fermentation

MDPI

Article

Effects of Inoculation with Lactic Acid Bacteria on the Preservation of *Nannochloropsis gaditana* Biomass in Wet Anaerobic Storage and Its Impact on Biomass Quality

Oluwatosin Oginni [1], Bradley Wahlen [1,*], Lynn Wendt [1], Michelle Walton [1], Thomas Dempster [2] and Henri Gerken [3]

[1] Energy & Environment Science & Technology, Idaho National Laboratory, Idaho Falls, ID 83415, USA; oluwatosin.oginni@inl.gov (O.O.); lynn.wendt@inl.gov (L.W.); michelle.walton@inl.gov (M.W.)
[2] DMS, Tempe, AZ 85284, USA; thomas.dempster@asu.edu
[3] Arizona Center for Algae Technology and Innovation, Arizona State University, Mesa, AZ 85212, USA; henri.gerken@asu.edu
* Correspondence: bradley.wahlen@inl.gov

Abstract: Wet anaerobic storage of algal biomass is a promising preservation approach that can ensure a continuous supply of these feedstocks to biorefineries year-round. An effective solution to preservation must ensure minimal dry matter loss and a change in biochemical composition during storage. Therefore, the objective of this study is to investigate the preservation of *Nannochloropsis gaditana* biomass through wet anaerobic storage and its impact on biomass quality. Prior to storage, the algae sample is inoculated with two different strains of lactic acid bacteria and thereafter stored for 30 and 180 days. Each inoculant limited the dry matter loss to <10% (dry basis) after the storage duration. Final pH values (4.3–4.8) indicate that the biomass samples are properly ensiled, achieving the acidic conditions necessary for preservation. Compositional analysis of the biomass after storage shows a reduction in carbohydrate content, a relative increase in lipid content, and no significant change in the protein fraction. Glucose and galactose were the most prevalent sugar monomers. The low dry matter loss and minimal compositional change indicate that wet anaerobic storage is an effective means of preserving algal biomass and ensuring a constant supply of algal biomass feedstock to a biorefinery.

Keywords: algae; wet anaerobic storage; dry matter loss; organic acid; lactic acid bacteria

Citation: Oginni, O.; Wahlen, B.; Wendt, L.; Walton, M.; Dempster, T.; Gerken, H. Effects of Inoculation with Lactic Acid Bacteria on the Preservation of *Nannochloropsis gaditana* Biomass in Wet Anaerobic Storage and Its Impact on Biomass Quality. *Fermentation* **2022**, *8*, 159. https://doi.org/10.3390/fermentation8040159

Academic Editor: Florian Delrue

Received: 2 March 2022
Accepted: 31 March 2022
Published: 2 April 2022

1. Introduction

Algal biomass is a versatile, viable, and sustainable biomass feedstock. This is due to its high biomass productivity, biochemical composition, ability to be cultivated on marginal lands, and utilization of non-potable water resources and carbon dioxide emitted from fossil fuel-based power plants [1–3]. Its biochemical composition (comprising of carbohydrates, proteins, and lipids) has made it a suitable feedstock for conversion into a wide range of biofuels and bioproducts. For example, Shakya et al. [4] reported that the hydrothermal liquefaction (at a temperature of 320 °C) of two *Nannochloropsis* species with high lipid content (49–55 wt.%) resulted in a high bio-oil yield of 57–66%. Alternatively, innovative processing schemes, such as combined algal processing and parallel algal processing, have been developed to produce multiple bioproducts from each of the algae biochemical macromolecules to make full use of the algal biomass in order to achieve cost parity with petroleum [5–7].

Like other biomass feedstocks, there is a time difference between algal biomass harvesting and conversion to biofuel and bioproducts, therefore necessitating proper handling after harvesting to ensure the maximum amount of biomass is utilized for conversion. Since algal biomass is a biological material with high moisture content, ineffective storage

can lead to significant degradation and changes in its biochemical composition. Microbial degradation of algal biomass can start immediately after harvest, resulting in dry matter loss of ~20% within a week of harvest [8]. Wendt et al. [9] reported dry matter losses of 36.8 and 44% (dry basis) for *Scenedesmus obliquus* algae strains (post-centrifugation solid concentration of 20%) stored aerobically and anaerobically at room temperature, respectively, for 30 days.

Davis et al. [10] stressed the importance of algal biomass preservation and biochemical composition in light of its economic implications on the minimum fuel selling price (MFSP). The authors estimated that by storing a portion of the algal biomass produced in the summer, the MFSP of *Scenedesmus* algae-derived biofuel can be reduced by 4–6%. Moreover, the authors highlighted the importance of biochemical composition, estimating that production of biofuels from high-lipid *Scenedesmus* biomass will reduce the MFSP by 14% compared to fuel obtained from high-carbohydrate *Scenedesmus* biomass due to the higher fuel yield of the former (141 gallons of gasoline equivalent (GGE)/ton compared to 116 GGE/ton). This implies that preservation of biomass composition will be a critical success factor of any storage operation.

Despite the potential of large-scale algae growth to serve as an alternative source of fuels and chemicals, seasonal variation in productivity and variability in biochemical composition pose significant economic challenges to the optimization of biorefinery conversion capacities. Wendt et al. [9] explain that even though cultivation and production of algal biomass occur throughout the year across various geographical locations, seasonal decreases in temperature and solar irradiation negatively impact growth rate and yield, preventing constant conversion throughput. To mitigate the seasonal variation in algal biomass productivity and ensure continuous feedstock supply to the biorefinery while maintaining dry matter stability and minimal change in biochemical composition, various preservation techniques such as drying, freezing, pasteurization, blending, and wet anaerobic storage have been explored and reported in the literature [1,8,9,11].

Many approaches to mitigating variability have challenges that prevent their full-scale adoption. Drying is utilized in many industries to stabilize high-moisture materials. However, drying algal biomass is considered economically unviable because of the high capital and operational costs, which can be equivalent to approximately 75% of the total cost of algal biomass harvesting [12]. Pereira et al. [13] investigated the blending of algae biomass with spent coffee grounds as a way to ensure a constant feedstock supply, increasing the amount of spent coffee grounds when algae productivity decreases. Although effective for locations where spent coffee grounds resources are proximal to algae cultivation and processing facilities, such an approach may not be practical for every location. Additionally, the inclusion of a second feedstock with a different composition at variable ratios could introduce unwelcome complexity to the conversion process.

