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Green Macroalgae Hydrolysate for Biofuel Production: Potential of *Ulva rigida*

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Abstract: In this study, the green macroalgae *Ulva rigida*, which contains 34.9% carbohydrates, underwent treatment with commercial hydrolytic enzymes. This treatment yielded a hydrolysate that contained 23 ± 0.6 g·L^{-1} of glucose, which was subsequently fermented with *Saccharomyces cerevisiae*. The fermentation process resulted in an ethanol concentration of 9.55 ± 0.20 g·L^{-1}. The optimal conditions for ethanol production by *S. cerevisiae* were identified as follows: non-sterilized conditions, an absence of enrichment, and using an inoculum size of 118 mg·L^{-1}. Under these conditions, the fermentation of the green macroalgal hydrolysate achieved a remarkable conversion efficiency of 80.78%. The ethanol o/t ratio, namely the ratios of the experimental to theoretical ethanol produced, for *Scheffersomyces stipitis*, *Candida guilliermondii*, *Kluyveromyces marxianus*, and *S. cerevisiae* after 48 h of fermentation were 52.25, 63.20, 70.49, and 82.87%, respectively. Furthermore, *S. cerevisiae* exhibited the best outcomes in terms of ethanol production (9.35 g·L^{-1}) and conversion efficiency (80.78%) after 24 h (optimal time) of fermentation.

Keywords: seaweed; *Ulva rigida*; ethanol; fermentation; biorefinery

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1. Introduction

Over the past few years, research has shifted towards high-tech alternate fuels in response to the ongoing consumption of fossil fuels, which has resulted in the depletion of these resources [1]. The role of fossil fuels is very important in energy sectors and the global economy [2,3], covering about 80–88% of the basic requirements [4,5]. This resource is recognized as non-sustainable as it contributes to the accumulation of greenhouse gases in the atmosphere, leading to global warming [6,7].

The depletion of fossil fuels not only raises their costs but also plays a role in global climate changes, contributing to increased carbon dioxide (CO_2) emissions [8]. This has spurred the necessity for alternate energy sources, prompting the development of biofuels in a bio-economical context [9,10]. Bioethanol has been considered to be an ideal candidate and a clean alternative fuel compared to fossil fuels [11,12]. It can be produced from many different biomass feedstocks, like date syrup [13], sugar cane, or corn starch (first-generation ethanol, 1G) [14,15]. However, due to the competition with feed production, the use of these resources for bioethanol production is limited. Indeed, using land plants for the manufacturing of alternative energy becomes a problem due to competition for food resources and the resulting higher price of cereals. Besides competition with food and feed, the increased use of biomass also has effects on land use and water availability [16,17]. These limitations have led to the development of alternative feedstocks, such as

lignocellulosic biomass: wood, agricultural, or forest residue (second-generation ethanol, 2G) [18]. But current technologies for lignocellulose fermentation need to overcome the cost of the complicated procedures required to release simple sugars from recalcitrant polysaccharides [19,20]. Moreover, the increasing need for energy consumption is anticipated to continue as the world population is predicted to increase. To fulfill the normal expanding demand for bioethanol, there is, therefore, a necessity to find alternative biomass sources.

Algal biomass has recently gained extensive world attention as a source of "third-generation biofuels" [21–26], due to its abundance, high photosynthetic efficiency, and production rate without the drawbacks of first- and second-generation biofuels. Initially, microalgae production with a high lipid content was conceived; however, this third biofuel generation presents some challenges in terms of production costs since it is under development. Within the third biofuel generation, a new concept can be proposed based on the use of seaweeds (macroalgae) as a promising 3G biomass material for 1G/2G "sugar-to-ethanol" processes [27,28].

Macroalgae are known to synthesize a great variety of polysaccharides, such as agar, carrageenan, or alginates, but also alkali-soluble hemicellulosic (β-(1,4)-D-glucuronan) and (β-(1,4)-D-glucoxylan) and amorphous α-cellulose with xylose residues [29,30]. It has also been reported that green algae of the Ulva type could produce ulvan, a sulfated polysaccharide mainly composed of glucuronic acid and sulfated rhamnose [31,32]. The oligosaccharides from *Ulva latuca* represent an important added-value income of algae biorefineries [33]. Furthermore, macroalgae have earned interest due to the absence of lignin in the cell wall, which facilitates the depolymerization of these polysaccharides [34]. After hydrolysis, a wide range of simple sugars, such as glucose, galactose, xylose, arabinose, and rhamnose, are provided [2,35]. Sugars from macroalgae could be obtained via hydrothermal systems [36]. Then, monosaccharides can serve as a substrate for bioethanol production. Macroalgae hydrolysates have already been studied with brown species such as *Sargassum* spp. [37] and *Laminaria digitata* [38], red species such as *Gelidium amansii* [39] and *Palmaria palmata* [40], and green species such as *Ulva lactuca* [41].

The production yields of macroalgae per unit area are significantly higher than those for terrestrial biomass [42]. Marine algae present relatively high photon conversion efficiency, enabling rapid biomass synthesis by assimilating abundant resources in nature such as sunlight, CO_2, and inorganic nutrients [43,44]. Notably, this biomass also requires no agricultural input such as fertilizer, pesticides, and water. In addition, it can be cultivated both on seawater and on-shore [45], so one does not necessarily encroach on agricultural land required for food crops. Indeed, seaweed crops can be integrated into municipal, agricultural, or industrial wastewaters [46], as well as within buildings [47], or in innovative ring-shaped systems for growth improvement [48]. However, it is noteworthy that open ponds are the most widely used system for large-scale outdoor seaweed cultivation; this preference is attributed to their cost-effectiveness, simplicity in construction, and ease of operation [49]. Naturally occurring algae are very low in density and relatively poor in carbohydrates, so mass culturing in controlled environments could be an interesting solution to ensure reliable high productivity. Faced with thermal stress or nutrient starvation, seaweed species can alter their metabolic pathways towards the formation and accumulation of intracellular compounds such as carbohydrates or lipids to endure unfavorable environmental conditions [50]. In a previous study, it was reported that *Ulva* sp. could accumulate high carbohydrates under nitrogen starvation [51]. Therefore, controlling crop conditions emerges as a pivotal factor in enhancing carbohydrate content and, consequently, in optimizing bioethanol production.

The green macroalga *Ulva* sp. (Chlorophyceae) is a common seaweed abundantly found worldwide that also thrives in eutrophicated coastal waters, providing a potential aquatic energy crop due to its high potential growth rate and relatively high carbohydrate content (36%) [52,53]. Recently, the research in [54] proposed an integrated process based on the production of ethanol and greenhouse gas consumption from macroalgae *Saccharina japonica.*

Nevertheless, bioethanol production does not only depend on sugar availability, but also on the choice of an adequate microorganism [55,56]. Among all microorganisms utilized for bioethanol production, *S. cerevisiae* is the most common yeast strain studied for ethanol conversion of brown, red, and green algal hydrolysate biomass [21,40,57]. This strain has a high ethanol tolerance, but also high yields and rates of fermentation. So, this yeast strain was selected as the reference for ethanol production in this study. However, other yeast strains could be used. For example, *Pichia stipitis* could be interesting for xylose fermentation [58,59] and *Kluyveromyces marxianus* is able to ferment mixed sugars comprising glucose, galactose, xylose, arabinose, and mannose from green macroalgae [40,60]. Due to its broad substrate spectrum (glucose, galactose, xylose, mannitol, and rhamnose), *Candida guilliermondii* is also an interesting strain for waste valorization in ethanol [40,61,62].

