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Effect of pH on *Schizochytrium limacinum* Production Grown Using Crude Glycerol and Biogas Digestate Effluent

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Abstract: The ability of microalgae, such as the rich in docosahexaenoic acid (DHA) heterotrophic marine microalgae *Schizochytrium limacinum* SR21, to utilize nutrients in their culture media derived from low-cost nutrient sources makes them a promising low-cost alternative source for the production of useful substances used in aqua feeds. The assessment of culture parameters, one of which is the pH, for every different nutrient growth medium used for microalgae cultivation is important, as they affect the biomass and secondary metabolite microalgae production. This study assessed the effect of different growth medium pH levels (6, 7, 8 and 9), at laboratory and pilot scale systems, on *S. limacinum* biomass productivity, lipid accumulation, proximate composition, carbon assimilation and DHA. The microalgae were cultivated in growth media containing two different alternative low-cost nutrient sources: (a) crude glycerol derived from biofuel industry as carbon source and (b) effluent digestate from biogas production of livestock decomposition as a source of nutrients and trace elements. It was found that a neutral pH (7) was the optimum level, as it enhanced biomass productivity of the lab and pilot scale cultivation systems at 44.9 g L^{-1} and 11 g L^{-1} and DHA content at 7.5% and 19% of the total lipid content, respectively.

Keywords: microalgae; docosahexaenoic acid; greenhouse production; aqua feed; sustainability; waste management; crude glycerol; effluent digestate

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1. Introduction

Industrialization of agriculture food production led to an abundance of agri-waste streams derived from conventional agriculture practices, in order to withstand the increasing global population and food scarcity [1]. These include crop residues from arable farming, food waste and manure from livestock farming [2] with the latter posing a great impact on environmental pollution. Improper management of livestock manure is responsible for nutrient leeching and pathogen contamination of the ground water [3]. Europe generates annually approximately 1500 million tons of animal manure [3], with pig manure management alone being responsible for 18% of the total greenhouse gas emissions derived from global livestock farming [4]. A potential solution in order to alleviate these environmental threats is the implementation of remediation practices.

Anaerobic digestion or co-digestion of livestock manure with agriculture crop residues is a process where microbial decomposition of organic matter produces biogas in order to generate electricity. Byproduct from this process is the decomposed organic matter or digestate which can be further utilized as crop fertilizers due to its high nutritional value, since it is rich in nitrogen [5], phosphorous [6], potassium [7], amino acids [8] and trace elements [9].

An alternative nutrient removal strategy, phycoremediation, is a process were the digestate is utilized as a source of organic nutrients in microalgae cultures [10]. Several researchers have successfully implemented this technology in order to reduce, to a substantial amount, ammonia and phosphate levels in digestates derived from different manure types. These include cattle manure [11], poultry litter [12], piggery manure [13] and waste water [14]. However, converting digestates into value added products can increase valorization, providing alternative routes of exploitation.

Microalgae serve as a promising green feedstock for the production of biodiesel in the biofuel industry due to their high content in triglycerides [15]. One of the by-products of the production of biodiesel is crude glycerol. Although it has applications in the pharmaceutical and cosmetic industry, its purification cost is high due the impurities [16]. Its low economical value in conjunction with the high production rate, since for each ton of biodiesel produced, 100 kg of crude glycerol is generated [17], have led researchers to utilize the particular waste stream as an alternative organic carbon source for microbial and microalgae fermentation [18]. Other high-value products include health food supplements (antioxidants, vitamins and food colorants) [19,20], sustainable protein sources for animal farming and aquaculture [21] and n–3 polyunsaturated fatty acids (PUFA) [22], with the latter generating interest in the pharmaceutical and aqua feed industry.

Schizochytrium sp. is a genus of non-photosynthetic unicellular eukaryotes in the family of *Thraustochytriaceae* [23] that synthesizes the highest yield of docosahexaenoic acid (DHA). The strain *Schizochytrium limacinum* SR21 accumulates up to 50% of its dry weight in lipids, yielding 35% DHA of its total lipid content in short production cycles [24]. DHA is an important and critical nutrient in feeds for the aquaculture industry, obtained primarily from conventional sources, such as fish oil, derived mainly from wild fish stocks that in some cases are not harvested with sustainable practices [25,26]. Therefore, *Schizochytrium* sp. is now considered as an alternative and sustainable source of DHA for replacing fish oil in aqua feeds [27], while it is considered safe in terms of toxicological hazard [28].

According to previous research, the DHA yield is closely related to the hydrogen ion concentration during the fermentation process [29]. Therefore, optimizing abiotic factors, such as the pH, is important, since it affects the growth, development and metabolic pathways of microalgae [18]. Proper regulation of the pH is a key parameter in microbial fermentation, considering that hydrogen ion concentration influences: (a) nutrient availability for proper biosynthesis, function and structure of different macromolecules (carbohydrates, lipids and proteins) [30], (b) enzyme activity, which is related to the production of secondary metabolites [31,32], (c) chemical reaction kinetics [33] and (d) intracellular pH, which is related to the production rate of adenosine triphosphate (ATP) during cellular respiration [34,35]. Previous research indicates that optimal hydrogen ion concentration is an important parameter to consider for proper growth and development of oleo genus algae, such as *Schizochytrium* sp. [36–38], while a hydrogen ion concentration beyond the optimal value significantly reduces biomass and lipid accumulation, ranging from 13–60% and 10–65%, respectively [28,39,40].

In our previous research, we successfully cultivated the strain *S. limacinum* SR21, utilizing crude glycerol as an alternative carbon source and effluent digestate as a source of nutrients and trace elements [36]. In this present study, we assess the effect of varying pH levels (6, 7, 8 and 9) of growth media containing crude glycerol and effluent digestate as main nutrient sources, on *S. limacinum* proximate composition, carbon assimilation and DHA content, at two batch experiments, conducted (1) at lab scale with aeriated shake flasks and (2) at pilot scale with open pond type reactors.

2. Materials and Methods

2.1. Microorganism, Activation and Seed Cultures

The strain SR21 of the marine heterotrophic microalgae *Schizochytrium limacinum* (*Aurantiochytrium limacinum* SR21, ATCC ® MYA–1381™, Washington D.C., WA, USA) was obtained from the American Type Culture Collection (ATCC). Activation of cells in order to

develop the seed inoculum was performed according to the ATCC protocol and described in detail in [36].

2.2. Growth Media Composition and Culture Conditions at Lab and Pilot Scale Experiments

In the present work, crude glycerol derived from the biofuel industry Ellin Verd (Volos, Greece) and effluent of digastate from biogas production of livestock decomposition from the biogass industry Seitis Bros Biogas S.A (Argyropouli, Greece), were used as main nutrient sources, crude glycerol as carbon and biogas digestate as nitrogen and trace elements sources.

