ethanol concentrations were measured using a refractometer (HP 1100 series, Agilent Technologies, Palo Alto, CA, USA) and an ethanol standard curve (0 to 100 g/L). The M8620AA OpenLAB Data Store Software A.02.01 was used to analyze the results.

2.3.2. Hydrogen and Volatile Fatty Acid Productions

For biohydrogen generation via anaerobic fermentation, the microbial consortium was chosen from a sewage treatment facility and treated with 2-bromoethanesulfonate in a complete medium to minimize the methane production from the archaea and to stabilize the community [26]. The composition of this culture medium was: K_2HPO_4 (18 g/L); KH_2PO_4 (18 g/L); $(NH_4)_2SO_4$ (72 g/L); NaCl (12 g/L); $MgSO_4$, $7H_2O$ (1.2 g/L); $CaCl_2$, $2H_2O$ (1.2 g/L); $MnSO_4$,$6H_2O$ (0.15 g/L); $CoCl_2$, $6H_2O$ (0.015 g/L); $FeSO_4$, $7H_2O$ (0.4 g/L); H_3BO_3, H2O (6 g/L); $ZnCl_2$ (8.2 g/L); $NiCl_2$, $6H_2O$ (2.6g/L); $CuCl_2$, $2H_2O$ (1.5 g/L); Na_2MoO_4, H_2O (2.6 g/L); $FeCl_2$, $4H_2O$ (75 g/L); hemin (0.12 g/L); biotin (0.24 g/L); PABA (0.12 g/L); resazurin (0.12 g/L).

This culture medium was supplemented with glucose at concentration of 20 and 10 g/L in, respectively, the pre-culture and final culture. Other components, such as L-cysteine (0.6 g/L) and carbonates (Na_2CO_3) (4.8 g/L), were also added to remove the oxygen and maintain the pH 6, respectively [41]. The pre-cultures were prepared in flasks (90 mL) with 10 mL of consortium for 12 h at 30 °C with stirring (150 rpm). The cultures were then degassed and sterilized before being inoculated with 10 mL of inoculum from pre-cultures, with biomass contents of 10 g/L. The fermentation took place at 30 °C with a stirring rate of 150 rpm [42]. A spectrophotometer (Biomate 3S, UV/vis spectrophotometer, Thermo Scientific, France) was used to measure the turbidity at 600 nm. After 24 h, 5 mL of the culture broth was sampled and centrifuged at 10,000× *g* for 5 min at 25 °C. One mL of the obtained supernatant was treated and analyzed by high-performance liquid chromatography (HPLC), as previously detailed (Section 2.3.1), to quantify the concentrations of the volatile fatty using acetate (1.25 to 20 g/L), propionate (1 to 20 g/L), and butyrate (1 to 20 g/L) as the standards.

After 24 h of anaerobic fermentation, the biogas volume was quantified using a Terumo® syringe. Its quality and composition, particularly the biohydrogen concentration, were determined by a micro-gas chromatograph (Agilent 3000 A, Agilent Technologies, France) with two channels and a thermal conductivity detector. The hydrogen content was measured in the first channel, which was outfitted with a 5 Å column at 60 °C and 30 psi. The obtained data were quantified using the Soprane software [26].