Therefore, the present study seeks to establish an effective and optimized approach for bioethanol production from the green macroalgae *U. rigida* using *S. cerevisiae* at first. After the characterization of the studied algae, different hydrolysate conditioning were tested, such as mineral and nitrogen enrichment as well as sterilization methods. The presence of endogenous species and their impact on bioethanol production was also investigated. Furthermore, the impact of varying inoculum sizes and yeast strains was examined to enhance the efficiency of the process.

2. Materials and Methods

2.1. Macroalgae and Determination of Their Composition

U. rigida was provided from the Algaplus company in Portugal (Ref. Algaplus U1. 2915.F; Ref. CEVA 2015-NV-381, Ílhavo, Portugal). Seaweed was cultivated in commercial outdoor open ponds, agitated by air injection under nitrogen starvation conditions for 21 days, by interrupting water renewal to increase the carbohydrate fraction. During cultivation, an increase from 7 to 24% dry matter (DM) of glucose content was observed due to intracellular production of starch under stress conditions. The seaweed samples underwent several processing steps; they were frozen, then subsequently thawed. Once thawed, the seaweed was milled using an URSCHEL machine (Urschel, Tiel, The Netherlands), resulting in fragments of about 2 cm in size. Dry matter was determined according to the gravimetric method by drying the macroalgae at 103 °C until all moisture had evaporated. Measurement of ash was made according to the reference standard NF V 18-101. Fibers were determined according to the Association of Official Analytical Chemist (AOAC) enzymatic–gravimetric method of [63]. Total Kjeldahl nitrogen (TNK) was measured using the normalized, regulation 152/2009 and a conversion factor of 6.5 was used (N × 6.5) to estimate the protein content [64].

A turbidimetric method was used for measuring total sulfates. A solution of barium chlorides and gelatin were added to a solution of sulfates in an acid medium, leading to a precipitate of the barium sulfate formed by the association of one mole of Ba^{2+} ions and one mole of SO_4^{2-} [65].

2.2. Microorganisms and Inoculum Preparation

The yeast *S. cerevisiae* CLIB 95 was obtained from the CIRM (Centre National de Ressources Microbiennes, Marseille, France). *Scheffersomyces stipitis* 3651, *Candida guilliermondii* 11947, and *Kluyveromyces marxianus* 11954 were obtained from DSMZ in Germany. Stock cultures were maintained on Petri dishes and the medium composition was (in g· L^{-1}): glucose, 10; peptone, 5; yeast extract, 3; malt extract, 3; and agar, 15. Cultures were maintained at 28 °C for 24 h and then stored at 4 °C. The inoculum preparation was well described by [66].

2.3. Endogenous Biomass Identification

Endogenous strains were isolated from algal hydrolysate as follows: after 48 h of incubation at 180 rpm and 28 °C, 1 mL of hydrolysate was resuspended in 9 mL KCl sterilized (150 mM), then diluted in cascade and seeded in a Sabouraud medium. Characterization

of the isolated strain was realized by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) Labocea (Fougères, France) [67].

2.4. Preparation of Algal Hydrolysates

The hydrolysis of algae was carried out in a pilot (Brouillon process) with 34.45 kg of defrosted *Ulva* biomass (20.8% dry matter). The biomass was added into 120 L concentration vessel containing water to obtain a 10% dry matter suspension and heated at 60 °C. The pH was adjusted at 5.5 by the addition of HCl (1 M). The gelatinization step was achieved by stirring and heating the biomass at 100 °C for 30 min. After cooling at room temperature, amylase enzyme was added in the reaction medium at 2.9% level based on the dry weight (DW) of starch, and then kept at 85 °C for 2 h to carry out the liquefaction step. Amyloglucosidase enzyme was added at 11.6% level based on the DW of starch and they were heated at 65 °C for 2 h under stirring to achieve the scarification step. Enzymes were deactivated by heating the mixture at 100 °C for 30 min. After they were cooled at 60 °C, the suspension was then transferred into a 100 L reactor. The pH was then adjusted to 0.88 using sulfuric acid 96%. To allow hydrolysis of co-extracted ulvans, the mixture was heated at 70 °C for 6 h, before being cooled at 40 °C to limit viscosity of the reaction mixture. pH was then adjusted to 5.0 by adding Na_2CO_3. Finally, the hydrolysate residues smaller than 11 µm sieve was recovered by filtration (Sweco Separator, Sweco, Nivelles, Belgium). The hydrolysate was autoclaved at 120 °C for 15 min, and the analysis of the biochemical composition of autoclaved and non-autoclaved hydrolysates was outsourced to the laboratory Agrobio (Vezin-le-Coquet, Brittany, France). Hydrolysate enrichment was carried out with NH_4Cl (1.07 $g{\cdot}L^{-1}$) or peptone (5 $g{\cdot}L^{-1}$).

2.5. Synthetic Medium

A synthetic medium was used as a model for the comparison to the hydrolysate-based medium. Its composition was a simple sugar (glucose) and salts at levels close to those found in the green algae *U. rigida* (NaCl, 0.25 M and SO_4^{2-}, 0.21 M). This medium was enriched with peptone (5 $g{\cdot}L^{-1}$) (source of nitrogen) and with mineral supplementation as described by [66].

2.6. Ethanol Fermentation

A rotating shaker (New Brunswick, INNOVA 40, Shirley, NJ, USA) was used for ethanol production assays at 180 rpm, 28 °C. The volume of the culture media was 250 mL and the culture time was 48 h. Inoculation levels were 0.1% or 1% (v/v). Samples were regularly withdrawn and centrifuged at 3000 rpm, 4 °C and 5 min. All experiments were duplicated. Fermentation efficiency corresponded to the ratio of the ethanol produced over the ethanol theoretically produced ratio ((Ethanol) o/t).

$$(\text{Ethanol})_{\text{theor}} = 2 \times (\text{glucose}) \times \left(\frac{M_{\text{ethanol}}}{M_{\text{glucose}}}\right);\ \text{Ethanol}_{\text{o/t}} = \frac{(\text{ethanol})_{\text{observed}}}{(\text{ethanol})_{\text{theor}}}$$

$$\text{Efficiency} = \frac{\text{Pratical yield of ethanol}}{\text{Theoretical yield}} \times 100$$

2.7. Analytical Methods

Analysis of total sugars and free sugars was determined using HPLC with an apolar column C18 after degradation of the polysaccharides by acidic methanolysis. Free sugar content was determined by the HPLC method with a column Rezex pb^{2+}. The HPLC involving an ion exclusion column HPX-87H (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) was used to measure the various metabolites produced by yeast including ethanol and glycerol. The analytical conditions were well described by Djelal et al. [66]. Assay of cell growth in the samples was measured with a spectrophotometer (SECOMAM, Ales, France) at 600 nm; this assay was performed twice, just directly after the levy and then samples were

centrifuged at 3000 rpm, 4 °C and 5 min; the difference between the two assays represented the cell growth. The pH was adjusted at 6 (pH meter 315i, WTW, Frankfurt, Germany) by the addition of sterile KOH 2 mol·L^{-1}. The salts founded in algae were analyzed with Dionex DX 120 (ThermoFisher Scientific, Waltham, MA, USA) equipped with a conductivity detector and Anion exchange column AS19 (4 × 250 mm) as the stationary phase. KOH was used at the mobile phase and the flow rate was set at 1 mL·min^{-1}. Analyses were carried out with a gradient elution mode. A simplified schematic representation for Section 2 is presented in Appendix A.