Wet anaerobic storage of algal biomass functions by the same principles as ensiling does in the preservation of forage for livestock feed [14,15] and has shown the potential to effectively preserve algae's biochemical composition and reduce dry matter loss. Wendt et al. [15] investigated wet anaerobic storage of *Scenedesmus acutus* in 100 mL and 500 mL storage reactors for 30 days and reported dry matter losses of 8.8 and 3.5% for the 100 mL scale and 500 mL scale storage, respectively. Similarly, the authors reported a dry matter loss of 7% for an algae/corn stover blend that was anaerobically stored for 30 days [9]. The low dry matter loss was attributed to microbial conversion of corn stover soluble sugars to organic acids. Lactic acid bacteria are considered to be very important in this process. They undergo homolactic (lactic acid only) or heterolactic (acetic and lactic acid) fermentation of algal sugars to produce organic acids. The reduced pH, along with the presence of the organic acids, limits the microbial activities of mold, yeast, and clostridia associated with biomass degradation [1,14].

The effectiveness of wet anaerobic storage in preserving the biochemical composition and dry matter content of algal biomass can be fully evaluated when this storage approach is utilized for various algae strains and the results are consistent. Previous work by the

authors has focused on wet anaerobic storage of freshwater algae strains (*S. obliquus*, *S. acutus*), algae blended with yard waste and corn stover [1,2,15,16]. However, this study is investigating the wet anaerobic storage of a saline algae strain, and the results from this study will contribute to the development of a holistic understanding of the effectiveness of wet anaerobic storage in preserving algal biomass feedstocks. Therefore, the aim of this study is to investigate the preservation of *Nannochloropsis gaditana* biomass through short-term (30 days) and long-term (180 days) storage durations, the impact of storage on biochemical composition, and whether preservation could be enhanced through inoculation with lactic acid bacteria.

2. Materials and Methods

2.1. Materials

Nannochloropsis gaditana algae strain used in this study was cultivated in a containment greenhouse at the Arizona Center for Algae Technology and Innovation (AzCATI, Mesa, AZ, USA) during the months of March and April of 2018. A 110 L vertical flat panel photobioreactor with a 2-inch light path using natural lighting (natural diurnal light/dark periods) was used in culturing the algal biomass. The culture medium used in the photobioreactor was F/2 media, adjusted to 35 g/L using oceanic sea salt (Oceanic Systems Inc., Dallas, TX, USA). The culture was grown over a 4-week period and harvested when culture density reached 3 g/L. The algal biomass was dewatered at 1800× *g* through Lavin 20–1160 V Centrifuges (AML Industries, Inc., Warren, OH, USA) with a flow rate of approximately 2 L/min. Dewatered algae (35.3% post-centrifugation solid concentration) were placed into Ziploc® bags, stored in a cooler on ice, and shipped overnight to Idaho National Laboratory, Idaho Falls, ID, USA.

Lactobacillus buchneri (B-1837) and *Tetragenococcus halophilus* (B-4244) were obtained from the Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA) as freeze-dried cultures. *L. buchneri* was revived in De Man, Rogosa, and Sharpe (MRS) broth and frozen at −80 °C in 20% glycerol MRS media. *T. halophilus* was revived in MRS media supplemented with 5% NaCl and preserved as a frozen stock in MRS-5% NaCl-20% glycerol.

2.2. Wet Anaerobic Storage

Storage experiments were performed in 125 mL reactors. A pictorial diagram of the reactor is provided in the Supplementary Materials. Algal biomass was stored for 30 and 180 days in triplicate at room temperature for each treatment condition and storage duration. For the untreated algal biomass, storage was initiated by filling the reactor 3/4 full to permit material expansion. The reactors were sealed with airtight lids fitted with a through-lid bulkhead tube adapter and a ball valve (P/N B-43S4, Swagelok, Solon, OH, USA). After sealing, the reactors were connected to a pump to evacuate air trapped in the reactors and filled with nitrogen gas to establish an anaerobic atmosphere. Gas collection bags were thereafter fitted to the ball valve with C-flex Ultra tubing (Masterflex, Cole-Parmer, Vernon Hills, IL, USA).

For all the treatment conditions, inoculants were added to an aliquot of biomass and mixed thoroughly prior to being stored in the reactors as described for the untreated biomass. For inoculant treated biomass, 200 µL of 1–2-day(s) cultures were added to ~300 mL of algal biomass at the following optical densities at 600 nm: *L. buchneri*, OD_{600} = 2.25, *T. halophilus*, OD_{600} = 0.46. Tryptone (7 mg tryptone/g wet biomass) was added to each inoculated experiment to serve as source of amino acids to support the growth of lactic acid bacteria. About 50 mL of the inoculant-treated biomass was added to each reactor and was then stored for 30 and 180 days. Lactic acid bacteria are fastidious microorganisms that rely on an external source for some amino acids. Tryptone was included as a precaution to support growth of lactic acid bacteria. Algae biomass is protein-rich and could very likely support the growth of fastidious lactic acid bacteria.

The moisture contents of initial and stored materials were determined gravimetrically by drying representative samples at 105 °C until reaching a constant weight. The mass of the algal biomass samples before and after storage were measured and dry matter loss was estimated as percentage of the initial materials using Equation (1).

$$\text{Dry matter loss (\%)} = (\text{Wi} - \text{Wf})/\text{Wi} \times 100 \quad (1)$$

where Wi and Wf are the initial biomass dry weight (g) before storage and final biomass dry weight (g) after storage.

2.3. Compositional Analysis

The NREL Laboratory Analytical Procedure for determination of total carbohydrates in algal biomass was followed in analyzing the carbohydrate compositions of fresh and stored algae samples [17] Briefly, samples were incubated with 72% (*w*/*w*) sulfuric acid for 1 h at 30 °C with frequent vortexing. Samples were thereafter diluted to 4% acid (*w*/*w*) and autoclaved for 1 h at 121 °C. Cooled samples were thereafter neutralized with calcium carbonate and filtered with a 0.2 µm syringe filter into two separate vials prior to high-performance liquid chromatography (HPLC) analysis.

The HPLC detector was calibrated using a five-component carbohydrate standard at 5 different levels (p/n 13528, Absolute Standards Inc., Hamden, CT, USA) and validated with a five-component check standard (p/n S-16632-R5, Accustandard, Inc., New Haven, CT, USA). Each sample was injected twice into the HPLC, and the analyte peaks from each sample were quantified using standards with matching retention times.

Similarly, NREL Laboratory Analytical Procedure for determination of total lipids as fatty acid methyl ester by in situ transesterification was followed in analyzing the fatty acid methyl ester (FAME) lipid content of the biomass samples [18]. Prior to the analysis, the biomass samples were lyophilized and ground with a mortar and pestle. Protein content of the biomass samples was estimated by multiplying the elemental nitrogen content by a conversion factor of 4.78 [19].

