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# From Microalgae to Biofuels: Investigating Valorization Pathways Towards Biorefinery Integration

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**Abstract:** The rapid growth of the world population led to an exponential growth in industrial activity all around the world. Consequently, $CO_2$ emissions have risen almost 400% since 1950 due to human activities. In this context, microalgae biomass has emerged as a renewable and sustainable feedstock for producing third-generation biofuels. This study explores the laboratory-scale production of bioethanol and biomethane from dried algal biomass. The first step was to evaluate and optimize the production of glucose from the biomass. Thus, three different techniques with three different solvents were tested to identify the most effective and efficient in terms of saccharification yield. With the assistance of an autoclave or a high-temperature water bath and 0.2 M NaOH as a solvent, yields of 79.16 ± 3.03% and 85.73 ± 3.23% were achieved which correspond to 9.24 and 9.80 g/L of glucose, respectively. Furthermore, the most efficient method from the pretreatment step was chosen to carry out a factorial design to produce bioethanol. The experiments showed that the loading of cellulase was of crucial importance to the optimization of the process. Optimized ethanolic fermentation yielded ethanol concentrations up to 4.40 ± 0.28 g/L (76.12 ± 4.90%) (0.3 M NaOH, 750 $\mu L/g_{cellulose}$ and 65 $\mu L/g_{starch}$), demonstrating the critical role of cellulase loading. Biomethane potential (BMP) assays on fermentation residues showed increased yields compared to untreated feedstock, with a maximum methane yield of 217.88 ± 10.40 mL/gVS. Combined energy production from bioethanol and biomethane was calculated at up to 1044.48 kWh/tn of algae feedstock, with biomethane contributing 75.26% to the total output. These findings highlight the potential of integrated algae-based biorefineries to provide scalable and sustainable biofuel solutions, aligning with circular economy principles.

**Keywords:** bioethanol; biomethane; enzymatic hydrolysis; microalgae



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## 1. Introduction

In the latest decade, the world population showed an exponential growth rate, from 7.51 billion in 2016 to 7.91 billion in 2021 and it is projected to reach 9.71 billion by 2050 [1] leading to a tremendous increase in energy demand, especially fossil fuels (i.e., coal, oil and natural gas). Nevertheless, the uncontrolled use of fossil fuels is a major concern due to their depletion as well as the negative effects on the environment [2].

Regarding energy consumption, world energy use showed a dramatic increase from 8589 million tonnes (Mtoe) recorded in 1955 to 13,147 Mtoe, recorded in 2015 [3]. Regarding primary energy consumption across Europe, based on data obtained from Eurostat [4] a significant decrease from approximately 1500 (Mtoe) recorded in 2006 to approximately 1250 (Mtoe) in 2020, was observed. According to the findings of [5], the total demand for primary energy is projected to be approximately 17,500 Mtoe in 2040. This rapid increase in

the demand for energy shall create an immense amount of stress in the energy production sector. For this reason, during the last decades, not only has research turned toward the development of new methods for energy production but also attempts have been made toward global policy changes. In this context, efficient alternatives for fossil fuels such as biofuels deriving from lignocellulosic biomass or other types of organic matter are currently trending among other environmental technologies [6,7].

In addition, since 2018, the EU revised Renewable Energy Directive (RED II) has been in effect. This directive focuses on emission prevention in the transport sector via the development and blending of advanced biofuels and conventional fuels. More specifically, in 2018, biofuels were blended with fossil fuels at a rate of 5.2%. Currently, the blending of conventional biofuels (food-based) is estimated at 4.1%, well below the 7% limit set in legislation up to 2030 (RED II). The blending of advanced (non-food based) biofuels is estimated at 1.2%. The majority of these fuels have derived (1%, group B) from waste such as fats and oils, while a small proportion (0.2%, group A) has derived from pine oil and cellulosic feedstocks [8,9].

On the other hand, the current scenario of anthropogenic pollution and unrestricted greenhouse gas emissions poses risks of exacerbating global warming, causing adverse impacts such as ocean acidification, desertification, and altered weather patterns. Global $CO_2$ emissions due to human activities have increased by almost 400% since 1950 and the high concentration of $CO_2$ in the atmosphere is predicted to continuously increase if the problem of $CO_2$ emission is not addressed [10]. According to the 2015 Paris Agreement, the rise in temperature of Earth should be kept below 2 °C in comparison to the preindustrial levels, and the increase in Earth's temperature should be limited to below 1.5 °C. To achieve this goal, hundreds of tons of $CO_2$ should be captured and stored annually until 2030 [2]. Despite all the efforts made, greenhouse gas emissions are still a pressing matter that needs to be resolved. Considering the decisions of the United Nations COP 28 for the importance of conserving, protecting, and restoring nature and ecosystems toward achieving the Paris Agreement temperature goal and preserving terrestrial and marine ecosystems serving as greenhouse gas sinks and reservoirs and biodiversity, a progressive shift away from fossil fuels may help ensure the survival of many ecosystems [11,12].

Considering all the above, biofuels represent a promising category of fuels derived from renewable sources, characterized by minimal environmental impact, widespread availability, sustainability, and reliability. Within this category, microalgae-based fuels emerge as a particularly environmentally friendly and promising option, demonstrating remarkable effectiveness in reducing global $CO_2$ emissions. Research suggests that one kilogram of microalgae biomass can sequester approximately 1.83 kg of $CO_2$ [13]. In addition, certain microalgae species could utilize sulfur oxides ($SO_x$) and nitrogen oxides ($NO_x$) as additional nutrient sources in addition to $CO_2$ [14]. Moreover, according to the existing literature, algal biomass typically contains large amounts of carbohydrates (5–23%), lipids (7–23%), and proteins (6–52%), but all those parameters are strongly species- and cultivation-dependent [15]. This fact indicates that biomass originating from algae could potentially be a very valuable substrate to produce different types of fuels.

The concept of biorefinery offers a sustainable framework for utilizing renewable biomass to produce a range of biofuels, energy, and high-value bioproducts while minimizing waste and environmental impact. Unlike conventional energy production systems, biorefineries integrate multiple processes to extract maximum value from the feedstock. Microalgae, with their diverse biochemical composition, are well suited for biorefinery applications, as they can be processed into multiple fuels such as bioethanol and biogas, alongside various co-products [16]. This approach aligns with the principles of the circular economy, ensuring efficient resource utilization and reduced greenhouse gas emissions. The present study adopts the biorefinery framework by investigating the sequential production of bioethanol through fermentation and biomethane via anaerobic digestion of fermentation residues, highlighting the integrated valorization of algae biomass. By integrating multiple conversion processes, the proposed treatment train aims to demonstrate a scalable and

sustainable pathway for maximizing energy output while minimizing waste, making it an ideal candidate for future biorefinery applications.