3. Results and Discussion

3.1. Global Composition of Ulva sp.

U. rigida composition was analyzed for its nitrogen and carbohydrate contents to obtain information regarding its potential as biomass for ethanol production. The global chemical composition of *U. rigida* is summarized in Table 1. *Ulva* contained an important quantity of fibers compared to other macroalgae; the fiber content reached 34.9% showing its relevance for bioenergy production [68]. The carbohydrate content of green macroalgae was 25–50% dry weight. This result is consistent with the findings of [69], who reported a carbohydrate content of 27.9 ± 0.4% in *U. rigida*.

Table 1. Chemical composition of cultivated *U. rigida*.

Proximate Composition	Relative % (Dry Weight Basis/Brut)
Dry matter	94.9 ± 0.05
Fibers	34.90 ± 0.05
Protein	5.68 ± 0.05
Ash	31.9 ± 0.05
N Kjeldahl	0.91 ± 0.05
Total sulfates	7.8 ± 0.05

Green algae contain glucans and sulfated polysaccharides (e.g., ulvan) as the major carbohydrate fraction. Ulvan is a water-soluble polysaccharide, constituted of repeated disaccharide units composed of sulfated rhamnose, glucuronic acid, iduronic acid, and xylose (Table 2). The composition of ulvan varies from one green algae to another. For example, ulvans from *Ulva fasciata (U. fasciata*) are mainly composed of rhamnose, xylose, and glucuronic acid [70,71]; whereas those from *Ulva armoricana* are mainly composed of rhamnose, glucuronic acid, and iduronic acid [32,72].

Table 2. Carbohydrate chemical composition of *U. rigida*.

Carbohydrate Content	Glucose	Rhamnose	Xylose	Iduronic Acid	Glucuronic Acid
Relative % (dry weight basis/brut)	25.6 ± 0.05	6.5 ± 0.05	2.1 ± 0.05	0.6 ± 0.05	2.8 ± 0.05

The protein content was 5.68%, which is not high compared to other green algae, for example *U. fasciata*, which presents a protein content of 14.4% [73]. The variation in the reported composition of Ulva species may be related to several environmental factors, such as water temperature, salinity, light, and nutrients which influence their ability to stimulate or inhibit the biosynthesis of several compounds [74].

Additionally, this macroalgae showed a high amount of ash (31.9%), which is in accordance with the usual values known for green algae ranging from 11% to 34% on a dry weight basis [69,75]. These results demonstrate that the macroalgae *U. rigida* are potentially good sources of polysaccharides and proteins.

3.2. Fermentation of Algal Hydrolysate with S. cerevisiae

The fermentation of the algal hydrolysate of *U. rigida* by *S. cerevisiae* with 0.1% (*v*/*v*) inoculation size was studied. The kinetics of the concentrations of glucose, ethanol, glycerol, and acetic acid during the fermentation are shown in Figure 1. Glucose was fermented in 48 h by *S. cerevisiae* and 9.55 g·L^{-1} of ethanol was produced. The concentration of glycerol and acetic acid produced increased during the fermentation and reached 1.3 and 0.36 g·L^{-1} at the end of the fermentation.

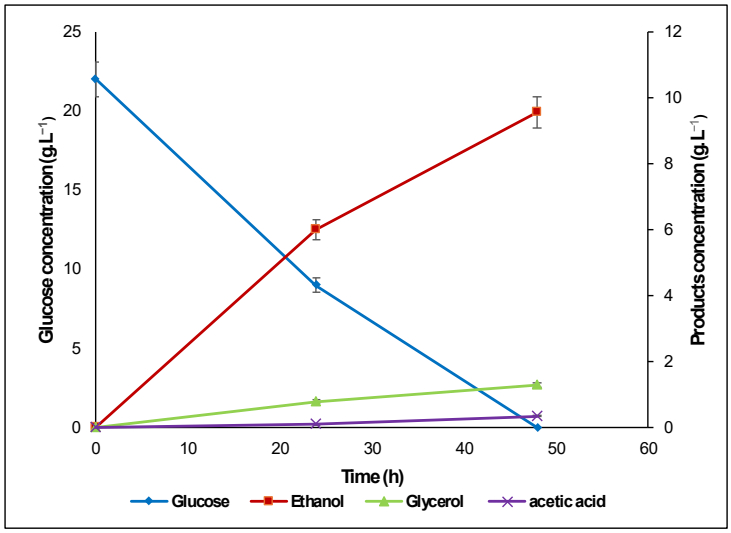


Figure 1. Glucose consumption, ethanol, acetic acid, and glycerol production during 48 h of fermentation of algal hydrolysate by *S. cerevisiae*.

The results obtained in the algal hydrolysate and the synthetic model medium are compared in Figure 2, showing that *S. cerevisiae* produces close yields of glycerol and acetic acid but more yields of ethanol with higher efficiency (experimental ethanol yield/theoretical ethanol yield) in the algal hydrolysate than those in the synthetic medium. The ethanol yields and the efficiency were 0.44 g·g^{-1} (0.57 C/C) reducing sugar and 85.33%, respectively, in the algal hydrolysate, whereas 0.38 g·g^{-1} and 74.35% were obtained in the synthetic medium. Therefore, algal hydrolysate is a performant source of carbon and nitrogen to produce bioethanol.

If compared to the related literature, it appears that the ethanol yield found (0.44 g·g^{-1} reducing sugar) was superior to those from *Eucheuma cottonii* and *Sargassum sagamianum* hydrolysates (0.33 and 0.39 g·g^{-1} reducing sugar) [76,77]; meanwhile, it was close to some other results. Indeed, 0.43 g·g^{-1} was obtained during the fermentation of the red algae *Gracilaria verrucosa* [78].

Lee et al. [79] obtained ethanol yields of 0.43–0.44 g·g^{-1} reducing sugar. An ethanol yield of 0.47 g·g^{-1} reducing sugar was obtained during the fermentation of the algal hydrolysate of the green algae *U. fasciata*, corresponding to 93.81% conversion efficiency [80]. Choi et al. [81] used the glucose contained in the hydrolysate of *Ulva pertusa Kjellman* for bioethanol production by *S. cerevisae* and the concentration of ethanol was approximately 90% of the maximum theoretical ethanol yield. It can be noticed from the ethanol yield and efficiency obtained, that algal hydrolysate of *U. rigida* has a great potential as a raw biomass for bioethanol production.