2.4. Organic Acid Determination

Organic acids present in the algae samples were determined by HPLC analysis. Prior to the analysis, the organic acids were extracted from the samples by adding refrigerated nano-pure water to wet algal biomass at a ratio of 10 mL (water) to 1 g (wet biomass) in 50 mL tube. Samples were vortexed and equilibrated overnight, and thereafter an aliquot was removed and filtered through a 0.2 µm syringe filter and acidified with 150 µL volume of 4N H_2SO_4.

Each extract was analyzed in duplicate using HPLC with a refractive index detector (1200 series, Agilent, Santa Clara, CA, USA). Individual organic acids were separated using an Aminex HPX 87H ion exclusion column (P/N 125-0140, Bio-Rad, Hercules, CA, USA). Refractive index detector was calibrated with a ten-component organic acid standard (P/N 95917, Absolute Standards Inc., Hamden, CT, USA) at five different levels. Sample analyte peaks with retention times matching a standard compound were identified and quantified using the calibration curve for the matching compound.

2.5. Energy, Proximate, and Ultimate Analysis

Higher heating values of the algae samples were measured using an isoperibolic bomb calorimetric system (LECO AC600, St. Joseph, MI, USA) following the ASTM D5865/D5865M–19 [20]. Samples were combusted in a combustion vessel under 450 psi of UHP O_2.

Prior to the proximate and ultimate analysis, the algae samples were freeze-dried and then ground into fine particles with a mortar and pestle. Proximate compositions of the algae samples were determined using a thermogravimetric analyzer (TGA 701 LECO, St. Joseph, MI, USA) following a standard protocol (ASTM D7582-15) [21]. ASTM standards D5373-10 [22] and ASTM D4239-10 [23] were followed in analyzing the elemental

compositions of the samples using a LECO TruSpec CHNS addon module (St. Joseph, MI, USA). Oxygen was determined by difference.

2.6. Statistical Analysis

A two-way ANOVA test was conducted on the experimental data using SAS JMP Pro software to test the impact of treatments and storage durations as well as the interaction effects between the two factors at a significant level of $p < 0.05$. The Tukey HSD test was used for the comparison of means and the results were reported as an average ± standard deviation.

3. Results

3.1. Storage Performance

Lactic acid bacteria (LAB) are non-aerobic, aerotolerant bacteria that efficiently ferment a variety of sugars to produce primarily lactic acid. Lactic acid bacteria can be further grouped according to their metabolism. Those that exclusively produce lactic acid are homofermenters, while those that produce both lactic and acetic acids are termed heterofermenters. The organic acids produced by LAB are inhibitory toward other bacteria, such as Clostridia, which has led to their use industrially to preserve food, feed, and forage. To evaluate how LAB inoculation would affect algae preservation, *N. gaditana* biomass was inoculated separately with a homofermentative (*Tetragenococcus halophilus*) strain and a heterofermentative strain (*Lactobacillus buchneri*). *T. halophilus* has the added benefit of being a halotolerant strain [24] that would be less likely to be negatively affected by the salt content of *N. gaditana* biomass.

N. gaditana biomass was stored anaerobically for 30 and 180 days without treatment and after inoculation with either *L. buchneri* or *T. halophilus*. The storage performance data can be found in Table 1. *Lactobacillus buchneri* treated biomass had the lowest dry matter loss of 4.5% (d.b.), which was significantly lower ($p < 0.05$) than the dry matter loss for the untreated and *T. halophilus* treated biomass samples after 30 days of storage. The lower dry matter loss recorded for *L. buchneri* treated biomass in comparison to the *T. halophilus* treated biomass is an indication that the heterofermentative metabolism of *L. buchneri* was more effective at limiting microbial degradation.

Table 1. Stability of *Nannochloropsis gaditana* after 30 and 180 days wet anaerobic storage.

Treatment	Storage Duration (Days)	Dry Matter Loss (% Dry Basis)	pH	Organic Acid (% Dry Basis)
Untreated	30	$6.2^{a} \pm 1.1$	$4.34^{c} \pm 0.10$	$9.8^{ab} \pm 0.7$
Tetragenococcus halophilus		$6.5^{a} \pm 0.3$	$4.60^{b} \pm 0.03$	$9.8^{ab} \pm 0.4$
Lactobacillus buchneri		$4.5^{b} \pm 0.2$	$4.76^{a} \pm 0.01$	$8.3^{b} \pm 0.3$
Untreated	180	$9.3^{a} \pm 0.8$	$4.29^{b} \pm 0.16$	$14.0^{b} \pm 0.4$
Tetragenococcus halophilus		$8.8^{a} \pm 0.5$	$4.29^{b} \pm 0.10$	$16.7^{a} \pm 1.3$
Lactobacillus buchneri		$7.1^{a} \pm 1.6$	$4.77^{a} \pm 0.12$	$13.2^{b} \pm 1.0$

Values in each column with the same superscript are not significantly different ($p < 0.05$).

There was no significant difference ($p < 0.05$) in the dry matter loss recorded for all the algae samples which were anaerobically stored for 180 days, and losses increased only moderately from 30 days. If the same rate of material loss in the first 30 days continued in the next 150 days, losses of 27% could be expected in the *L. buchneri* inoculated biomass. This decreasing rate of degradation has been previously observed in the ensiling of forage crops, where most of the dry matter loss occurs within the first days and weeks of storage [25]. Although inoculation with *L. buchneri* was beneficial for short duration storage, this advantage disappeared with long-term storage, with biomass experiencing the same level of preservation regardless of treatment. This includes the no treatment control, where the stability achieved indicates that sufficient lactic acid bacteria were already present within the algae biomass. This is further supported by the composition of organic acids produced in storage, which will be discussed later. Achieving stabilizing conditions with-

out requiring specialized inoculants will simplify the process of preserving algae biomass at industrial scales.

Dry matter loss is a key performance indicator for evaluating the preservation performance of wet anaerobic storage and primarily occurs through the loss of material as CO_2. The gas composition consisted of primarily CO_2 with trace amounts of hydrogen. While the dry matter loss values after 180 days of storage were significantly higher ($p < 0.05$) than after 30, they did remain less than 10% (d.b.) of the initial biomass, which was an initial objective of the study. Preventing losses from exceeding 10% can be attributed to lactic acid fermentation of soluble sugars to lactic acid and other organic acids [1,14].

