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Enhancing Lipid Production of *Chlorella* sp. by Mixotrophic Cultivation Optimization

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Abstract: Mixotrophic microalgal cultivation can utilize CO_2 and organic carbon sources. This study optimized the cultivation nitrogen source (peptone, urea, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3), carbon combination (glucose, glycerol, sucrose), $(NH_4)_2SO_4$ nitrogen source and pH (6–11) in a local microalgae species with three characteristics (high pH-resistant, high growth rate and high lipid content). *Chlorella* sp. G3H3-1-2 biomass production and lipid accumulation were estimated using the fatty acid methyl esters (FAMEs) concentration. The *Chlorella* sp. G3H3-1-2 FAME content was strongly influenced by the carbon, nitrogen sources and pH variations. The pH ranged from 6.0 to 8.0, which produced the highest specific growth rate of 1.22 day^{-1} for *Chlorella* sp. G3H3-1-2 while using glucose as the single carbon source. However, the highest total FAMEs content of 59% in the *Chlorella* sp. G3H3-1-2 biomass of 1.75 g/L was obtained while using the combination of 1 g-glucose/L as the carbon source and 0.2 g-$(NH_4)_2SO_4$/L as the nitrogen source at the high pH value of 10.

Keywords: microalgae; lipid accumulation; carbon source; nitrogen source; pH

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1. Introduction

Due to the continuous and increasing excessive use of fossil fuels to satisfy the world's major energy requirements, the amount of anthropogenic greenhouse gases such as CO_2 in the atmosphere has increased in recent decades. Global climate change and recurrent energy crises have threatened food security and ecological stability. Searching for applicable alternative energy sources is an urgent task at present. Utilizing renewable biomass to yield alternative energy sources can simultaneously decrease the use of fossil fuels and reduce CO_2 emissions [1]. CO_2 in both the air and exhaust gases (power plants, transport, refineries industries) emitted from the fossil fuel combustion is offset by CO_2 fixed in the atmosphere via photosynthesis. Therefore, it is beneficial to develop renewable energy sources that can capture/sequester atmospheric CO_2 while decreasing the dependency on fossil reserves to protect the natural environment [2].

Rising oil prices and increasing greenhouse gas emissions have brought increasing attention to large-scale biodiesel production [3]. Biodiesel is a mono alkyl ester consisting of long-chain fatty acids produced by transesterification from renewable feedstocks. It is a non-toxic fuel that releases less gaseous pollutants (such as dioxide, sulfur, etc.) into the atmosphere than conventional fossil fuel. However, most common commercial biodiesel feedstocks are crop oil and waste cooking oil, which will lead to increased commercial competition and food crop shortages.

Biodiesel production using microalgae provides several advantages, including higher growth rates, high lipid contents, higher photosynthetic efficiency compared to other conventional energy crops and decreased agricultural land requirement for growth [4]. Microalgae can be used as a renewable energy resource because they can convert atmospheric CO_2 into organic material through photosynthesis/photoautotrophic mechanisms, as well as reducing CO_2 emissions [5]. The microalgal biomass has the excellent ability to fix CO_2 (1.83 kg of CO_2/kg of dry microalgae); thus, microalgal cultures are full-fledged carbon capture and storage (CCS) processes [6]. When a light is supplied as the energy source, microalgae can use both organic carbon and inorganic carbon (CO_2) simultaneously, conducting a mixotrophic metabolism [7].

CO_2 bio fixation by microalgae in photobioreactors is thought to be a feasible strategy to reduce CO_2 emissions on Earth [8]. The flue gas CO_2 concentrations are relatively high and able to supply the ideal carbon source for microalgae growth [8]. Moreover, CO_2 can be directly passed through the photobioreactors without the requirement to be separated in advance. Microalgae can use CO_2 in the raw flue gases emitted from industrial exhaust gases as a carbon source [9]. However, the pH in the culture medium will decrease significantly from continuous exposure to high CO_2 concentrations. This exposure can adversely affect the microalgal physiology [10]. Moreover, the high alkalinity increases CO_2 solubility as well as creates a decreased pH buffer effect in the culture medium. If the microalgae strain is applied to reduce the CO_2 in flue gases in the future, screening for microalgae strains with high pH resistance (above 9) is a prerequisite. It will be relevant to apply CO_2 from flue gases to decrease greenhouse gas emissions and produce biodiesel economically from microalgae [8].