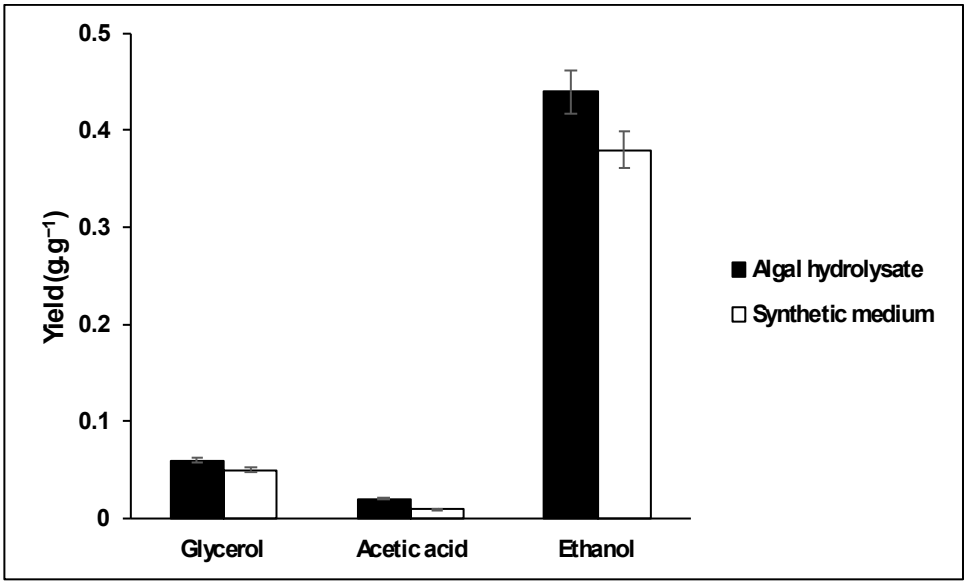


Figure 2. Glycerol, acetic acid, and ethanol yields ($g \cdot g^{-1}$), after 48 h of fermentation by *S. cerevisiae* in the algal hydrolysate (■) and the synthetic medium (□).

3.3. Optimization of the Fermentation with S. cerevisiae

3.3.1. Effect of Medium Enrichment

For comparative purposes, two types of enrichment were tested; a mineral one, NH_4CL with mineral supplementation as described by [65], and an organic one, peptone. The corresponding results recorded during *S. cerevisiae* culture (0.1% *v*/*v* inoculum) are summarized in Table 3.

Table 3. Effect of minerals, ammonium chloride, and peptone on fermentation of algal hydrolysate by *S. cerevisiae*, after 48 h of fermentation.

Conditions	Cell Density (600 nm)	Ethanol ($g \cdot L^{-1}$)	Productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Glycerol Yield (%) (*c*/*c*)	Ethanol Yields (%) (*c*/*c*)	Efficiency (%)
Without nutrients, ammonium chloride and peptone	9.35 ±0.05	9.55 ± 0.05	0.20 ± 0.05	5.87 ± 0.05	56.83 ± 0.05	85.33 ± 0.05
With nutrients and ammonium chloride	9.10 ±0.05	9.41 ± 0.05	0.20 ± 0.05	6.87 ± 0.05	55.92 ± 0.05	83.96 ± 0.05
With peptone	9.30 ±0.05	9.40 ± 0.05	0.20 ± 0.05	6.03 ± 0.05	55.79 ± 0.05	83.76 ± 0.05

S. cerevisiae showed the same fermentation time of glucose (48 h) in the presence or absence of enrichment, even with a mineral or organic nitrogen source. Adding nitrogen had no impact on the rate of ethanol production which was 0.20 $g \cdot L^{-1} \cdot h^{-1}$ in all cases. Ethanol and glycerol yields were not improved by nitrogen supplementation. *S. cerevisiae* showed the same trend of growth regardless of the type of enrichment.

The high content of proteins and minerals in algae should account for the absence of the impact of mineral and nitrogen additional sources on *S. cerevisiae* growth; in fact, algae are sources of proteins [82]. The amount of proteins that can be found in the raw material may increase after hydrolysis, Hou et al. [38], who studied *Laminaria digitata,* found that the protein content in the solid residues after fermentation was enriched 2.7 fold, and they found that amino acids contained in peptone were also abundant in this macroalgae. Moreover, the authors of [73] found that the enrichment of culture medium with yeast extract and peptone during fermentation of *U. fasciata* had no impact on ethanol yields,

which was supported by the high protein content of the seaweed *U. fasciata*, 14.4 ± 2.2% on dry basis. Therefore, the use of an additional source of nitrogen in the fermentation of algal hydrolysates is not needed. Consequently, the hydrolysate obtained after the enzymatic saccharification could be fermented directly without the addition of nitrogen sources and minerals.

3.3.2. Inoculum Size Effect

The amount of inoculum used is one of the main factors that influence fermentation, specific consumption, and production rate. The 0.1 and 1% (*v*/*v*) inoculum sizes were investigated to determine whether they could affect ethanol fermentation. Glucose was totally consumed (100%) in 24 h of fermentation in the case of 1% inoculation, whereas for the 0.1% inoculation level this percentage decreased to 58% (Table 4). Maximum ethanol productivity (0.40 $g \cdot L^{-1} \cdot h^{-1}$) was also obtained with 1% inoculation, leading to 9.64 $g \cdot L^{-1}$ of ethanol produced, while these results were divided by two in the case of 0.1% inoculation.

Table 4. Inoculum size effect on fermentation by *S. cerevisiae* over 24 h of fermentation.

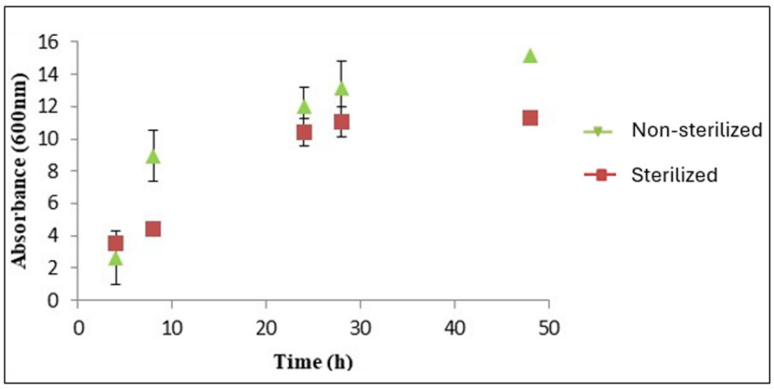
Inoculum Size % (*v*/*v*)	Ethanol ($g \cdot L^{-1}$)	Sugar Consumed %	Productivity $g \cdot L^{-1} \cdot h^{-1}$	Glucose Consumption Rate $g \cdot L^{-1} \cdot h^{-1}$	Y Ethanol (%) (*c*/*c*)	(Ethanol)o/t Ratio (%)
0.1	5.54 ± 0.05	58 ± 0.05	0.23 ± 0.05	0.54 ± 0.05	56.19 ± 0.05	84.38 ± 0.05
1	9.64 ± 0.05	100 ± 0.05	0.40 ± 0.05	0.95 ± 0.05	55.20 ± 0.05	82.89 ± 0.05

