processes

MDPI

*Article*

# Optimizing the Enzymatic Hydrolysis of Bioflocculated Microalgae for Bioethanol Production

**Viviane Simon, João Felipe Freitag, Júlia Lorenzato da Silva and Luciane Maria Colla ***

Graduate Program in Civil and Environmental Engineering (PPGEng), University of Passo Fundo (UPF), Campus I, L1 Building, BR 285, Bairro São José, Passo Fundo 99052-900, RS, Brazil; 158662@upf.br (J.F.F.); 170930@upf.br (J.L.d.S.)

* Correspondence: lmcolla@upf.br; Tel.: +55-(54)-3316-8193

**Abstract:** *Spirulina platensis* is a promising microalga, but biomass harvesting remains a challenge. Fungal bioflocculation offers a potential solution, facilitating the production of valuable bioproducts like bioethanol. Effective cell disruption methods, including physical-chemical and enzymatic treatments, can enhance biomass utilization. However, commercial enzymes are not optimized for microalgae, necessitating research on ideal operational conditions. This study evaluated physical and enzymatic processes to hydrolyze bioflocculated microalgae for bioethanol production. The microalga was harvested using a fungal bioflocculant produced via submerged fermentation. Biomass hydrolysis involved physical methods (autoclaving, ultrasound + autoclaving, ultrasound + gelatinization, and gelatinization) combined with enzymes (amylase, amyloglucosidase, cellulase, and xylanase), optimized for pH, temperature, and enzyme load. Hydrolysates were then used for bioethanol production. Results showed a microalgae harvest efficiency of 99.7% with a 1:8 fungus-to-microalgae ratio. Enzyme optimization identified ideal conditions (e.g., pH 4.5; 60 °C for amylase/amyloglucosidase, 70 °C for cellulase, and 50 °C for xylanase). Combined enzymatic treatments achieved approximately 70% hydrolysis efficiency, yielding 19.06 g/L glucose and 7.29 g/L ethanol (~79% conversion). Ethanol productivity was ~0.6 g per 1 g bioflocculated biomass $L^{-1}$·hr. These findings highlight the potential of enzymatic hydrolysis for complex biomasses, although further studies are needed to refine enzyme applications for better biomass utilization.

**Keywords:** biomass valorization; fungal-assisted bioflocculation; pretreatment; enzymatic saccharification; enzyme association; bioenergy

check for updates

Academic Editors: Marija Koprivica, Jelena Petrović and Marija Simic

Received: 31 December 2024
Revised: 23 January 2025
Accepted: 25 January 2025
Published: 28 January 2025

**Citation:** Simon, V.; Freitag, J.F.; da Silva, J.L.; Colla, L.M. Optimizing the Enzymatic Hydrolysis of Bioflocculated Microalgae for Bioethanol Production. *Processes* **2025**, *13*, 364. https://doi.org/10.3390/pr13020364

**Copyright:** © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

## 1. Introduction

With the increasing demand for energy, sustainable alternatives are being sought to meet global needs and reduce the environmental impacts of fossil fuels. The use of biomass, such as microalgae, is a viable environmental alternative due to its ability to cycle atmospheric carbon through photosynthesis, where it captures carbon dioxide and produces oxygen [1]. Moreover, compared to biomass used in second-generation fuels (which rely on lignocellulosic plant biomass, such as straw, bagasse, and leaves), microalgae overcome technological barriers related to lignin recalcitrance, which hinders consolidation due to the complexity of converting raw materials into biofuel [2].

Microalgal biomass can be used in integrated biorefineries due to its ability to accumulate reserve compounds such as carbohydrates (between 22% and 60%) and lipids (between 4% and 75%) [3–5], as well as other industrially important biomolecules (proteins, pigments, vitamins, and other bioactive compounds) [6,7]. By fully utilizing the cellular

composition of microalgae, biorefineries offer a profitable and competitive approach for the co-production of biofuels and high-value bioproducts [3,8].

One of the limitations of the microalgae cultivation process is biomass harvesting, which is associated with high operational costs, especially on a large scale [9]. Generally, techniques such as centrifugation, filtration, and coagulation/flocculation are used, but these methods are considered costly, and in the case of coagulation/flocculation, detrimental to biomass conversion processes into bioproducts due to the use of chemicals [10–12]. In light of this, bioflocculation has emerged as a promising technique that has been studied.

Bioflocculation consists of microalgal biomass flocculation using microbial biomass [13]. This technique is considered ecofriendly and does not contaminate the final biomass with the use of chemicals [14–16]. Through bioflocculation, biomass can also be enhanced by incorporating fungi, bacteria, or algae as bioflocculants. However, the resulting biomass will exhibit varied chemical compositions, necessitating the study of downstream processes linked to subsequent applications.

Regarding its application, bioflocculation requires specific studies since the same microalgae, even when cultivated under standardized environmental and nutritional conditions, can exhibit different compositions that alter the optimal bioflocculation conditions. Similarly, the growth phase of the microalgae at the time of harvest can affect charge distribution in the medium, impacting bioflocculation efficiency [9].

Carbohydrates are the main product of the microalgal photosynthetic pathway and are also present in other microorganisms used in bioflocculation. Microalgal polysaccharides have been widely studied for the production of bioethanol, biohydrogen, and other bioproducts [17,18]. However, with bioflocculation, microbial biomass from different microorganisms is obtained, which presents challenges in evaluating biotechnological conversion routes, particularly in biomass hydrolysis for the release of intracellular carbohydrates.

Bioflocculated biomass may contain prokaryotic and eukaryotic microorganisms, which primarily differ in their cell wall composition. Prokaryotic microorganisms have cell walls mainly composed of glucose polysaccharides, making bond breaking easier, whereas eukaryotic microorganisms have structures with a matrix composed of biopolymers that are resistant to cell rupture processes [19,20]. Therefore, it is essential to use treatments capable of hydrolyzing compounds present in both the microalgal cell wall and other microorganisms used in bioflocculation to enhance biomass conversion efficiency.

Physical methods can be employed to rupture the cell wall and improve the yield of intracellular compound release [21,22]. These methods include ultrasound, microwaves, electric field pulses, mechanical methods, autoclaving, and freezing/thawing [23]. Additionally, chemical or enzymatic methods can be used, commonly associated with physical methods as pretreatments to increase hydrolysis efficiency. Enzymatic hydrolysis has advantages over chemical hydrolysis because it does not involve chemicals or high temperatures, operates under milder conditions with lower energy consumption, offers greater substrate specificity, and does not produce toxic byproducts [24,25].

For microalgal biomass hydrolysis, the most commonly used enzymes are α-amylases and amyloglucosidases (starch-degrading), as well as cellulases (cell wall-degrading) [26,27]. In bioflocculated biomass containing various microorganisms, different enzymes can contribute to the breakdown of polysaccharides [28,29]. Algal biomass hydrolysis has been widely studied for bioenergy production, particularly for its potential in producing bioethanol and biohydrogen. However, regarding bioflocculated microalgal biomass, further studies are needed to ensure complete biomass hydrolysis and maximize energy production efficiency. In this context, the aim of this study was to investigate different physical and enzymatic methods for the hydrolysis of bioflocculated biomass from the microalga *Spirulina platensis* using the fungus *Aspergillus niger* to produce bioethanol.

## 2. Materials and Methods

### 2.1. Fungal Bioflocculation

#### 2.1.1. Microalgal Cultivation Conditions

The microalga *Spirulina platensis* was cultivated in Zarrouk medium [30] at a concentration of 20% (*w*/*v*), supplemented with micronutrient solutions A5 ($H_3BO_3$; $MnCl_2.4H_2O$; $ZnSO_4.7H_2O$; $CuCO_4.5H_2O$; $Na_2MoO_4.2H_2O$) and B6 ($NH_4VO_3$; $KCr(SO_4).12H_2O$; $NiSO_4.6H_2O$; $Na_2WO_4.2H_2O$; $CO(NO_3)_2.6H_2O$). The cultivation was conducted in a 180 L raceway with an initial inoculum concentration of 0.2 $g{\cdot}L^{-1}$. Cultivation took place in a hydroponic greenhouse, with agitation provided by rotating blades (0.35 $m.s^{-1}$), temperature control maintained between 20 °C and 30 °C, and natural light supplied within the hydroponic greenhouse. The microalgae were cultured until the end of the stationary growth phase, when they reached a stable cell density, which was reported to result in the highest carbohydrate accumulation [31].

A semi-continuous cultivation was performed, involving the removal of 90 L of culture medium at the end of the stationary growth phase for fungal bioflocculation, followed by the addition of 90 L of 20% (*w*/*v*) Zarrouk medium to continue cultivation without the need for preparing a new inoculum. Microalgal growth was monitored by sampling three times a week to determine pH using potentiometry and to assess microalgal biomass concentration via spectrophotometry, which utilizes a pre-established relationship between absorbance at 670 nm and the dry weight of microalgal biomass, both performed in triplicate.

#### 2.1.2. Production of Fungal Bioflocculant

The production of fungal bioflocculant was carried out using a methodology adapted from [15]. For this study, a strain of the fungus *Aspergillus niger* DAOM was used [32]. The fungus was transferred from test tubes containing *A. niger* maintained in potato dextrose agar (PDA) culture medium at 4 °C to Petri dishes containing 15 mL of PDA medium. To facilitate this transfer, 5 mL of sterile 0.1% Tween solution was added to the tubes containing the fungus. From this suspension of fungal spores, 0.5 mL was transferred to the Petri dishes containing PDA medium, which were then incubated in a thermostatic oven at 30 °C for 5 days. The obtained inoculum was used to inoculate the submerged fermentation (SF) culture medium to increase biomass.

The submerged fermentation culture medium was prepared using a suspension of wheat bran at 10% (*w*/*v*) in distilled water. The suspension was boiled for 30 min at 100 °C, followed by filtration to remove insoluble suspended solids. Submerged fermentation was carried out in 250 mL Erlenmeyer flasks, with the addition of 100 mL of culture medium, 1% sodium nitrate as a supplementary nitrogen source, and a pH adjusted to 7. The media were inoculated with the addition of 2 mycelial disks, each 1 cm in diameter, from an *A. niger* inoculum prepared in Petri dishes into each Erlenmeyer flask. SF was performed over 72 h on an orbital shaker (model TE-421, Tecnal, Piracicaba, Brazil) at 30 °C and 0.97 g (120 rpm). The resulting wet biomass was initially used as a bioflocculant for the microalga *S. platensis*.

#### 2.1.3. Harvesting Microalgae

Tests of bioflocculation conditions were conducted by varying the concentration of wet bioflocculant (microalgae/wet fungus biomass) while adjusting the millivoltage (approximately zero) using 20% glacial acetic acid. Control tests were performed with the addition of bioflocculant without millivoltage adjustment and with millivoltage adjustment without the addition of bioflocculant. The added bioflocculant contained 90% moisture. The microalgae/fungus ratio (dry basis) was determined by considering the moisture content of the fungal biomass. Table 1 presents the experimental conditions of the bioflocculation assays.

**Table 1.** Experimental conditions of the bioflocculation assays.

| Experiments [1] | Ratio (Microalgae/Fungus) [2] | Millivoltage Adjustment (mV) |
|---|---|---|
| 1 | 8:1 | with adjustment |
| 2 | 5:1 | with adjustment |
| 3 | without fungus | with adjustment |
| 4 | 8:1 | without adjustment |
| 5 | 5:1 | without adjustment |

[1] The experiments were conducted in duplicate. [2] Concentration was measured on a dry basis.