More specifically, microalgae are considered a third-generation feedstock for the production of sustainable biofuels not only due to their abundance in various aquatic environments but also because they do not require arable land for their cultivation [17]. The main fuels indicated in the literature that can be produced from microalgae include the production of alcohols (i.e., bioethanol), biodiesel, and biogas although there is a plethora of byproducts from algae biomass [18,19].

The production of bioethanol is achieved simply by fermenting simple sugars such as glucose and maltose into alcohol with the assistance of certain species of yeast, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* [20]. The conventional technique to produce bioethanol requires a pretreatment step, the hydrolysis of polysaccharides into monosaccharides, and finally yeast fermentation [21–23].

In addition, algae biomass could also be a quality feedstock to produce biogas via anaerobic digestion (AD). Usually before AD, the feedstock (i.e., conventional plants) undergoes a pretreatment step, chemical or mechanical in some cases, in order to break down their rigid structure [24].

Considering the pressing need for sustainable alternatives to fossil fuels, the present study aims to develop and evaluate a comprehensive biorefinery framework for the sequential production of bioethanol and biomethane from microalgae biomass. Specifically, the objectives of this research are: (i) To identify and optimize pretreatment techniques that maximize the saccharification yield of algae biomass for glucose production, evaluating different methods and solvents to determine the most effective approach; (ii) To investigate the ethanolic fermentation process by utilizing the optimal pretreatment method and applying factorial experimental design to optimize ethanol yields, focusing on critical parameters such as enzyme loading and process conditions; (iii) To assess the biomethane potential (BMP) of fermentation residues derived from the ethanolic fermentation process, evaluating the viability of integrating anaerobic digestion into the treatment framework and (iv) To quantify the overall energy potential of the algae-based biorefinery by combining the outputs of bioethanol and biomethane production, demonstrating the feasibility and scalability of the proposed approach for sustainable biofuel production.

By addressing these objectives, the study seeks to contribute to the advancement of microalgae-based biofuel production systems and provide insights into the circular economy principles for energy recovery and waste minimization, aligning with the holistic goals of biorefineries.

## 2. Materials and Methods

### *2.1. Materials*

The algae biomass that was utilized throughout the study was kindly provided by Algen, Slovenia. The biomass was received at the Unit of Environmental Science and Technology, School of Chemical Engineering, National Technical University of Athens, Athens, Greece, for analysis and processing. In total, about 500 g of feedstock were received and characterized in terms of physicochemical composition. All the utilized chemicals were of analytical quality. Regarding the use of enzymes, Novozymes® (Frederiksberg, Denmark) kindly supplied us with Spirizyme Excel XHS (Novozymes®, Frederiksberg, Denmark) (2337 U/mL) and CellicTec3 XHS (Novozymes®, Frederiksberg, Denmark) (171.7 FPU/mL). For the purpose of enzymatic hydrolysis as a first step for the production of fermentable sugars, the enzyme loadings remained constant throughout the pretreatment investigation at 45 $\mu L/g_{starch}$ for amylase and 500 $\mu L/g_{cellulose}$ for cellulase based on preliminary experiments. As far as the fermentation process is concerned, the yeast strain employed was *Saccharomyces cerevisiae* (*S. cerevisiae*, baker's yeast) which also remained constant at 2% (d.b.).

### 2.2. Analytical Methods

For the estimation of total and water-soluble solids, hemicellulose, cellulose, and insoluble residue in algal biomass (as received and pretreated), the National Renewable Energy Laboratory (NREL) procedure (National Renewable Energy Laboratory, Golden, CO, USA) was applied [25]. For total starch determination, the Total Starch (AA/AMG) test kit (e.g., Megazyme, Wicklow, Ireland) was used (AACC Method 76-13.01). The Soxhlet standard method (5520E) was utilized for the quantification of fats and lipids [26,27]. Marketable kits (Glucose oxidase–peroxidase method (GOD/PAP), Biosis SA, Athens, Greece; Spectro-quant Volatile Organic Acids Test 1,018,909 by Merck KGaA Millipore, Darmstadt, Germany; Ethanol Assay Kit, K-EtOHLQR, Megazymes) were used for the photometric determination of glucose, volatile fatty acids, and ethanol in the liquid fraction, respectively. All analyses took place in triplicate. The KERN DAB 100-3 Moisture Analyzer was utilized to determine the moisture content of the substrate prior to its treatment. This approach was employed to obtain reliable measurements of the dry matter to estimate the solids, enzymes, and yeast loadings.

### 2.3. Experimental Methods

#### 2.3.1. Pretreatment and Saccharification

Regarding the methods used for the pretreatment of biomass, according to the literature, there is a vast selection of pretreatment methods that could be utilized in order to maximize the potential sugars from algae biomass such as supercritical carbon dioxide, ammonia fiber explosion, ultrasonication, acid pretreatment, and alkaline pretreatment [28–33].

Considering the requirements for equipment and chemicals for each of the proposed pretreatment techniques in the literature, it was decided to conduct a series of experiments combining some of the most effective techniques, according to the recent updates in research. All of the experiments were carried out on a laboratory scale (250 mL boro-bottles with a 100 mL final volume). The solid loading and enzyme loadings were kept constant at 10%$w/w$, 45 μL/$g_{starch,}$ and 500 μL/$g_{cellulose}$ to maintain comparability of results.

Three different pretreatment techniques were employed in this research study. (A) The samples were hydrothermally pretreated using an autoclave (ISOLAB Laborgerate GmbH) at 121 °C for 30 min. (B) The samples were treated in a water bath at 90 °C for 75 min. (C) The samples were ultrasonicated at 150 W for 10 min using an ultrasonic probe (Branson Ultrasonics™ Sonifier™ SFX550 Cell Disruptor, Emerson, MO, USA). For each pretreatment three different cases were examined as solvents: (1) Distilled water, (2) Alkaline solution of NaOH (0.2 M), and (3) Acid solution of $H_2SO_4$ (1% $v/v$). All the above are summarized in Table 1.