The elemental composition of crude glycerol, which was used in order to replace conventional carbon sources, had a purity of 80.4% w/w, containing 0.7% w/w methanol and 11.9% w/w moisture. The composition of pre-treated waste effluent (in mg L^{-1}) was total N (161), P (91), Ca (1000), Mg (260), S (330), Cu (5.6), Co (30), Zn (12), Mn (11) and Fe (110). The physical characteristics were total solids (2.2 w/w), conductivity (15.05 mS cm L^{-1}), optical density at 660 nm (0.7), pH (7.7), ash (1 w/w), moisture (97.8 w/w) and COD (1 g L^{-1}). Pre-treatment and characterization methods with regards to effluent digestate and crude glycerol are described in detail in [19].

2.2.1. Experiment at Lab Scale in Aerated Shake Flasks

In the experiment at lab scale, the microorganisms were cultivated in four different initial growth media pH levels: 6, 7, 8 and 9. The experiment was carried out in 500 mL shake flasks (DURAN ® GLS 80 ® Laboratory Bottle Wide Mouth) with 400 mL working volume containing 10% v/v inoculum of the seed cultures. The culture media contained 48% v/v pretreated effluent digestate, 120 g L^{-1} crude glycerol and 12.5 g L^{-1} NH4CL mixed in ASW containing 20 g L^{-1} sea salts, optimum concentrations of effluent, crude glycerol and NH_4CL, which in our previous research [19] were standardized to promote cell proliferation and PUFA synthesis.

Cultures were incubated in a growth chamber at 25 °C for 7 days on an orbital shaker set at 120 rpm. Ionic concentration was constantly corrected with 2M KOH. Oxygen was sparged into the medium with compressed air at a rate of 150 L h^{-1}. During the experiment, the dissolved oxygen (DO) level was maintained at 50% of saturation by regulating the oxygen supply. Prior to inoculation, the pH of the medium was adjusted to designated target values with 2M KOH or 1N HCL and autoclaved at 121 °C for 15 min. The experiment was carried out for 7 days.

2.2.2. Experiment at Pilot Scale in Open Pond Type Reactors

In the experiment at pilot scale, microorganisms were cultivated in square-shaped closed pond systems with sides of 3 m and a height of 0.5 m. The depth was fixed at 0.1 m in order to achieve a working volume of 1000 L. The interior of the ponds was covered with single use retractable PVC waterproof geomebranes which covered the heating system of the ponds, a radiant floor heating system constituted of 25 mm diameter PVC heating pipe network installed across the bottom. The radiant heating system was connected to an air-to-water heat exchanger (BCOOL LTD, model BAHW 05 HD, cooling capacity: 14.6 kW, heating capacity: 17.0 kW). Temperature was controlled and monitored via temperature probes (01CT–1LH, Belimo, Wien, Austria) immersed inside the ponds. Oxygen was sparged into the medium with a side channel blower (ASC0140–2ST161, AIRTECH Europe GmbH, Schweinfurt, Germany) connected to a network of 16 mm width perforated PVC pipes installed at the bottom of the ponds. Dissolved oxygen (DO) level was maintained at 50% of saturation by regulating the air supply. Mixing of the nutrient solution was performed with a vertical axial agitator (T63A6, Viborg Electro, Viborg, Germany) operating at 50 rpm. The pH was regulated with 2M KOH. Correction and monitoring of the pH was performed via a custom made controller coupled with industrial grade pH probes (GE 100, GREISNGER, Regenstauf, Germany). The seed culture, in order to inoculate the ponds, was cultivated in a 600 L airlift vortex fermentor with a 400 L working volume.

The seed culture medium contained 12 g L^{-1} NH_4CL, 120 g L^{-1} crude glycerol, 1.5 g L^{-1} KH_2PO_3, 0.7 g L^{-1} $MgSO_4$ $7H_2O$ and 5 mL L^{-1} micronutrients. All ingredients were mixed in ASW containing 20 g L^{-1} sea salts. Seed cultures were inoculated with 10% v/v inoculum, whereas media formulation and culture parameters were the same as described in experiment 1. In experiment 2, all culture media prior to inoculation were sterilized with the use of a commercial UV sterilizer (P2–110 W UV Sterilizer, Tropical Marine Centre Ltd., Hertfordshire, UK) for 24 h. Cultures were grown at growth media pH 6, 7, 8 and 9.

The culture media in pilot scale experiments were regulated at 120 g L^{-1} crude glycerol 12.5% v/v pretreated effluent digestate containing 1.44 g L^{-1} NH_4^+ mixed in ASW containing 20 g L^{-1} sea salts. NH_4CL was not added to the culture, since the excess nitrogen content of the lab culture media resulted in high biomass productivity in expense to lipid productivity. Moreover, the sterilization process at pilot scale was altered with the use of a UV sterilizer that kept the $NH4^+$ ions, since in our previous research, the application of heat in autoclave sterilization in order to sterilize the culture medium, which contains NH_4CL, led to ammonia volatilization and loss of nitrogen to the environment [20].

The regulation to use 12.5% v/v of effluent instead of 50% v/v was based on the fact that at preliminary experiments previously conducted at pilot scale, the addition of 50% w/w effluent concentration that was necessary to achieve optimal biomass productivity in conjunction with high aeriation rate, resulted in excess foam formation, while minimal foam formation was achieved when the concentration of effluent was reduced to 12.5% v/v. That is a frequent problem which is encountered during the fermentation process that results in reduced biomass productivity, reduced oxygen content and evidently contamination, leading to a collapse of the cell culture. A common practice to counter foam production is the use of chemical antifoam agents, increased mechanical agitation and ultrasonic foam disruption [21,22], a fact that was manageable at lab scale level with the addition of 1% v/v antifoam agents; however, this was not feasible at the pilot scale phase.

2.3. Measurements of Algal Biomass and Cell Content

Biomass productivity was determined gravimetrically by cell dry weight, total lipids were extracted and analyzed according to [23] and fatty acids were prepared by acid catalyzed transesterification according to [24], purified by thin layer chromatography (TLC) and separated and quantified by glass-liquid chromatography (GLC). All the previously mentioned methods and proximate composition measurements are described in detail in [19]. Carbon and nitrogen assimilation were determined measuring the TOC and $NH4^+$ with the photometric method (HACH device) of the growth medium every 12 h.