2.3.3. Biodiesel Production

The *Yarrowia lipolytica* (MUCL 28849) was from the BCCM/MUCL (Agro) Industrial Fungi and Yeasts Collection in Belgium [43]. The *Y. lipolytica* was kept at 4 °C on potato dextrose agar. The growth medium was made up of: K_2HPO_4 (3 g/L); Na_2HPO_4, H_2O (3 g/L); $(NH_4)_2SO_4$ (3 g/L); $MgSO_4$, $7H_2O$ (1 g/L); $ZnSO_4$, $7H_2O$ (0.040 g/L); $FeSO_4$, $7H_2O$ (0.016 g/L); $MnSO_4$, H_2O (0.0038 g/L); $CaCl_2$, $2H_2O$ (0.023 g/L); $CoCl_2$, $6H_2O$ (0.5 mg/L); $CuSO_4$, $5H_2O$ (0.0009 g/L); Na_2MoO_4, $2H_2O$ (0.00006 g/L); and H_3BO_3 (0.003 g/L), supplemented by 10 mL/L of a mineral stock solution containing $MnSO_4$ (0.38 g/L); H_3BO_3 (0.3 g/L); $ZnSO_4$, $7H_2O$ (4 g/L); FeSO4, $7H_2O$ (1.6 g/L); $CaCl_2$, $2H_2O$ (2.3 g/L); and 1 ml/L of metals' stock solution composed of Na_2MoO_4, $2H_2O$ (0.06 g/L); $CoCl_2$, $6H_2O$ (0.5 g/L); and $CuSO_4$, $5H_2O$ (0.9 g/L) [44]. To prevent precipitation, the pH was lowered to 4.5 with orthophosphoric acid 85% before sterilization and subsequently to 6 with KOH 1 M following sterilization. The yeast preculture was prepared by inoculating 200 mL of medium culture containing glucose (4 g/L) with the *Y. lipolytica* colonies picked from an agar plate and keep for 24 h at 30 °C in a rotary incubator shaker at 100 rpm (INFORS HT, Multitron Standard, Switzerland). Ten mL of pre-culture were used to make cultures with various substrates, RPS, BP1-PS, and BP2-F (2 g/L). Glucose (2 g/L) was used as the positive control.

The cultures were incubated at 30 °C at 200 rpm in the same rotary shaker, and 5 mL of each culture was collected every 5 h. High pressure homogenization was used to disrupt the cells in the samples, through a nozzle at 2500 bar pressure (Constant System Limited, Low March, Daventry NN11 4SD, UK). Half a mL of each collected sample was homogenized with 4 mL of chloroform, 2 mL of methanol, and 1 mL of distilled water. The mixture was then separated into an aqueous superior phase and a lower phase containing the extracted lipids by a centrifugation for 10 min at 2000× *g*. The organic phase was then pooled in pre-weighed tubes in 1 mL increments. After evaporating the solvent, the lipids were recovered using a speed-vacuum at 40 °C for 120 min until reaching a constant weight. The lipids were suspended in chloroform and kept at −20 °C. The transesterification was completed, and the fatty acid methyl esters' profile was analyzed, using the method of Morrison and Smith [36]. One hundred mL of the sample were combined with 800 mL of $MeOH/BF_3$ (14%) and placed in a water bath at 100 °C for 15 min. After cooling the mixture, 1.5 mL of water was mixed with 750 mL of hexane containing heptadecane, 1% (*v*/*v*) was added, and 1 mL of the mixture was finally injected and analyzed by gas chromatography

(HP6890, Hewlett Packard, Palo Alto, CA, USA) equipped with a Supelco SPB-5 (Supelco Inc., Bellefonte, PA, USA) capillary column (30 m × 0.32 mm × df 0.25 mm), using nitrogen as a gas vector at 0.80 mL/min and flame ionization as a detector (280 °C). The rise in the temperature was from 170 °C to 220 °C, at a rate of 3 °C/min, for 3 min. The injector was set at 250 °C. The identification of the methyl esters was made by comparing the retention times with known standards. The obtained data were analyzed using MestReNova 7.1.

2.4. Statistical Analyses

All the statistics were determined using the statistical software SigmaPlot 12.5 (Systat Software, SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation of three replicates. A *t*-test and post hoc procedures (Tukey test) were performed to compare the data and analyze the pairwise differences. The differences were considered significant for $p < 0.05$.

3. Results and Discussion

3.1. Chemical Constituents

3.1.1. Ash, Moisture, Proteins, Fats, and Phenolic Contents

The *H. scoparia* biomass and by-product compositions are given in Table 1. A qualitative study of *H. scoparia* shown that the raw biomass contains 3% fat and 7% proteins. According to the literature, the protein content in brown algae can range from 3% to 15% [45], but the fat content is around 5%, as with the total lipids content in *A. nodosum*, 4.46% [46]. The ash and moisture contents of the dried *H. scoparia* were, respectively, 43% and 7%, which is consistent with previous studies reporting that the ashes could vary from 24% to 49% and the moisture between 4% and more than 18%, depending on the season and the genus of the brown seaweed [47–49]. For instance, *H. scoparia* presented a low moisture content which agreed with the values reported for *Undaria pinatifida*, a seaweed used for biofuel production [50]. Furthermore, this macroalgae had 4.4% of phenolic content which is close to the content found for another brown Tunisian seaweed, e.g., *Cystoseira compressa*, with a value of 4.8% [51,52]. It is noteworthy that the harvesting period is an important parameter that can indirectly affect the macroalgal biomass availability and its biochemical composition [53].