On the contrary, inoculum size did not influence ethanol yield, which was 56.19 and 55.20% (*c*/*c*) for 0.1 and 1% (*v*/*v*) inoculation levels, respectively. In terms of ratio, (Ethanol) o/t and ethanol yield 0.1 and 1% *v*/*v* inoculation showed very similar results. Many studies [83,84] have reported that the size of the inoculum enhanced the rate of 2,3-butanediol formation but not its product on carbon substrate yield. Glucose consumption rate also increased with the inoculation level; it rose from 0.54 to 0.95 $g \cdot L^{-1} \cdot h^{-1}$; the productivity was also influenced by the quantity of inoculated cells. However, a study of the optimization of the fermentation of glucose by *Bacillus licheniformis*, probably carried out using a factorial design [85], demonstrated that an increase in the size of the inoculum also had a positive effect on the yield on butanediol production. In the present work, raising the inoculation level increased the production rate, but not the amount of ethanol yield. High ethanol productivity is an economically relevant factor for an industrial purpose and hence the 1% inoculation level was selected and considered thereafter.

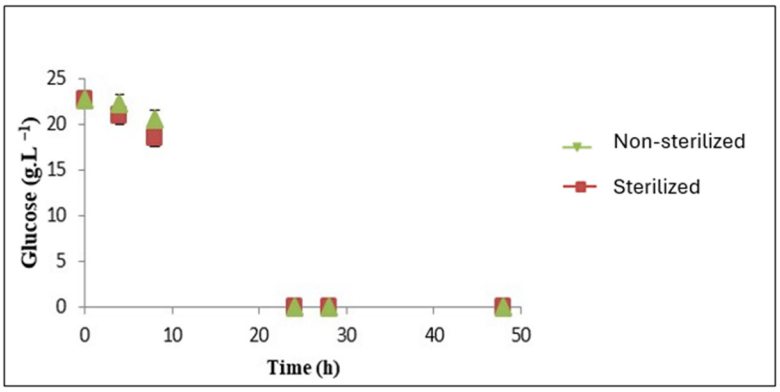
3.3.3. Sterilization Effect

In many microbial processes, sterilization costs impact significantly the total production cost [86]. Ethanol production under non-sterilized conditions has gained high attention for energy saving considerations. The impact of this parameter was therefore examined. *S. cerevisiae* was cultured in sterilized (autoclaved at 120 °C for 15 min) and non-sterilized hydrolysate with 1% (*v*/*v*) inoculum size, at pH 5. Indeed, only a very slight decrease in lipid, carbohydrate, and calcium was observed, showing the stability of the hydrolysates under the considered sterilization conditions. Ethanol o/t ratio, the ratio of the experimental to the theoretical ethanol produced, was found to be 82.7% in the sterilized medium and 80.7% in the non-sterilized medium. This slight difference was potentially caused by the small number of endogenous bacteria present in the medium due to the unsterilized conditions, which can also be the reason for the difference in growth observed in Figure 3a. Maillard reactions could be processed by the autoclave, leading to negative effects, such as the generation of undesired furfural compounds, other nutritional elements, and the degradation of sugars [87]. The glucose concentrations before and after the sterilization process were 22.75 $g \cdot L^{-1}$ and 22.65 $g \cdot L^{-1}$, respectively, showing no sugar loss and hence indicating the absence of significant impact of the Maillard

reactions between glucose and nitrogen sources as often observed during culture media sterilization (Figure 3b). Regarding the ethanol concentration, it was also not significantly affected by the sterilization (Figure 3c); indeed, the ethanol concentrations were 9.99 $g \cdot L^{-1}$ and 9.59 $g \cdot L^{-1}$ in the sterilized and non-sterilized medium, respectively. It agrees with other findings [88] that demonstrated the feasibility and the potential of the non-sterile fermentation process of oil mill wastewater. Ethanol production in non-sterile fermentation by *S. cerevisiae* was successfully achieved in their study; no significant statistical differences were observed for both biomass and ethanol production between sterile and non-sterile cultures. Contrarily, most of the related studies found an impact of sterilization on the fermentation performances. Glucose concentration decreased by 5% after autoclaving during ethanol production by *Zymomonas mobilis*. This loss was attributed to the Maillard reactions between glucose and the nitrogen sources, leading to an increase in the ethanol yield for the non-autoclaved process, from 70 to 73 $g \cdot L^{-1}$ compared with the autoclaved process (fermentation at pH = 4.5); the rate of glucose utilization in the non-sterilized media was also found to be higher compared to autoclaved media [89]. The impact of the sterilization on the composition of the hydrolysate was examined, showing its negligible impact (Table 5). From non-sterile and not seeded algal hydrolysate, bacteria were isolated and identified as *Pseudomonas putida*, an aerobic strict, Gram-negative bacterium. This resistant microorganism could be largely encountered in soil and water. However, its impact could be neglected, since in the absence of inoculation, only 5% (result not shown) of the glucose present in the hydrolysate was consumed after 48 h. Consequently, the presence of this resistant bacterium did not significantly impact ethanol fermentation. The absence of sterilization constitutes a significant advantage to the process for its subsequent implementation on an industrial scale [89,90].

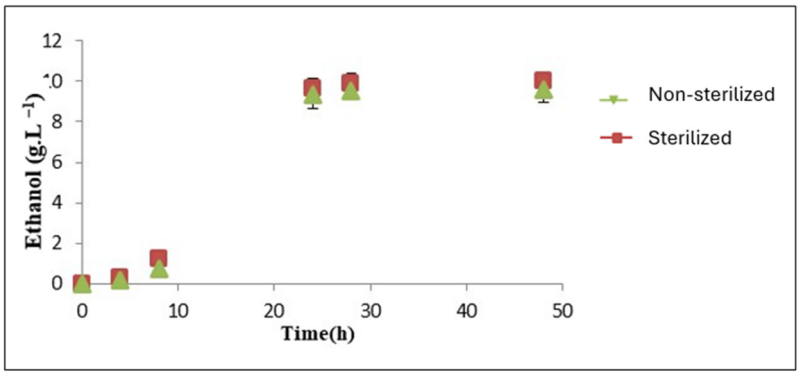


(**a**)



(**b**)

Figure 3. *Cont.*



(**c**)

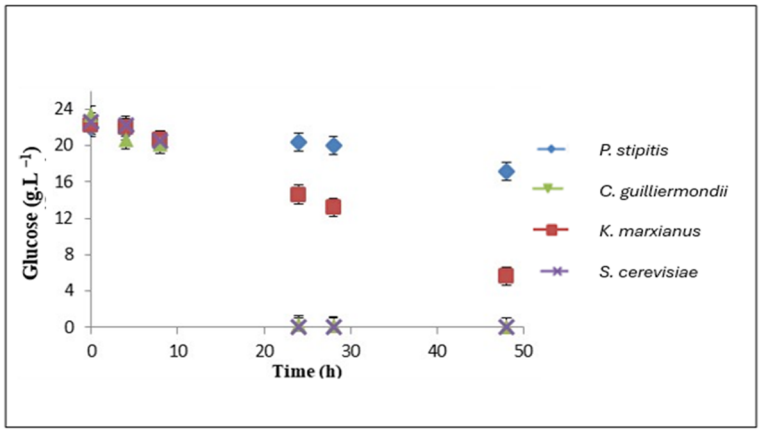
Figure 3. Growth rate (**a**), glucose consumption (**b**), and ethanol production (**c**) for *S. cerevisiae* with the non-sterilized and the sterilized algal hydrolysate.