The final pH of an ensiled material is dependent on the concentrations of individual organic acids. For example, lactic acid has a pka of 3.8 and therefore ensiled material containing predominantly homofermentative LAB would be expected to have a lower pH than similar biomass containing primarily heterofermentative LAB due to acetic acid's higher pka of 4.8. The final pH values of all the stored algae samples were 4.29–4.77. At both storage durations, the biomass with the higher pH had been inoculated with *L. buchneri*, a heterofermentative organism. This outcome is consistent with the heterofermentative metabolism of *L. buchneri* that yields both lactic and acetic acids. The final pH values are all within the expected range for well-ensiled biomass [26].

Organic acid production and the decrease in pH values function to inhibit microbial activity, leading to well preserved biomass. The data presented here indicate that wet anaerobic storage can successfully preserve algae biomass with a high moisture content (65%, wet basis) for more than six months, the length of storage required to mitigate for seasonal variation in biomass productivity.

Total organic acids produced after 30 and 180 days of storage were 8.3–9.8% and 13.2–16.7% (d.b.), respectively (Table 1). There was a significant increase in the total organic acid produced as the storage duration increased from 30 to 180 days, as well as a shift in their relative concentrations (Figure 1). In both the untreated and the *T. halophilus* inoculated algae biomass samples, lactic acid was the dominant organic acid at both short and long storage durations. However, they differed in the second most abundant organic acid, with isovaleric being prominent in untreated biomass and propionic acid with *T. halophilus* inoculation. *L. buchneri* treated biomass had the lowest lactic acid and the highest acetic acid production for both storage durations, consistent with its heterofermentative metabolism. With continued storage, each storage condition experienced additional lactic acid production. Butyric acid, commonly associated with Clostridia metabolism and poor preservation, appeared to be below 1% for all samples and did not increase with additional storage for the untreated and *T. halophilus* inoculated biomass. For all treatments, lactic, propionic, and isovaleric acids were the predominant organic acids. Acetic acid was only a substantial component of the *L. buchneri* inoculated algae biomass.

Cultivation of algae strains tolerant of water with a wide salinity range is important to reducing the cost of algal biomass production and enhancing its sustainability. Evaporation of water from open ponds increases salinity throughout the day, and a strain tolerant of high dissolved salt (35–50 ppt) permits the use of brackish or saline water to replace water lost to evaporation [27], avoiding costly consumption of freshwater. Prior to this study, the effect of higher salt concentration on the microbial community responsible for establishing conditions for the ensiling of algal biomass was uncertain and poor stability had been observed with saline algae biomass (unpublished results). The outcome of storage stability studies reported here, low dry matter loss, post-storage pH of 4.75 or lower, and effective lactic acid production, indicates the compatibility of this approach to preservation with algal biomass cultivated in saline conditions. The algal biomass for this study was cultivated in closed flat panel reactors where evaporative water loss and contamination were minimized. Additional research is needed to verify that the variable salinity and increased microbial contamination inherent to open raceway ponds do not adversely impact preservation in wet anaerobic storage of saline strains.

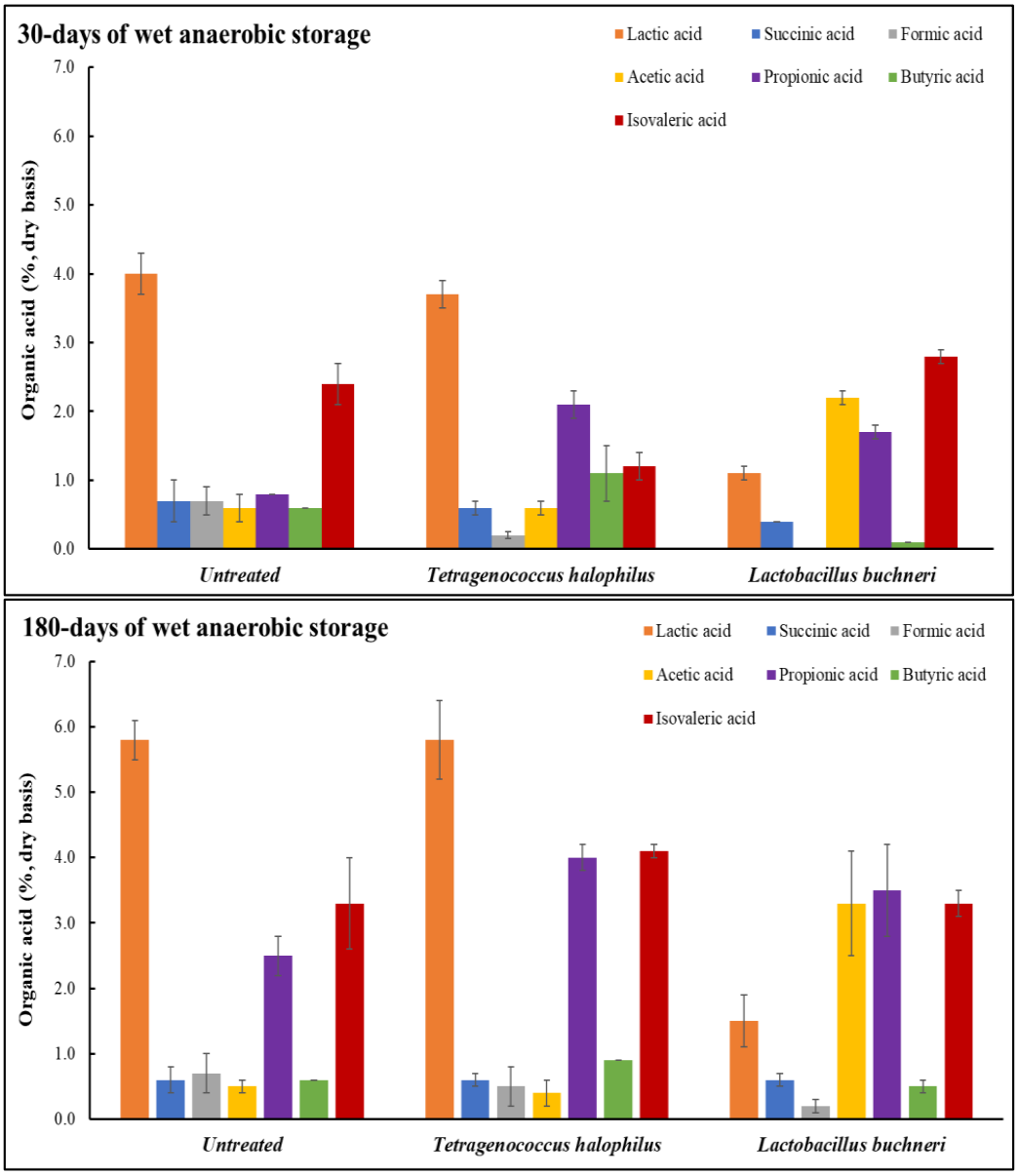

Figure 1. Effect of storage treatment on the organic acid composition of *Nannochloropsis gaditana* biomass after 30 and 180 days of wet anaerobic storage. *Nannochloropsis gaditana* biomass was either stored without treatment (Untreated) or after inoculation with *T. halophilus* or *L. buchneri*.