2.4. Statistical Analysis

The experiment was carried out with a total randomized design, with four different treatments and three repetitions per treatment. Mean differences between treatments were assessed by one-way ANOVAs, to examine the interaction between hydrogen ion concentration, biomass productivity, crude proteins, total lipids, ash content, crude carbohydrates and calories. The difference between each group was tested using the least significant difference (LSD) from the (Turkey-HSD) test. Prior to the ANOVA, the homogeneity of the variances and the normality of the residues were tested using the Fligner-Killeen test and Shapiro-Wilk test, respectively. In all statistical tests, the level of significance was set at $p = 0.05$. Experimental error was quantified according to standard deviation. Data were analyzed with R software for statistical analysis (R Foundation for Statistical).

3. Results

3.1. Biomass Productivity

The use of pre-treated effluent at different pH levels had a pronounced effect on biomass productivity of *S. limacinum*. As shown in Figure 1, the particular microalgae species has the ability to grow at a wide range of pH Levels. At lab scale, the highest ($p < 0.05$) final biomass productivity was 44.9 $\pm$ 3.6 g L^{-1} when the pH was set at 7. Higher

pH levels (8 and 9) resulted in approximately 49% lower biomass productivity, namely, 11.6 ± 0.7 g L^{-1} and 11.7 ± 1.5 g, respectively (Figure 1a). The same trend was observed at pilot scale when the pH level was set at 7. Biomass productivity was maximized at 120 h, reaching a cell dry weight of 11 ± 0.5 g L^{-1}, whereas at higher pH values (8 and 9) cell proliferation showed a plateau that was lower ($p < 0.05$), i.e., approximately 40–50% compared to that observed at pH 7 (Figure 1b). At pH 6, the produced final dry biomass (g L^{-1}) of *S. limacinum* was significantly higher than values obtained at pH 8 and 9, but significantly lower than that produced at pH 7.

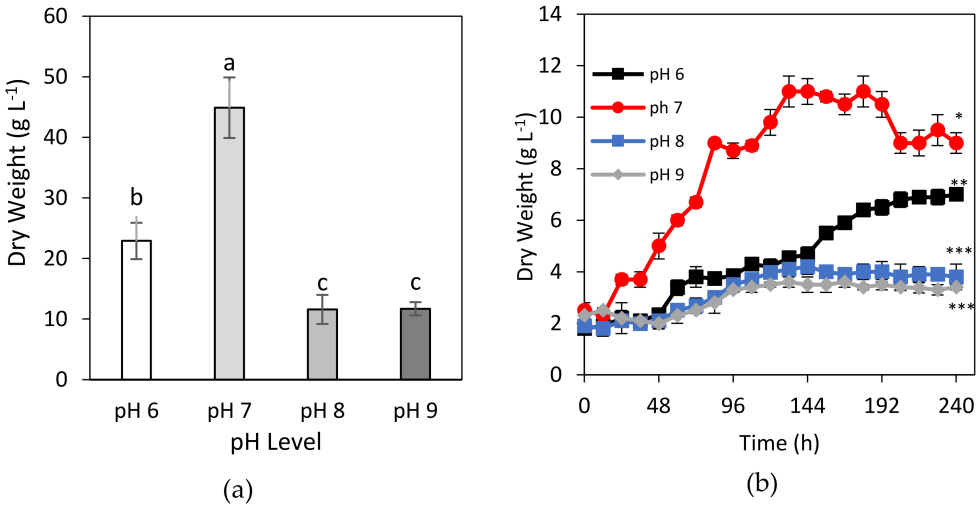

Figure 1. Dried biomass productivity of *S. limacinum* grown at varying pH levels (6, 7, 8 and 9) in growth media containing (**a**) 48% (*v*/*v*) effluent digestate and crude glycerol after 7 days of cultivation at lab scale and (**b**) 12.5% (*v*/*v*) effluent digestate and crude glycerol after 10 days (240 h) of cultivation at pilot scale. Values are represented as mean ± standard deviation of triplicates. Letters (a, b and c) indicate statistical differences analyzed at a level of $p < 0.05$, while asterisks indicate significant differences for $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

3.2. Total Lipids and DHA Yield

Lipid accumulation expressed in terms of total lipid content differed with respect to varying levels of pH at lab and pilot scale. At lab scale, the highest lipid productivity was achieved at pH 7 with a total lipid content of 7.76 ± 1 g L^{-1}, whereas elevated pH (8 and 9) levels resulted in a considerable decline of the total lipid content, 3.24 ± 0.5 g L^{-1} and 3.68 ± 0.8 g L^{-1}, respectively (Figure 2a). The same trend was observed with respect to DHA yield, since the cultivation of the microorganisms at neutral pH levels yielded 582 ± 43 mg L^{-1}, while at elevated hydrogen ion concentrations (pH 9), DHA was not detectable (Figure 2b). Lipid productivity increased gradually during the fermentation process at all pH levels when cells were cultured at the open pond reactors. However, lipid production was maximized ($p < 0.05$) at the end of the experiment when the microorganisms were cultivated at pH 7, yielding 980 mg L^{-1} (Figure 2c). A similar trend was observed with regards to their DHA content. Specifically, the highest ($p < 0.05$) DHA content was observed at the end of the fermentation process at pH 7, yielding 186.7 mg L^{-1} (Figure 2d), while hydrogen ion concentrations other than 7 resulted in poor DHA contents, ranging from 1.5 mg L^{-1} ± 0.15 to 36.2 mg L^{-1} ± 0.5 at the end of the experiment (Figure 2d).

3.3. Carbon and Nitrogen Assimilation

At lab scale, the total carbon content of the growth media was reduced significantly at approximately 36% when the pH was set at 7, indicating a higher carbon assimilation from the microorganisms, while at higher pH levels, glycerol was assimilated to a lesser extend ranging from 13.6–16.2% (Figure 3a). At pilot scale, the highest carbon assimilation was observed when cells were grown at pH 7, which resulted in a total carbon content reduction

of 54% from the initial concentration at the end of the experiment (Figure 3b). With regards to the nitrogen source, ammonia content at laboratory conditions was significantly reduced at all pH levels. However, the lowest ammonia content was observed when cells were grown at pH 8 and 9 (Figure 3d). At pilot scale, ammonia was completely depleted from the cell culture media at all pH levels. However, when the pH of the media was set at 9, ammonia concentration declined rapidly until depletion at hour 108 h (Figure 3d).