Table 5. Chemical composition of autoclaved and non-autoclaved hydrolysates.

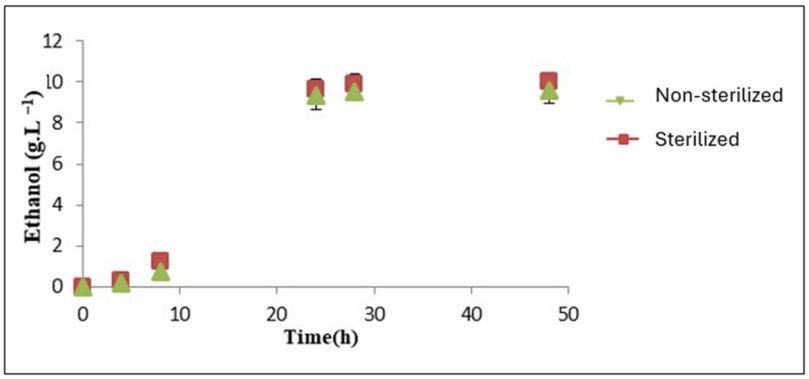
	Composition % (Dry Weight/Dry Weight)					
	Ash	**Moisture**	**Lipids**	**Total Nitrogen**	**Carbohydrates**	**Calcium (mg/100 g)**
Hydrolysate	5.63 ± 0.05	88.8 ± 0.05	0.3 ± 0.05	0.4 ± 0.05	4.9 ± 0.05	55.2 ± 0.05
Autoclaved hydrolysate	5.52 ± 0.05	89.2 ± 0.05	0.1 ± 0.05	0.4 ± 0.05	4.8 ± 0.05	54.0 ± 0.05

3.4. Comparison with Three Other Yeast Strains

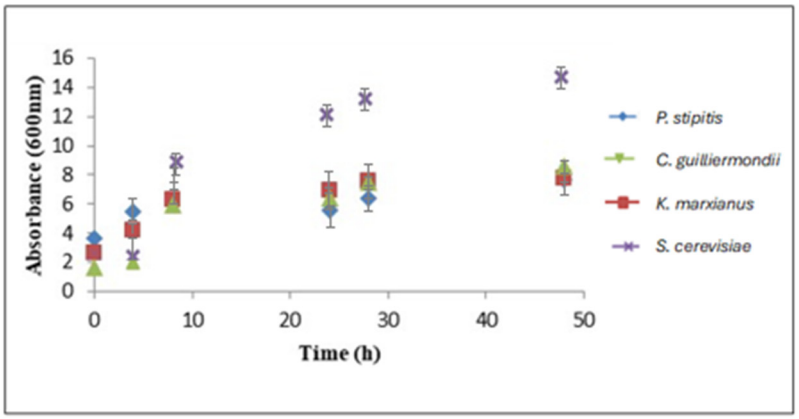
The hydrolysate was subjected to fermentation without sterilization or enrichment and for an inoculum size of 1% (*v*/*v*). Batch cultures were carried out using four different yeasts: *S. cerevisiae*, *K. marxianus*, *P. stipites*, and *C. guilliermondii.* These yeasts have proven their relevance for ethanol fermentation from various biomasses [77,91,92]. Fermentation of the algal hydrolysate using the four yeasts was compared to synthetic medium to demonstrate the potentiality of the algal hydrolysate in the production of ethanol. The glucose consumption from algal hydrolysate varied according to the evaluated yeast. Glucose was completely consumed after 24 h by *S. cerevisiae* and *K. marxianus*; meanwhile, after 48 h, *C. guilliermondii* consumed 74.95% of the glucose present in the hydrolysate, and *P. stipitis* showed a slow consumption, only 22% of the available glucose (Figure 4a). Because of the differences in glucose consumption, bioethanol production varied for each yeast (Figure 4b), and the four yeasts can be classified according to the level of ethanol production as follows: *S. cerevisiae* > *K. marxianus* > *C. guilliermondii* and *P. stipitis. S. cerevisiae* exhibited the highest and fastest growth, because of its higher and faster consumption of sugars compared to the other strains; despite its low glucose consumption, the growth observed for *P. stipitis* was like that observed for *C. guillermondii* and *K. marxianus* (Figure 4c). For *S. cerevisiae* and *K. marxianus*, after 22 h of fermentation, all the glucose was depleted and ethanol production ceased after 28 h of culture, while growth continued until the end of fermentation (48 h). This can be caused by the richness of proteins and vitamins of the algal hydrolysate. As regards the ethanol o/t ratio, the results were found to be maximal with *S. cerevisiae* with a value of 82.87% after 48 h of fermentation; meanwhile, *K. marxianus* and *C. guillermondii* showed 70.49 and 63.20%, respectively, and only 52.25% for *P. stipitis* (Table 6).



(**a**)



(**b**)



(**c**)

Figure 4. Glucose consumption (**a**), ethanol production (**b**), and cell density (**c**) for *P. stipitis*, *C. guilliermondii*, *K. marxianus*, and *S. cerevisiae* during fermentation of algal hydrolysate without sterilization or enrichment and for an inoculum size of 1% (*v*/*v*).

Moreover and unlike the other strains, *P. stipitis* displayed a very low ethanol production rate and glucose consumption rate in the algal hydrolysate if compared to the other strains; this should be related to the sensibility of this strain to the aeration conditions and its need for a micro-oxygenation of the medium, essentially given by a high agitation [93]. Studies [94,95] have shown that oxygen concentration is an important factor for *P. stipitis* to ferment glucose and xylose into ethanol. Under anaerobic conditions, *P. stipitis* cells suffered a decrease in ethanol yield and fermentation rate [96]. The algal hydrolysate of *U.*

rigida contains glucose as the main sugar, and *P. stipitis* is recognized for its higher ethanol productivity growing on glucose than on xylose [97]; for that, it was chosen for this study. But the aeration conditions applied for the fermentation were not appropriate for ethanol production by *P. stipitis*.

Table 6. Comparison of the results obtained during yeasts' growth on synthetic medium and algal hydrolysate at 48 h of fermentation.