Mixotrophic microalgae are able to utilize CO_2 and organic carbon as carbon sources [11–14]. Microalgae usually grow in a two-stage mode during mixotrophic cultivation. In the event of a high initial organic carbon content, organic carbon is the preferred carbon source during the first heterotrophic stage. When the organic carbon decreases into a convinced concentration, the microalgae metabolism is shifted toward photosynthesis by converting CO_2 into biomass in the second stage [15]. Mixotrophic cultivation can increase the microalgae biomass and lipid accumulation [16], which is an added advantage for microalgal biodiesel production. Monosaccharides are considered a more suitable organic carbon source for *C. pyrenoidosa* mixotrophic growth than disaccharides [17]. Wan et al. [18] reported that the highest *Chlorella* biomass concentration was obtained under mixotrophic growth with glucose 10 g/L. This was 4.2 times the value from photoautotrophic conditions. Furthermore, for *Chlorella* growth, other nutrients such as nitrogen and phosphorus are equally vital. Wastewater contains some critical carbon and various nutrients, all considered as potential culture mediums for microalgal cell growth. Hence, carbon and nutrient concentration optimization are crucial for mixotrophic *Chlorella* cultivation. The various cultivation approaches, e.g., wavelengths and light intensity illustration, carbon source, temperature, nitrogen source and phosphate nutrients, heavy metals stress, salinity stress, etc., are applied to promote lipid production [19]. Nutrient limitation is a promising strategy to shift the biochemical pathways and lipid accumulation in the microalgae cell. This technique has been developed by researchers and industries to control and adjust the nutrient composition in synthetic or real industrial wastewater [8].

As described above, this study isolated a microalgae strain with three characteristics (high pH resistance, high growth rate and high lipid content). In addition, it is important to establish a suitable culture medium and cultivation conditions to obtain a biomass with certain production characteristics. The objective of this study is first to isolate a local microalgae species, *Chlorella* sp. G3H3-1-2, in Taiwan, and capture the CO_2 from the flue gases. To reach peak cell growth and the highest lipid accumulation to develop the feasibility of utilizing microalgae for biodiesel production, the cultivation conditions including nitrogen source, combination ratios of carbon and nitrogen sources and pH for *Chlorella* sp. G3H3-1-2 were optimized. The FAMEs profile of the microalgal lipid was also analyzed to evaluate the microalgal potential for biodiesel production.

2. Materials and Methods

2.1. Collection of Samples, Establishment and Identification of Algal Strains

The microalgae samples were collected from the seacoast in Taiwan (23.714173, 120.173138) and stored in sterile centrifugal tubes (Appendix A). These microalgae were cultivated using Wayne's medium containing full-strength seawater, agar 18 g/L and glucose 1 g/L in a plate at 30 °C for 2 to 7 d. Single colonies were extracted and carefully transferred to a new plate. These algal strains were identified according to the 18S rRNA gene sequences and PCR amplification extracted DNA using F Primer 597F (5′-3: Cgg gCA gAK Tgc AAg ATC gTA A) adopted with five different types of barcode and R Primer 598R (5′-3: TTA AAg AgT ATC gAT WgT TTC gAA TTC). The morphological characteristics were observed using a light microscope (ESPA, Taiwan). Isolated *Chlorella* sp. G3H3-1-2 (G3H3-1-2) was cultivated in modified Wayne's medium (malt salt 30 g/L, $NaH_2PO_4 \cdot 2H_2O$ 2 mg/L, Na_2EDTA 4.5 mg/L, H_3BO_3 3.36 mg/L, $MnCl_2 \cdot 4H_2O$ 0.036 mg/L, $FeCl_3 \cdot 6H_2O$ 0.13 mg/L and urea 0.03 g/L) at 30 °C and pH 8.0 to study the microalgal growth and FAMEs accumulation without rotation.