**Table 1.** Conditions and solvents used for the pretreatment step prior to enzymatic hydrolysis.

| Experiment | Conditions | Solvent |
|---|---|---|
| A.1 | Hydrothermal at 121 °C for 30 min | Distilled $H_2O$ |
| A.2 | | NaOH (0.2 M) |
| A.3 | | $H_2SO_4$ (1% $v/v$) |
| B.1 | Water bath at 90 °C for 75 min | Distilled $H_2O$ |
| B.2 | | NaOH (0.2 M) |
| B.3 | | $H_2SO_4$ (1% $v/v$) |
| C.1 | Ultrasonication at 150 W for 10 min | Distilled $H_2O$ |
| C.2 | | NaOH (0.2 M) |
| C.3 | | $H_2SO_4$ (1% $v/v$) |

In addition, an experiment using just distilled water was conducted as blank. It is worth mentioning that each experiment was carried out in duplicate for more reliable results. After the pretreatment phase, the pH of each sample was set at approximately 5.5 and the enzymes were added to the mixture for the saccharification process. The enzymatic

hydrolysis conditions were the following: 72 h retention time at 50 °C and 150 rpm using a shaker (KS 3000 i control, IKA, Staufen, Germany). Also, the glucose concentration was monitored throughout the 72 h period of each experiment and the maximum concentration was recorded. At the end of each experiment, the whole mixture was transferred to falcon tubes for the separation of solid from the liquid fraction via centrifugation at 3.5 k rpm for 10 min. The solid part was dried at 35 °C in a Carbolite AX30 hot air oven, for 24 h and it was fully characterized in terms of total solids, moisture content, volatile solids (VS), ash, cellulose, hemicellulose, starch, and insoluble residue. In the liquid fraction, ethanol, glucose, volatile organic acid, and phenol concentrations were measured.

The saccharification yield was estimated to assess the efficiency of the process for each case. The term saccharification yield denotes the ratio of the quantity of sugars produced from the degradation of polysaccharides to the theoretical sugar content present in the feedstock (Equation (1)) [34]:

$$Y_{\text{Saccharification}} = \frac{\text{Maximum Produced Sugars (g)}}{\text{Theoretical Sugars (g)}} \times 100\% \quad (1)$$

To confirm the efficiency of the saccharification process, the degradation efficiencies of both the solid and major polysaccharides, i.e., cellulose and starch were calculated.

#### 2.3.2. Bioethanol Production

To produce bioethanol on a lab scale, the simultaneous saccharification and fermentation (SSF) mode was applied to the algae biomass, as determined by the preliminary experiments carried out. At first, all experiments were conducted in 250 mL autoclavable bottles, using a shaker (KS 3000 i control, IKA, Staufen, Germany). For the optimization trials, the experiments were performed under different concentrations of chemicals, and different enzyme loadings (Cellulolytic and Amylolytic enzymes) by applying the principles of factorial design. Enzyme loadings of 25, 45, and 65 $\mu L/g_{starch}$ for Spirizyme Excel XHS and 250, 500 and 750 $\mu L/g_{cellulose}$ for the CellicTec3 were tested by applying a constant solid loading of 10% $w/w$. The chemical means and the pretreatment method were selected during the pretreatment investigation step. The same pretreatment was applied to all the trials. The fermentation step was conducted at 35 °C for 24 h using 2% $w/w$ of yeast *S. cerevisiae*.

To quantify the efficiency of the process, two parameters were measured at t = 0 h and t = 24 h. The first parameter was the ethanol content which was also the goal of this step. The second parameter was the glucose content to determine if the fermentation was successful or if there was excess glucose at 24 h which could indicate the effect of an inhibitor.

The equation for the calculation of ethanol yield is taking into consideration the ethanol produced from the process of SSF and the theoretical ethanol that could be produced if all glucose in the feedstock was metabolized to ethanol (Equation (2)) [35,36]:

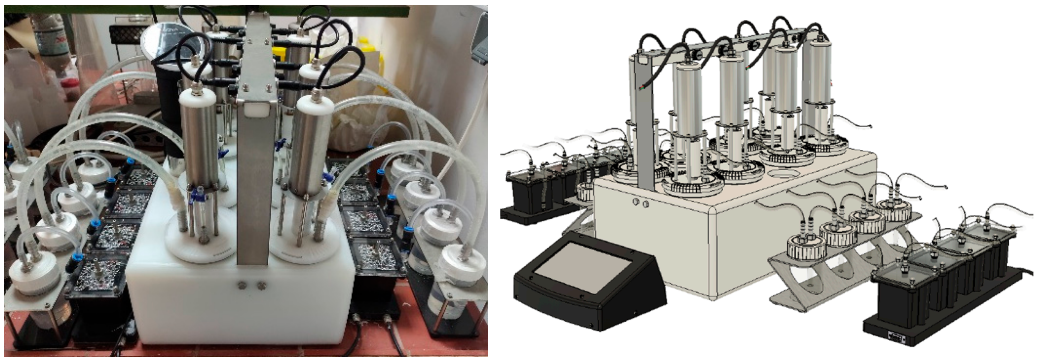
$$Y_{\text{etOH}} = \frac{\text{Produced Ethanol (g)}}{\text{Theoretical Ethanol (g)}} \times 100\% \quad (2)$$

#### 2.3.3. Biochemical Methane Potential Assay

The biochemical methane potential assay was conducted to assess the biodegradability of the dried feedstock as well as the derived stillage from the ethanolic fermentation conducted in the previous step. For this purpose, a lab-scale BMP continuously agitated batch reactor manufactured by CJC LABS, with a working volume of 1 L (Figure 1), was utilized along with a small scrubbing unit to estimate the produced methane. The assay was conducted according to the protocol developed by Angelidaki [37]. The apparatus could conduct 16 simultaneous batch experiments. In each cycle, two vessels were utilized to conduct one blank and one control experiment to verify the activity of the inoculum. The control was conducted with granulated cellulose to replicate the whole process of

the degradation. The retention time of each cycle was set to 30 days, and the cumulative methane content was measured with the assistance of the integrated data logger of the reactor. Each vessel of the apparatus was partially filled, and the chosen vs. ratio of the inoculum and substrate was set to 1:4. The latter was chosen based on the quantity of the stillages derived from ethanolic fermentation. The inoculum was received from an existing anaerobic digestion plant treating municipal sewage sludge in Athens, Greece (VS 20.8 g/L). Upon the completion of each cycle, the corresponding yield for each case was calculated according to the following equation:

$$\text{Yield}_{CH_4} = \frac{\text{Cummulative Produced } CH_4 \text{ (mL)}}{\text{VS of substrate added (g)}} \quad (3)$$



(**a**) (**b**)

**Figure 1.** (**a**) BMP Reactor; (**b**) Reactor schematic.

## 3. Results and Discussion

### *3.1. Chemical Composition*

The first step was to determine the composition of the received dried biomass. All the measured parameters according to Section 2.2 are presented in Table 2. As can be observed, the feedstock had an elevated content of acid-insoluble residue and a lower content of carbohydrates, i.e., cellulose and starch.