3.2. Elemental and Proximate Compositions

Table 2 shows the elemental composition, energy, and ash content of the algae samples. The ash content of the unstored biomass was 7.0%. The storage durations had no effect on the ash content of the untreated biomass. However, the ash content slightly increased to ~8% for the biomass samples inoculated with *L. buchneri* and *T. halophilus* during the 30 and 180 days of storage. Wahlen et al. [1] reported a consistent increase in the ash content of wet, anaerobically stored *S. obliquus* blended with corn stover. Wendt et al. [2] also reported an increase in the ash content (3.50–4.36%, d.b.) of *S. acutus* mixed with glycosidase and glucose oxidase, which were anaerobically stored for 30 and 180 days. The high ash content reported in this study can be attributed to the cultivation conditions of *N. gaditana*, which, as a marine strain, has a higher ash content compared to freshwater strains, which is expected due to the much higher dissolved solid content of seawater [16]. It is imperative to note that high ash content in algal biomass, or in any biomass, is an undesirable property. Elevated ash content can adversely impact conversion operations to produce biofuels by reducing

fuel yield, requiring greater throughput due to unconvertible material, and can have an adverse effect on equipment operation (e.g., wear, slagging, scaling, etc.).

Table 2. Elemental and proximate compositions of *Nannochloropsis gaditana* stored for 30 and 180 days.

Treatment	Storage Duration	Ash (% d.b.)	C (% d.b.)	H (% d.b.)	N (% d.b.)	O (% d.b.)	S (% d.b.)	HHV (MJ/kg)
Unstored	0	7.0	57.6 ^{c} ± 0.6	9.1 ^{b} ± 0.1	2.5 ^{b} ± 0.0	23.5 ^{a} ± 0.7	0.31 ^{a} ± 0.02	28.0 ^{ab} ± 0.2
Untreated	30	7.0	58.3 ^{c} ± 0.1	9.2 ^{b} ± 0.0	2.7 ^{b} ± 0.0	22.5 ^{b} ± 0.1	0.35 ^{a} ± 0.01	27.8 ^{bc} ± 0.1
L. buchneri		7.0	58.3 ^{c} ± 0.1	9.0 ^{c} ± 0.1	2.9 ^{ab} ± 0.0	22.5 ^{b} ± 0.1	0.32 ^{a} ± 0.03	28.2 ^{a} ± 0.2
T. halophilus		8.1	58.4 ^{ab} ± 0.1	9.1 ^{b} ± 0.1	3.0 ^{a} ± 0.0	21.1 ^{c} ± 0.2	0.29 ^{a} ± 0.02	27.6 ^{c} ± 0.2
Untreated	180	7.3	59.1 ^{a} ± 0.1	9.3 ^{b} ± 0.0	2.8 ^{b} ± 0.0	21.2 ^{c} ± 0.1	0.32 ^{a} ± 0.04	28.2 ^{a} ± 0.2
L. buchneri		8.4	58.9 ^{a} ± 0.2	9.3 ^{b} ± 0.0	3.0 ^{a} ± 0.0	20.2 ^{d} ± 0.2	0.29 ^{a} ± 0.03	28.5 ^{a} ± 0.2
T. halophilus		7.7	58.9 ^{a} ± 0.3	9.4 ^{a} ± 0.0	3.0 ^{a} ± 0.0	20.6 ^{cd} ± 0.3	0.36 ^{a} ± 0.05	28.5 ^{a} ± 0.3

All values were measured in triplicate and are displayed as a percentage of dry biomass (dry basis, d.b.). Values in each column with the same superscript are not significantly different ($p < 0.05$).

The higher heating values of the algae samples ranged between 27.6–28.5 MJ/kg. There was no significant difference ($p < 0.05$) in the higher heating values of algal biomass stored for 180 days in comparison to the unstored biomass. However, the higher heating value (27.6 MJ/kg) of *T. halophilus* treated biomass after 30 days of storage was significantly lower ($p < 0.05$) than the higher heating value (28.0 MJ/kg) of the unstored biomass. This reduction can be attributed to the higher ash content (8.1% d.b.) of *T. halophilus* treated biomass. The small difference (<1.0 MJ/kg) in the higher heating values of the stored and unstored biomass samples is an indication that the wet anaerobic storage was effective in preserving the algal biomass.

There was a slight increase in the elemental compositions (Carbon, Hydrogen, and Nitrogen) of the algae samples after the storage durations (30 and 180 days) in comparison to the unstored sample. Meanwhile, elemental oxygen content was significantly reduced ($p < 0.05$) for both storage durations and all treatments. This reduction may be attributed to the production of CO_2 occurring during storage due to microbial activity. There was no significant difference ($p < 0.05$) in the sulfur content in any of the algae samples.

Algae biomass has high nitrogen content, which can be problematic for thermochemical conversion approaches such as hydrothermal liquefaction, but is less of a concern for fermentation approaches to fuel production. The high concentration of nitrogen (2.5–3.0%, d.b.) in this biomass is an indication that the utilization of the biofuel derived from this algae feedstock may result in NOx emission or require substantial hydrogenation. However, the carbon (57–59%, d.b.) and hydrogen contents (~9.0%, d.b.) of the algae samples were higher and the oxygen contents (20–23%, d.b.) were lower than the ones reported for lignocellulosic biomass feedstocks such as Miscanthus, Switchgrass, and Arundo [28,29], pinewood and pine bark [30], yard wastes [16], and forest residues [31,32].