2.2. Serum Bottle Cultivation of Isolated Chlorella sp. G3H3-1-2

The *Chlorella* sp. G3H3-1-2 was cultured in 1 L serum bottle at 40 °C using an exponentially growing seed culture. Light intensity of 4300 lux was adopted. Aeration was achieved by sparging air enriched with 10% CO_2 at 0.5 vvm [8]. The *Chlorella* sp. G3H3-1-2 growth was studied using different nitrogen sources (peptone, urea, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3) at 1 g/L concentration with and without aeration for 7 days. A selected concentration of urea and $(NH_4)_2SO_4$ (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 g/L) was then investigated at the optimum concentration. Moreover, the initial cultivation pHs (6, 7, 8, 9, 10, 11) were applied to study the pH effects on microalgal growth with glucose 1 g/L and $(NH_4)_2SO_4$ 0.2 g/L. During microalgal growth, the liquid sample was collected from the serum bottle with respect to time to determine the microalgal biomass concentration, pH, NH_4^+-N concentration, residual sugar concentration and FAMEs content of the microalgal biomass. The detailed information on cultivations and the experimental design are shown in Table 1.

Table 1. Experimental design for microalgae *Chlorella* sp. G3H3-1-2 cultivation condition optimization.

Experiment	Nitrogen Sources	Carbon Sources	Initial pH
1. Nitrogen sources effect	Peptone, urea, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3 (1 g/L)	Sucrose 1.0 g/L	9
2. Nitrogen sources concentrations effect	$(NH_4)_2SO_4$ 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 g/L	Sucrose 1.0 g/L	9
3. Carbon and nitrogen sources combination effect	$(NH_4)_2SO_4$ 0.2, 0.4, 0.6 g/L	Glucose, glycerol, sucrose (1 g/L)	9
4. Cultivation pH effect	$(NH_4)_2SO_4$ 0.2 g/L	Glucose 1 g/L	6, 7, 8, 9, 10, 11

2.3. Microscopic Observation

The lipid accumulation in the microalgal biomass was observed using the Nile red staining method using fluorescence microscopy. The microalgae sample was centrifuged at 1500 rpm for 10 min. The cell pellets were washed with saline water several times to remove unsuspended particles. Subsequently, the microalgae samples were treated with 0.5 mL of Nile red solution and incubated at 40 °C. After 15 min of incubation in darkness, the stained microalgae samples were washed in distilled water successively to eliminate the unstained dye particles. The intracellular lipid contents were observed using fluorescent microscopy (ESPA, Taichung, Taiwan) at a wavelength of 470 nm.

2.4. Analytical Method

The biomass concentration was quantified using a spectrophotometer at 680 nm (OD 680) according to the standard curve between dry biomass concentration and OD 680

value. The total carbohydrate concentration and the glycerol concentration were estimated using the phenol–sulfuric acid assay method and high-performance liquid chromatography (HPLC; Young Lin Acme 9000 HPLC), respectively, which were presented in our previous study [5].

Based on the curves for the correlations between OD680 and dry biomass concentration, the changes in the specific growth rate under different culture conditions were determined. The maximum specific growth rate (μ_m, d^{-1}) was evaluated by cultivating *Chlorella* sp. G3H3-1-2 in batch systems and subsequently elaborating on the experimental data obtained during the exponential growth phase. The μ_m of *Chlorella* sp. G3H3-1-2 was calculated using Equation (1) [20,21].

$$\mu_m = \frac{lnN_2 - lnN_1}{t_2 - t_1} \quad (1)$$

where N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2) during the exponential growth phase, respectively.

2.5. Total Fatty Acids Methyl Esters (FAMEs)

The lipid content in the microalgal biomass was estimated using the fatty acid methyl esters (FAMEs) concentration via the direct transesterification method followed by gas chromatography (YL6100 GC, Young Lin Instrument Co., Ltd., Anyang, Republic of Korea), as reported in our previous study [8]. The FAMEs profile including C16:0, C16:1, C18:0, C18:1, C18:2, C18:3 and C20:0 was quantified according to their peak area relative to the C17:0 fatty acid internal standard and expressed as a percentage of the total fatty acid content.