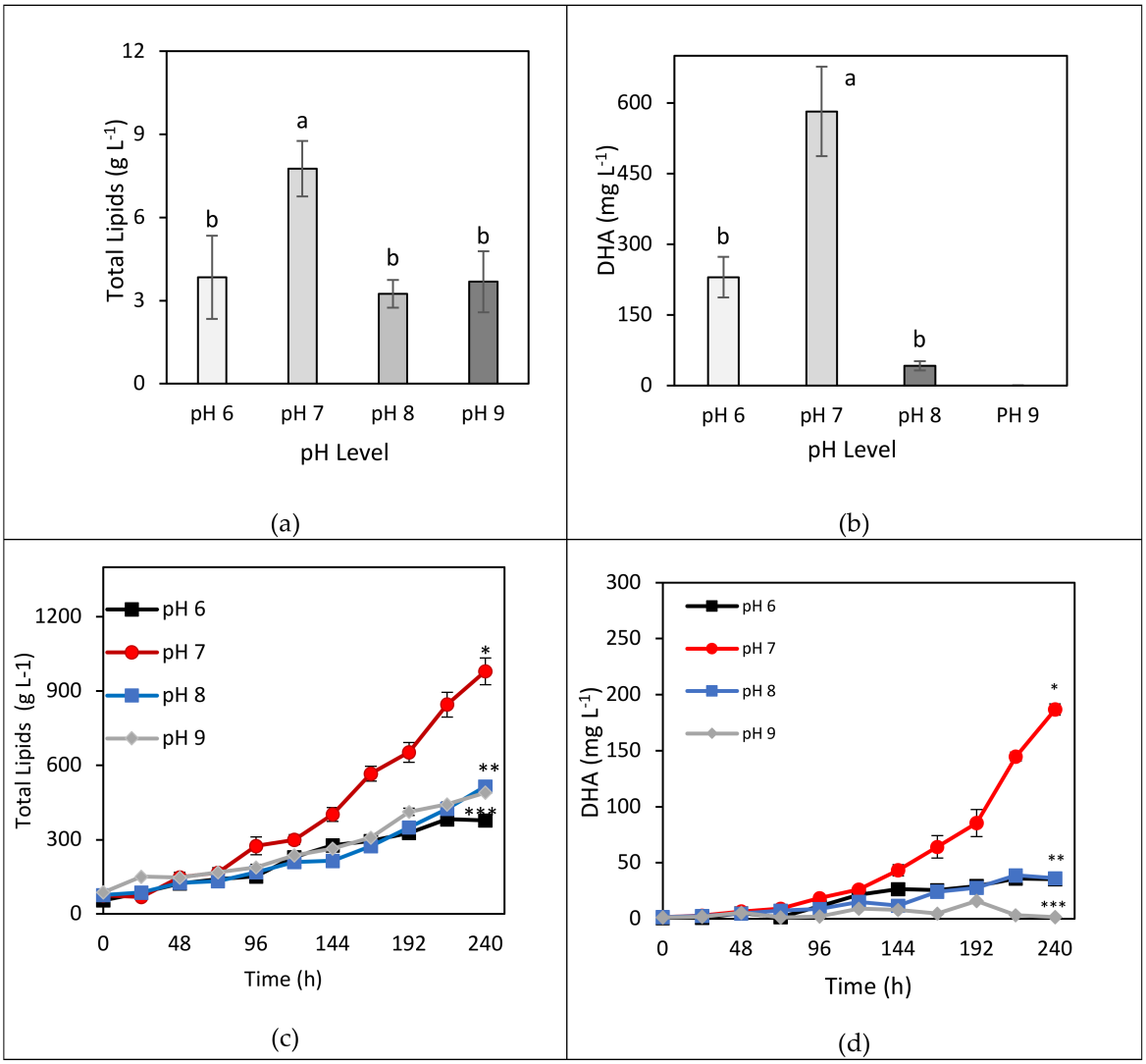

Figure 2. Total lipid content and DHA yield of *S. limacinum* grown at varying pH levels (6, 7, 8 and 9) grown in growth media containing: (**a**,**b**) 48% (*v*/*v*) effluent digestate and crude glycerol after 7 days of cultivation at lab scale and (**c**,**d**) 12.5% (*v*/*v*) effluent digestate and crude glycerol after 10 days (240 h) of cultivation at pilot scale. Values are represented as mean ± standard deviation of triplicates. Letters (a and b) indicate statistical differences analyzed at a level of $p < 0.05$, while asterisks indicate significant differences for $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

3.4. Proximate Composition

After 7 days of cultivation at laboratory conditions, significantly lower values of lipid and energy and higher values of carbohydrates were found in *S. limacinum* cultured at pH values 6 and 7, compared to culture at pH 8 and 9 (Table 1). The highest ($p < 0.05$) protein content was observed at pH 6 and the lowest ($p < 0.05$) at pH 7, whereas protein contents of *S. limacium* cultured at pH 8 and 9 exhibited intermediate values (Table 1).