The bioflocculation assays were initially conducted in 250 mL beakers containing 200 mL of microalgal culture medium, with agitation performed using a mechanical stirrer (200 rpm for 1.5 min and 20 rpm for 10 min). Subsequently, under the best flocculation efficiency conditions, further assays were performed on a larger volume scale, using 2 L jar test containers (at the established bioflocculant concentration and millivoltage) containing cultures of *S. platensis*. The jar test mechanical stirrer (model 713, Fisatom, São Paulo, Brazil) was used to perform rapid mixing (1.5 min), followed by slow mixing (10 min) [33,34]. After slow mixing, the medium was allowed to settle for 30 min to facilitate microalgal biomass separation, followed by filtration, from which an aliquot was taken to determine the bioflocculation efficiency, which was assessed using spectrophotometry. The harvesting efficiency was determined using Equation (1).

$$\text{Harvesting efficiency}(HE, \%) = \frac{\left(C_0 - C_f\right)}{C_0} \times 100 \quad (1)$$

Here, HE = harvesting efficiency (%); $C_0$ = initial concentration of *S. platensis* ($g \cdot L^{-1}$); and $C_f$ = final concentration of *S. platensis* ($g \cdot L^{-1}$).

#### 2.1.4. Characterization of Biomasses

The biomasses (microalgae and fungi) were characterized using centesimal composition methodology [35], focusing on the contents of total proteins, lipids, ash, moisture, and total carbohydrates.

### *2.2. Cell Disruption and Enzymatic Hydrolysis*

The characterization of commercial enzymes was carried out, mainly due to the complexity of the bioflocculated biomass, with the aim of achieving higher hydrolysis efficiencies. Different enzymes were characterized to determine the optimal pH and temperature, enzymatic load, and optimal hydrolysis times. The enzymes tested included α-amylase (LpHera), amyloglucosidase—AMG (Amylase AG XXL and Saczyme GO 2), cellulase (Celluclast 1.5L), and xylanase (Ultraflo Max) from Novozymes®, provided by the company LNF Enzimas (RS, Brazil). The enzymes had the following characteristics: LpHera exhibited an activity of 440 KNU–T/g at a pH between 4.5 and 6.5; Saczyme GO 2 showed an activity of 1725 AGU/g at a pH between 3.0 and 5.0; Celluclast 1.5L had an activity of 700 EGU/g at a pH between 4.0 and 6.0; and Ultraflo Max also had an activity of 700 EGU/g at a pH between 4.0 and 5.5. For all enzymes, the manufacturer recommended application concentrations between 100 mL/$m^3$ and 300 mL/$m^3$. These enzymes were selected according to the cellular composition of the bioflocculated biomass. The enzyme LpHera hydrolyzes 1,4-α-D-glycosidic bonds in amylose polysaccharides, the enzymes Amylase AG XXL and Saczyme GO 2 hydrolyze 1,4 and 1,6-α-D-glucose bonds at the non-reducing ends of polysaccharides, the enzyme Celluclast 1.5L hydrolyzes 1,4-β-D-glycosidic bonds and other β-glucans, and Ultraflo Max hydrolyzes 1,4-β-D-xylosidic bonds, forming xylans.

#### 2.2.1. Enzyme Characterization Studies: Determination of Optimal pH and Temperature

The enzymes used in biomass hydrolysis (amylase, amyloglucosidase—AMG, cellulase, and xylanase) were subjected to enzymatic activity analysis using their respective standard substrates, as enzymes can lose activity over time and different batches can exhibit different enzymatic activities. To characterize the optimal pH and temperature, a 3k experimental design (k = 2) was employed, with variations in pH (4.5, 5.5, and 6.5) and temperature (50 °C, 60 °C, and 70 °C) for all enzymes, except for LpHera (α-amylase), which was tested at temperatures of 50 °C, 70 °C, and 90 °C. The temperature range values were established by the manufacturer according to the enzymes' technical datasheet.

The activity of the enzymes amylase (LpHera) and amyloglucosidase—AMG (Amylase AG XXL and Saczyme GO 2) was assessed using a methodology adapted from [36] using starch as the standard substrate (1% solution in 0.2 M phosphate buffer). Similarly, the activity of the enzymes cellulase (Celluclast 1.5 L) and xylanase (Ultraflo Max) was evaluated, using carboxymethyl cellulose (CMC) and filter paper as substrates, respectively. Both activities were carried out in 0.2 M phosphate buffer at a concentration of 1%. Enzymatic activities were evaluated through the determination of total reducing sugars (TRS) using the 3,5-dinitrosalicylic acid (DNS) method [37], following protein precipitation with Carrez I and II. For all three determination methods, enzymatic activities were calculated using Equation (2) [38].

$$EA = \frac{RS}{t} * \frac{1000}{180} * DF \quad (2)$$

Here, [RS] = concentration of reducing sugars (mg/mL); t = reaction time (30 min); and DF = dilution factor.

#### 2.2.2. Study of Enzyme Concentration and Hydrolysis Times

After characterizing the enzymes regarding their optimal pH and temperatures, a study was conducted to determine the enzymatic concentration necessary for the hydrolysis of bioflocculated biomass. Under optimized conditions for each enzyme, concentrations of 0.01%, 0.03%, and 0.05% ($v/v$) were tested, with 0.01% corresponding to 100 mL/m$^3$. The experiments were performed in duplicate using 250 mL Erlenmeyer flasks containing a 10% ($w/v$) suspension of bioflocculated biomass in 0.2 M phosphate buffer, with a working volume of 50 mL. The biomass underwent a cell disruption process and carbohydrate gelatinization in an autoclave for 20 min at 121 °C [39]. The reactors were incubated in a water bath at 150 rpm, with samples collected every hour for analysis of reducing sugars. The initial time (t0) was without enzyme addition to evaluate the efficiency of the pretreatment and subsequent times involved enzyme addition for a period of 6 h (Saczyme GO 2 and Amylase XXL) and 4 h (LpHera, Celluclast 1.5 L, and Ultraflo Max) until the results of RS stabilized. Both the pH and temperatures used were defined based on previous optimization assays.

#### 2.2.3. Study of Combined Use of Enzymes

After establishing the optimal conditions for enzyme usage (temperature, pH, concentration, and hydrolysis time), different enzyme combinations were tested for the hydrolysis of bioflocculated biomass. For the assays, 50 mL of volume in 250 mL Erlenmeyer flasks were prepared with a 10% biomass suspension. The suspension underwent pretreatment for cell disruption, followed by the addition of enzymes.

The pretreatment methods (autoclaving, ultrasound, ultrasound + autoclaving, and gelatinization) were evaluated to assess whether different cell disruption methods would increase total reducing sugar yields at the end of the process. Gelatinization was used as a

control since all methods undergo subsequent gelatinization except for autoclaving, which already utilizes high temperatures.

These methods were chosen for their ability to alter the physical and chemical characteristics of biomass, promoting greater enzyme accessibility to the polymeric structures, such as polysaccharides, present in microalgae and fungi. The use of an autoclave, for example, involves increasing pressure and temperature, which can break resistant chemical bonds and facilitate enzymatic hydrolysis. Ultrasound, on the other hand, generates high-frequency waves that can promote cavitation, breaking the cells and facilitating the release of intracellular components that are crucial for the hydrolysis process. The combination of ultrasound with gelatinization, which involves heating starch to its gelation point, can further improve the efficiency of hydrolysis by making the polysaccharides more susceptible to enzymatic action. Isolated gelatinization can also modify the structure of starch and other compounds, increasing the efficiency of the bioethanol conversion process [40]. The different pretreatment methods are tested to identify the most effective combination that increases the hydrolysis rate and maximizes ethanol production while considering factors such as environmental sustainability and the economic feasibility of the process.

Three duplicate assays were conducted: Blend 1: α-amylase + AMG; Blend 2: α-amylase + AMG + cellulase; Blend 3: α-amylase + AMG + xylanase. Specific enzymes were selected from those tested in previous stages based on their action on the microbial cell wall and their operational range, including α-amylase (LpHera), AMG (Saczyme GO 2), cellulase (Celluclast 1.5 L), and xylanase (Ultraflo Max). The enzyme combinations were tested across all pretreatment methods. All assays initially used α-amylase at a temperature of 90 °C for 2 h, followed by the addition of the other enzymes at 60 °C until stability in total reducing sugar yields was achieved. Hydrolysis efficiency was calculated using the ratio of the polysaccharide composition present in the initial biomass in g/100 g and after hydrolysis, also in g/100 g. This ratio was presented as a percentage (%). Equation (3) was used to calculate biomass hydrolysis efficiency.

$$HE(\%) = (CHOi/CHOf)*100 \quad (3)$$

Here, HE (%) = hydrolysis efficiency; CHOi = percentage of initial carbohydrates; and CHOf = percentage of carbohydrates after hydrolysis.

For comparison, based on the best conditions obtained in these assays, the hydrolysis of biomass from the microalgae *Spirulina platensis* and the fungus *Aspergillus niger* was conducted individually.

### *2.3. Bioethanol Production*

#### 2.3.1. Enzymatic Hydrolysis

Based on the best results from the enzyme synergy tests, scale-up was carried out for subsequent bioethanol production. Saccharification assays were conducted in a 1.2 L stainless steel reactor (TECNAL) with benchtop agitation at 150 rpm. Enzymes were added at a ratio of 100 mL/m$^3$. α-Amylase was added once the medium reached 90 °C and acted individually for 1 h. The temperature was then reduced to 60 °C for the addition of amyloglucosidase and cellulase, which continued for an additional 2 h, followed by enzyme inactivation at 100 °C for 15 min. The determination of reducing sugars was performed using the 3,5-dinitrosalicylic acid (DNS) method [37]. At the end of saccharification, the mixture was centrifuged for 15 min at 1320× *g*, and the liquid fraction was used for alcoholic fermentation assays.

#### 2.3.2. Microorganism and Inoculum Preparation

The yeast strain *S. cerevisiae* CAT-1, obtained from the strain bank of the Laboratory of Biochemistry and Bioprocesses at the University of Passo Fundo, was used for ethanol production. This yeast strain was maintained at 4 °C in a refrigerated environment on solid slant medium with the following composition: 1% yeast extract, 2% peptone, 2% glucose, and 2% agar.

To activate the cells, a loopful of the microorganism was incubated in a previously sterilized medium consisting of 1% yeast extract, 2% peptone, and 2% glucose (100 mL of medium in a 250 mL Erlenmeyer flask), referred to as the pre-inoculum. This pre-inoculum was incubated on an orbital shaker at 150 rpm and 30 °C for 48 h.

Subsequently, the inoculant was prepared by adding 10% ($v/v$) of the pre-inoculum to the cultivation medium (2% glucose, 1% yeast extract, and 2% peptone). The inocula were prepared in 1 L Erlenmeyer flasks (with a working volume of 500 mL) and incubated on an orbital shaker at 150 rpm for 10 h, which was sufficient time for the yeast to reach the exponential growth phase. Before adding the yeast to the hydrolyzed cultivation medium, the inoculum density was measured at 600 nm using a spectrophotometer, and the inoculum was only added when the absorbance was close to 0.8.

#### 2.3.3. Alcoholic Fermentation

The fermentations were conducted in 1 L Erlenmeyer flasks containing 500 mL of medium. The pH of the hydrolyzed medium was adjusted to between 4.5 and 5.0 by adding acetic acid. The process was carried out in duplicate. The fermentation medium was sterilized and subsequently inoculated with 10% ($v/v$) of the previously prepared inoculum of *S. cerevisiae* CAT 1. Samples of 40 mL were collected at 0, 6, 12, 18, and 24 h for the determination of reducing sugar (RS) and ethanol concentrations. The reducing sugar concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method with a glucose standard curve [37].