	Glucose Consumption Rate at 48 h ($g \cdot L^{-1} \cdot h^{-1}$)		Ethanol Production Rate ($g \cdot L^{-1} \cdot h^{-1}$)		(Ethanol) o/t Ratio	
	Hydrolysate	**Synthetic Medium**	**Hydrolysate**	**Synthetic Medium**	**Hydrolysate**	**Synthetic Medium**
K. marxianus	0.49 ± 0.05	0.43 ± 0.05	0.18 ± 0.05	0.14 ± 0.05	70.49 ± 0.05	64.10 ± 0.05
P. stipitis	0.10 ± 0.05	0.41 ± 0.05	0.03 ± 0.05	0.10 ± 0.05	52.25 ± 0.05	49.37 ± 0.05
C. guilliermondii	0.35 ± 0.05	0.43 ± 0.05	0.11 ± 0.05	0.09 ± 0.05	63.20 ± 0.05	42.97 ± 0.05
S. cerevisiae	0.47 ± 0.05	0.42 ± 0.05	0.20 ± 0.05	0.16 ± 0.05	82.87 ± 0.05	74.35 ± 0.05

In addition, a decrease in the performances was observed for *P. stipitis* in the algal hydrolysate if compared to the synthetic medium; the glucose consumption rate decreased from 0.10 to 0.03 $g \cdot L^{-1} \cdot h^{-1}$ and ethanol production rate was divided by four. On the other hand, a faster fermentation in the hydrolysate compared to the synthetic model medium was observed for the other strains. Rouhollah et al. [98] tested the fermentation of sugars by *P. stipitis, S. Cerevisiae* and *K. marxianus,* and observed that *P. stipitis* ferments glucose more slowly (30 times of fermentation) than the two other yeasts. However, in the present study, *P. stipitis* showed similar rates of production (approximately 0.12 $g \cdot L^{-1} \cdot h^{-1}$) and consumption (0.42 $g \cdot L^{-1} \cdot h^{-1}$) in the synthetic model medium compared to the other strains. The fermentation process was therefore affected by the composition of the algal hydrolysate; nutrients such as trace elements or vitamins can be required for *P. stipitis* to achieve rapid fermentation, or the presence of some inhibitors have decreased the rate of production of ethanol and consumption of glucose by *P. stipitis.*

Based on ethanol yields, namely ethanol produced over glucose consumed (expressed in carbon/carbon), the results were as follows: *S. cerevisiae* (55% C/C) > *K. marxianus* (47%) > *C. guilliermondii* (42% C/C) > *P. stipitis* (35%) (Figure 5). From this, *S. cerevisiae* appeared to be the most promising candidate for the valorization of glucose contained in algal hydrolysates. It should be noted that during fermentation acetic acid and glycerol were secreted by the yeasts. For acetic acid yields, *S. cerevisiae* was found to be the highest producer with (3% C/C), whereas *P. stipitis* did not produce this acid. Contrarily, this latter species produced the highest yields of glycerol, 8% C/C; while *S. cerevisiae*, *K. marxianus,* and *C. guilliermondii* produced 6, 3, and 1% C/C, respectively. This high glycerol yield showed an attempt to adapt their metabolism to the algal hydrolysate.

S. cerevisiae, C. guilliermondii, and *K. marxianus* were therefore able to ferment algal hydrolysate and produce ethanol, as well as *P. stipitis* but at a lower production rate. *S. cerevisiae* showed relevant results, in agreement with the related literature. Indeed, some studies [99] have reported that *S. cerevisiae* was able to produce 7.2 $g \cdot L^{-1}$ of ethanol by fermenting *Ulva pertusa* hydrolysate. Furthermore, some research [100] recorded high levels of ethanol produced by a wild *S. cerevisiae* strain growing on *Sargassum* spp. hydrolysate based on glucose as substrate. To save time, the fermentation was stopped at 24 h and despite this, *S. cerevisiae,* the most studied for ethanol conversion of cellulosic and lignocellulosic biomass [101,102], still gave the best results in terms of ethanol produced (9.35 $g \cdot L^{-1}$) and conversion efficiency (80.78%).

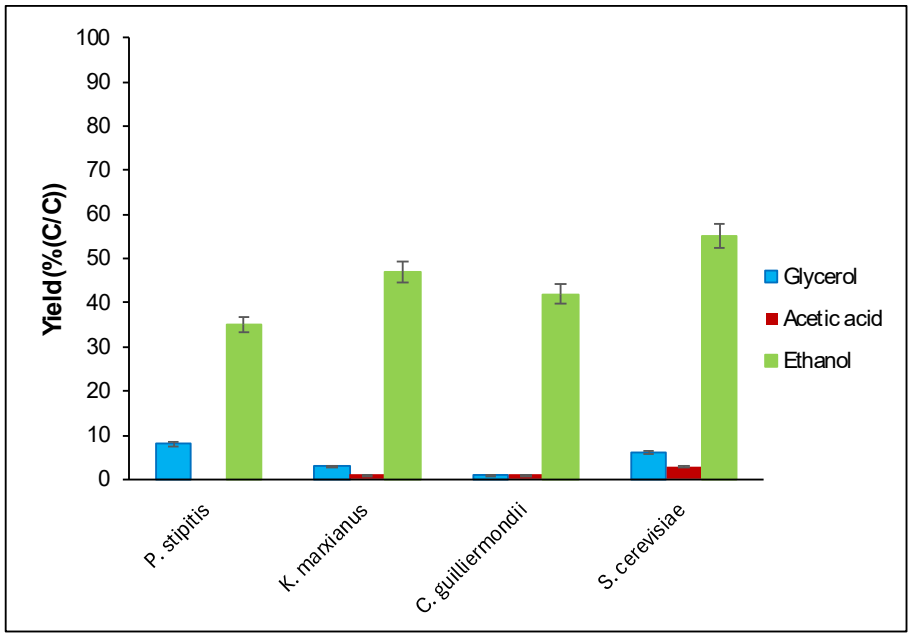


Figure 5. Glycerol, acetic acid, and ethanol yields obtained with the various yeasts at 48 h of fermentation.

3.5. Comparison of Ethanol Yields for Different Green Algal Feedstocks

The culture performances achieved with *S. cerevisiae* (1% *v*/*v* inoculum, non-sterilized, and non-enriched hydrolysate) were compared to the performances reported for various algal feedstocks (Table 7). The ethanol yield obtained in this study (0.41 $g \cdot g^{-1}$) exceeded the values reported for the green seaweed *U. rigida* by [103] and [52], which were 0.37 $g \cdot g^{-1}$ and 0.33 $g \cdot g^{-1}$, respectively. Similarly, it outperformed the red seaweed *Kappaphycus alvarezii* (0.39 $g \cdot g^{-1}$) despite using the same *S. cerevisiae* strain, enriching the algal hydrolysate, and employing a higher inoculum size [104]. This result was in agreement with other findings [105] during the fermentation of the green algae *Chaetomorpha linum* (0.41 $g \cdot g^{-1}$). However, it was lower than those of other studies [78]. The potential of algal biomass appears therefore highly promising; nonetheless, it is imperative to assess the current state of the resource, considering the imperative to avoid any alteration to the biodiversity of the encompassing ecosystem.