3.3. Biochemical Composition

The impact of wet anaerobic storage on the biochemical composition of *N. gaditana* is presented in Table 3. The carbohydrate content of the unstored algae sample was 21.4% (d.b.) and the carbohydrate content reduced significantly ($p < 0.05$) as the storage duration increased from 30 to 180 days. The carbohydrate content of the untreated algae sample decreased from 16.6 to 9.7% (d.b.) with continued storage. Similarly, the total carbohydrate content of *L. buchneri* and *T. halophilus* treated biomass samples decreased from 16.7 to 11.7% and from 13.9 to 9.1% (d.b.), respectively, from 30 to 180 days of storage. This reduction in carbohydrate content with increased storage duration is not ideal and can be attributed to continued fermentation of accessible and non-recalcitrant carbohydrates present in the biomass to organic acids under anaerobic conditions. Ensiling as a method of preservation necessarily leads to some decomposition of biomass in order to produce sufficient organic acids to limit microbial metabolism and minimize degradation [2,9]. Of the treatments evaluated in this study, *L. buchneri* inoculation was the most effective at preservation, as

this biomass experienced the least amount of dry matter loss after 180 days and also had the highest carbohydrate content after storage.

Table 3. Effect of wet anaerobic storage on biochemical composition of *Nannochloropsis gaditana*.

Treatment	Storage Duration	Carbohydrates (%, d.b.)	Protein (%, d.b.)	FAME (%, d.b.)
Unstored	0	21.4 ^{a} ± 0.8	12.1 ^{c} ± 0.2	41.1 ^{c} ± 0.3
Untreated	30	16.6 ^{b} ± 0.1	12.7 ^{c} ± 0.1	45.1 ^{a} ± 0.3
Lactobacillus buchneri		16.7 ^{b} ± 0.2	13.9 ^{b} ± 0.1	40.6 ^{c} ± 0.6
Tetragenococcus halophilus		13.9 ^{c} ± 0.2	14.2 ^{a} ± 0.1	46.1 ^{a} ± 2.1
Untreated	180	9.7 ^{e} ± 0.4	13.3 ^{b} ± 0.1	46.9 ^{a} ± 0.8
Lactobacillus buchneri		11.7 ^{d} ± 0.1	14.3 ^{a} ± 0.1	44.3 ^{b} ± 0.8
Tetragenococcus halophilus		9.1 ^{e} ± 0.1	14.5 ^{a} ± 0.1	44.5 ^{a} ± 2.1

All values were measured in triplicate and are displayed as a percentage of dry biomass (dry basis, d.b.). Values in each column with the same superscript are not significantly different ($p < 0.05$).

The protein content of the algae samples increased in apparent concentration as the storage duration increased from 30 to 180 days. The protein content of the unstored algae was 12.1% (d.b.) and after storage, the untreated algae had protein contents of 12.7% (30 days) and 13.3% (180 days, d.b.). Increases in protein content observed for the treated algae samples were not substantial as the increase was <5% higher than the protein content of the unstored algae sample. A similar increase in protein content was reported for *S. acutus* biomass after storage and various treatments [2]. The conclusion in this study that protein is unaffected by storage is not conclusive and this is due to limitations in the standard method for protein measurement [33]. This method is not a direct measurement of protein but a measurement of nitrogen content. The protein content is then determined by multiplying the nitrogen content by an experimentally determined conversion factor [34]. It is possible for proteins to be degraded without the bulk nitrogen content changing. Future research will explore protein stability through direct measurements of protein content. The apparent increase in protein content can be attributed to the relative increase in nitrogen content during storage. As carbon and oxygen are lost as CO_2 from the consumption of carbohydrates, nitrogen becomes enriched.

The FAME lipid content was influenced by the storage duration, as there was a significant difference ($p < 0.05$) between the lipid content of unstored algae and the algae samples stored for 30 and 180 days, except in the case of *L. buchneri* inoculated biomass after 30 days of storage. However, there was no significant difference ($p < 0.05$) between the lipid contents of the algae samples stored for 30 and 180 days, except for the *L. buchneri* treated biomass, in which its lipid content increased from 40.6 to 44.3% (d.b.) as the storage duration increased from 30 to 180 days. The relative increase in the FAME lipid content is attributed to changes in the carbohydrate fraction of the algae samples during storage. Degradation of any one of the three biomass macromolecules (carbohydrate, lipid, and protein) during storage can cause the others to increase in proportion to the total. Changes in biochemical composition could affect the value of the algal biomass and influence the distribution of the biofuel and bioproducts obtained from the biomass [1].

3.4. Carbohydrate Composition

Storage has the greatest effect on carbohydrate composition (Table 4), with glucose being affected to a greater extent than other sugar monomers. Glucose (4.2–15.2%, d.b.) and galactose (3.8–4.5%, d.b.) were the primary sugar monomers identified in *N. gaditana* biomass, while xylose (0.6–0.8%, d.b.), galactose (3.8–4.5%, d.b.), arabinose (0.2–0.4%, d.b.), and mannose (0.6–0.9%, d.b.) were identified as minor sugar monomers. A comparison of the sugar monomers of the algae samples after 30 and 180 days of storage, demonstrated that glucose content was significantly reduced from its initial value of 15.2% (d.b.), reaching values of 10.4% (d.b.) and 4.2% (d.b.) for untreated algal biomass stored anaerobically for

30 and 180 days, respectively. A similar reduction in glucose content was observed for all other treatments as well. Surprisingly, there were no significant changes in the contents of other carbohydrate monomers. Given that glucose constitutes about 40–71% of the total carbohydrate content of any of the algae samples, it can be inferred that the wet anaerobic storage had a significant effect on the carbohydrate composition of *N. gaditana* biomass.

Table 4. Effect of wet anaerobic storage on carbohydrate composition of *Nannochloropsis gaditana*.

Treatment	Storage Days	Glucose (%, d.b.)	Xylose (%, d.b.)	Galactose (%, d.b.)	Arabinose (%, d.b.)	Mannose (%, d.b.)
Unstored	0	15.2 ^{a} ± 0.6	0.63 ^{c} ± 0.04	4.68 ^{a} ± 0.25	0.39 ^{ab} ± 0.02	0.69 ^{a} ± 0.06
Untreated	30	10.4 ^{c} ± 0.03	0.70 ^{b} ± 0.01	4.46 ^{ab} ± 0.09	0.40 ^{ab} ± 0.02	0.77 ^{a} ± 0.04
L. buchneri		10.8 ^{b} ± 0.05	0.66 ^{bc} ± 0.01	4.17 ^{c} ± 0.05	0.40 ^{ab} ± 0.04	0.64 ^{a} ± 0.04
T. halophilus		8.5 ^{e} ± 0.02	0.61 ^{c} ± 0.02	3.84 ^{de} ± 0.05	0.32 ^{b} ± 0.10	0.64 ^{a} ± 0.1
Untreated	180	4.2 ^{g} ± 0.1	0.63 ^{bc} ± 0.03	3.80 ^{e} ± 0.05	0.40 ^{ab} ± 0.04	0.74 ^{a} ± 0.1
L. buchneri		5.5 ^{f} ± 0.03	0.83 ^{a} ± 0.02	4.04 ^{cd} ± 0.09	0.52 ^{a} ± 0.06	0.76 ^{a} ± 0.01
T. halophilus		3.6 ^{h} ± 0.04	0.65 ^{bc} ± 0.02	3.92 ^{de} ± 0.01	0.31 ^{b} ± 0.03	0.64 ^{a} ± 0.04

All values were measured in triplicate and are displayed as a percentage of dry biomass (dry basis, d.b.). Values in each column with the same superscript are not significantly different ($p < 0.05$).