Samples of fermented mash were distilled using a benchtop micro distiller. Ethanol content was evaluated using the spectrophotometric method proposed by [41] for determining alcohol content in hydroalcoholic mixtures, based on the absorbance of samples at 600 nm after distillation and the subsequent reaction following the addition of potassium dichromate at 60 °C for 30 min. The efficiency (η) of ethanol formation was calculated using Equation (4) [42], and productivity was determined using Equation (5).

$$\eta(\%) = \frac{\Delta E}{0.511 \times (s_0 - s)} \times 100 \quad (4)$$

Here, η: ethanol formation efficiency; ΔE: change in ethanol concentration ($g \cdot L^{-1}$); $S_0$: initial concentration of total reducing sugars ($g \cdot L^{-1}$); S: final concentration of total reducing sugars ($g \cdot L^{-1}$); and 0.511: stoichiometric coefficient of the reaction.

$$Pethanol\left(g \cdot L^{-1} \cdot h^{-1}\right) = \frac{\Delta E}{\Delta t} \quad (5)$$

Here, ΔE: change in ethanol concentration (g/L); and Δt: change in fermentation time (h).

### 2.4. Data Processing

Data processing was performed using Statistica 10 software, version 10.0.228.2 (Tulsa, OK, USA) through analysis of variance (ANOVA), followed by Tukey's test for mean comparison, with a confidence level of 95%.

## 3. Results and Discussions

Through the analysis of the growth curve of the microalgae *S. platensis*, it was observed that the adaptation phase of the microalgae occurred during the first 7 days (lag phase), followed by the exponential growth phase (log phase), which saw a significant increase in the cell concentration of the microalgae until the 15th day of cultivation. Subsequently, after entering the stationary phase, it remained for 10 days until it began to show signs of decline and cell death. Thus, on the 25th day, the cultures were terminated with a concentration of $2.18 \pm 0.008\ g \cdot L^{-1}$.

Ref. [36] conducted the cultivation of the microalgae *Spirulina platensis* for bioethanol production. The cultures were carried out in 10 L mini tanks using the Zarrouk 20% culture medium, similar to this study, but without the addition of vitamins A5 and B6. The growth of the microalgae exhibited a similar behavior to that observed in this study, with well-defined growth phases, albeit shorter, reaching the end of the stationary growth phase in 12 days of cultivation. According to [43], micronutrients such as iron, manganese, zinc, copper, molybdenum, cobalt, vanadium, and nickel are essential for the functioning of microalgal cellular organelles, with each species having specific physiological characteristics in terms of absorbing and eliminating micronutrients. For the microalgae harvest, fungal bioflocculation was used with the aim of biomass enhancement and separation efficiency improvement.

### 3.1. Fungal Bioflocculation Trials

Figure 1 presents the bioflocculation efficiency results for the different treatments applied. The highest bioflocculation efficiencies were observed for trials 1 and 2 (99.70% and 99.41%, respectively; $p > 0.05$). Experiments 3 and 4 yielded lower, but statistically equal results (97.19% and 97.70%, respectively). The lowest efficiency was obtained for trial 5 (89.60%), which was conducted without the use of bioflocculants. Some microalgae, such as *Spirulina*, have the potential for auto-flocculation, which can occur naturally. This phenomenon refers to cellular aggregation and/or cell adhesion in a liquid medium [44,45]. The tests yielded results similar to those obtained by [15], which ranged from 95.9% to 98.8%. In that study, different pH values (4, 6, 8, and no pH adjustment) were used; however, millivoltage was not evaluated. The best results were achieved in control experiments without pH adjustment (values between 10 and 11). Coagulation/flocculation assays are commonly conducted with consideration only of the pH of the medium, without millivoltage control. Ref. [46] described the importance of the surface charge of algae in their coagulation process. The study suggested that the coagulation mechanism occurred through mutual attraction and neutralization of the charges of both the algae and the bioflocculant used. In this study, millivoltage control was conducted to assess at which point/pH the neutralization of the medium charges would be achieved. The isoelectric potential was obtained at a pH of approximately 6.82, similar to the results obtained by [47], which ranged between 6.5 and 6.9.

Overall, the tests exhibited high efficiency values; however, when evaluating floc formation, experiments 1 and 2 demonstrated greater stability compared to the others and could be filtered using a common sieve. In the case of the other treatments, when the liquid was agitated, the separated biomass mixed back into the medium, requiring another separation method, such as centrifugation.

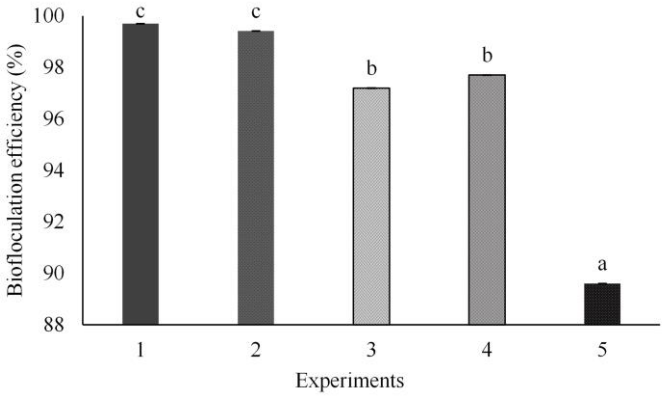



**Figure 1.** Influence of the percentage of bioflocculant and millivoltage adjustment on bioflocculation efficiency (%) in the harvest of *Spirulina platensis* microalgae using fungal biomass. Experiments: 1—8:1 ratio of bioflocculant WITH millivoltage adjustment (mV); 2—5:1 ratio of bioflocculant WITH mV adjustment; 3—WITHOUT the addition of bioflocculant and WITH mV adjustment; 4—8:1 ratio of bioflocculant and WITHOUT mV adjustment; 5—5:1 ratio of bioflocculant and WITHOUT mV adjustment. Means ± standard deviation. Equal letters indicate no statistical difference between the experiments, while different letters indicate statistical difference between the experiments ($p < 0.05$).

As there was no statistical difference ($p < 0.05$) between using an 8:1 and a 5:1 (microalgae/fungus on a dry basis) ratio of fungal bioflocculant, it was decided to proceed with the scale-up experiments using an 8:1 ratio (8:1 fraction of microalgae/fungus on a dry basis) of bioflocculant with millivoltage adjustment, following experiment 1. Larger quantities of microalgae were used relative to the fungus, primarily considering future biotechnological processes, given the complexity of the fungal cell wall compared to that of microalgae. The fungal cell wall is composed of various types of alpha-glucans. The most common of these polysaccharides is glycogen, which consists of glycosyl units linked by α-1,4 and α-1,6 bonds, and glucans in which the glycosyl units are linked only by α-1,3 bonds, or α-1,3 and α-1,4 bonds [48–50]. As mentioned above, glycogen is an energy storage material that accumulates in the form of grains in the cytoplasm [51]. Polysaccharides from other classes are components of the cell wall, serving as structural elements [49] and providing resistance.

When the same conditions were applied in 2 L jar test tanks, the efficiencies were maintained, reaching values between 99.5% and 99.7%. Furthermore, when scaling up the process, under the proposed conditions, neither rapid nor slow agitation was necessary, as biomass separation occurred within 30 min.

In the bioflocculation process, fungal hyphae and mycelia contain polysaccharides with active sites that enable bioadsorption and cluster formation [52]. The main phenomenon enabling floc aggregation is related to the negative charges of the microalgae and the positive charges of the fungal hyphae [53], which can also be adjusted through millivoltage adjustment.

According to [13], when *S. platensis* begins to show cellular death, destabilization of charges on the cell surface can occur, which is one of the requirements for biomass aggregation in the bioflocculation process. In this context, even at the end of the stationary growth phase, with the use of fungal bioflocculant and millivoltage adjustment, efficiencies close to 100% were achieved. This suggests that, regardless of the growth phase of *S. platensis* microalgae, with the bioflocculation conditions established in this study, it is possible to achieve high bioflocculation efficiencies.

Moreover, the physical and chemical interaction between *S. platensis* and *A. niger* can explain the high bioflocculation efficiency. Ref. [15] performed FTIR analyses to evaluate these interactions. The FTIR results indicate significant changes in the absorption spectra of the biomass after the application of the fungal bioflocculant. The analysis revealed

interactions between the functional groups of *S. platensis* (such as amine, carboxyl, and hydroxyl groups) and the components of *A. niger* (such as the polysaccharides present in fungal hyphae). The spectral bands associated with functional groups like –OH, $–NH_2$, and –COOH, present in both the microalgae and the fungus, showed variations in absorption intensities, suggesting a physical and chemical interaction between the two organisms. These interactions promote the formation of more stable flocs, facilitating the bioflocculation harvesting process. This interaction was corroborated by the SEM analysis conducted by [54], which demonstrated the formation of a porous and structured matrix resulting from the intertwining of fungal hyphae with microalgal cells, providing greater exposure of active sites for enzymatic action.

In the context of scaling up, the fungus-to-microalgae ratio could be optimized, as fungal production through submerged fermentation can be adapted to a more industrial and cost-effective process. Additionally, larger-scale systems might benefit from further optimization of operational parameters, such as aeration, nutrient supply, and mixing strategies, to enhance fungal bioflocculant production and maintain high separation efficiency. Moreover, integrating continuous or semi-continuous processes could improve scalability and productivity, reducing downtime and making the process more viable for commercial applications [55].

Conventionally employed techniques in microalgae harvesting, such as centrifugation, flotation, filtration, and flocculation, are not economically viable when applied to industrial bioethanol production. These technologies account for up to 50% of the total production cost and are not environmentally sustainable, as they require high energy consumption, and the addition of chemicals limits medium recycling and the polysaccharide hydrolysis process, potentially causing damage to the cells due to shear stress. Bioflocculation, on the other hand, stands out as an ecofriendly technique, offering high harvesting efficiency, lower energy consumption, and greater technological feasibility [10–12].

### *3.2. Characterization of the Biomasses*

The biomass used in the study (microalgae with fungi) was characterized for its proximal composition because sugar yields and hydrolysis efficiency are based on the carbohydrate composition of the biomass. The bioflocculated biomass contained approximately 29.2 ± 4.06% carbohydrates, 27.02 ± 3.14% proteins, 32.02 ± 5.08% lipids, 0.35 ± 0.01% ash, and 7.41 ± 0.04% moisture. These results were calculated on a dry basis for use in enzymatic hydrolysis assays.

### *3.3. Cell Disruption and Enzymatic Hydrolysis*

#### 3.3.1. Enzyme Characterization

Figure 2 presents the results of the enzyme characterization regarding pH and temperature.

The enzyme LpHera (α-amylase) exhibits variable enzymatic activities under different pH and temperature conditions. Optimal activity (3.77 ± 0.065 U) was observed at pH 6.5 and 90 °C, similar to the activity at pH 5.5 and 90 °C (3.72 ± 0.060 U), while reduced activity was noted at pH 6.5 and 50 °C (3.06 ± 0.044 U). Figure 2 illustrates LpHera's activity results, highlighting its effectiveness under gelatinization conditions due to its robust thermal stability, which is crucial for its integration at this stage of starch processing.