Table 1. *H. scoparia* (RPS) and by-products' composition after carbohydrate (BP1-PS) and fucoxanthin (BP2-F) extractions.

Composition % (*w*/*w*)	*H. scoparia* (RPS)	By-Product after Carbohydrate Extraction (BP1-PS)	By-Products after Fucoxanthin Extraction (BP2-F)
Fat	2.92 ± 0.28	1.5 ± 0.52	0.93 ± 0.6
Protein	6.53 ± 0.56	1 ± 0.5	0.87 ± 0.71
Ash	42.63 ± 2.2	47.34 ± 1.4	35.41 ± 2.5
Moisture	6.79 ± 0.16	7.57 ± 0.16	9.06 ± 0.45
Phenolic content	4.4 ± 0.005	0.43 ± 0.0003	0.29 ± 0.0005

This biochemical composition provides information that could be useful in performing the appropriate pretreatments to make *H. scoparia* more suitable for energy production. According to Suutari et al., the protein level can impact the microbial bioconversion of macroalgae [54]. For example, a high protein content (7–20%) can reduce the biohydrogen production [55]. Furthermore, it has been shown that the bioconversion of macroalgae with a high soluble protein content can produce toxic ammonia [55]. In the case of brown macroalgae, the microbial degradation of the constitutive proteins was inhibited, due to their cellular localization and linkages with cell wall polysaccharides [56]. Hou et al. reported that the bioethanol production after macroalgae bioconversion was correlated with their carbohydrate contents, rather with their protein contents [57]. In addition, because of the low lipid concentrations, the production of biofuels from *H. scoparia* primarily depends

on their carbohydrate conversion to bioethanol production, and secondly on their fatty acids' transesterification capacity to produce biodiesel.

The ash content from *H. scoparia* seemed to be high and reflected its mineral content. It can provide functional micronutrients serving to enhance biological conversion, such as anaerobic digestion [58]. In this regard, in comparison to the thermal conversion, biological processing is the most ideal conversion procedure for algae since the algae are the most tolerant of ash content [59]. Regarding the literature, the high quantity of ash increases the onset of the pyrolysis process and then the bio-oil yields. So, *H. scoparia* could produce a low bio-oil yield via fast pyrolysis behavior, due to its high content of ash. According to Bae et al., some of the pretreatments are beneficial for ash reduction [60], but excessive quantities of ash (> 20%) can generate slagging and fouling, which is a key issue for biofuels production [61].

3.1.2. Composition in Chemical Elements

H. scoparia (RPS) contains macro-, microelements, and heavy metals (Table 2). According to the literature, the levels of these elements can significantly change depending on the species [45]. *H. scoparia* contained lower levels of different micro-, macroelements, and heavy metals than in the *F. vesiculosus* or other French edible macroalgae that are considered suitable for food. This composition highlighted the low food potential of this seaweed and encouraged investigating its bioconversion potential [61]. Note that *H. scoparia* also contained alkali metals, such as K and Na. According to Ross et al., these elements derived from the macroalgae biomass may cause problems with fouling and slagging in gasification and biohydrogen production [62]. The high alkali metal concentrations may also cause the inhibition of bioethanol production [62]. Thus, it is of the utmost importance to identify and quantify these various chemical elements, which allows the determination of whether a particular pretreatment must be applied to the seaweed to reduce the concentration of ash and undesirable chemical elements [62].

Table 2. Composition in chemical elements of *H. scoparia* (RPS).

Chemical Elements	mg/kg DW [1]
P	29.950 ± 0.008
Ca	19.919 ± 0.003
K	15.689 ± 0.037
Na	10.350 ± 0.097
Mg	8137.5 ± 0.056
Fe	3946.75 ± 0.023
Mn	51.5 ± 0.005
Ni	23.75 ± 0.008
Zn	23.55 ± 0.012
Cu	6.95 ± 0.01
Pb	4 ± 0.01

[1] DW: dry weight.