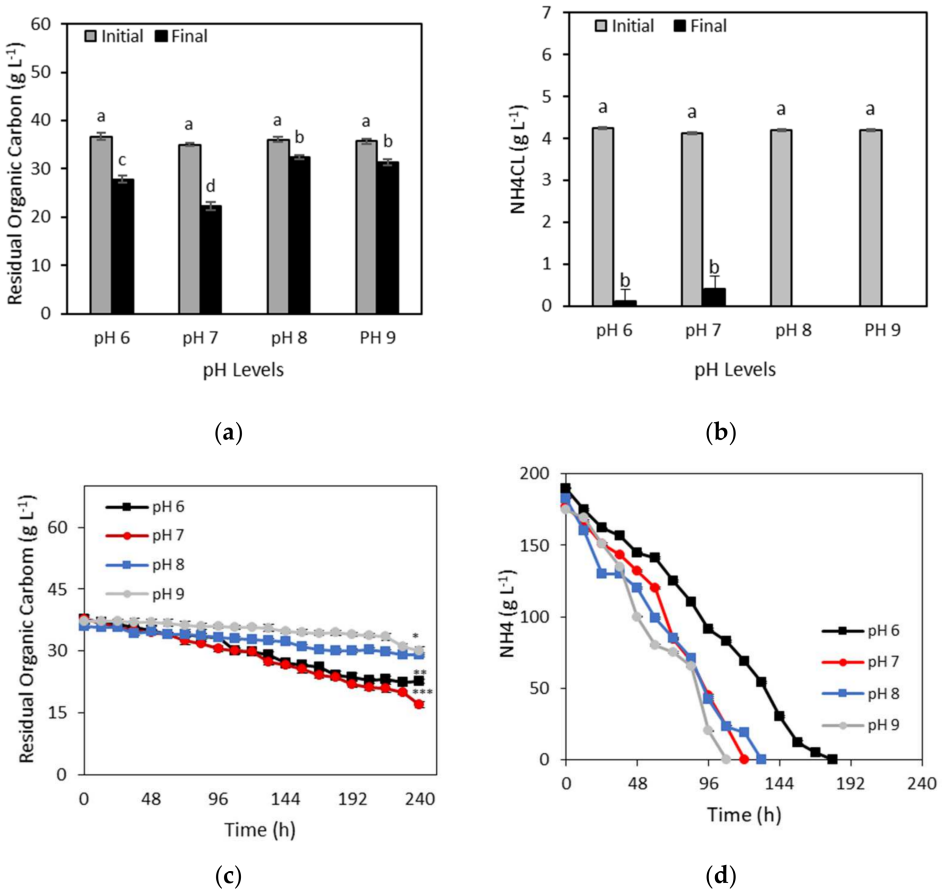

Figure 3. Residual of organic carbon and $NH4^+$ content of growth media at varying pH levels (6, 7, 8 and 9) for the cultivation of *S. limacinum*, containing: (**a**,**b**) 48% *v*/*v* effluent digestate and crude glycerol after 7 days of cultivation at lab scale and (**c**,**d**) 12.5% (*v*/*v*) effluent digestate and crude glycerol after 10 days (240 h) of cultivation at pilot scale. Values are represented as mean ± standard deviation of triplicates. Letters (a and b) indicate statistical differences analyzed at a level of $p < 0.05$, while asterisks indicate significant differences for $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Table 1. Proximate composition of dried algal biomass of *S. limacinum* grown in Lab scale at growth medium containing 48% *v*/*v* effluent digestate and crude glycerol at varying pH levels (6, 7, 8 and 9) after 7 days of cultivation. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c and d) indicate statistical differences analyzed at a level of $p < 0.05$.

pH Level	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
6	16.8 ± 0.6 ^{a}	44.0 ± 3.70 ^{a}	30.83 ± 3.1 ^{a}	8.0 ± 0.3 ^{a}	0.37 ± 0.03 ^{a}	15.0 ± 0.1 ^{a}
7	17.0 ± 3.5 ^{a}	25.0 ± 0.04 ^{b}	27.0 ± 2.8 ^{b}	30.8 ± 7.1 ^{b}	0.30 ± 0.08 ^{a}	15.8 ± 1.2 ^{b}
8	28.0 ± 5.3 ^{c}	36.5 ± 0.78 ^{c}	8.57 ± 1.3 ^{c}	26.5 ± 0.2 ^{c}	0.27 ± 0.01 ^{a}	18.8 ± 2.5 ^{c}
9	31.5 ± 2.0 ^{c}	36.0 ± 0.19 ^{c}	3.37 ± 0.5 ^{d}	29.0 ± 1.0 ^{d}	0.51 ± 0.05 ^{a}	19.4 ± 2.1 ^{c}

At pilot scale, lipid content exhibited a gradual increase during the cultivation period. Lipid content was maximized at the last day of the experiment at all pH levels, with the highest values ($p < 0.05$) being observed when cells were cultured at high pH levels (8 and 9), ranging from 13.0 ± 0.1 to 14. ± 1.4% (Table 2). With regards to the protein content, all pH levels led to a reduced protein content, exhibiting a gradual decline during the fermentation process (Tables 3–6). When the cultivation was performed at pH 9, cells exhibited the lowest protein content, 12 ± 1.12% ($p < 0.05$) (Tables 2 and 6), while lower

pH levels resulted in a significantly higher protein synthesis ranging approximately from 13–17% (Table 2).

Table 2. Proximate composition of dried algal biomass of *S. limacinum* grown in growth medium containing 12.5% *v*/*v* effluent digestate and crude glycerol at varying pH levels (6, 7, 8 and 9) after 10 days of cultivation. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c and d) indicate statistical differences analyzed at a level of $p < 0.05$.

pH Level	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
6	9.0 ± 1.9 ^{b}	17.0 ± 0.8 ^{a}	61.9 ± 0.3 ^{c}	9.0 ± 0.5 ^{a}	3.1 ± 0.4 ^{a}	16.6 ± 0.5 ^{c}
7	10.0 ± 0.8 ^{b}	13.1 ± 0.9 ^{c}	69.3 ± 2.7 ^{a}	5.6 ± 0.7 ^{c}	2.0 ± 0.6 ^{b}	17.7 ± 0.4 ^{b}
8	13.0 ± 0.1 ^{a}	16.0 ± 0.7 ^{b}	59.6 ± 0.9 ^{c}	8.0 ± 0.8 ^{b}	3.4 ± 0.5 ^{a}	18.1 ± 0.9 ^{a}
9	14.0 ± 1.4 ^{a}	12.0 ± 0.12 ^{d}	63.3 ± 1.0 ^{b}	7.8 ± 0.3 ^{b}	2.9 ± 0.33 ^{a}	18.9 ± 1.0 ^{a}

Table 3. Proximate composition of dried algal biomass of *S. limacinum* grown in growth medium containing 12.5% *v*/*v* effluent digestate and crude glycerol at pH 6, after 240 h of cultivation at pilot scale. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c, d, e and f) indicate statistical differences analyzed at a level of $p < 0.05$.

Time (h)	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
0	3.1 ± 0.5 ^{f}	26 ± 1.43 ^{a}	60.8 ± 2.96 ^{b}	7 ± 0.27 ^{d}	3.1 ± 0.35 ^{c}	15.4 ± 0.5 ^{b}
24	4.5 ± 0.48 ^{e}	25.5 ± 1.39 ^{a}	59.3 ± 1.03 ^{b}	7.9 ± 0.97 ^{b}	2.8 ± 0.15 ^{b}	16.4 ± 1.60 ^{a}
48	5.6 ± 1.06 ^{f}	24 ± 1.74 ^{b}	60.1 ± 2.68 ^{b}	7.8 ± 0.81 ^{c}	2.5 ± 0.67 ^{b}	15.5 ± 0.72 ^{c}
72	6.7 ± 1.25 ^{f}	24.9 ± 1.3 ^{b}	58.2 ± 1.18 ^{c}	7.4 ± 0.09 ^{c}	2.8 ± 0.1 ^{b}	16.5 ± 1.97 ^{a}
96	6.5 ± 0.65 ^{f}	23.8 ± 1.42 ^{b}	59.1 ± 1.21 ^{b}	7.7 ± 0.67 ^{c}	2.9 ± 1.03 ^{b}	15.9 ± 0.8 ^{b}
120	6.8 ± 0.18 ^{e}	21 ± 1.69 ^{c}	61.8 ± 1.14 ^{a}	8.2 ± 0.58 ^{b}	2.2 ± 0.08 ^{c}	16 ± 1.33 ^{a}
144	7.3 ± 1.14 ^{d}	20 ± 1.89 ^{c}	60.4 ± 1.05 ^{ab}	8.9 ± 0.83 ^{b}	3.4 ± 1.08 ^{b}	16.7 ± 1.24 ^{a}
168	7.9 ± 0.37 ^{c}	19.1± 1.98 ^{c}	59.9 ± 2.77 ^{b}	9.4 ± 1.25	3.7 ± 1.20 ^{a}	15.8 ± 1.11 ^{a}
192	8.5 ± 0.68 ^{b}	18.8 ± 1.40 ^{c}	59.5 ± 1.37 ^{b}	9.6 ± 0.49 ^{a}	3.6 ± 0.88 ^{a}	16.4 ± 2 ^{a}
216	8.9 ± 0.9 ^{a}	17.9 ± 1.33 ^{d}	60.4 ± 2.85 ^{ab}	9.7 ± 0.89 ^{a}	3.1 ± 0.37 ^{b}	16.1 ± 0.78 ^{b}
240	9 ± 1.93 ^{a}	17 ± 1.83 ^{d}	61.9 ± 0.3 ^{a}	9 ± 0.47 ^{a}	3.1 ± 0.44 ^{b}	16.6 ±0.53 ^{a}