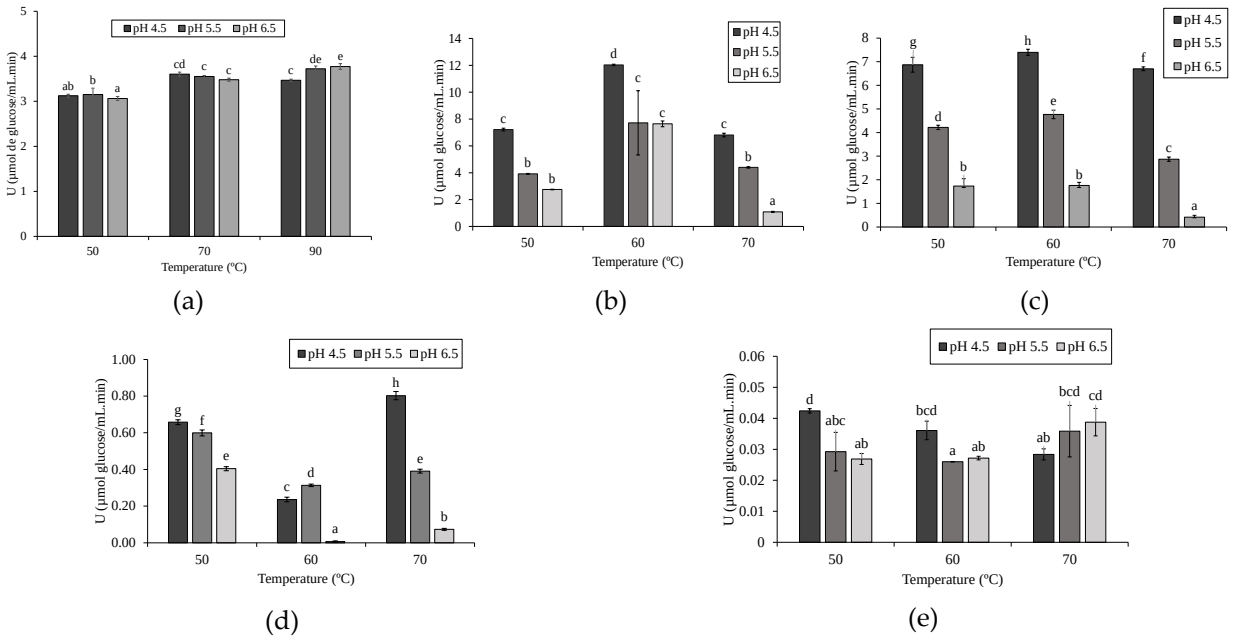



**Figure 2.** Results of enzyme characterization tests regarding pH and temperature. (**a**) Amylase = LpHera; (**b**) AMG = Saczyme GO 2; (**c**) Amylase = Amylase AG XXL; (**d**) Cellulase = Celluclast 1.5 L; (**e**) Xylanase = Ultraflo Max. Means ± standard deviation. Equal letters indicate no statistical difference between assays, while different letters indicate statistical difference between assays ($p < 0.05$).

Known for its application in ethanol production, LpHera possesses intermediate acid stability, enabling starch hydrolysis within the pH range of 4.5–4.8, unlike commercially used α-amylases, which are typically active within the pH range of 5.0–6.5 [56,57]. Employing pH 4.5 during saccharification optimizes enzyme activity, thereby reducing chemical requirements and potentially increasing starch sugar yields by up to 0.2%, which enhances process economics.

The enzyme Saczyme GO 2 (AMG) demonstrates superior enzymatic activity at pH 4.5 and 60 °C (12.03 ± 0.049 U), with reduced activity at pH 6.5 and 70 °C (1.08 ± 0.035 U). It is widely used in lignocellulosic hydrolysis and lipid extraction from microalgae, although this occurs under the ideal pH and temperature conditions specified by the manufacturer [34,58]. However, optimizing enzyme characterization for specific batches and storage durations could potentially enhance overall efficiency.

The amylase AG XXL shows peak activity at pH 4.5 and 60 °C (7.39 ± 0.130 U). Although there are no studies on microalgal hydrolysis, it shares operational pH and temperature ranges with AMG 300L for potential applications in microalgae biomass saccharification [16,59].

Celuclast 1.5 L achieves maximum activity on CMC substrate at pH 4.5 and 70 °C (0.8026 ± 0.022 U), indicating potential for improved carbohydrate release under lower pH and higher temperature conditions in microalgal contexts [60].

Ultraflo Max exhibits varied enzymatic activities across different pH and temperature settings, emphasizing its versatility in enzymatic processes despite the presence of diverse optimal conditions (data from enzyme characterization studies).

These enzymes play fundamental roles in optimizing saccharification processes across diverse biotechnological applications, offering insights into their operational ranges and potential for enhancing bioconversion efficiencies in industrial settings.

Figure 3 presents the results of the enzyme characterization regarding enzyme load and hydrolysis time.

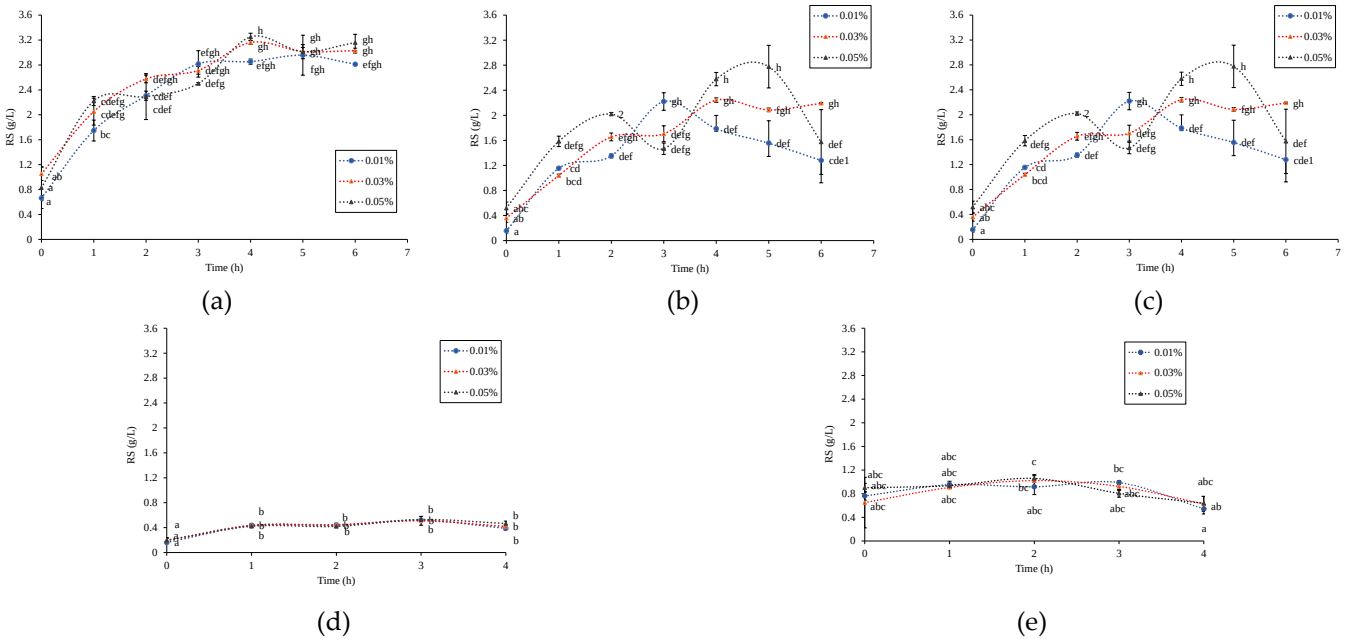



**Figure 3.** Results of enzyme loading tests and hydrolysis times. (**a**) Amylase = LpHera; (**b**) AMG = Saczyme GO 2; (**c**) Amylase = Amylase AG XXL; (**d**) Cellulase = Celluclast 1.5 L; (**e**) Xylanase = Ultraflo Max. Means ± standard deviation. Equal letters indicate no statistical difference between assays, while different letters indicate statistical difference between assays ($p < 0.05$).

During the characterization of the enzymes LpHera, Saczyme GO 2, Amylase AG XXL, Celluclast 1.5 L, and Ultraflo Max for hydrolyzing bioflocculated microalgal biomass, distinct patterns of reducing sugar release were observed under varying enzyme concentrations and assay times. For LpHera, the highest means ($1.05 \pm 0.060$ g·L$^{-1}$) were achieved with a 0.05% enzyme concentration at 2 h, maintaining stable yields over 4 h, unlike lower means at 4 h with the addition of 0.01% enzyme. Similarly, Saczyme GO 2 exhibited peak averages ($3.25 \pm 0.059$ g·L$^{-1}$) at 4 h with 0.05% enzyme, underscoring stable yields across concentrations. Amylase AG XXL mirrored these trends, showing optimal results ($2.58 \pm 0.106$ g·L$^{-1}$) at 4 h with 0.05% enzyme, along with comparable yields at 0.03% concentration.

In contrast, Celluclast 1.5 L displayed its highest mean ($0.76 \pm 0.22$ g·L$^{-1}$) at 3 h with 0.03% enzyme, maintaining statistical equivalence across most assays, except for the addition of 0.01% at 0 h. Ultraflo Max, although there was no statistical difference between treatments at 0 h, stabilized reducing sugar release from the first hour of testing.

In comparison with studies focusing solely on microalgae, these results suggest challenges in fungal cell wall disruption, which affects hydrolysis efficiency even under optimized pH and temperature conditions [61,62]. Strategies for enzyme application vary, with studies employing sequential addition methods, such as starting with amylases followed by cellulases or vice versa, based on enzyme activity windows [63–65]. For this study, enzymes were selected based on prior characterization data and microbial cell wall attributes, which guided their sequential addition. Initially, LpHera will act for 2 h at 90 °C, followed by a blend of Saczyme GO 2, Celluclast 1.5 L, and Ultraflo Max for another 2 h at 60 °C, all maintained at pH 4.5.

Given that there are no statistical differences across different enzyme percentages, a standard concentration of 0.01% enzyme was adopted for all tests. This methodology aims to optimize enzymatic hydrolysis efficiencies for bioflocculated microalgal biomass, anticipating increased sugar yield reduction through sequential enzyme application.

Based on the results of enzyme characterization, specific enzymes were selected based on the conducted tests and the microbial cell wall to evaluate their combined usage. The LpHera enzyme (α-amylase) was selected and added at the beginning of the enzymatic hydrolysis process for a period of 2 h, with the subsequent addition of associated enzymes (Saczyme GO 2, Celluclast 1.5 L, and Ultraflo Max), in different blends for an additional 2 h of testing. The pH was maintained at 4.5, and the temperatures were 90 °C for the LpHera enzyme and 60 °C for the other enzymes. Overall, there was no statistical difference in the utilization of different enzyme percentages in the tests; therefore, it was decided to standardize the use of 0.01% for all enzymes.

### 3.3.2. Study of Combined Use of Enzyme

Figure 4 presents the results obtained from the combined enzyme assays for Blend 1: LpHera (α-amylase) and Saczyme GO 2 (AMG).

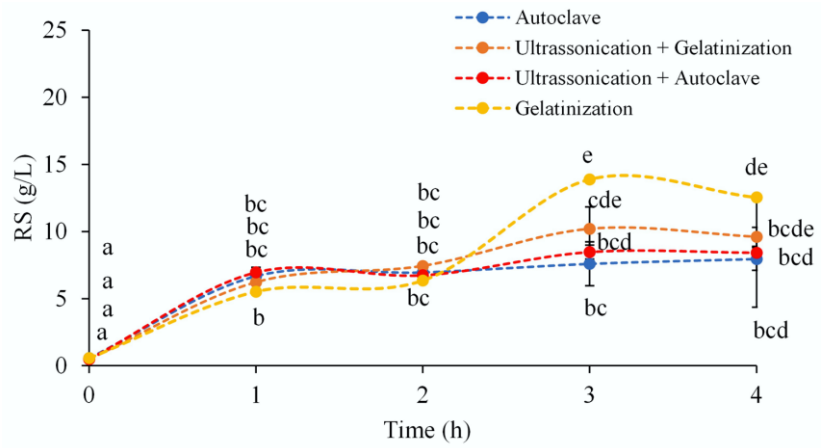



**Figure 4.** Total reducing sugar yield [1] using LpHera and Saczyme GO2 enzymes. [1] Means ± standard deviation. Equal letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

In the different pretreatments used for this enzyme blend, the highest mean values of total reducing sugars were observed at 3 h of assay, after the addition of the Saczyme GO 2 enzyme (13.88 ± 0.12 g·L$^{-1}$), using gelatinization, with the results remaining stable at 4 h for the same pretreatment. The LpHera (α-amylase) achieved maximum yields in the first hour of assays, demonstrating statistical equality for all analyzed pretreatments, showing stability in the following two hours after the amyloglucosidase was added. The lowest means were observed at time zero (t0h), when there was no enzyme addition, only the release of sugars through the pretreatments used.