3.1.3. Sugars' Contents

As shown in Table 3, the total carbohydrate content in *H. scoparia* (RPS) represented 133.9 g/kg, which was much higher than for the carbohydrates' content reported from *S. vulgare* (31.03 ± 6.59 g/kg) [46], or from *C. barbata* (9.6 g/kg) [47]. Note that the brown seaweeds were reported as showing a higher content of carbohydrates during the spring [48]. The BP1-PS and BP2-F fractions were still composed of carbohydrates with, respectively, 79.02 ± 0.03 g/kg and 113.20 ± 0.04 g/kg. The significant difference ($p < 0.05$) between each fraction comes from the extraction methodology, where the BP2-F (obtained after pigment extraction) was not depleted in carbohydrates in comparison to the BP1-PS. These carbohydrate contents, as well as the reducing sugars' values for the by-products, were still close to the quantities reported for raw brown macroalgae, such as *Sargassum obtusifolium*

and *Rosenvingea nhatrangensis* (123 and 12.6 g/kg, respectively) [49], which highlights their potential as feedstock materials for fermentation, etc.

Table 3. Total and reducing sugars contents.

Samples (g/kg DW)	Total Sugars (g/kg)	Reducing Sugars (g/kg)
RPS	133.9 ± 0.12	39.5 ± 0.05
BP1-PS	79.02 ± 0.03	15.73 ± 0.09
BP2-F	113.20 ± 0.04	15.26 ± 0.02

3.2. Biofuels' Production from H. scoparia and By-Products of Extraction

3.2.1. Production of Bioethanol

The RPS, BP1-PS and BP2-F were subjected to solid state fermentation and used as substrates to generate bioethanol from *Saccharomyces cerevisiae* ATCC7754. This strain was selected owing to its ability to produce important ethanol concentrations, regarding its low sensibility to a high concentration of ethanol, high selectivity for fermentable sugars such as glucose, galactose, and mannose during fermentation, and the capacity to produce a low quantity of by-products [50,51]. Figure 1 shows the evolution of bioethanol production simultaneously with the consumption of fermentable sugars from the RPS, BP1-PS and BP2-F fractions for 72 h. According to the theoretical yield of Gay-Lussac for ethanol production from glucose (0.51 g ethanol/g glucose), around 5.2 g/L of ethanol was produced for the positive control.