**Table 2.** Composition of the received dried algal biomass.

| Parameter (% d.b.) | Feedstock |
|---|---|
| Total Solids | 91.96 ± 0.78 |
| Moisture | 8.04 ± 0.78 |
| Volatile Solids | 65.79 ± 0.66 |
| Ash | 34.21 ± 0.66 |
| Oils | 0.95 ± 0.00 |
| Water Soluble Solids | 12.25 ± 0.04 |
| Free Glucose | 0.08 ± 0.01 |
| Starch | 1.78 ± 0.16 |
| Cellulose | 9.21± 0.57 |
| Hemicellulose | 17.52 ± 1.21 |
| Acid Insoluble Residue | 26.72 ± 4.38 |
| Total Nitrogen (Kjeldahl) | 4.18 ± 0.10 |

The results obtained from the characterization of the feedstock showed a slight deviation from those that can be found in the literature. More specifically, algae biomass typically contains high lipid content in a range between 8 and 77% (d.b.) depending on the strain [24]. In our case, the lipid content did not exceed 1% (d.b.). Also, the carbohydrate content was within the low limits of the literature. Depending on the strain, the range of the carbohydrate content of algal species is between 11 and 50% (d.b.) [22] while

our feedstock was measured at 10.99 ± 0.73% (d.b.). Considering that the received algae biomass was cultivated in an open raceway pond neither the purity of the dominant strain nor the cultivation conditions can be ensured, consequently, the composition of the derived biomass could have fluctuations compared with those of pure strain cultivations. In general, the chemical composition of algae biomass, particularly lipid and carbohydrate content, is species- and cultivation dependent, potentially affecting process efficiency and consistency [38].

### *3.2. Lab-Scale Pretreatment Method Investigation*

To initiate the pretreatment experiments, a trial was conducted as blank by using distilled water and the enzyme mixture (45 μL/$g_{starch}$ and 500 μL/$g_{cellulose}$ for Spirizyme Excel XHS and CellicTec3, respectively). This trial resulted as expected in a saccharification yield of lower than 1% according to Equation (1). Consequently, the application of a pretreatment method was deemed necessary. The results of the 72 h period of monitoring the glucose concentration in all the experiments of Table 1 are presented in Figures 2–4.

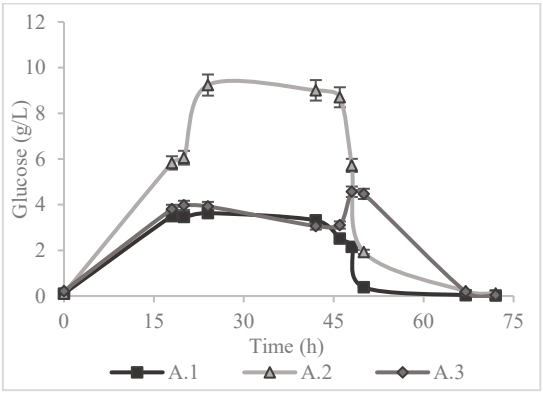



**Figure 2.** Glucose concentration throughout the 72 h enzymatic hydrolysis of algae after pretreatment in autoclave at 121 °C for 30 min.

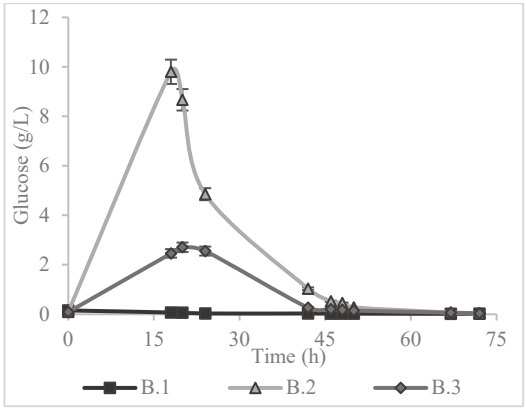



**Figure 3.** Glucose concentration throughout the 72 h enzymatic hydrolysis of algae after pretreatment in water bath at 90 °C for 75 min.

The saccharification yields of these experiments are shown in Figure 5. In the saccharification process, two experiments were the most effective in terms of yield; A.2 and B.2 which correspond to alkaline hydrothermal pretreatment and alkaline thermal pretreatment, respectively. A.2 and B.2 yielded 79.16 ± 3.03% with a concentration of 9.24 g/L and 85.73 ± 3.23% with a glucose concentration of 9.80 g/L, respectively. In addition, a notable conclusion is that when distilled water was used i.e., B.1 and C.1 despite the hydrothermal pretreatment, the yields were below 1%, indicating the effect of the solvents in the production of sugars. This fact supports the research of Kassim [28] who concluded that

the alkaline pretreatment in combination with high temperatures favored the production of reducing sugars, i.e., glucose. In addition, according to Kumar [18], acid and alkali pretreatment are widely acceptable methods because they are less energy-intensive and at the same time-efficient in removing unwanted materials from biomass.

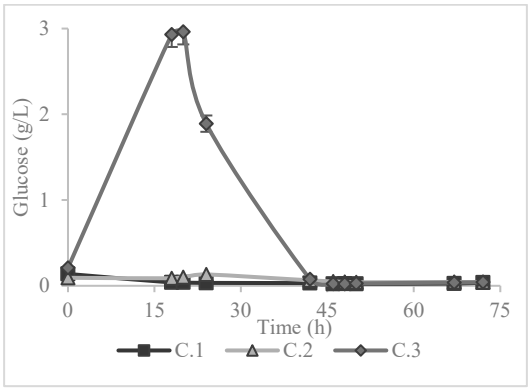



**Figure 4.** Glucose concentration throughout 72 h enzymatic hydrolysis of algae after ultrasonic pretreatment at 150 W for 10 min.