Wendt et al. [2] reported a similar change in carbohydrate composition for the freshwater strain *S. acutus* treated with sulfuric acid, glycosidase, and glucose oxidase. The authors reported a reduction in the glucose, galactose, and mannose content of the enzyme-treated biomass. This reduction was attributed to the conversion of complex carbohydrates into simple sugars that were then easily utilized as a carbon source. Wahlen et al. [1], however, reported glucose and xylose as the major carbohydrates (constituting about 90–93% of the total sugars) while galactose, arabinose, and mannose were the minor carbohydrate components found in a *S. obliquus* algae biomass blended with corn stover.

4. Conclusions

This study has demonstrated that wet anaerobic storage of *Nannochloropsis gaditana* algal biomass is a promising preservation approach to ensure a continuous supply of the algal biomass to biorefineries. Dry matter loss in long-term storage (180 days) of less than 10% (d.b.) for all treatment conditions and post-storage pH values measuring less than 4.8 indicates that wet anaerobic storage is an effective means of preserving saline microalgal biomass. The minimal change in the higher heating values, reduction of oxygen content, and preservation of lipids indicate that the wet anaerobic approach to preserving algal biomass will preserve feedstock quality for high temperature conversion approaches, such as hydrothermal liquefaction. The loss of carbohydrates in storage could impact fuel yields through biochemical approaches to conversion. The loss in yield could partly be recovered by the value of organic acids that are produced in well-ensiled algal biomass. Utilizing this preservation technique will not only stabilize the dry matter and biochemical composition of saline algae strains, but it will be a cost-effective preservation approach which will help to mitigate the seasonal variation in the productivity of algal biomass feedstocks.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8040159/s1, Figure S1: Pictorial diagram of the storage reactors.

Author Contributions: Conceptualization, B.W. and L.W.; Formal analysis, O.O., B.W. and L.W.; Funding acquisition, B.W. and L.W.; Investigation, O.O., B.W., L.W. and M.W.; Methodology, B.W., L.W. and M.W.; Project administration, B.W. and L.W.; Resources, T.D. and H.G.; Supervision, B.W. and L.W.; Visualization, O.O.; Writing—original draft, O.O., B.W. and L.W.; Writing—review and editing, O.O. and B.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the U.S. Department of Energy (DOE), Office of Energy Efficiency and Renewable Energy (EERE), Bioenergy Technologies Office (BETO), under Idaho National Laboratory Award No. DE-AC07-05ID14517. The views expressed in the article do not

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors acknowledge Kastli Schaller and Bradley Thomas at the Idaho National Laboratory for analytical support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, collection, analyses, or interpretation of data, writing of the manuscript, or in the decision to publish the results.

References

1. Wahlen, B.D.; Wendt, L.M.; Murphy, J.A.; Seibel, F. Mitigation of variable seasonal productivity in algae biomass through blending and ensiling: An assessment of compositional changes in storage. *Algal Res.* **2019**, *42*, 101584. [CrossRef]
2. Wendt, L.M.; Kinchin, C.; Wahlen, B.D.; Davis, R.; Dempster, T.A.; Gerken, H. Assessing the stability and techno-economic implications for wet storage of harvested microalgae to manage seasonal variability. *Biotechnol. Biofuels* **2019**, *12*, 1–14. [CrossRef] [PubMed]
3. Langholtz, M.H.; Stokes, B.J.; Eaton, L.M. *2016 Billion-Ton Report: Advancing Domestic Resources for a Thriving Bioeconomy, Volume 1: Economic Availability of Feedstocks*; Oak Ridge National Laboratory: Oak Ridge, TN, USA, 2016; 448p.
4. Shakya, R.; Adhikari, S.; Mahadevan, R.; Shanmugam, S.R.; Nam, H.; Hassan, E.B.; Dempster, T.A. Influence of biochemical composition during hydrothermal liquefaction of algae on product yields and fuel properties. *Bioresour. Technol.* **2017**, *243*, 1112–1120. [CrossRef] [PubMed]
5. Dong, T.; Knoshaug, E.P.; Davis, R.; Laurens, L.M.L.; Van Wychen, S.; Pienkos, P.T.; Nagle, N. Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts. *Algal Res.* **2016**, *19*, 316–323. [CrossRef]
6. Li, Y.; Leow, S.; Dong, T.; Nagle, N.J.; Knoshaug, E.P.; Laurens, L.M.L.; Pienkos, P.T.; Guest, J.S.; Strathmann, T.J. Demonstration and Evaluation of Hybrid Microalgae Aqueous Conversion Systems for Biofuel Production. *ACS Sustain. Chem. Eng.* **2019**, *7*, 5835–5844. [CrossRef]
7. Knoshaug, E.P.; Mohagheghi, A.; Nagle, N.J.; Stickel, J.J.; Dong, T.; Karp, E.M.; Kruger, J.S.; Brandner, D.G.; Manker, L.P.; Rorrer, N.A.; et al. Demonstration of parallel algal processing: Production of renewable diesel blendstock and a high-value chemical intermediate. *Green Chem.* **2018**, *20*, 457–468. [CrossRef]
8. Wahlen, B.D.; Roni, M.S.; Cafferty, K.G.; Wendt, L.M.; Westover, T.L.; Stevens, D.M.; Newby, D.T. Managing variability in algal biomass production through drying and stabilization of feedstock blends. *Algal Res.* **2017**, *24*, 9–18. [CrossRef]
9. Wendt, L.M.; Wahlen, B.D.; Li, C.; Kachurin, G.; Ogden, K.L.; Murphy, J.A. Evaluation of a high-moisture stabilization strategy for harvested microalgae blended with herbaceous biomass: Part I—Storage performance. *Algal Res.* **2017**, *25*, 567–575. [CrossRef]
10. Davis, R.; Kinchin, C.; Markham, J.; Tan, E.; Laurens, L.; Sexton, D.; Knorr, D.; Schoen, P.; Lukas, J. *Process Design and Economics for the Conversion of Algal Biomass to Biofuels: Algal Biomass Fractionation to Lipid- and Carbohydrate-Derived Fuel Products*; NREL/TP-5100-62368 United States 10.2172/1159351 NREL English; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2014; 110p.
11. Nelson, J.A. *Postharvest Degradation of Microalgae: Effect of Temperature and Water Activity*; Utah State University: Logan, UT, USA, 2015. [CrossRef]
12. Aliyu, A.; Lee, J.G.M.; Harvey, A.P. Microalgae for biofuels via thermochemical conversion processes: A review of cultivation, harvesting and drying processes, and the associated opportunities for integrated production. *Bioresour. Technol. Rep.* **2021**, *14*, 100676. [CrossRef]
13. Pereira, A.P.; Dong, T.; Knoshaug, E.P.; Nagle, N.; Spiller, R.; Panczak, B.; Chuck, C.J.; Pienkos, P.T. An alternative biorefinery approach to address microalgal seasonality: Blending with spent coffee grounds. *Sustain. Energy Fuels* **2020**, *4*, 3400–3408. [CrossRef]
14. Borreani, G.; Tabacco, E.; Schmidt, R.J.; Holmes, B.J.; Muck, R.E. Silage review: Factors affecting dry matter and quality losses in silages. *J. Dairy Sci.* **2018**, *101*, 3952–3979. [CrossRef] [PubMed]
15. Wendt, L.M.; Wahlen, B.D.; Knoshaug, E.P.; Nagle, N.J.; Dong, T.; Spiller, R.; Panczak, B.; Van Wychen, S.; Dempster, T.A.; Gerken, H.; et al. Anaerobic Storage and Conversion of Microalgal Biomass to Manage Seasonal Variation in Cultivation. *ACS Sustain. Chem. Eng.* **2020**, *8*, 13310–13317. [CrossRef]
16. Wahlen, B.D.; Wendt, L.M.; Murphy, A.; Thompson, V.S.; Hartley, D.S.; Dempster, T.; Gerken, H. Preservation of Microalgae, Lignocellulosic Biomass Blends by Ensiling to Enable Consistent Year-Round Feedstock Supply for Thermochemical Conversion to Biofuels. *Front. Bioeng. Biotech.* **2020**, *8*, 316. [CrossRef] [PubMed]
17. Wychen, S.V.; Laurens, L. *Determination of Total Carbohydrates in Algal Biomass: Laboratory Analytical Procedure (LAP)*; No. NREL/TP-5100-60957; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2016.