2.6. Statistical Analysis

All statistical analyses were conducted using IBM SPSS (Statistical Package for the Social Sciences) statistics 22 software. Variables are reported as significant at either 95% confidence (p-value less than or equal to 0.05) or 90% confidence (p-value between 0.05 and 0.10).

3. Results and Discussion

3.1. Nitrogen Sources Effect

Nitrogen is essential for all organisms, primarily consisting of cell material (proteins, amino acids and nucleic acids) and other nitrogen-containing molecules [22]. Therefore, to investigate the most appropriate nitrogen source for cell growth and FAMEs accumulation for *Chlorella* sp. G3H3-1-2, inorganic and organic nitrogen were tested simultaneously.

As shown in Figure 1, $(NH_4)_2SO_4$ was the best nitrogen source among the tested compounds, such as peptone, urea, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3. The maximum biomass concentration obtained with $(NH_4)_2SO_4$ as the nitrogen source was approximately 2.3 g/L, which was significantly higher and doubling than that obtained with NH_4Cl, $NaNO_3$ and KNO_3, which were approximately 1.0 g/L. The final biomass observed with peptone and yeast extract were approximately 1.5 g/L. In contrast, the FAMEs content and FAMEs yield from algal cells are illustrated in Figure 2. The algal biomass obtained from the medium that lacked nitrogen source produced the highest FAMEs content of 40%, followed by 16% using $(NH_4)_2SO_4$. The highest FAMEs productivity of about 0.5 g/L/d was obtained using the $(NH_4)_2SO_4$ 0.04 g/L nitrogen source. Figure 2 also reveals that the main compositions of the FAMEs were C16:0, C18:1, C18:2 and C18:3, regardless of aeration. Under aeration conditions, the proportion of these fatty acids was significantly affected by the type of nitrogen source. The unsaturated fatty acids content in the G3H3-1-2 microalgae cells is generally higher than that of saturated fatty acids, regardless of aeration.

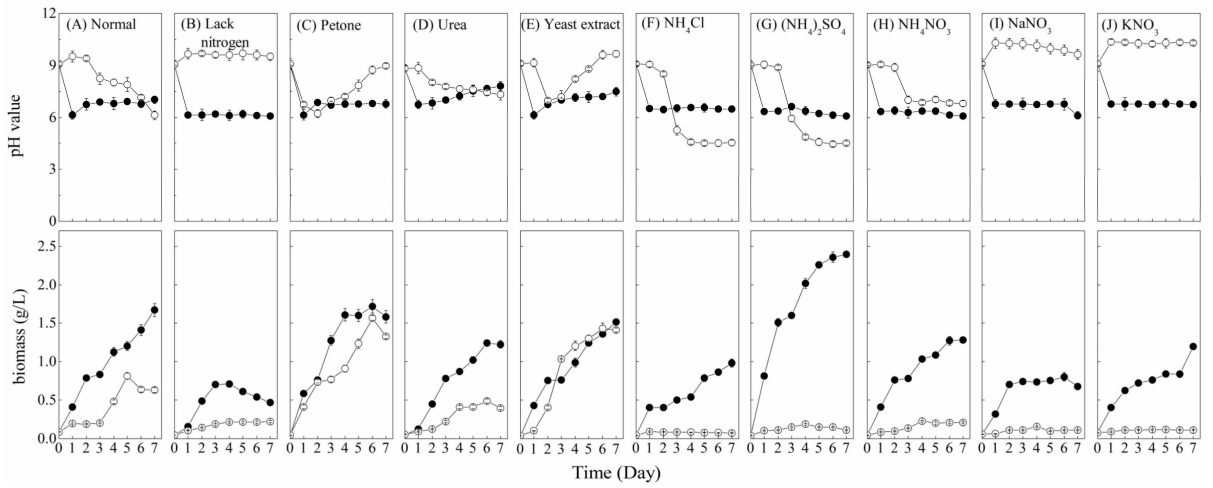

Figure 1. *Chlorella* sp. G3H3-1-2 biomass and pH variation at various nitrogen sources. Culture condition: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; carbon sources: sucrose 1.0 g/L; various nitrogen sources; aeration: 10% CO_2; n = 3. Note: (**A**,**B**): autotrophic cultivation; (**C**–**J**): mixotrophic cultivation. (●) aeration rate 0.5 vvm, 10% CO_2; (○) with no aeration.