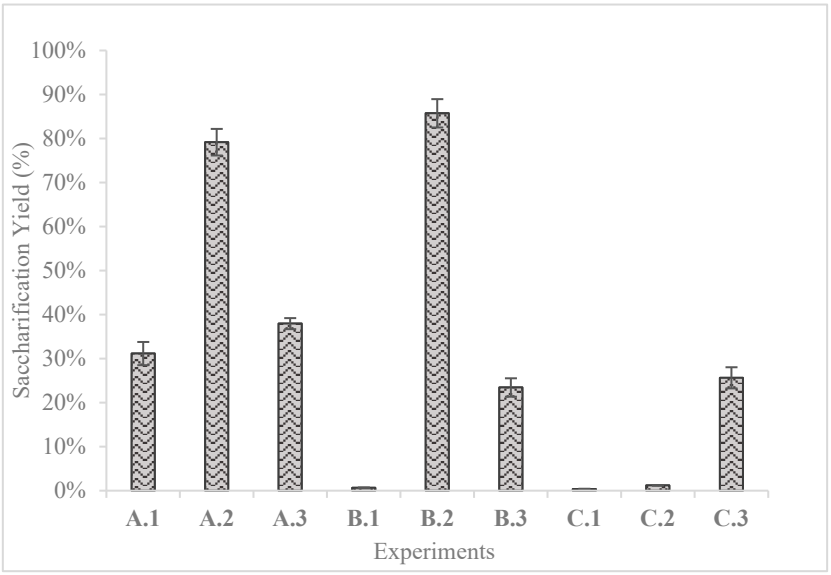



**Figure 5.** Saccharification yields per pretreatment method and solvent.

In Figures 6 and 7 the concentrations of phenolic compounds and volatile organic acids at the end of the enzymatic hydrolysis are presented.

The highest phenolic concentrations recorded were observed in experiments A.2 and C.2 (53.30 ± 4.67 and 48.80 ± 5.09 mg/L) with the use of NaOH and autoclave or ultrasonication, respectively. The slightly elevated phenolic concentrations are due to the fact that these chemical pretreatments of algae disrupted cell walls and hydrolyzed macromolecules, releasing phenolic compounds such as phlorotannins, flavonoids, and simple phenolics [34]. Nevertheless, these concentrations did not seem to inhibit the enzymatic hydrolysis [39,40].

On the other hand, the volatile organic acid concentrations were significantly higher in 72 h of the experiment, giving concentrations from 2.71 ± 0.19 to 6.19 ± 0.36 g/L with the highest being at experiment B.2. This increase in the concentration of VOAs could be due to the 72 h retention time of enzymatic hydrolysis [41]. More specifically, during the enzymatic hydrolysis, at a specific point, the concentration of glucose dropped dramatically

as shown in Figures 2–4 while at the end of each experiment, the concentration of VOAs spiked. This fact could be due to glucose oxidation to VOA compounds.

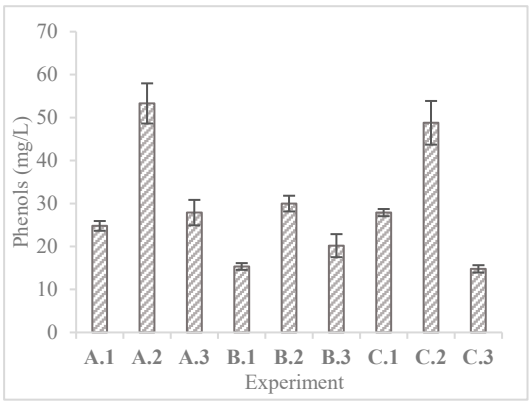



**Figure 6.** Phenolic content after 72 h enzymatic hydrolysis.

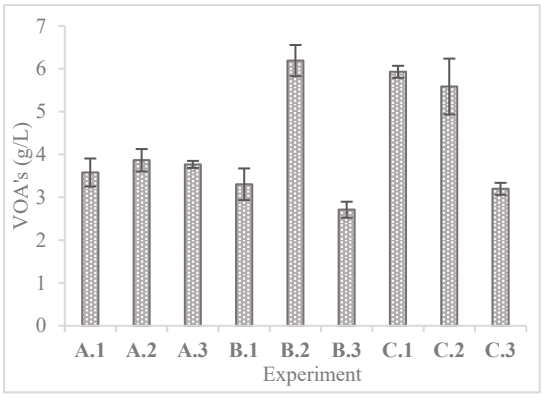



**Figure 7.** Volatile Organic Acid concentration after 72 h enzymatic hydrolysis.

As far as the solid fraction of each experiment is concerned, the results are presented in Figure 8.

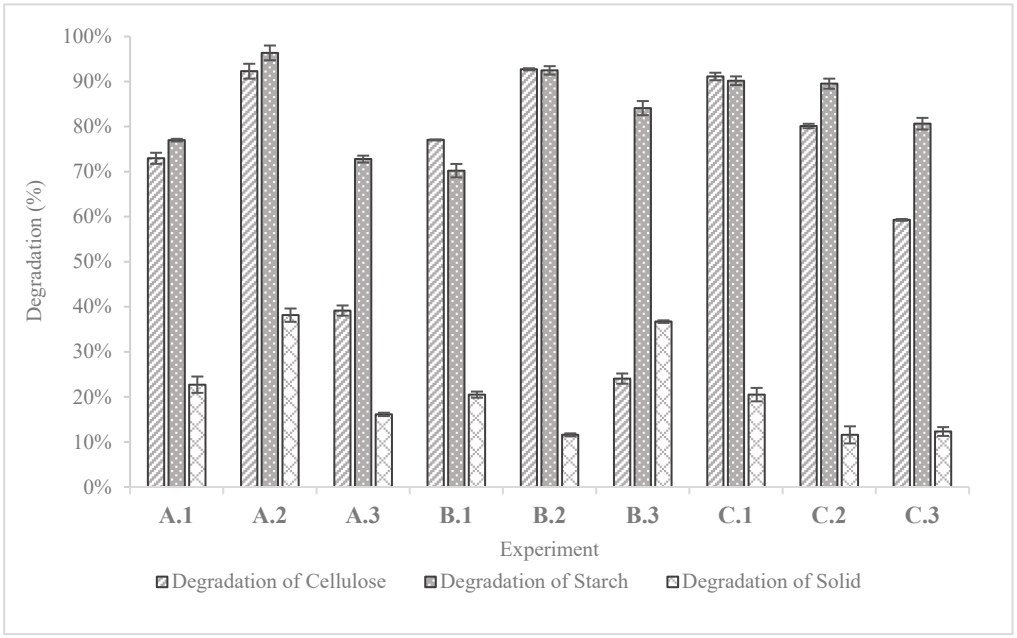



**Figure 8.** Degradation of solid and major polysaccharides after enzymatic hydrolysis.

According to Figure 8, the degradation efficiencies of both starch and cellulose were quite elevated; in all cases, the efficiencies ranged from 59.30 to 96.35%. This result indicates both the effectiveness of pretreatment methods and enzymatic hydrolysis. The highest degradation of cellulose and starch recorded was 92.73% and 96.35% in experiments B.2 and A.2, respectively.

Regarding the degradation of solid, it ranged from 11.58% to 38.17% in experiment A.2. The high degradation of solid in experiment A.2 is justified due to the elevated pressure in the autoclave that favors the breakdown of the solid.