The enzyme α-amylase acts by transforming starch into dextrins and oligosaccharides through the hydrolysis of α-1,4 linkages, and then α-1,4 and α-1,6 linkages are hydrolyzed by the action of the amylolytic enzyme, transforming oligosaccharides into low molecular weight glucose monomers [64].

Figure 5 presents the results obtained from the assays of combined enzyme usage for the enzymes in Blend 2: LpHera (α-amylase), Saczyme GO 2, and Celluclast 1.5 L.

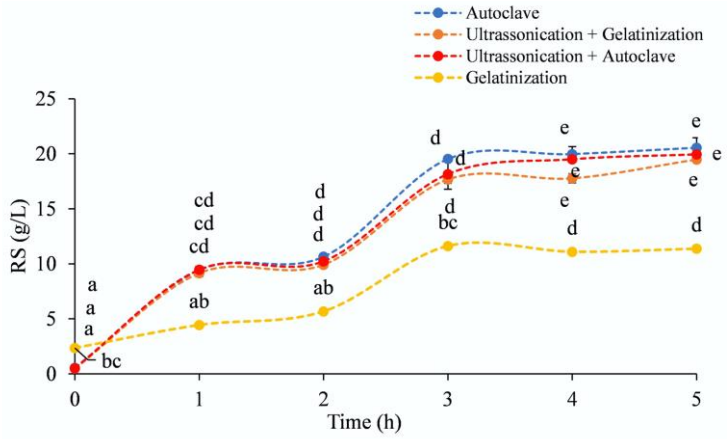



**Figure 5.** Total reducing sugars yield [1] using the enzymes LpHera, Saczyme GO 2, and Celluclast 1.5 L. [1] Means ± standard deviation. Equal letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

When combining the enzymes α-amylase, AMG, and cellulase, higher yields of reducing sugars were obtained compared to assays using only α-amylase and AMG. The highest means were observed at 5 h (20.55 ± 0.90 $g \cdot L^{-1}$) for assays using autoclaving as pretreatment. Assays using gelatinization as pretreatment yielded the lowest amounts over time compared to the others. Autoclaving, ultrasound + autoclaving, and ultrasound + gelatinization pretreatments showed the same behavior over time, with statistical equality at all analyzed times ($p < 0.05$).

In the study conducted by [61], the enzymes α-amylase and amyloglucosidase (AMG) were used together to determine the optimal conditions for the enzymatic hydrolysis process of protein extraction residues from *S. platensis* cells. The results indicate that with both amylolytic enzymes added simultaneously in the enzymatic hydrolysis process, a higher release of reducing sugars in the medium was obtained, making this strategy promising for ethanol conversion.

Figure 6 presents the results obtained from the combined enzyme use assays for the enzymes in Blend 3: LpHera (α-amylase), Saczyme GO 2, and Ultraflo Max. In assays using autoclaving, ultrasound + autoclave, and ultrasound + gelatinization, there was stability in reducing sugar yields after 3 h of assays, with the first two pretreatments showing statistical equality ($p < 0.05$) at the same described times.

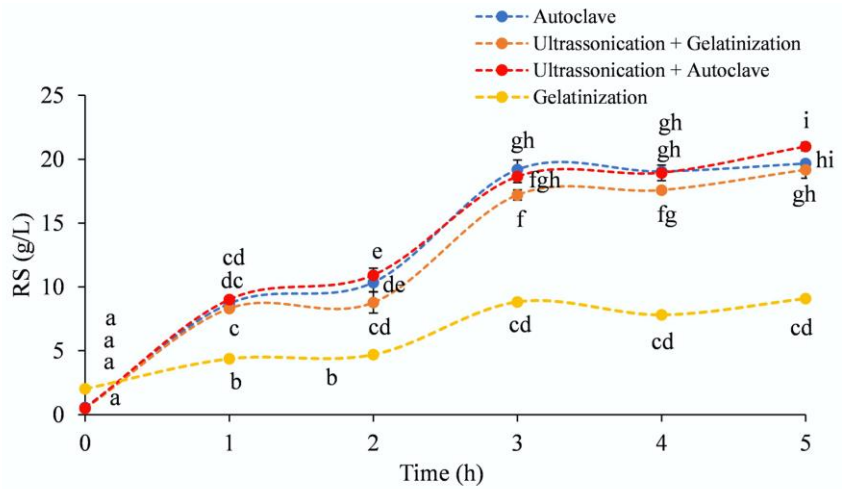



**Figure 6.** Total reducing sugar yield [1] using the enzymes LpHera, Saczyme GO 2, and Ultraflo Max. [1] Means ± standard deviation. Identical letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

Overall, the enzymatic combinations exhibited similar behavior, demonstrating almost immediate action after addition. The enzyme LpHera (α-amylase) showed stable results after 1 h of experimentation, and after the addition of the other enzymes, regardless of the blend used, there was stability in reducing sugar yields after another hour of assays. According to [66], the action of the α-amylase enzyme occurs in two stages. The first stage involves the rapid release of reducing sugars through the breakdown of more complex sugars, converting starch into dextrins. Subsequently, saccharification occurs more slowly, requiring the addition of another catalyst. The α-amylase acts on internal 1,4 linkages without interfering with the 1,6 linkages, thereby reducing the viscosity of the medium and allowing the action of the amiloglucosidase enzyme to follow.

As observed in the obtained results, to evaluate which enzyme combination and pretreatment is more efficient, statistical analysis was performed at the 3 h time point, where stability in reducing sugar yields was observed for most assays. Figure 7 presents the results obtained for the different enzyme blends at this 3 h time point.

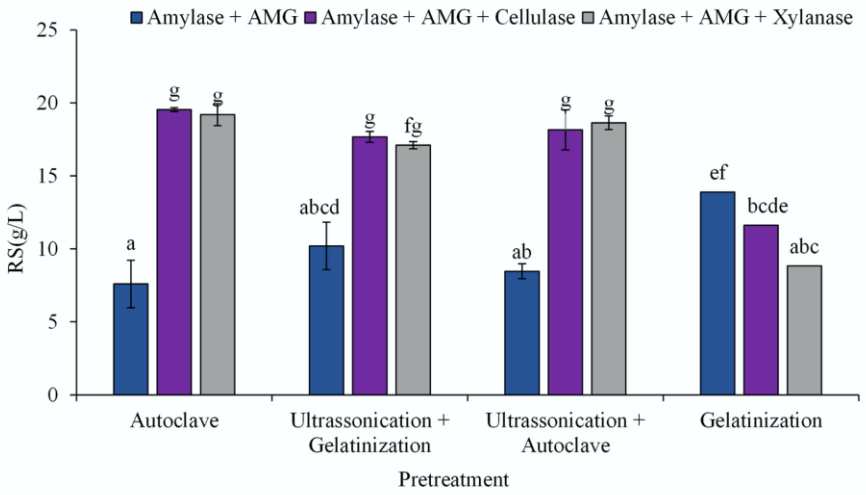



**Figure 7.** Total reducing sugar yields [1] for the different enzyme combinations at the 3 h time point. [1] Means ± standard deviation. The same letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

The highest averages were obtained using the enzymes LpHera + Saczyme GO 2 + Celluclast 1.5 L (19.53 ± 0.13 g·L$^{-1}$), with autoclaving as the pretreatment. The same blend of enzymes showed statistical equality for treatments using ultrasound + gelatinization and ultrasound + autoclaving. Furthermore, when using the combination of LpHera + Saczyme GO 2 + Ultraflo Max, statistically equal results were obtained for the same pretreatments ($p < 0.05$). The lowest yields were obtained using the other enzyme blends, regardless of the pretreatment used.

In addition, the hydrolysis efficiency was calculated based on the carbohydrates present in the biomass. Using the results of proximate analysis, the enzymatic hydrolysis efficiency was calculated for the same 3 h period across different enzyme blends and pretreatments. The results show only the action of the enzymes, with the t0 time corresponding to the action of the pretreatments.

Figure 8 presents the results of enzymatic hydrolysis efficiency. The pretreatments showed an efficiency at t0 (without enzyme addition) of approximately 4%. For enzymatic action, the highest means were observed using the enzymes LpHera + Saczyme GO 2 + Celluclast 1.5 L (65.13 ± 0.46%). There was no statistical difference ($p < 0.05$) when using ultrasound or ultrasound + autoclaving. Assays using the enzymes LpHera + Saczyme GO 2 + Ultraflo Max showed similar results and statistical equality with assays from the previous blend. Regarding hydrolysis efficiency for different biomasses, ref. [15] obtained results of

56.93 ± 3.64%, 96.61 ± 20.26%, and 45.68 ± 0.22% for the biomass of *A. niger*, *S. platensis*, and *A. niger* + *S. platensis*, respectively. Concerning the low hydrolysis efficiency for the bioflocculated biomass containing fungal cells, it is noteworthy that the freezing/thawing + gelatinization pretreatment showed no effectiveness in breaking down the most resistant matrices of the fungal cell wall, which are composed mainly of polysaccharides [67].

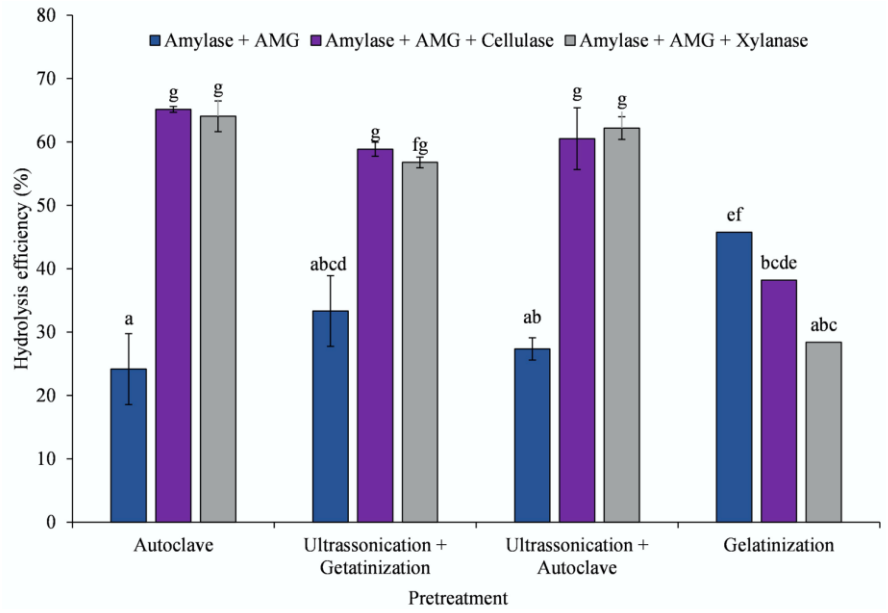



**Figure 8.** Enzymatic hydrolysis efficiency [1] for different treatments at 3 h. [1] Means ± standard deviation. The same letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

The high hydrolysis efficiency (~70%) of bioflocculated biomass can be explained by the enzyme-microalgae-fungus interaction. According to a study conducted by [54], when subjected to enzymatic hydrolysis, the bioflocculated biomass exhibited a significantly higher release of reducing sugars, such as glucose and xylose, compared to biomass from isolated microalgae or fungi, with a conversion efficiency exceeding 85%. The assays demonstrated high efficiency in the release of reducing sugars, indicating that the enzymes facilitated access to the polysaccharides present in the microalgae-fungus biomass. This performance was attributed to the production of lignocellulolytic enzymes (cellulases, hemicellulases, and ligninases, assessed through enzymatic activity) by the fungus *A. niger* during the bioflocculation process, as well as the porous matrix of the bioflocculated biomass (analyzed using Scanning Electron Microscopy—SEM), which preserved enzymatic activity by reducing protein denaturation and facilitating substrate access. Raman spectroscopy confirmed the degradation of structural polysaccharides in the bioflocculated biomass after hydrolysis. Furthermore, the release of reducing sugars from the bioflocculated biomass was approximately twice as high as that from isolated microalgae biomass, highlighting the importance of the physical and chemical interactions between microorganisms. The bioflocculated system also proved advantageous by eliminating the need for external enzyme addition, as the fungus produced enzymes directly during the process, thereby reducing costs and increasing the system's sustainability.