Table 4. Proximate composition of dried algal biomass of *S. limacinum* grown in growth medium containing 12.5% *v*/*v* effluent digestate and crude glycerol at pH 7, after 240 h of cultivation at pilot scale. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c and d) indicate statistical differences analyzed at a level of $p < 0.05$.

Time (h)	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
0	3 ±0.5 ^{d}	30 ± 1.47 ^{a}	59.5 ± 1.66 ^{c}	6 ± 0.78 ^{a}	1.5 ± 0.41 ^{b}	15.8 ± 0.89 ^{c}
24	3 ± 0.92 ^{d}	25 ± 2.85 ^{b}	63.5 ± 2.83 ^{b}	6.7 ± 0.40 $_{a}$	1.8 ± 0.88 ^{a}	16.3 ± 0.82 ^{b}
48	4 ± 0.94 ^{d}	23.4 ± 1.29 ^{b}	64.1 ± 1.97 ^{b}	6.8 ± 0.34 ^{a}	1.7 ± 0.28 ^{a}	16.4 ± 0.62 ^{b}
72	4.5 ± 0.73 ^{d}	20.5 ± 0.89 ^{b}	68.6 ± 2.56 ^{c}	5 ± 0.5 ^{b}	1.4 ± 0.65 ^{b}	16.4 ± 0.53 ^{b}
96	5.5 ± 0.91 ^{d}	17.3 ± 2.73 ^{c}	69.6 ± 1.72 ^{c}	5.8 ± 0.9 ^{b}	1.8 ± 0.41 ^{b}	16.8 ± 0.73 ^{a}
120	5 ± 0.55 ^{d}	18 ± 1.69 ^{c}	69.4 ± 2.17 ^{c}	5.7 ± 0.6 ^{b}	1.9 ± 0.77 ^{a}	16 ± 0.64 ^{b}
144	6 ± 0.69 ^{c}	15.3 ± 0.89 ^{d}	71.1 ± 2.62 ^{c}	5.5 ± 0.26 ^{b}	2.1 ± 0.25 ^{a}	16.4 ± 0.46 ^{b}
168	6.3 ± 0.23 ^{c}	14 ± 1.26 ^{d}	71.3 ± 2.32 ^{c}	5.4 ± 0.85 ^{c}	2.4 ± 0.38 ^{a}	16.5 ± 1.18 ^{a}
192	7.5 ± 0.76 ^{b}	14.25 ± 0.79 ^{d}	70.95 ± 1.52 ^{c}	5.3 ± 0.41 ^{c}	2 ± 0.51 ^{b}	16.8 ± 0.71 ^{a}
216	9.5 ± 0.54 ^{a}	13.8 ± 1.73 ^{d}	68 ± 1.86 ^{c}	5.7 ± 0.43 ^{b}	3 ± 0.91 ^{a}	17.3 ± 0.83 ^{a}
240	10 ± 0.8 ^{a}	13.11 ± 0.92 ^{d}	69.3 ± 2.72 ^{c}	5.6 ± 0.75 ^{b}	2 ±0.59 ^{b}	17.7 ± 0.42 ^{a}

Table 5. Proximate composition of dried algal biomass of *S. limacinum* grown on medium containing 12.5% *v*/*v* effluent digestate and crude glycerol at pH 8, after 240 h of cultivation at pilot scale. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c, d, e, f and g) indicate statistical differences analyzed at a level of $p < 0.05$.

Time (h)	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
0	4 ± 0.08 ^{g}	29 ± 2.48 ^{a}	58.2 ± 2.31 ^{c}	5.9 ± 1.42 ^{c}	2.9 ± 0.74 ^{b}	16.2 ± 0.87 ^{d}
24	4.8 ± 0.41 ^{f}	25 ± 2.51 ^{b}	61.1 ± 2.26 ^{b}	6.3 ± 1.54 ^{c}	2.8 ± 0.19 ^{b}	16.3 ± 0.64 ^{d}
48	6 ± 0.80 ^{e}	23 ± 2.84 ^{b}	61.5 ± 1.98 ^{b}	6.8 ±1.08 ^{b}	2.7 ± 0.97 ^{b}	16.7 ± 0.36 ^{c}
72	6.7 ± 0.74 ^{e}	26 ± 2.54 ^{b}	58.5 ± 1.25 ^{c}	6.4 ± 0.81 ^{b}	2.4 ± 0.34 ^{c}	16.7 ± 0.26 ^{c}
96	8 ± 0.12 ^{d}	24 ± 2.61 ^{b}	58.2 ± 2.1 ^{c}	7.2 ± 0.34 ^{b}	2.6 ± 0.12 ^{c}	16.8 ± 0.26 ^{c}
120	8.4 ± 0.83 ^{c}	24 ± 1.42 ^{b}	57.5 ± 1.17 ^{c}	7.3 ± 1.01 ^{b}	2.8 ± 0.28 ^{b}	16.9 ± 0.16 ^{c}
144	8 ± 0.18 ^{c}	23 ± 1.59 ^{b}	59.8 ± 1.13 ^{c}	7.4 ± 0.29 ^{b}	1.8 ± 0.59 ^{d}	17.2 ± 0.45 ^{b}
168	9.1 ± 1.07 ^{c}	20 ± 1.86 ^{c}	60.4 ±2.37 ^{c}	7.8 ± 0.97 ^{a}	2.7 ± 0.94 ^{b}	17.2 ± 0.65 ^{b}
192	10 ± 0.29 ^{c}	17 ± 0.79 ^{d}	62.7 ± 2.4 ^{b}	7.7 ± 0.12 ^{a}	2.6 ± 0.91 ^{b}	17.6 ± 0.59 ^{b}
216	11.5 ± 0.42 ^{b}	14 ± 1.48 ^{e}	63 ± 0.99 ^{a}	7.8 ± 0.9 ^{a}	3.7 ± 0.18 ^{a}	17.3 ± 0.92 ^{b}
240	13 ±0.12 ^{a}	16 ± 0.66 ^{d}	59.6 ± 0.88 ^{c}	8 ± 0.78 ^{a}	3.4 ± 0.46 ^{a}	18.1 ± 0.88 ^{a}