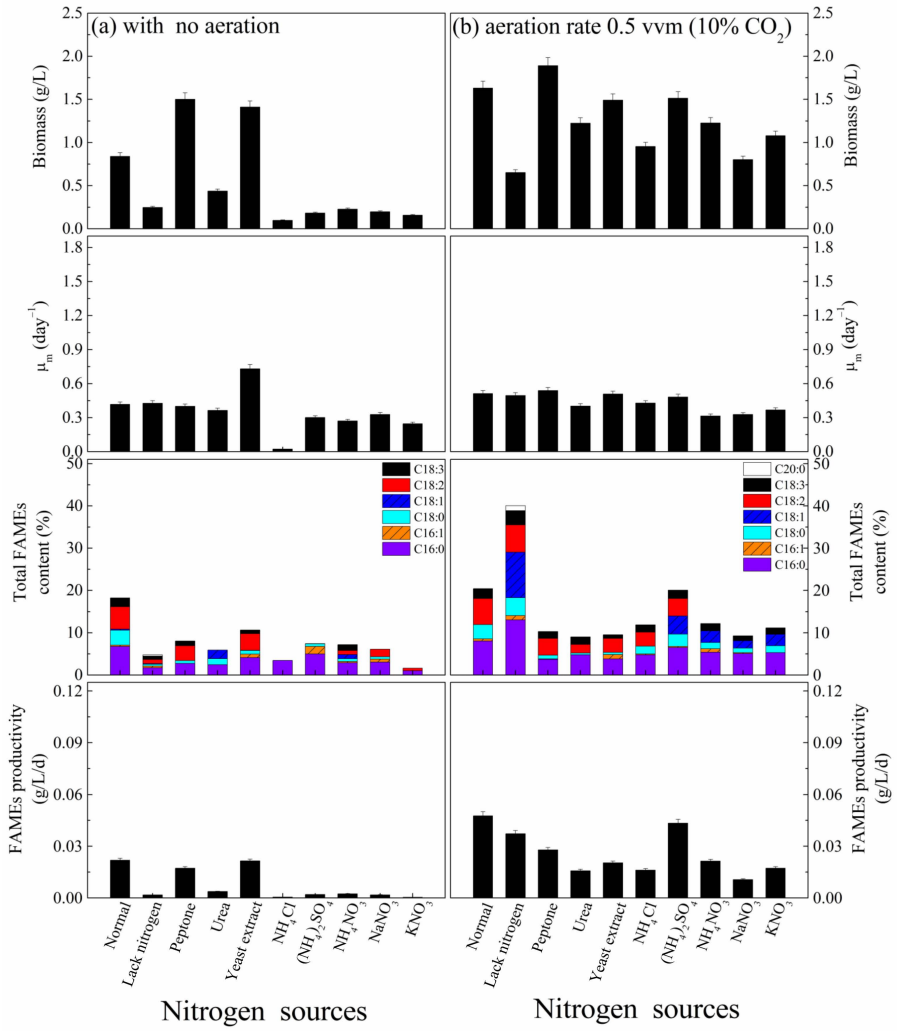

Figure 2. *Chlorella* sp. G3H3-1-2 biomass growth and total FAMEs performances at various nitrogen sources. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; carbon sources: sucrose 1.0 g/L; nitrogen sources: 1.0 g/L; aeration: 10% CO_2; n = 3. Note: (**a**): without aeration; (**b**): aeration rate 0.5 vvm with 10% CO_2.

The highest μ_m of 0.7 day^{-1} was obtained using the yeast extract nitrogen source with no aeration. This value is much higher than the values from 0.2–0.3 day^{-1} while adding other nitrogen sources (peptone, urea, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and KNO_3) with the no-aeration condition. Under the CO_2 exposure condition, the μ_m values (about 0.3 day^{-1}) were lower when culturing with NO_3^- nitrogen sources (NH_4NO_3, $NaNO_3$ and KNO_3).