Studies addressing the enzymatic hydrolysis of mixed biomass are scarce, especially those involving the use of enzymatic blends. Incorporating computational modeling or molecular simulations could even be used to predict how enzymes interact with the polymeric structures present in the biomass and identify preferential binding sites. Molecular dynamics simulations, for instance, can reveal how factors such as surface charge, structural conformation, and hydrophobic interactions influence hydrolysis efficiency. Additionally,

computational models can help optimize process conditions, such as temperature, pH, and enzyme concentrations, to maximize saccharification yield. By integrating experimental data with computational simulations, it is possible not only to understand the molecular mechanisms underlying saccharification but also to design more efficient and targeted processes for producing bioproducts, such as bioethanol, in mixed biomass systems [63,64,68].

Based on the obtained results, it was decided to carry out the hydrolysis of microalgae and fungus using the enzyme association LpHera + Saczyme GO 2 + Celluclast 1.5 L, under the same concentration conditions and action times. This blend was chosen for its efficiency and its action on the chemical bonds present in the microbial cell wall. Both cellulase and xylanase act on (1,4)-β-D-glucosidic linkages, but cellulase exhibits a specific action on cellulose, which is present in both microalgal and fungal cells [23,69].

### *3.4. Production of Bioethanol*

Figure 9 shows the results of ethanol production and total reducing sugars throughout the alcoholic fermentation process of biofloculated biomass containing the microalga *Spirulina platensis* and the fungus *Aspergillus niger*.

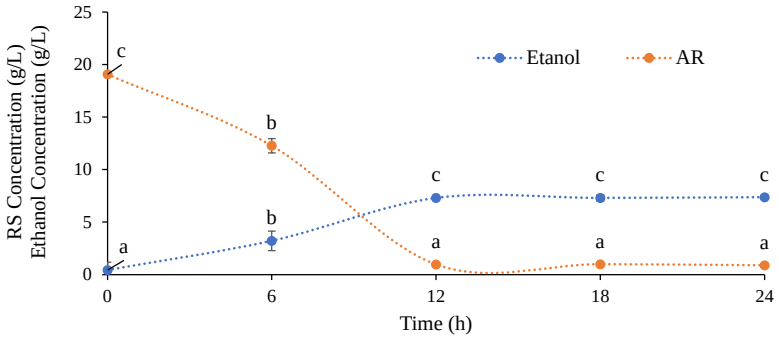



**Figure 9.** Ethanol concentration and total reducing sugars [1] over time. [1] Means ± standard deviation. The same letters indicate no statistical difference between the assays, while different letters indicate a statistical difference between the assays ($p < 0.05$).

The present study achieved similar results using biofloculated biomass. The lower conversion efficiencies of biofloculated biomass are attributed to the presence of the fungus *A. niger*, which has a complex cell wall composed of a matrix of polysaccharides, including chitin, glucans (mainly β-1,3-glucan and β-1,6-glucan), and proteins, forming a rigid network resistant to enzymatic degradation. Chitin, a polymer of N-acetylglucosamine, contributes to this rigidity and resistance due to its crystalline structure and β-1,4 linkages, which are difficult to hydrolyze. The heterogeneous cell wall, containing multiple layers and types of polysaccharides, requires a specific enzyme cocktail for complete hydrolysis. Bound proteins such as glycoproteins also increase resistance, as they are heavily glycosylated, hindering enzyme action. Additionally, the dense and intertwined structure limits enzyme accessibility to internal substrates, reducing hydrolysis efficiency [70].

As the highest yields of total reducing sugars were observed using Blend 2: α-amylase + amyloglucosidase + cellulase, these enzymes were employed for the enzymatic hydrolysis of biofloculated biomass containing *Spirulina platensis* and *Aspergillus niger* for ethanol production. The enzymatic hydrolysis produced 19.06 ± 0.11 g/L of glucose during saccharification. The fermented mash yielded 7.29 ± 0.24 g/L of ethanol after 12 h of fermentation, with no statistically significant difference observed in the results up to 24 h of testing, corresponding to a 79.21 ± 0.38% conversion of glucose into ethanol. This represents a productivity of approximately 0.6 g of ethanol per 1 g of biofloculated biomass per $L^{-1}.h^{-1}$. In the study by [71], the authors conducted enzymatic hydrolysis of *Spirulina platensis* to produce ethanol using α-amylase + amyloglucosidase + cellulase enzymes,

achieving 27 g/L of fermentable sugars that were converted with 92% efficiency to 12.7 g/L of ethanol after 9 h of fermentation.

In a study by [72], ethanol production from the carbohydrate-enriched biomass of the microalga *S. platensis* was explored. Different acids ($H_2SO_4$, $HNO_3$, HCl, and $H_3PO_4$) were tested for saccharification. Higher acid concentrations resulted in greater yields of reducing sugars; however, the authors noted that the presence of acids during polysaccharide hydrolysis significantly affected the ethanol production processes. These findings underscore the importance of studies that involve enzymatic processes for biomass hydrolysis in ethanol production.

In terms of values, the study by [73] showed that bioethanol production from algae using chemical treatment (thermal acid hydrolysis) costs approximately USD 26/ton of dry algae at the bioethanol production plant, while enzymatic treatment, which employs a simpler process, results in a cost of USD 64.6/ton at a production scale of 80,000 tons per year. When scaled up to 400,000 tons per year, production costs for enzymatic treatment increase to USD 91.3/ton, suggesting that, despite greater energy efficiency, enzyme production costs still represent a significant challenge. Regarding the selling price of bioethanol, the values for the enzymatic process ranged from USD 2.39/gallon for 80,000 tons per year to USD 2.08/gallon for 400,000 tons, indicating a slight improvement in economic viability at a larger scale, but still remaining high compared to chemical treatments. The sensitivity analysis in the study highlighted that the price of algae has the greatest impact on the final cost of bioethanol, emphasizing that cost reduction in large-scale algae production is crucial for the economic success of third-generation bioethanol production.

Ref. [74] conducted a techno-economic analysis on the use of microalgae as a source for biofuel production, exploring various integrated biorefinery options. They demonstrated that an integrated system could generate multiple biological products, thereby improving economic returns and facilitating large-scale commercial implementation. Considering that the production of *S. platensis* is already established industrially, integrating it with other microorganisms, such as fungi, could enable the deployment of third-generation biorefineries.

The integration of enzymatic hydrolysis and bioethanol production from biomass, particularly from microalgae or mixed microbial systems, presents significant potential for reducing greenhouse gas (GHG) emissions and enhancing the economic viability of biorefineries. This approach supports renewable biofuel production and aligns with global sustainability goals, making it an essential area for development.

From a GHG reduction perspective, bioethanol derived from algae biomass is considered carbon-neutral, as the carbon dioxide ($CO_2$) released during combustion is offset by the $CO_2$ absorbed by the algae during its growth cycle. Additionally, algae cultivation does not require arable land or freshwater resources, and its carbon sequestration capabilities further contribute to GHG emission reductions. When combined with energy-efficient enzymatic hydrolysis processes, which consume less energy than chemical treatments, the overall life cycle emissions of bioethanol are significantly minimized [75–77].

Economically, this technology diversifies and strengthens the bioeconomy by reducing reliance on food crop-based biofuels and avoiding the food vs. fuel dilemma. Utilizing algal biomass or waste biomass materials decreases raw material costs, particularly as algae and other non-food biomass are renewable and widely available. Moreover, the avoided costs associated with GHG emissions in biorefineries using enzymatic hydrolysis are noteworthy. These processes benefit from potential savings tied to carbon credits or environmental sustainability incentives. As governments implement stricter regulations on carbon emissions and promote green technologies, biorefineries that adopt energy-efficient, low-emission technologies may gain economic advantages through carbon trading schemes or subsidies [78,79].

Integrating enzymatic hydrolysis into biorefinery systems offers a dual advantage: it achieves substantial GHG emissions reductions while lowering production costs and creating opportunities in renewable energy markets. This comprehensive approach represents a pivotal step toward sustainable energy production and climate change mitigation.

## 4. Conclusions

The use of fungal bioflocculant associated with millivoltage adjustment for charge neutralization between microalgae and fungus allowed for the separation of microalgal biomass with high efficiency (~100%). Millivoltage adjustment enables efficient separation of microalgae at different growth phases, as charge neutralization causes microorganisms to aggregate into more stable flocs. Additionally, using different concentrations of bioflocculant with millivoltage adjustment did not interfere with biomass separation. Thus, lower concentrations of bioflocculant were selected for future biotechnological processes, given the complexity of the cell wall of the fungus *A. niger*.

For the hydrolysis of the bioflocculated biomass (*S. platensis* + *A. niger*), autoclaving was selected as the optimal pretreatment for cell wall disruption. This process was used to release intracellular carbohydrates, which were subsequently hydrolyzed using different commercial enzymes. Among the various enzyme blends applied, the combined use of $\alpha$-amylase (LpHera), amyloglucosidase (Saczyme GO2), and cellulase (Celluclast 1.5 L) produced the highest yields of reducing sugars. The hydrolysis process achieved an efficiency of approximately 70%, with 4% attributed to the pretreatment and the remainder to the specific enzymes used.

Bioflocculation is a process that does not interfere with the final biomass, as it does not use chemical reagents for separation. Therefore, this biomass can be used in hydrolysis processes to obtain high-value bioproducts such as bioethanol. The study of microbial cell walls and different methods of pretreatment and enzymatic hydrolysis has resulted in high hydrolysis efficiency. Currently, methods for the hydrolysis and conversion of microalgal biomass into bioethanol are being studied, with few studies focusing on bioflocculated biomass.

This study was able to produce bioethanol competitively using bioflocculated microalgal biomass with fungi, achieving a conversion efficiency of approximately 80%. These results are promising, given the complexity of the raw material and in comparison to studies that use only microalgal biomass for bioethanol production. The addition of fungal biomass to the process addresses issues such as the interference of chemicals used in microalgae harvesting and hydrolysis processes during bioethanol production.

For future studies, it is suggested that biofloc formulation be optimized by investigating combinations of microalgae and fungi that maximize enzymatic hydrolysis efficiency and bioethanol yields. The development of more specific enzymes, including optimized enzymatic blends and genetically modified strains, could reduce costs and improve outcomes. Studies on enzyme stability and reuse, such as enzyme immobilization, would also be beneficial for reducing production costs. Additionally, detailed analyses of economic feasibility at different scales and environmental impact through life cycle assessments are important for identifying opportunities for improvement. Integrating biofloc production with carbon capture systems could reduce greenhouse gas emissions, while exploring byproducts generated during the process could add value to the production chain. The use of computational modeling and molecular simulations could predict enzymatic interactions with substrates and optimize operational conditions. Finally, studies on public policies, economic incentives, and carbon credits could improve economic viability and encourage large-scale adoption. These suggestions encompass technological and operational improvements, as

well as environmental and economic impact analyses, providing a holistic pathway for advancing the use of fungal bioflocculants in sustainable bioethanol production.

**Author Contributions:** Formal analysis, V.S. and L.M.C.; funding acquisition, L.M.C.; investigation, V.S., J.F.F. and J.L.d.S.; methodology, V.S., J.F.F. and L.M.C.; supervision, L.M.C.; writing—original draft, V.S.; writing—review and editing, L.M.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was financed by the Coordination for the Improvement of Higher Education Personnel (CAPES) (finance code 001), National Council for Scientific and Technological Development (Process number 403786/2021-5) and FAPERGS - Research Support Foundation of the State of RS (Process number 21/2551-0002233-7).