18. Van Wychen, S.; Ramirez, K.; Laurens, L.M.L. *Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by In Situ Transesterification: Laboratory Analytical Procedure (LAP)*; NREL/TP-5100-60958 United States 10.2172/1118085 NREL English; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2016.
19. Lourenço, S.O.; Barbarino, E.; Lavín, P.L.; Lanfer Marquez, U.M.; Aidar, E. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. *Eur. J. Phycol.* **2004**, *39*, 17–32. [CrossRef]
20. *ASTM D5865/D5865M-19*; Standard Test Method for Gross Calorific Value of Coal and Coke. ASTM International: West Conshohocken, PA, USA, 2019.
21. *ASTM D7582-15*; Standard Test Methods for Proximate Analysis of Coal and Coke by Macro Thermogravimetric Analysis. ASTM International: West Conshohocken, PA, USA, 2015.
22. *ASTM D5373-10*; Standard Test Methods for Determination of Carbon, Hydrogen and Nitrogen in Analysis Samples of Coal and Carbon in Analysis Samples of Coal and Coke. ASTM International: West Conshohocken, PA, USA, 2010.
23. *ASTM D4239-10*; Standard Test Method for Sulfur in the Analysis Sample of Coal and Coke Using High-Temperature Tube Furnace Combustion. ASTM International: West Conshohocken, PA, USA, 2010.
24. Kumazawa, T.; Nishimura, A.; Asai, N.; Adachi, T. Isolation of immune-regulatory *Tetragenococcus halophilus* from miso. *PLoS ONE* **2018**, *13*, e0208821. [CrossRef] [PubMed]
25. McDonald, P.; Henderson, N.; Heron, S. *The Biochemistry of Silage*, 2nd ed.; Cambrian Printers, Ltd.: Aberystwyth, UK, 1981. [CrossRef]
26. Weinberg, Z.G.; Muck, R.E. New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol. Rev.* **1996**, *19*, 53–68. [CrossRef]
27. Krishnan, A.; Likhogrud, M.; Cano, M.; Edmundson, S.; Melanson, J.B.; Huesemann, M.; McGowen, J.; Weissman, J.C.; Posewitz, M.C. *Picochlorum celeri* as a model system for robust outdoor algal growth in seawater. *Sci. Rep.* **2021**, *11*, 11649. [CrossRef]
28. Oginni, O.; Singh, K.; Zondlo, J.W. Pyrolysis of dedicated bioenergy crops grown on reclaimed mine land in West Virginia. *J. Anal. Appl. Pyrolysis* **2017**, *123*, 319–329. [CrossRef]
29. Oginni, O.; Singh, K. Pyrolysis characteristics of *Arundo donax* harvested from a reclaimed mine land. *Ind. Crops Prod.* **2019**, *133*, 44–53. [CrossRef]
30. Ren, X.; Meng, J.; Chang, J.; Kelley, S.S.; Jameel, H.; Park, S. Effect of blending ratio of loblolly pine wood and bark on the properties of pyrolysis bio-oils. *Fuel Process. Technol.* **2017**, *167*, 43–49. [CrossRef]
31. Oginni, O.; Singh, K. Effect of carbonization temperature on fuel and caffeine adsorption characteristics of white pine and Norway spruce needle derived biochars. *Ind. Crops Prod.* **2021**, *162*, 113261. [CrossRef]
32. Oasmaa, A.; Solantausta, Y.; Arpiainen, V.; Kuoppala, E.; Sipilä, K. Fast Pyrolysis Bio-Oils from Wood and Agricultural Residues. *Energy Fuels* **2010**, *24*, 1380–1388. [CrossRef]
33. Laurens, L.M.L. Summative Mass Analysis of Algal Biomass—Integration of Analytical Procedures. *NREL* **2013**, *303*, 275–3000.
34. Templeton, D.W.; Laurens, L.M.L. Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed, and fuel. *Algal Res.* **2015**, *11*, 359–367. [CrossRef]