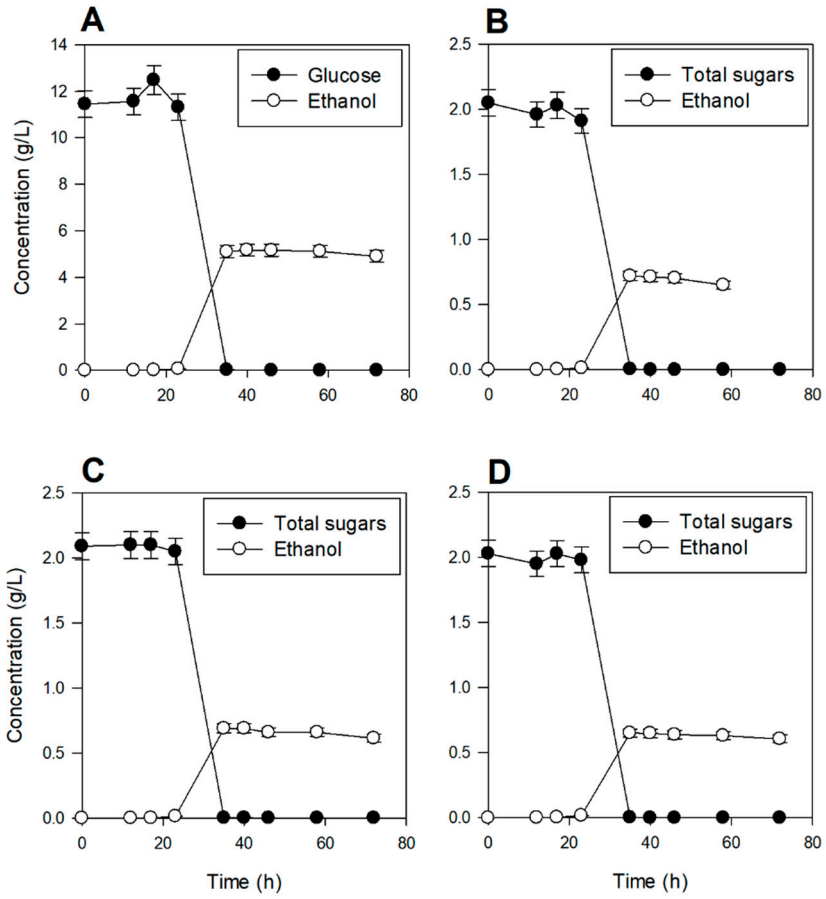

Figure 1. Production of ethanol from (**A**) glucose (positive control); (**B**) RPS; (**C**) BP1-PS; and (**D**) BP2-F fractions. The standard deviations were lower than 5%.

Due to their content of reducing sugars (Table 3), each of the other substrates allowed the production of ethanol, with concentrations ranging from 0.65 to 0.72 g/L during the

three days of fermentation, without a significant difference ($p > 0.05$). This corresponded to 0.35, 0.345, and 0.325 g ethanol/g DW for RPS, BP1-PS, and BP2-F, respectively. The difference between the ethanol production from BP1-PS and BP2-F was explained by the slight difference in the initial content of the reducing sugars. The difference between the yield of ethanol produced from BP1-PS and BP2-F and the yield of RPS could be due to the phenolic compounds present in RPS, which is higher than those of the two by-products (4.4%). Chen et al. showed that the phenolic compounds can affect the fermentation ability of *S. cereviseae* and reduce ethanol production due to their antimicrobial properties [63]. These results were higher than those reported for the untreated macroalgae *Saccharina japonica* (0.281 g ethanol/g DW) or *Laminaria japonica* (0.28 g ethanol/g DW) [52]. According to Obata et al., this change could be explained by the difference in the constitutive fermentable sugar characteristics of each macroalgae [64].

Better results were reported for the pretreated (mechanical, acid pretreatments, and enzymatic hydrolysis) brown seaweed materials from *Sargassum vulgar* or *Padina tetrastromatica*, with some yields close to 0.38 and up to 0.66 g ethanol/g DW [12]. For instance, washing, drying, milling, and heating (121 °C for 20 min in an autoclave) macroalgae can be efficient in releasing fermentable biomolecules, by breaking down the outer layer of the cell wall and increasing the surface area [53,54]. Combining the mechanical pretreatment with the use of enzymes can also reduce the generation of fermentation inhibitors [65,66]. Thus, further experiments should be planned to enhance the ethanol production by pretreatment, in particular RPS for more exposing and/or generating of fermentable polymers, oligo- and monomers [65–67]. Other studies highlighted that not only the enzymatic steps can affect the fermentation yields, since additional elements can change the bioethanol production. Mutripah et al. showed that an acid hydrolysis of the seaweed using 0.2 M H_2SO_4 before treatment at 121 °C for 15 min allowed the generation of the reducing sugars' content of about 60 g/kg DW [68]. *P. australis* was also composed of around 145 g/kg DW total carbohydrates, which was close to the values for *H. scoparia* (133 g/kg DW). However, the use of the *H. scoparia* biomass as a fermentation substrate resulted in a higher ethanol yield (0.35 g ethanol/g DW) than that reported for *P. australis* (0.002 g ethanol/g DW). This finding could be due to the high level of 5-HMF and levulinic acid obtained after hydrolysis using H_2SO_4, which could inhibit the fermentation process [68]. It clearly shows the importance of choosing the appropriate hydrolysis pretreatment for significantly enhancing the bioethanol production. Using various enzymes in the mix, such as recombinant alginate lyases, oligo-alginate lyases, and cellulases, can increase the ethanol yields, as reported for the *M. pyrifera* biomass [69]. The authors confirmed that enzymatic treatments could be sufficient for increasing glucose liberation, avoiding the fermentative issues caused by acid and/or thermal pretreatment [69].