Table 6. Proximate composition of dried algal biomass of *S. limacinum* grown on medium containing 12.5% *v*/*v* effluent digestate and crude glycerol at pH 9, after 240 h of cultivation at pilot scale. Values (percentages) are represented as the mean ± standard deviation of triplicates, whereas letters (a, b, c, d, e and f) indicate statistical differences analyzed at a level of $p < 0.05$.

Time (h)	Crude Lipid (%)	Crude Protein (%)	Crude Carbohydrates (%)	Ash Content (%)	Moisture Content (%)	Gross Energy (MJ kg^{-1})
0	3.8 ± 0.62 ^{f}	31 ± 1.76 ^{a}	55.2 ± 0.96 ^{b}	6.5 ± 1.61 ^{d}	3.5 ± 0.86 ^{a}	15.7 ± 0.48 ^{c}
24	6 ± 1.60 ^{e}	27 ± 2.5 ^{b}	56.9 ± 0.82 ^{b}	6.8 ± 1.54 ^{c}	3.3 ± 0.62 ^{a}	16 ± 0.18 ^{c}
48	6.7 ± 0.27 ^{e}	21 ± 2.28 ^{c}	62.5 ± 0.94 ^{a}	6.7 ± 0.41 ^{c}	3.1 ± 0.99 ^{a}	17.4 ± 0.25 ^{b}
72	8 ± 0.38 ^{d}	19 ± 2.34 ^{c}	63.9 ± 1.54 ^{b}	6.4 ± 0.08 ^{c}	2.7 ± 0.73 ^{b}	17.5 ± 0.17 ^{b}
96	9.5 ± 1.5 ^{c}	19.5 ± 1.7 ^{c}	62.1 ± 1.3 ^{a}	6.8 ± 0.38 ^{c}	2.1 ± 0.55 ^{c}	17.3 ± 0.21 ^{b}
120	10.3 ± 1.3 ^{c}	17 ± 1.95 ^{d}	63.5 ± 1.1 ^{a}	6.7 ± 1.69 ^{c}	2.5 ± 0.27 ^{b}	17.8 ± 0.89 ^{b}
144	10.6 ± 1.38 ^{c}	16 ± 2.51 ^{d}	64.5 ± 0.9 ^{a}	7.3 ± 1.54 ^{c}	1.6 ± 0.73 ^{d}	18.3 ± 0.26 ^{a}
168	11 ± 1.36 ^{b}	15.8 ± 2.14 ^{d}	63.8 ± 0.68	7.5 ± 0.32 ^{b}	1.9 ± 0.33 ^{d}	18.4 ± 1.07 ^{a}
192	12.5 ± 1.5 ^{b}	14 ± 1.74 ^{e}	62.2 ± 1.52 ^{a}	7.8 ± 1.69 ^{a}	3.5 ± 0.95 ^{a}	18.7 ± 0.55 ^{a}
216	13 ± 0.41 ^{a}	13.8 ± 1.82 ^{e}	62.4 ± 0.57 ^{a}	7.7 ± 1.57 ^{a}	3.1 ± 0.45 ^{a}	18.6 ± 0.06 ^{a}
240	14 ± 0.44 ^{a}	12 ± 0.12 ^{f}	63.3 ± 0.98 ^{a}	7.8 ± 0.31 ^{a}	2.9 ± 0.33 ^{a}	18.9 ± 0.97 ^{a}

With regards to the carbohydrate content, pH levels higher than 7 reduced the intracellular carbohydrate content exhibiting a gradual decline during the fermentation process (Tables 3, 5 and 6), while the opposite trend was observed when cells were cultivated at a neutral pH (Table 4), where the highest accumulation of carbohydrates was observed (Table 2). With respect to the ash content, microorganisms grown at a pH 6 exhibited the highest ash content, 11–37.5% higher compared to the rest of the treatments, while gross energy showed an increasing trend with the highest calorific value observed at elevated pH levels ranging between 18.1 ± 0.88 and 18.9 ± 0.97 MJ kg^{-1} ($p < 0.05$) (Table 2).

4. Discussion

4.1. Effect of Varying pH Concentration on Biomass, Lipid Productivity, Carbon and Nitrogen Assimilation

According to previous research, optimal hydrogen ion concentration is an important parameter to consider in microbial fermentation for proper growth and development of oleo genus fungi and algae. Jiang et al. [37] cultivated the particular microorganism utilizing different combinations of inorganic and organic nitrogen sources (yeast extract, peptone, monosodium glutamate and sodium nitrate) in the cultivation media with a pH value of 6 achieving a biomass productivity, ranging from 15–55 g/L with a total lipid content of 13.8 to 55%. Chi et al. [38] cultivated the same strain in a cultivation media

with different carbon sources (glucose, glycerol and crude glycerol) with the pH set at 8, reaching a biomass productivity of 18 g/L and 50% lipid accumulation. In another study, Chen et al. [29] cultivated a similar strain of the family of *Thraustochytrids, Schizochytrium* sp. *S31*, using a conventional nitrogen source (yeast extract) with the pH set at 6.8. Applying a stepwise dissolved oxygen strategy, they achieved a biomass productivity of 40 g L^{-1} and lipid accumulation of 50%. Interesting is the research conducted by Nakahara, et al. [39], which cultivated the strain *Schizochytrium* sp. using glucose and corn steep liquor with the pH set at 4. Despite the low hydrogen ion concentration, the microorganism reached a biomass productivity of 9 g L^{-1} and 50% lipid accumulation. However, when Zhu et al. [40] cultivated *Schizochytrium limacinum* with the pH set at 3, growth and development was completely inhibited. According to their results, the optimal hydrogen concentration was 7. When the pH was set below the optimum value, biomass productivity increased from 0–13%, whereas lipid accumulation exhibited a downward trend ranging from 9–18%. Hydrogen ion concentration beyond the optimal value reduced significantly biomass and lipid accumulation ranging from 13–60% and 10–65%, respectively. A similar trend was also observed by Zhao et al. [27] with regards increased pH values. Consequently, optimal pH values could be species-specific but for the family of *Thraustochytrids* and the several substrains, a culture medium with neutral pH is considered optimal for balanced growth and development. However, when the strain *S. limacinum* SR21 was cultivated in a culture media containing acetic acid as an alternative carbon source and the pH was set to 6, biomass productivity was reduced by 27%, whereas lipid and DHA productivity declined severely, by 40% and 50%, respectively [41]. Consequently, optimal pH value of the culture media is also determined by the individual ingredients.