Table 7. Comparison of ethanol yields obtained during *S. cerevisiae* growth on various algae.

Algae	Fermenting Strain	Enrichment	Size of Inoculum	Ethanol Yield	References
U. rigida	*S. cerevisiae* CLIB 95	Without enrichment	1% *v*/*v* (0.12 g)	0.54% (*c*/*c*) (0.41 $g \cdot g^{-1}$ reducing sugar)	Present study
U. rigida	*Pachysolen tannophilus*	-	5%	0.37 $g \cdot g^{-1}$	[103]
Colpomenia sinuosa	*Meyerozyma guilliermondii*,	-	10%	0.26 $g \cdot g^{-1}$	[106]
Kappaphycus alvarezii	*S. cerevisiae* (NCIM 3455)	With nitrogen source	5% *v*/*v*	0.39 $g \cdot g^{-1}$	[104]
Chaetomorpha linum	*S. cerevisiae (Baker's yeast)*	Yeast extract Peptone medium	10% *v*/*v*	0.41 $g \cdot g^{-1}$	[105]
U. rigida	*S. cerevisiae (beaker)*	-	0.5 g	333.3 $mg \cdot g^{-1}$	[52]
Gracilaria sp.	*S. cerevisiae*	2 $g \cdot L^{-1}$ yeast extract	10% *v*/*v*	0.47 $g \cdot g^{-1}$	[107]
Gracilaria verrucosa	*S. cerevisiae*	3 $g \cdot L^{-1}$ yeast extract and 0.25 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$	6% *v*/*v*	0.43 g·g-$^{-1}$	[78]

Khambhaty and colleagues [104] estimated the ethanol production from the red algae *Kappaphycus alvarezii* at 2.3 kg/1000 kg fresh weight; the percentage of ethanol reached 2.46%. In the present study, ethanol production from the green algae *U. rigida* was estimated at 90 kg/1000 kg dry weight corresponding to an ethanol content of 1.04%.

This comparison showed that the results of the present study were comparable to those reported in the literature; therefore, the green algae *U. rigida* holds potential as a feedstock for bioethanol production.

Ethanol production under non-sterilized conditions has gained the attention of many researchers since it can save 30–40% energy consumption in cooking starch and sterilization during ethanol production, which also simplifies the process [89]. In addition, nitrogen, trace elements, and vitamins are needed to achieve rapid fermentation and high levels of ethanol, but they increase production costs, especially on an industrial scale. In this study, fermentation experiments were carried out without sterilization or enrichment; so, the combination of the energy saving of the non-sterilized and non-enriched process on the one hand and the efficiency of ethanol production by *S. cerevisiae* on the other hand is promising for future implementation on a larger scale.

4. Conclusions

Algal seaweed exhibits significant potential as one of the most important renewable energy sources. This present study successfully demonstrated that the green algae *U. rigida* serves as an attractive biomass material that can be readily converted into ethanol. Following enzymatic hydrolysis, we evaluated fermentation parameters. The results indicate that the optimal conditions for the fermentation of the green macroalgal hydrolysate, resulting in an 80.78% conversion efficiency, were as follows: using *S. cerevisiae* under non-sterilized conditions, without enrichment, and with an inoculum size of 1% (v/v). Among the strains studied, *S. cerevisiae* appeared to be the most promising for fermenting algal hydrolysates.

Author Contributions: Conceptualization, A.C., W.S. and M.B.; methodology, A.C., W.S. and M.B.; validation, H.D., A.A. and R.P.; formal analysis, W.S. and M.B.; investigation, W.S., A.C. and M.B.; writing—original draft preparation, W.S., A.C. and M.B.; writing—review and editing, A.S., H.D. and A.A.; supervision, H.D., A.A. and R.P.; funding acquisition, R.P. and A.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

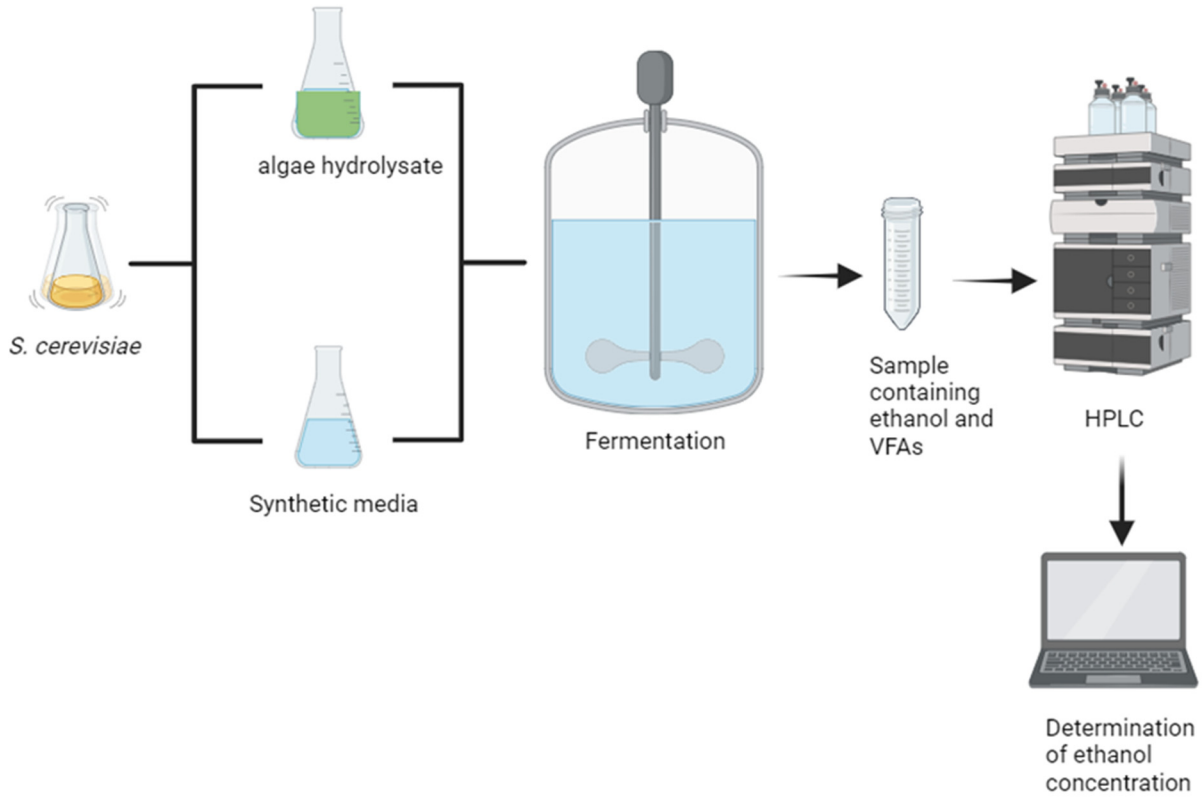

Figure A1. Simplified design for the protocol used to produce ethanol.

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