The selection of suitable fungal or microbial strains is a crucial aspect of the fermentation process. Identifying the organisms able to convert heterogeneous sugars is still a challenge in bioethanol production from macroalgae [70]. Indigenous halotolerant marine yeast could be used in the fermentation process, considering their tolerance to high contents of salts in ash [71]. It has been outlined that the use of *Candida* sp. could increase the ethanol yield by up to 1.76% for the macroalgae *Kappaphycus alvarezii*, despite the high salt concentrations [71]. Offei et al. [70] reported that acclimatizing yeasts to higher concentrations of some substrates was a way of increasing ethanol output. This was accomplished employing a two-stage fermentation of the hydrolysates from *S. japonica* [72]. *S. cerevisiae* KCCM 1129 was used in the first fermentation stage of *S. japonica* to convert the glucose content into ethanol, while *P. angophorae* KCTC 17574 was utilized in the second fermentation stage to convert the mannitol content into ethanol. The acclimatized *P. angophorae* KCTC 17574 was able to produce about 0.3 g ethanol/g mannitol, instead of 0.13 g ethanol/g mannitol for the non-acclimatized one.

Moreover, the fermentation technique, which simultaneously used saccharification and fermentation (SSF), is reported to be a more productive method than splitting hydrolysis and fermentation (SHF). It saves time, reduces the number of needed unit operations, and

decreases the contamination issues. The SSF method may enhance the level of bioethanol production by avoiding end-product inhibition and the potential loss of sugars [73].

3.2.2. Production of Hydrogen and Volatile Fatty Acids

Figure 2 illustrates the volatile fatty acids (VFAs) and the biohydrogen production of the bacterial consortium for 24 h (dark fermentation), using RPS, BP1-PS, and BP2-F fractions as the substrates. Glucose was the positive control (1.85 g/L butyrate, 479 mg/L acetate, 72 mg/L propionate, 40 mL H_2/g glucose corresponding to 43% H_2 in the total biogas). The results highlighted that each fraction can be used as feedstocks for generating biohydrogen, i.e., 1.3 mL H_2/g DW (around 1/3 of the yield by using glucose).

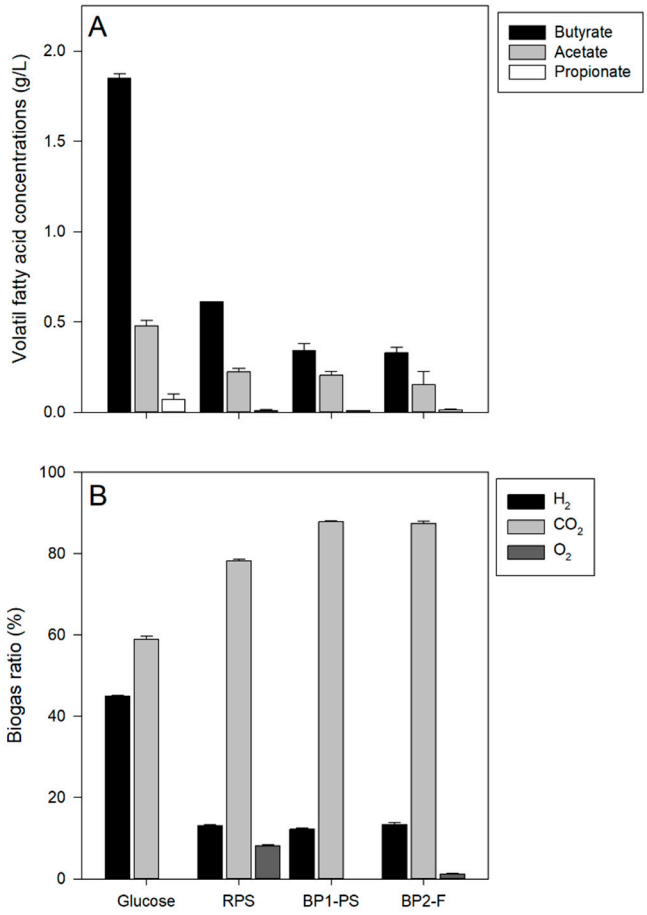

Figure 2. Production of (**A**) VFAs and (**B**) bio-hydrogen from RPS, BP1-PS, and BP2-F fractions, using glucose as positive control.