Our results are in agreement with the previously mentioned research with regards to the optimum hydrogen ion concentration, since in both experiments, culture media with pH other than 7 resulted in reduced cell dry weight. However, the sharp decline of the biomass productivity cannot be attributed only due to the increasing pH values. According to Wang, et al. [42], an alternative method to purify municipal wastewaters from NH4–N is "air striping", a process by which the liquid is agitated with a stream of air which carries away the volatile compounds such as ammonia. This process is enhanced with increasing pH levels, above 7 leading to an ammonia removal efficiency of approximately 10–25% in 60 min [43]. Consequently, the increased aeriation rate in our experimental set up in conjunction with increasing pH values probably removed part of the ammonia nitrogen from the culture media resulting in decreased biomass productivity due to insufficient nitrogen concentration, which was evident from the low ammonia concentration, both at lab and pilot scale. This can also explain the increasing lipid content at elevated pH values, since nitrogen starvation ceases cell proliferation while the carbon source continues to be assimilated [44]. With regards the residual total organic carbon content, which was high at elevated pH levels, this could be attributed to the low biomass productivity, which was not able to completely assimilate crude glycerol, in combination with increased hydrogen ion concentration, which could inhibit the glycerol metabolism due to decreased enzymatic activity in the pathway [45].

4.2. Effect of Varying pH Concentrations on Proximate Composition

The nutritional value of the different species which belong to the family of *Thraustochytrids* is species-specific and differs with respect to culture conditions and culture media composition [46]. According to our results, at laboratory conditions, the total protein content exhibited an elevated fraction at high pH levels with reduced biomass productivity. This is in agreement with our previous results when the species was cultivated with 0% and 8% effluent concentration [36], where the protein content was negatively correlated to the biomass productivity and vice versa. This indicates that assimilated nitrogen was directed towards deposition rather than growth.

Nevertheless, microalgae cultivation targeting high protein content could be used as an alternative future protein supplement for the aquaculture industry considering the fact

that fish diets require high protein content feeds ranging from 40–55% [47]. Interestingly, at a pH level of 6, the microorganisms exhibited 43% higher total protein content compared to those cultured at neutral pH levels. This could be used as an alternative dual phase fermentation strategy in order to enhance protein synthesis by reducing the hydrogen ion concertation below 7 after biomass productivity has been maximized. However, at pilot scale where no additional inorganic nitrogen source was added, lipid productivity and protein content were substantial lower with an increased carbohydrate fraction compared to the laboratory conditions. This indicates that the depletion of nitrogen probably induced the influx of glycerol. A common fermentation strategy in order to trigger lipid accumulation in oleaginous fungi and algae is nitrogen limitation [37]. When nitrogen becomes exhausted, cell proliferation is ceased; however, the carbon source continues to be consumed and converted into triglycerols, which is the main lipid class used in the fatty acid synthesize pathway for DHA production [48]. Moreover, marine fungi and algae grown in high salinity cultivation media [49] in conjunction with nitrogen starvation [50] have the ability to synthesize glycerol, which is retained in the plasmalemma in order to regulate the intracellular water potential. This could explain the increased carbohydrate content since the cells were cultivated in high saline artificial sea water. The ash content was high when cells were cultured at pH values beyond 7. This can be attributed due to the inorganic salts NH_4Cl and KH_2PO_3 added to the culture media, which was the case in our previous research [36]. However, the lower ash content when microorganisms were grown at pilot scale exhibited lower values compared to the lab scale due to the lower effluent in conjunction with the absence of the inorganic salts NH_4Cl and KH_2PO_3.

Furthermore, the high lipid content at lab scale when cells were cultivated at high pH levels proves that the assimilated carbon was probably directed towards lipid accumulation, which can also be reflected by their higher calorific value, whereas at pilot scale, the higher gross energy content probably derived from the lipids in conjunction with high carbohydrate content.

4.3. Effect of Varying pH Concentration on DHA Productivity

DHA productivity can be influenced by several abiotic factors, especially with regards to the optimal hydrogen ion concentration of the culture media. Zhao et al. [27] indicated that very low acidic conditions can have a negative effect on cell growth and development of *Schizochytrium* sp., since several enzymes are impeded resulting in low membrane permeability of the cell walls. On the contrary, under alkaline culture conditions, some key metal ions related to enzymes responsible for lipogenesis and PUFA synthesis remain undissolved and, therefore, unavailable for the cells [27]. According to previous research, when a similar strain was cultivated other than the optimal pH, glycerol was probably utilized by the microorganisms for maintenance of their physiological conditions and survival rather than DHA production [51]. This is in line with our results, since cultivation of the microorganisms under acidic or alkaline conditions resulted in a reduced DHA content both at laboratory and pilot scale, ranging from 18–80% and 50–63%, respectively, while at a very high hydrogen ion concentration, DHA was either extremely low or no detectable.

5. Conclusions

This study achieved to recover successfully nutrients contained in the effluent digestate and crude glycerol both waste streams derived from the biofuel industry in order to cultivate the marine microalgae *S. limacinum* SR21 in the frame of the circular economy concept, converting wastes into a potential value-added product. It was evidenced that the strain *S. limacinum* SR21 can successfully grow at a wide range of pH levels. DHA productivity was maximized when cells were cultivated at a neutral pH level at laboratory conditions and pilot scale, yielding 582 mg L^{-1} and 186.7 mg L^{-1}. Although biomass productivity was considerably lower when the marine microalgae *S. limacinum* SR21 was cultivated at pilot scale, it was shown that inorganic nutrients can be completely replaced with the addition of 12.5% effluent digestate. Since this study did not reach industrial

production standards with regards to the DHA content at laboratory conditions and pilot scale, a further assessment of other culture parameters (nitrogen source and concentration, dissolved oxygen content and temperature) is needed to optimize DHA production and to produce supplements for aquaculture feeds using as growth medium alternative and low-cost nutrient sources such as crude glycerol and effluent digestate from the biofuel industry.

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