Figure 3 shows the microalgal lipid visualization after staining with Nile red. The presence of a high lipid content in the algal biomass was confirmed through examination with a fluorescent microscope. The Nile-red-stained microalgae cells had yellow-gold globules inside. The cell appearance indicated the existence of lipid droplets. This Nile method is a rapid and safer lipid content quantification in *Chlorella* sp. The yellow-gold fluorescence in the *Chlorella* sp. G3H3-1-2 cells showed the presence of lipid droplets. According to the results in Figure 2, the FAMEs content could reach 16–40% with various nitrogen sources with and without aeration. The high FAMEs content is indicated with the red arrow.

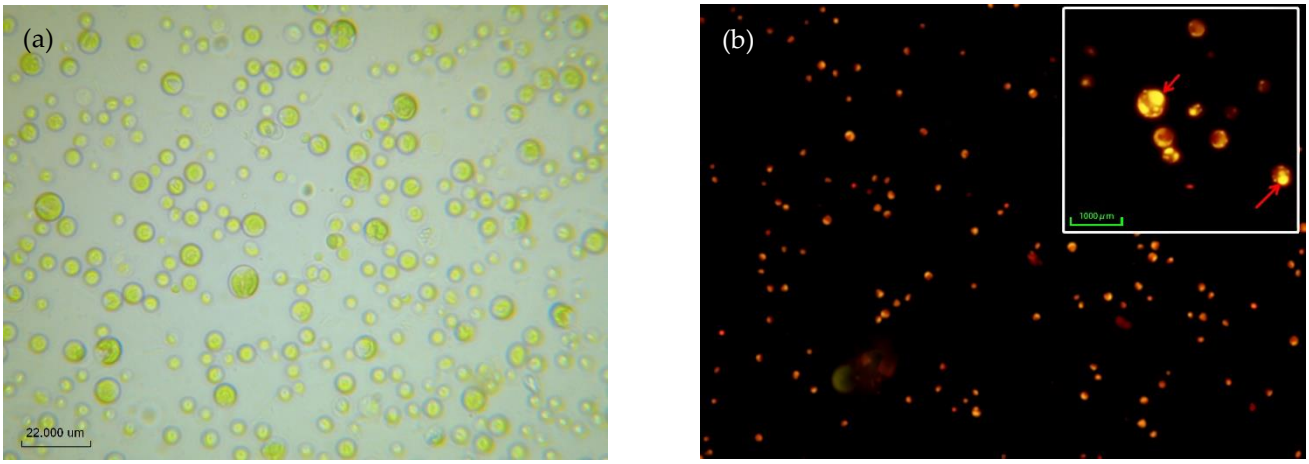

Figure 3. Microscopic images of *Chlorella* sp. G3H3-1-2 cells stained with Nile red staining: (**a**) bright field, (**b**) fluorescence microscopy.

Our results demonstrate that $(NH_4)_2SO_4$ was a superior nitrogen source for *Chlorella* sp. G3H3-1-2 cell growth and FAMEs accumulation under the investigated conditions. This result was consistent with the findings from a previous report [17] that ammonium was the most favorable nitrogen source for microalgae because it consumed less energy than other nitrogen sources when carrying out assimilation. Moreover, the organic nitrogen sources were inferior nitrogen sources for *Chlorella* sp. G3H3-1-2 compared to ammonium, although they have generally been more appropriate for microalgal growth without causing drastic pH variations. Similar results shown in another study [23] pointed out that a higher FAMEs content of about 0.38 g/g was obtained by culturing *Neochloris oleoabundans* with the nitrate (FAMEs content) nitrogen source. This was greater than that obtained using the urea (FAMEs content was about 0.17 g/g) and ammonium (FAMEs content was about 0.18 g/g) nitrogen sources. Ammonium sulfate provides another attempt to reduce the microalgal biodiesel production cost because it is much cheaper than other organic or inorganic nitrogen sources. This will be more economical for large-scale commercial biodiesel production.