Regarding the literature, these results were lower than those observed, for example, by using *Huzikia husiforme* sp. (10.3 mL H_2/g DW) [74] or the brown seaweed *L. japonica* (58.5 mL H_2/g DW) as substrates, noting that pretreatments have been applied (acid, alkali, or enzymatic hydrolysis step) for generating more sugars to enhance the level of biohydrogen production [75,76]. However, the yield of H_2 produced from *H. scoparia* (13 mL H_2/g DW) was slightly higher than the one reported from non-treated *L. japonica* (10 mL H_2/g DW) [77]. The H_2 production was coupled in the experiments with two preferential biosynthesis pathways for the bacterial consortium [20], one producing acetate, and the second one butyrate. A significant difference ($p < 0.05$) was observed regarding the butyrate concentration (612 mg/L) which was two times higher using the RPS than the BP1-PS and BP2-F fractions. This result comes from the content of reducing sugars, which represented 39.5 g/kg DW in the RPS. The acetate concentrations were close to 154, 205, and 225 mg/L for BP2-F, BP1-PS, and RPS, respectively, without a significant difference. These

data were slightly lower than those reported, for example, from *L. japonica*, *P. elliptica*, and *E. critina* to produce butyric acid (1.9, 2.5, and 2.8 g/L, respectively), acetic acid (4.5, 2.2, and 2.8 g/L, respectively) or propionate (0.6, 0.4, and 0.4 g/L, respectively) [20,74,75]. Having a balanced C:N ratio of 20–30 should help in increasing the productivity, as reported by some papers [20,76–78]. According to the literature, many factors, such as pH, can affect the yield of H_2 production from macroalgae. Thus, the optimal pH to produce biohydrogen from various biomasses can vary between 5.2 to 7, butyrate being the main VFA, produced at pH 7 [79]. So, the butyrate pathway was preferentially used by our consortium to produce biogas from *H. scoparia;* the acetate and propionate being produced in a second step [79].

To increase the H_2 production, heat pretreatments of the biomass could be of crucial importance, in particular to liberate specific alditols and sugars, which could be finally converted into pyruvate, H_2, butyric acid, and acetate. This pretreatment step seems beneficial when considering the difficulty of mannitol fermenting in anaerobic conditions. Liu et al. showed that the heat pretreatment of *L. japonica* (200 °C) produced a maximum of H_2 yield close to 85.45 mL H_2/g DW, in comparison with the untreated biomass (10 mL H_2/g DW) [80]. The relative amount of H_2 produced from *H. scoparia* may also be due to the low initial substrate concentration (1%). Indeed, many of the studies have reported the effect of substrate concentrations on H_2 production. For example, using *L. japonica* (pH = 6), the optimal substrate concentration was 2% to produce the maximum yield of biohydrogen (75.88 mL H_2/g DW) [80]. Thus, further experiments should be planned to enhance the biohydrogen production from RPS, BP1-PS and BP2-f and by-products for biofuel production since it can still be considered a potential but mid-term suitable alternative for energy production.

3.2.3. Biodiesel Production

The time-course measurements of lipids produced by *Y. lipolytica* are shown in Figure 3. The positive control allowed the release of 155 mg/L of lipids after 84 h. The RPS, BP1-PS and BP2-F fractions showed significant differences ($p < 0.05$) in terms of the lipids' concentrations, with some values of 30.5, 1.6 and 13.3 mg/L, respectively. These substrates showed a low potential, probably because of the presence of high nitrogen concentrations in the media and the unbalanced initial C:N ratio, leading to a low yield in the lipid contents [76–78]. Moreover, *Y. lipolytica* is not able to use the VFAs produced in the medium culture for making intracellular lipids. The total lipids content is the principal indicator of biodiesel production from macroalgae. Thus, *H. scoparia* is characterized by a lipid content close to 3% DW. This low total lipid content was in concordance with the literature. Song et al. reported that the level of lipids from brown macroalgae was below 5% DW [81]. In addition, it was observed that the brown macroalgae *S. trinodis* present 1.25% of total lipids, highlighting the low potential of this seaweed for biodiesel production [82]. Regarding literature, the fluctuation in the lipids' levels is influenced by the environmental conditions as well as the intrinsic potential of macroalgae [82].

It is noteworthy that the compositions of fatty acids, as described in Table 4, were closely the same for all the fractions with linoleic (C18:2) and oleic (C18:1) acids representing around 80%, which was consistent with the literature [20]. Liang et al. reported that the composition of fatty acids from soybean and Jatropha oils used for generating biodiesel in the United States and European Union was like the composition of fatty acids obtained from lipids, using *H. scoparia* as a carbon source [83]. Fei et al. also showed that the profile of fatty acids, using glucose or VFAs as a carbon source of the lipid accumulation from *C. albidus*, was close to the profile given in Table 4 [83].