**Data Availability Statement:** Data is contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Bocken, N.M.P.; Short, S.W. Unsustainable Business Models—Recognising and Resolving Institutionalised Social and Environmental Harm. *J. Clean. Prod.* **2021**, *312*, 127828. [CrossRef]
2. Brennan, L.; Owende, P. Biofuels from Microalgae—A Review of Technologies for Production, Processing, and Extractions of Biofuels and Co-Products. *Renew. Sustain. Energy Rev.* **2010**, *14*, 557–577. [CrossRef]
3. Kandasamy, S.; Zhang, B.; He, Z.; Bhuvanendran, N.; EL-Seesy, A.I.; Wang, Q.; Narayanan, M.; Thangavel, P.; Dar, M.A. Microalgae as a Multipotential Role in Commercial Applications: Current Scenario and Future Perspectives. *Fuel* **2022**, *308*, 122053. [CrossRef]
4. Mata, T.M.; Martins, A.A.; Caetano, N.S. Microalgae for Biodiesel Production and Other Applications: A Review. *Renew. Sustain. Energy Rev.* **2010**, *14*, 217–232. [CrossRef]
5. De Farias Silva, C.E.; Bertucco, A. Bioethanol from Microalgae and Cyanobacteria: A Review and Technological Outlook. *Process Biochem.* **2016**, *51*, 1833–1842. [CrossRef]
6. Park, Y.H.; Han, S.-I.; Oh, B.; Kim, H.S.; Jeon, M.S.; Kim, S.; Choi, Y.-E. Microalgal Secondary Metabolite Productions as a Component of Biorefinery: A Review. *Bioresour. Technol.* **2022**, *344*, 126206. [CrossRef]
7. Figueroa-Torres, G.M.; Pittman, J.K.; Theodoropoulos, C. Optimisation of Microalgal Cultivation via Nutrient-Enhanced Strategies: The Biorefinery Paradigm. *Biotechnol. Biofuels* **2021**, *14*, 64. [CrossRef]
8. Stiles, W.A.V.; Styles, D.; Chapman, S.P.; Esteves, S.; Bywater, A.; Melville, L.; Silkina, A.; Lupatsch, I.; Fuentes Grünewald, C.; Lovitt, R.; et al. Using Microalgae in the Circular Economy to Valorise Anaerobic Digestate: Challenges and Opportunities. *Bioresour. Technol.* **2018**, *267*, 732–742. [CrossRef]
9. Barros, A.I.; Gonçalves, A.L.; Simões, M.; Pires, J.C. Harvesting techniques applied to microalgae: A review. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1489–1500. [CrossRef]
10. Vonshak, A.; Richmond, A. Mass Production of the Blue-Green Alga Spirulina: An Overview. *Biomass* **1988**, *15*, 233–247. [CrossRef]
11. Zhuang, L.-L.; Azimi, Y.; Yu, D.; Wang, W.-L.; Wu, Y.-H.; Dao, G.-H.; Hu, H.-Y. Enhanced Attached Growth of Microalgae Scenedesmus. LX1 through Ambient Bacterial Pre-Coating of Cotton Fiber Carriers. *Bioresour. Technol.* **2016**, *218*, 643–649. [CrossRef] [PubMed]
12. Vandamme, D.; Pontes, S.C.V.; Goiris, K.; Foubert, I.; Pinoy, L.J.J.; Muylaert, K. Evaluation of Electro-coagulation–Flocculation for Harvesting Marine and Freshwater Microalgae. *Biotechnol. Bioeng.* **2011**, *108*, 2320–2329. [CrossRef]
13. Alam, M.A.; Vandamme, D.; Chun, W.; Zhao, X.; Foubert, I.; Wang, Z.; Muylaert, K.; Yuan, Z. Bioflocculation as an Innovative Harvesting Strategy for Microalgae. *Rev. Environ. Sci. Biotechnol.* **2016**, *15*, 573–583. [CrossRef]
14. Chua, E.T.; Shekh, A.Y.; Eltanahy, E.; Thomas-Hall, S.R.; Schenk, P.M. Effective Harvesting of Nannochloropsis Microalgae Using Mushroom Chitosan: A Pilot-Scale Study. *Front. Bioeng. Biotechnol.* **2020**, *8*, 771. [CrossRef]
15. Nazari, M.T.; Rigueto, C.V.T.; Rempel, A.; Colla, L.M. Harvesting of Spirulina Platensis Using an Eco-Friendly Fungal Bioflocculant Produced from Agro-Industrial by-Products. *Bioresour. Technol.* **2021**, *322*, 124525. [CrossRef]
16. Lee, O.K.; Kim, A.L.; Seong, D.H.; Lee, C.G.; Jung, Y.T.; Lee, J.W.; Lee, E.Y. Chemo-Enzymatic Saccharification and Bioethanol Fermentation of Lipid-Extracted Residual Biomass of the Microalga, Dunaliella Tertiolecta. *Bioresour. Technol.* **2013**, *132*, 197–201. [CrossRef]
17. Virmond, E.; Rocha, J.D.; Moreira, R.F.P.M.; José, H.J. Valorization of Agroindustrial Solid Residues and Residues from Biofuel Production Chains by Thermochemical Conversion: A Review, Citing Brazil as a Case Study. *Braz. J. Chem. Eng.* **2013**, *30*, 197–230. [CrossRef]

18. Cheirsilp, B.; Maneechote, W. Insight on Zero Waste Approach for Sustainable Microalgae Biorefinery: Sequential Fractionation, Conversion and Applications for High-to-Low Value-Added Products. *Bioresour. Technol. Rep.* **2022**, *18*, 101003. [CrossRef]
19. Voigt, J.; Stolarczyk, A.; Zych, M.; Malec, P.; Burczyk, J. The Cell-Wall Glycoproteins of the Green Alga Scenedesmus Obliquus. The Predominant Cell-Wall Polypeptide of Scenedesmus Obliquus Is Related to the Cell-Wall Glycoprotein Gp3 of Chlamydomonas Reinhardtii. *Plant Sci.* **2014**, *215–216*, 39–47. [CrossRef]
20. Rodríguez, R.; Jiménez, A.; Guillén, R.; Heredia, A.; Fernández-Bolaños, J. Postharvest Changes in White Asparagus Cell Wall during Refrigerated Storage. *J. Agric. Food Chem.* **1999**, *47*, 3551–3557. [CrossRef]
21. Ha, G.-S.; Saha, S.; Basak, B.; Kurade, M.B.; Kim, G.-U.; Ji, M.-K.; Ahn, Y.; Salama, E.-S.; Woong Chang, S.; Jeon, B.-H. High-Throughput Integrated Pretreatment Strategies to Convert High-Solid Loading Microalgae into High-Concentration Biofuels. *Bioresour. Technol.* **2021**, *340*, 125651. [CrossRef] [PubMed]
22. Keris-Sen, U.D.; Gurol, M.D. Using Ozone for Microalgal Cell Disruption to Improve Enzymatic Saccharification of Cellular Carbohydrates. *Biomass Bioenergy* **2017**, *105*, 59–65. [CrossRef]
23. Velazquez-Lucio, J.; Rodríguez-Jasso, R.M.; Colla, L.M.; Sáenz-Galindo, A.; Cervantes-Cisneros, D.E.; Aguilar, C.N.; Fernandes, B.D.; Ruiz, H.A. Microalgal Biomass Pretreatment for Bioethanol Production: A Review. *Biofuel Res. J.* **2018**, *5*, 780–791. [CrossRef]
24. Arumugam, A.; Malolan, V.V.; Ponnusami, V. Contemporary Pretreatment Strategies for Bioethanol Production from Corncobs: A Comprehensive Review. *Waste Biomass Valorization* **2021**, *12*, 577–612. [CrossRef]
25. Zabed, H.M.; Akter, S.; Yun, J.; Zhang, G.; Awad, F.N.; Qi, X.; Sahu, J.N. Recent Advances in Biological Pretreatment of Microalgae and Lignocellulosic Biomass for Biofuel Production. *Renew. Sustain. Energy Rev.* **2019**, *105*, 105–128. [CrossRef]
26. Sriyod, K.; Reungsang, A.; Plangklang, P. Single and Combined Enzymatic Saccharification and Biohydrogen Production from Chlorella Sp. Biomass. *Bioenergy Res.* **2021**, *14*, 940–953. [CrossRef]
27. Demuez, M.; Mahdy, A.; Tomás-Pejó, E.; González-Fernández, C.; Ballesteros, M. Enzymatic Cell Disruption of Microalgae Biomass in Biorefinery Processes. *Biotechnol. Bioeng.* **2015**, *112*, 1955–1966. [CrossRef]
28. Alavijeh, R.S.; Karimi, K.; Wijffels, R.H.; van den Berg, C.; Eppink, M. Combined Bead Milling and Enzymatic Hydrolysis for Efficient Fractionation of Lipids, Proteins, and Carbohydrates of Chlorella Vulgaris Microalgae. *Bioresour. Technol.* **2020**, *309*, 123321. [CrossRef]
29. Zhang, Y.; Kang, X.; Zhen, F.; Wang, Z.; Kong, X.; Sun, Y. Assessment of Enzyme Addition Strategies on the Enhancement of Lipid Yield from Microalgae. *Biochem. Eng. J.* **2022**, *177*, 108198. [CrossRef]
30. Zarrouk, C. Contribution A L'etude D'une Cyanophycee. Influence de Divers Facteurs Physiques et Chimiques Sur La Croissance et La Photosynthese de Spirulina Mixima. Ph.D. Thesis, University of Paris, Paris, France, 1996.
31. Magro, F.G.; Margarites, A.C.; Reinehr, C.O.; Gonçalves, G.C.; Rodigheri, G.; Costa, J.A.V.; Colla, L.M. *Spirulina Platensis* Biomass Composition Is Influenced by the Light Availability and Harvest Phase in Raceway Ponds. *Environ. Technol.* **2018**, *39*, 1868–1877. [CrossRef]
32. Colla, L.M.; Ficanha, A.M.M.; Rizzardi, J.; Bertolin, T.E.; Reinehr, C.O.; Costa, J.A.V. Production and Characterization of Lipases by Two New Isolates of Aspergillus through Solid-State and Submerged Fermentation. *Biomed. Res. Int.* **2015**, *2015*, 725959. [CrossRef] [PubMed]
33. Chen, J.; Leng, L.; Ye, C.; Lu, Q.; Addy, M.; Wang, J.; Liu, J.; Chen, P.; Ruan, R.; Zhou, W. A Comparative Study between Fungal Pellet- and Spore-Assisted Microalgae Harvesting Methods for Algae Bioflocculation. *Bioresour. Technol.* **2018**, *259*, 181–190. [CrossRef] [PubMed]
34. Miranda, R.; Latour, I.; Hörsken, A.; Jarabo, R.; Blanco, A. Enhanced Silica Removal by Polyamine- and Polyacrylamide-Polyaluminum Hybrid Coagulants. *Chem. Eng. Technol.* **2015**, *38*, 2045–2053. [CrossRef]
35. AOAC - ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. *Official Methods of Analysis of the Association of Official Analytical Chemists International*, 17th ed.; AOAC: Washington, DC, USA, 2000; pp. 1–2.
36. Rempel, A.; Machado, T.; Treichel, H.; Colla, E.; Margarites, A.C.; Colla, L.M. Saccharification of Spirulina Platensis Biomass Using Free and Immobilized Amylolytic Enzymes. *Bioresour. Technol.* **2018**, *263*, 163–171. [CrossRef]
37. Miller, G.L. Modified DNS Method for Reducing Sugar. *Anal. Chem. J.* **1959**, *31*, 426–428. [CrossRef]
38. Alva, S.; Anupama, J.; Savla, J.; Chiu, Y.Y.; Vyshali, P.; Shruti, M.; Yogeetha, B.S.; Bhavya, D.; Purvi, J.; Ruchi, K.; et al. Production and Characterization of Fungal Amylase Enzyme Isolated from Aspergillus Sp. JGI 12 in Solid State Culture. *Artic. Afr. J. Biotechnol.* **2007**, *6*, 576–581.
39. Ho, S.H.; Huang, S.W.; Chen, C.Y.; Hasunuma, T.; Kondo, A.; Chang, J.S. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresour. Technol.* **2013**, *135*, 191–198. [CrossRef]
40. Phwan, C.K.; Ong, H.C.; Chen, W.-H.; Ling, T.C.; Ng, E.P.; Show, P.L. Overview: Comparison of Pretreatment Technologies and Fermentation Processes of Bioethanol from Microalgae. *Energy Convers. Manag.* **2018**, *173*, 81–94. [CrossRef]
41. Salik, F.L.; Povh, N.P. Método Espectrofotométrico Para Determinação de Teores Alcoólicos Em Misturas Hidroalcoólicas. In Proceedings of the Congresso Nacional da STAB 5, São Paulo, Brazil, August; 1993; pp. 262–266.