To conclude, the degradation efficiencies that were calculated confirm the results that had derived from the saccharification yields. The goal of this step was to identify the most effective pretreatment combination to produce sugars that could stand as a viable substrate for alcoholic fermentation. From all the experiments of this step and considering the future scalability of the process, the combination of alkaline pretreatment and 90 °C water bath was selected to be used as the pretreatment step.

### *3.3. Factorial Design for Bioethanol Production*

Utilizing the optimal pretreatment method from the previous step, a factorial design was performed, as mentioned in paragraph 2.3.2 for dried algae biomass to assess the bioethanol production, focusing on bioethanol yield. Consequently, the liquid phase of the residues after fermentation was analyzed in terms of ethanol and residual glucose concentrations. These results are shown in Table 3. The ethanol yield for each experiment was calculated according to Equation (2).

**Table 3.** Ethanol concentrations and ethanol yield after alkali pretreatment and 24 h of SSF for dried algae biomass.

| No. | Conditions | | | Liquid Phase After Fermentation | | Yield |
|---|---|---|---|---|---|---|
| | NaOH (M) | CellicTec3 ($\mu L/g_{cellulose}$) | Spirizyme Excel XHS ($\mu L/g_{starch}$) | Ethanol Concentration (g/L) | Glucose Concentration (g/L) | Ethanol Yield (%) |
| 1 | 0.1 | 250 | 25 | 3.10 ± 0.42 | 0.08 ± 0.01 | 53.63 ± 7.35 |
| 2 | 0.1 | 750 | 25 | 3.30 ± 0.14 | 0.09 ± 0.01 | 57.09 ± 2.44 |
| 3 | 0.1 | 250 | 65 | 3.19 ± 1.42 | 0.13 ± 0.04 | 54.65 ± 2.84 |
| 4 | 0.1 | 750 | 65 | 3.58 ± 1.44 | 0.12 ± 0.03 | 61.26 ± 2.56 |
| 5 | 0.3 | 250 | 25 | 2.80 + 0.28 | 0.11 ± 0.00 | 48.43 ± 4.88 |
| 6 | 0.3 | 750 | 25 | 3.90 ± 0.14 | 0.11 ± 0.00 | 67.47 ± 2.45 |
| 7 | 0.3 | 250 | 65 | 3.70 ± 0.14 | 0.10 ± 0.00 | 64.00 ± 2.44 |
| 8 | 0.3 | 750 | 65 | 4.40 ± 0.28 | 0.10 ± 0.02 | 76.12 ± 4.90 |
| Center | 0.2 | 500 | 45 | 2.95 ± 0.25 | 0.06 ± 0.00 | 50.26 ± 4.96 |

In addition, mean values and standard deviations were calculated, in order to assess random errors with 95% statistical significance. The Cochran criterion was applied to validate the homogeneity of fluctuations. Furthermore, a mathematical model was developed, showing the impact and significance of the chosen factor on the optimization parameter. In this procedure, ethanol yield was examined as the optimization parameter. Checking of developed mathematical model adequacy was achieved by the Fisher criterion.

Algae-dried biomass seems to offer the highest ethanol yield (76.12 ± 4.90%) corresponding to 4.40 ± 0.28 g/L of ethanol when 0.3 M NaOH was added along with 65 μL Spirizyme Excel XHS/$g_{starch}$ and 750 μL CellicTec3/$g_{cellulose}$. It is important to note that after each experiment as shown in Table 3, the concentration of glucose is very close to zero, indicating that at the end of the fermentation, *S. cerevisiae* had completely metabolized the glucose produced into ethanol.

Based on the results of the factorial experiment presented in Table 3 and the mathematical processing and advanced statistical tools, such as ANOVA for the evaluation of the statistical significance of results, [34,35] the following equations were constructed both in

coded and in physical values to indicate the impact of the operational conditions chosen for the maximization of ethanol yield.

Coded values:

$$Y_{etOH} = 0.5999 + 0.0532\ X3 \quad (4)$$

Physical Values:

$$Y_{etOH} = 0.33815 + 0.0002128\ CellicTec3 \quad (5)$$

The analysis of the factorial design revealed that the highest ethanol yield was achieved with high levels of NaOH, amylase, and cellulase. Additionally, the analysis highlighted that the ethanol production from algae-dried biomass is positively impacted just by the concentration of cellulase within the studied range. This means that the increase in the cellulase concentration would overall favor the production of ethanol from algae-dried biomass.

### *3.4. Biomethane Potential*

The biomethane yields were calculated in accordance with Equation (3). The results obtained from the assay are presented in Figure 9.

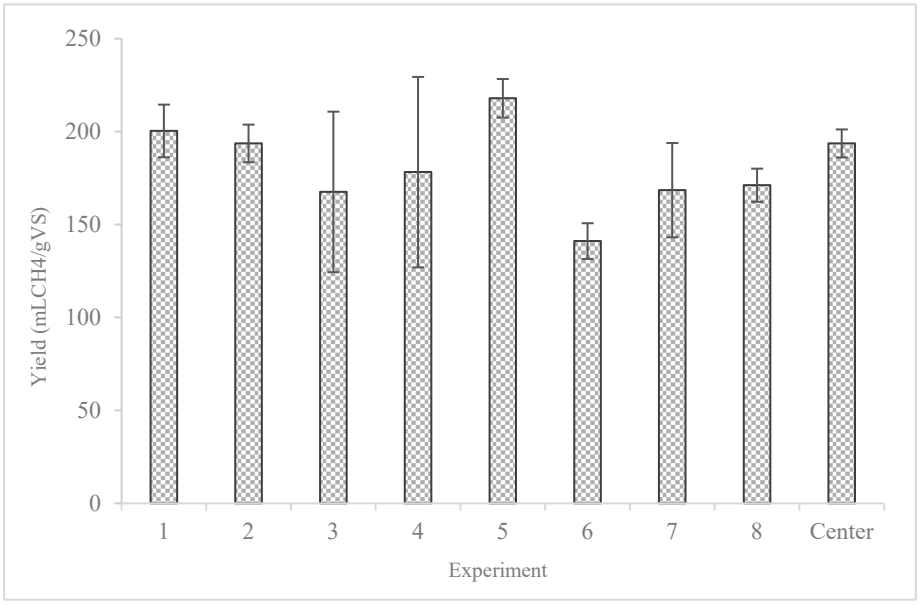



**Figure 9.** Total produced biomethane from the BMP experiments.

These results were satisfactory since according to Ward [42] all the yields were within the range mentioned in the literature. For example, the methane yield from the mixed culture of *Scenedesmus* sp. and *Chlorella* sp. was 143 mL/gVS. In addition, the methane yield obtained from the digestion of the dried feedstock without any treatment was measured at 122.29 ± 16.54 mL/gVS. The highest methane yield obtained from the assays was recorded in experiment 5 corresponding to 217.88 ± 10.40 mL/gVS followed by experiment 2 with 200.31 ± 14.7 mL/gVS. This fact reveals that ethanolic fermentation prior to anaerobic digestion favors biomethane production, especially in the case of experiment 5 (0.3 M NaOH, 750 $\mu L/g_{cellulose}$ and 65 $\mu L/g_{starch}$), the ethanol yields almost doubled. It is worth mentioning that the maximum yield was obtained from the experiment that only one factor (NaOH) was at the high level during the factorial experiment, while the other two (amylase and cellulase) were at the lower level of the design.