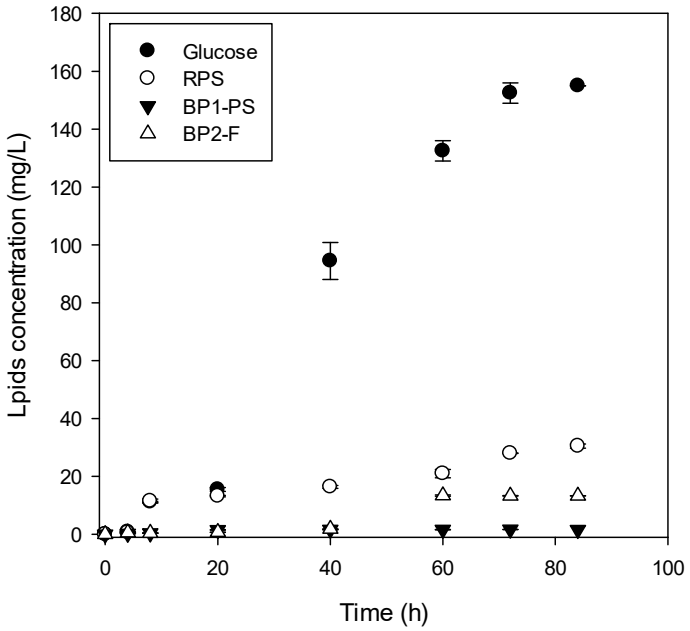

Figure 3. Production of lipids by *Y. lipolytica* fermentation from medium enriched with RPS, BP1-PS, and BP2-F fractions, and using glucose as positive control.

Table 4. Fatty acids' composition of lipids produced by *Yarrowia lipolytica* from RPS, BP1-PS, and BP2-F.

Fatty Acids Composition	%
Palmitic acid C16:0	11
Palmitoleic acid C16:1	10
Stearic acid C18:0	1
Oleic acid C18:1	28
Linoleic acid C18:2	51
Linolenic acid C18:3	1

These authors also showed that pretreatments are a crucial step to achieve high yields of carbohydrates (alginate and mannitol) or VFAs to be used by oleaginous yeast, such as *Y. lipolytica*, to produce high quality biodiesel. Indeed, the type of fatty acids and the ratio between the SFAs (saturated fatty acids) and UFAs (unsaturated fatty acids) play an important role in determining the quality of biodiesel produced from macroalgae. The *H. scoparia* showed a UFAs > SFAs ratio [82]. The SFAs from *H. scoparia* were higher (11%) than the SFAs' contents reported from *S. trinodis* (1.167%) [82]. High quantities of the SFAs could also improve the stability of biodiesel, whereas high levels of UFAs may enhance the biodiesel fluidity. Thus, combining them correctly could lead and serve to producing biodiesel of a higher quality [82].

4. Conclusions

Transesterification, thermochemical, and biochemical are the principal processes to convert biomass into biofuels; the choice of one of these technologies still being related to the so-called bioconversion. *Halopteris scoparia*, which is an abundant brown seaweed found in Tunisia, showed a significant potential for being used as feedstock for energy conversion. Without an optimal run and specific pretreatment, it was possible to produce ethanol (0.35 g/g DW), H_2 (1.3 mL H_2/g DW), VFAs (612 mg butyrate/L, 225 mg acetate/L, 11 mg propionate/L) and a small quantity of lipids (30.5 g/L) from the raw macroalgae fraction (RPS). The feasibility of producing energy vectors by using some of the by-products from the extraction processes of high value molecules (polysaccharides, pigments) was also assessed. Further experiments should be planned, for instance by enhancing yields (e.g., pretreatments for upgrading the saccharification), directing metabolic flux using

various experimental parameters, or by changing the content of the inputs. Overall, this work completes the proof of concept for generating alternative and cleaner energy by setting up new processing sectors or making the concept economically viable of biorefinery from North African brown seaweeds. In addition, the processing of by-products to energy can reduce environmental impacts and increase the capacity of energy recovery in power generation.

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