42. Hang, Y.D.; Lee, C.Y.; Woodams, E.E.; Cooley, H.J. Production of Alcohol from Apple Pomace. *Appl. Environ. Microbiol.* **1981**, *42*, 1128–1129. [CrossRef]
43. Quigg, A. Micronutrients. In *The Physiology of Microalgae*; Springer International Publishing: Cham, Switzerland, 2016; pp. 211–231.
44. Cheng, P.; Chen, D.; Liu, W.; Cobb, K.; Zhou, N.; Liu, Y.; Liu, H.; Wang, Q.; Chen, P.; Zhou, C.; et al. Auto-Flocculation Microalgae Species Tribonema Sp. and Synechocystis Sp. with T-IPL Pretreatment to Improve Swine Wastewater Nutrient Removal. *Sci. Total Environ.* **2020**, *725*, 138263. [CrossRef]
45. Uba, K.I.; Gaid, G.D.; Perales, J.M.L.; Bongga, F.C.; Gaid, R.D. Use of Aluminium Sulphate as Flocculant for Spirulina (Arthrospira Platensis) with Notes on the Use of Antioxidants for Preservation under Refrigerated Conditions. *J. Fish.* **2022**, *10*, 103203. [CrossRef]
46. Ives, K.J. The Significance of Surface Electric Charge on Algae in Water Purification. *J. Biochem. Microbiol. Technol. Eng.* **1959**, *1*, 37–47. [CrossRef]
47. Veneu, D.M.; da Silva, M.P.M.; Di Salvio, A.M.; Delphino, I.A.Z. Tratamento de água eutrofizada através dos processos de pré-oxidação, coagulação e floculação. *Engevista* **2015**, *17*, 175–186. [CrossRef]
48. Bacon, J.S.D.; Farmer, V.C.; Jones, D.; Taylor, I.F. The Glucan Components of the Cell Wall of Baker's Yeast (*Saccharomyces Cerevisiae*) Considered in Relation to Its Ultrastructure. *Biochem. J.* **1969**, *114*, 557–567. [CrossRef]
49. Hochstenbach, F.; Klis, F.M.; van den Ende, H.; van Donselaar, E.; Peters, P.J.; Klausner, R.D. Identification of a Putative Alpha-Glucan Synthase Essential for Cell Wall Construction and Morphogenesis in Fission Yeast. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9161–9166. [CrossRef] [PubMed]
50. Grun, C.H. The Structure of Cell Wall -Glucan from Fission Yeast. *Glycobiology* **2004**, *15*, 245–257. [CrossRef]
51. Ruiz-Herrera, J.; Ortiz-Castellanos, L. Cell Wall Glucans of Fungi. A Review. *Cell Surf.* **2019**, *5*, 100022. [CrossRef]
52. Zhang, J.; Hu, B. A Novel Method to Harvest Microalgae via Co-Culture of Filamentous Fungi to Form Cell Pellets. *Bioresour. Technol.* **2012**, *114*, 529–535. [CrossRef]
53. Zhou, W.; Cheng, Y.; Li, Y.; Wan, Y.; Liu, Y.; Lin, X.; Ruan, R. Novel Fungal Pelletization-Assisted Technology for Algae Harvesting and Wastewater Treatment. *Appl. Biochem. Biotechnol.* **2012**, *167*, 214–228. [CrossRef]
54. Civzele, A.; Mezule, L. Fungal—Assisted Microalgae Flocculation and Simultaneous Lignocellulolytic Enzyme Production in Wastewater Treatment Systems. *Biotechnol. Rep.* **2025**, *45*, e00875. [CrossRef]
55. Ray, A.; Banerjee, S.; Das, D. Microalgal Bio-Flocculation: Present Scenario and Prospects for Commercialization. *Environ. Sci. Pollut. Res.* **2021**, *28*, 26294–26312. [CrossRef] [PubMed]
56. Phuong, H.; Massé, A.; Dumay, J.; Vandanjon, L.; Mith, H.; Legrand, J.; Arhaliass, A. Enhanced Liberation of Soluble Sugar, Protein, and R-Phycoerythrin Under Enzyme-Assisted Extraction on Dried and Fresh Gracilaria Gracilis Biomass. *Front. Chem. Eng.* **2022**, *4*, 718857. [CrossRef]
57. Eshra, D.; El-Iraki, S.; Abo Bakr, T. Performance of Starch Hydrolysis and Production of Corn Syrup Using Some Commercial Enzymes. *Int. Food Res. J.* **2014**, *21*, 815–821.
58. Castejón, N.; Marko, D. Fatty Acid Composition and Cytotoxic Activity of Lipid Extracts from Nannochloropsis Gaditana Produced by Green Technologies. *Molecules* **2022**, *27*, 3710. [CrossRef]
59. Pancha, I.; Chokshi, K.; Maurya, R.; Bhattacharya, S.; Bachani, P.; Mishra, S. Comparative Evaluation of Chemical and Enzymatic Saccharification of Mixotrophically Grown De-Oiled Microalgal Biomass for Reducing Sugar Production. *Bioresour. Technol.* **2016**, *204*, 9–16. [CrossRef]
60. Rojo, E.M.; Piedra, I.; González, A.M.; Vega, M.; Bolado, S. Effect of Process Parameters on the Valorization of Components from Microalgal and Microalgal-Bacteria Biomass by Enzymatic Hydrolysis. *Bioresour. Technol.* **2021**, *335*, 125256. [CrossRef]
61. Peron-Schlosser, B.; Stobienia, M.; Bispo, L.d.O.; Colla, L.M.; Baraldi, I.J.; Colla, E. Residual Fractions from *Arthrospira Platensis* Protein Extraction as Feedstock for Ethanol Production. *Biocatal Biotransform.* **2021**, *39*, 378–388. [CrossRef]
62. Onay, M. Bioethanol Production via Different Saccharification Strategies from H. Tetrachotoma ME03 Grown at Various Concentrations of Municipal Wastewater in a Flat-Photobioreactor. *Fuel* **2019**, *239*, 1315–1323. [CrossRef]
63. Shokrkar, H.; Ebrahimi, S.; Zamani, M. Enzymatic Hydrolysis of Microalgal Cellulose for Bioethanol Production, Modeling and Sensitivity Analysis. *Fuel* **2018**, *228*, 30–38. [CrossRef]
64. Shokrkar, H.; Ebrahimi, S. Evaluation of Different Enzymatic Treatment Procedures on Sugar Extraction from Microalgal Biomass, Experimental and Kinetic Study. *Energy* **2018**, *148*, 258–268. [CrossRef]
65. Constantino, A.; Rodrigues, B.; Leon, R.; Barros, R.; Raposo, S. Alternative Chemo-Enzymatic Hydrolysis Strategy Applied to Different Microalgae Species for Bioethanol Production. *Algal Res.* **2021**, *56*, 102329. [CrossRef]
66. Reguly, J.C. *Biotecnologia Dos Processos Fermentativos*; 1ª.; Editora Universitária/UFPel: Pelotas, Brazil, 1996.
67. Yoshimi, A.; Miyazawa, K.; Abe, K. Cell Wall Structure and Biogenesis in Aspergillus Species. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 1700–1711. [CrossRef] [PubMed]

68. Shokrkar, H. Model-based evaluation of enzymatic hydrolysis of microalgal cellulose and different cellulosic materials. Cellul Chem Technol. *Cellul. Chem. Technol.* **2023**, *57*, 97–106. [CrossRef]
69. Fontaine, T.; Simenel, C.; Dubreucq, G.; Adam, O.; Delepierre, M.; Lemoine, J.; Vorgias, C.E.; Diaquin, M.; Latgé, J.-P. Molecular Organization of the Alkali-Insoluble Fraction of Aspergillus Fumigatus Cell Wall. *J. Biol. Chem.* **2000**, *275*, 27594–27607. [CrossRef]
70. Gonzalez Bautista, E.; Laroche, C. Arthrospira Platensis as a Feasible Feedstock for Bioethanol Production. *Appl. Sci.* **2021**, *11*, 6756. [CrossRef]
71. Werlang, E.B.; Julich, J.; Muller, M.V.G.; de Farias Neves, F.; Sierra-Ibarra, E.; Martinez, A.; Schneider, R.d.C.d.S. Bioethanol from Hydrolyzed Spirulina (Arthrospira Platensis) Biomass Using Ethanologenic Bacteria. *Bioresour. Bioprocess.* **2020**, *7*, 27. [CrossRef]
72. Markou, G.; Angelidaki, I.; Nerantzis, E.; Georgakakis, D. Bioethanol Production by Carbohydrate-Enriched Biomass of Arthrospira (Spirulina) Platensis. *Energies* **2013**, *6*, 3937–3950. [CrossRef]
73. Fasahati, P.; Woo, H.C.; Liu, J.J. Industrial-Scale Bioethanol Production from Brown Algae: Effects of Pretreatment Processes on Plant Economics. *Appl. Energy* **2015**, *139*, 175–187. [CrossRef]
74. Rajesh Banu, J.; Preethi; Kavitha, S.; Tyagi, V.K.; Gunasekaran, M.; Karthikeyan, O.P.; Kumar, G. Lignocellulosic Biomass Based Biorefinery: A Successful Platform towards Circular Bioeconomy. *Fuel* **2021**, *302*, 121086. [CrossRef]
75. Ho, S.-H.; Chen, Y.-D.; Chang, C.-Y.; Lai, Y.-Y.; Chen, C.-Y.; Kondo, A.; Ren, N.-Q.; Chang, J.-S. Feasibility of CO2 Mitigation and Carbohydrate Production by Microalga Scenedesmus Obliquus CNW-N Used for Bioethanol Fermentation under Outdoor Conditions: Effects of Seasonal Changes. *Biotechnol. Biofuels* **2017**, *10*, 27. [CrossRef]
76. Hossain, N.; Haji Zaini, J.; Mahlia, T.M.I. A Review of Bioethanol Production from Plant-Based Waste Biomass by Yeast Fermentation. *Int. J. Technol.* **2017**, *8*, 5. [CrossRef]
77. Soudagar, M.E.M.; Kiong, T.S.; Jathar, L.; Nik Ghazali, N.N.; Ramesh, S.; Awasarmol, U.; Ong, H.C. Perspectives on Cultivation and Harvesting Technologies of Microalgae, towards Environmental Sustainability and Life Cycle Analysis. *Chemosphere* **2024**, *353*, 141540. [CrossRef] [PubMed]
78. Hossain, N.; Mahlia, T.M.I.; Zaini, J.; Saidur, R. Techno-economics and Sensitivity Analysis of Microalgae as Commercial Feedstock for Bioethanol Production. *Environ. Prog. Sustain. Energy* **2019**, *38*, 13157. [CrossRef]
79. Slade, R.; Bauen, A. Micro-Algae Cultivation for Biofuels: Cost, Energy Balance, Environmental Impacts and Future Prospects. *Biomass Bioenergy* **2013**, *53*, 29–38. [CrossRef]


**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.