### *3.5. Energy Production Routes*

Based on the results obtained from all the previously described processes, two scenarios were developed for the potential energy production from algae biomass. These scenarios considered the valorization of products from both bioethanol and biogas production.

First Route: This scenario involved solely biogas production from the received dry biomass without any pretreatment. As outlined in Section 3.4, the untreated feedstock yielded 123.74 ± 14.54 mL of biomethane per gram of volatile solids (mL/gVS) during the BMP assay. Using this yield and considering the lower heating value (LHV) of biomethane as 10 kWh/m$^3$ [43], the total energy potential from biogas production was calculated as follows:

$$\text{Energy } (\text{kWh/tn dry algae}) = \text{BMP yield } (\text{mL/gVS}) \cdot \text{Feedstock vs. } (\text{g}) \times \frac{\text{LHV } (\text{kWh/m}^3)}{1000}$$

Substituting the values, the energy potential was estimated to be 683.03 ± 63.54 kWh/tn of dried feedstock.

Second Route: This scenario involved both bioethanol fermentation and biomethane production from the fermentation residues. The energy yield for bioethanol was derived from the ethanol concentration obtained in the factorial design experiments (e.g., 4.40 ± 0.28 g/L from experiment 5), the density, and LHV of ethanol (7.44 kWh/L). The total energy from ethanol production was calculated as:

$$\text{Energy from bioethanol } (\text{kWh/tn}) = \text{Ethanol concentration } (\text{g/L}) \cdot \text{Volume of broth } (\text{L}) \times \text{LHV } (\text{kWh/L})$$

For the biogas component, the BMP assay results for the fermentation stillages were used. Experiment 5, for instance, yielded 217.88 ± 10.40 mL/gVS from the stillages. The energy calculation followed the same formula as in the first route, using the LHV of biomethane.

By summing the energy from bioethanol and biomethane, the total energy yield for each experiment was obtained. The values for all experiments are presented in Figure 10, with the highest energy output recorded in Experiment 5 (1045 kWh/tn of algae) and the lowest in Experiment 2 (885 kWh/tn of algae). These calculations assume that all ethanol and biomethane produced are fully valorized and converted into usable energy.

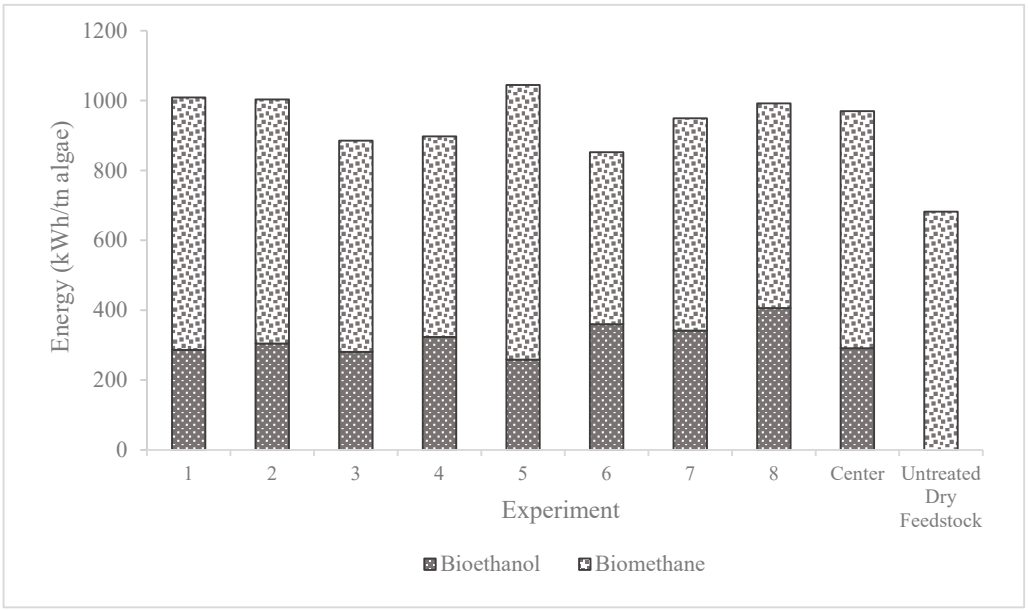



**Figure 10.** Potential energy produced from ethanolic fermentation and biomethane.

Table 4 provides a detailed breakdown of the individual energy contributions from bioethanol and biomethane for each experiment.

**Table 4.** Fractions of the total potentially produced energy.

| No. | Bioethanol | Biomethane | Total Energy | Bioethanol (%) | Biomethane (%) |
|---|---|---|---|---|---|
| | kWh/tn Algae | | | | |
| 1 | 286.14 | 722.68 | 1008.82 | 28.36 | 71.64 |
| 2 | 304.60 | 698.54 | 1003.14 | 30.36 | 69.64 |
| 3 | 280.70 | 604.46 | 885.16 | 31.71 | 68.29 |
| 4 | 323.22 | 574.20 | 897.42 | 36.02 | 63.98 |
| 5 | 258.39 | 786.08 | 1044.48 | 24.74 | 75.26 |
| 6 | 359.98 | 492.43 | 852.41 | 42.23 | 57.77 |
| 7 | 341.47 | 607.90 | 949.36 | 35.97 | 64.03 |
| 8 | 406.13 | 585.89 | 992.02 | 40.94 | 59.06 |
| Center | 290.94 | 678.70 | 969.64 | 30.01 | 69.99 |

## 4. Discussion

The results of this study highlight the potential of algae biomass as a feedstock for bioethanol and biomethane production, but a broader comparison with other strains and cultivation conditions is essential for understanding its applicability in diverse settings. Previous studies have shown that different microalgae species exhibit significant variations in their carbohydrate, lipid, and protein content, which can influence positively both the efficiency of bioethanol production and the methane yield from anaerobic digestion. For example, species such as *Chlorella vulgaris* and *Scenedesmus obliquus* have been reported to yield higher ethanol concentrations due to their more readily fermentable sugars compared to other species like *Spirulina* or *Nannochloropsis* [44–47]. Moreover, cultivation conditions, such as light intensity, temperature, and nutrient availability, may further impact the overall biofuel yield. Optimizing these conditions can lead to a more sustainable and efficient production process, but it is important to recognize that the suitability of a given strain or cultivation method will depend on regional factors and resource availability.

In terms of pretreatment, the study focused on alkaline and thermal methods due to their proven effectiveness in maximizing saccharification yields. However, emerging pretreatment technologies, such as enzymatic or microbial treatments, present promising alternatives that could significantly reduce energy and chemical requirements, although the economics of the process as well as its simplicity are also critical factors. Enzymatic pretreatment, for example, employs specific enzymes to break down the complex structure often under milder conditions than those required for thermal or alkaline methods. Studies have demonstrated that the application of xylanases, or laccases could reduce the energy consumption associated with biomass pretreatment, while also minimizing the formation of inhibitory by-products that can hinder fermentation processes [48,49]. Microbial pretreatments, which utilize bacteria or fungi to degrade cellulose, are another emerging area of research with the potential to provide more sustainable, low-energy alternatives to chemical-based methods [50]. While these technologies are still under development, they could complement or replace traditional pretreatment methods, leading to more efficient and environmentally friendly biofuel production processes.

Furthermore, several limitations must be considered when interpreting the results of this study. First, laboratory-scale conditions may differ significantly from industrial-scale operations, and scaling up the pretreatment, fermentation, and digestion processes could introduce inefficiencies and technical obstacles. Additionally, alkaline and thermal pretreatments, while effective at the laboratory scale, may face operational challenges and waste management issues when implemented on a larger scale. The economic feasibility of bioethanol and biomethane production from algae biomass must also be carefully evaluated to ensure it is competitive with traditional fossil fuels and other biofuels. Finally, the treatment and disposal of alkaline by-products and residues remain a concern, as

improper management could lead to environmental and ecological harm. These factors should be addressed in future research to ensure the commercial viability and sustainability of algae-based biofuel production.

## 5. Conclusions

To sum up, the primary objective of this study was to explore and demonstrate alternative pathways for the valorization of algae biomass, emphasizing its potential to serve as a sustainable resource for bio-based products. The chemical composition of the algae feedstock was identified as a critical determinant for the efficiency and success of the proposed processes, particularly for bioethanol production. This is because the availability and composition of fermentable sugars in the biomass directly influence the yield and efficiency of the subsequent fermentation processes.

Furthermore, the investigation into various pretreatment methods highlighted the importance of optimizing pretreatment strategies to maximize sugar release during enzymatic hydrolysis. Among the different approaches evaluated, a combination of thermal and alkali pretreatment was found to be the most effective in enhancing sugar production. Specifically, the study demonstrated that employing thermal pretreatment techniques such as autoclaving or using a water bath, in conjunction with alkali treatment using sodium hydroxide (NaOH), resulted in significant glucose concentrations. The glucose yield reached 9.24 g/L with autoclave pretreatment and 9.80 g/L with water bath pretreatment, both using NaOH.

When considering the potential for scaling up the process for industrial applications, the most practical and energy-efficient pretreatment method was selected for the fermentation step. Based on its simplicity, cost-effectiveness, and relatively high glucose yield, the combination of a water bath at 90 °C and NaOH at a concentration of 0.2 M was determined to be the most viable option. This choice balances the technical performance with economic and operational feasibility, paving the way for further developments in algae-based bioethanol production.

Regarding the fermentation step, a factorial design experiment was conducted to systematically evaluate the influence of various parameters on the fermentation performance. The findings were particularly insightful, revealing that only one critical parameter, cellulase loading, significantly influenced the outcomes within the studied range. Specifically, the concentration of cellulase was identified as the key determinant for maximizing the fermentation efficiency. Notably, the highest observed yield was $76.12 \pm 4.90\%$, achieved under conditions where 0.3 M NaOH was used in combination with a cellulase loading of 750 $\mu L/g_{cellulose}$ and 65 $\mu L/g_{starch}$. This highlights the importance of enzyme loading optimization for improving fermentation yields.

In addition to the fermentation results, biochemical methane potential (BMP) assays were performed to evaluate the feasibility of applying anaerobic digestion to the residual algae biomass (stillage) for biomethane production. The assays demonstrated promising potential for converting algae stillage into a renewable energy source. The maximum methane yield recorded was $217.88 \pm 10.40$ mL/gVS, which was obtained from the residue of experiment 5 in the factorial design. These results indicate that anaerobic digestion can effectively utilize the residual biomass, contributing to the overall efficiency of the process.

Lastly, energy production scenarios based on the proposed treatment train revealed significant benefits from integrating ethanolic fermentation with anaerobic digestion. The combined approach not only enhanced biomethane production but also significantly boosted the total energy output. It was estimated that more than 1000 kWh/tn algae biomass could be generated through this integrated process. This highlights the potential of the proposed treatment chain to maximize energy recovery from algae biomass, offering a sustainable and efficient pathway for bioenergy production.

This study exemplifies the biorefinery approach by integrating multiple processes to maximize the valorization of algae biomass. The sequential production of bioethanol and biomethane demonstrates an efficient and sustainable method for deriving energy

and value-added products from renewable feedstocks. The findings suggest that the combined application of bioethanol fermentation and anaerobic digestion not only enhances the overall energy yield but also minimizes waste, supporting the principles of the circular economy. This integration underscores the potential of algae-based biorefineries to contribute to global efforts toward energy sustainability and greenhouse gas reduction. Future studies could explore further optimization and scaling of this integrated process, reinforcing its feasibility for industrial applications.

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## Abbreviations

| Abbreviation | Description |
|---|---|
| SOx | Sulfur Oxides |
| AD | Anaerobic Digestion |
| NOx | Nitrogen Oxides |
| BMP test | Biochemical Methane Potential Assay |
| NREL | National Renewable Energy Laboratory |
| $Y_{Saccharification}$ | Saccharification Yield |
| $Y_{etOH}$ | Ethanol Yield |
| VOA | Volatile Organic Acids |
| SSF | Simultaneous Saccharification and Fermentation |
| LHV | Lower Heating Value |
| VS | Volatile Solids |

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