3.2. Nitrogen Concentrations Effect

To confirm the optimal nitrogen source concentration in culture medium, containing 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 g/L $(NH_4)_2SO_4$, tests were carried out while operational temperature, air flux, light intensity and the other parameters were kept at the same conditions. As shown in Figure 4, the *Chlorella* sp. G3H3-1-2 biomass increased significantly from 1.2 to 2.3 g/L when the $(NH_4)_2SO_4$ concentration increased from 0 to 0.6 g/L. However,

the biomass concentration did not increase further even when the $(NH_4)_2SO_4$ concentration increased from 0.6 to 2.0 g/L. Additionally, the highest specific *Chlorella* sp. G3H3-1-2 growth rate obtained was 0.7 day^{-1} when the $(NH_4)_2SO_4$ concentration was 2.0 g/L, whereas the other $(NH_4)_2SO_4$ concentration did not significantly affect specific growth rates (Figure 5). In contrast to biomass, increasing the $(NH_4)_2SO_4$ concentration in the culture medium resulted in reducing the FAMEs content of the microalgal cells. As shown in Figure 5, the FAMEs content visibly decreased from 52 to 15% when the $(NH_4)_2SO_4$ concentration was increased from 0 to 0.8 g/L.

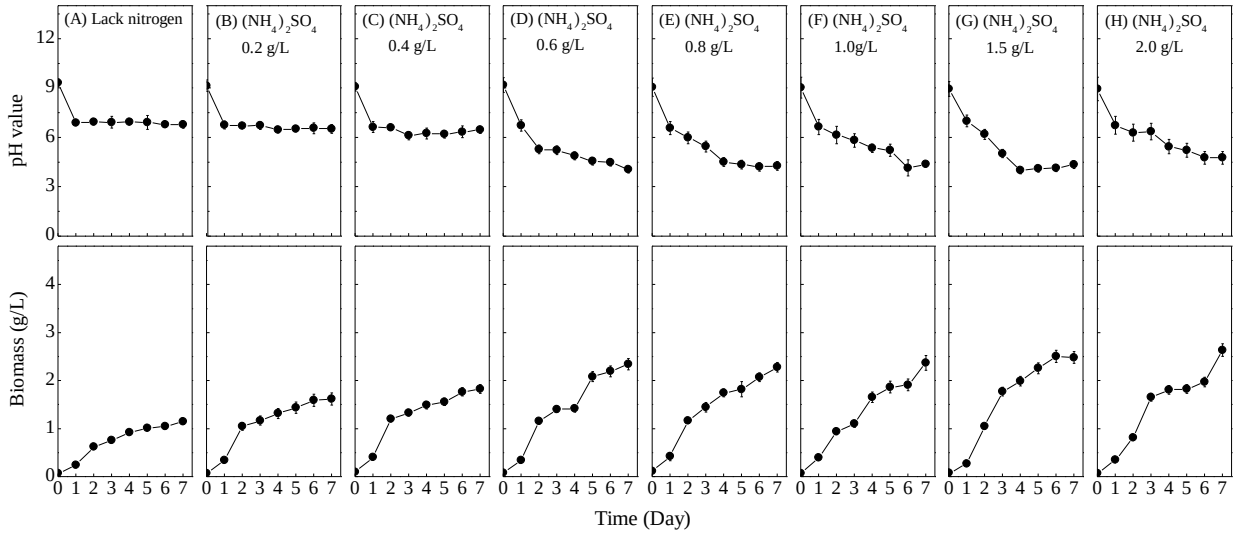

Figure 4. *Chlorella* sp. G3H3-1-2 biomass and pH variation at various $(NH_4)_2SO_4$ concentrations. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; carbon sources: sucrose 1.0 g/L; various $(NH_4)_2SO_4$ concentrations; aeration: 10% CO_2; Aeration rate 0.5 vvm, 10% CO_2; n = 3. Note: (**A**): autotrophic cultivation. (**B**–**H**): mixotrophic cultivation.

Nitrogen limitation conditions may activate diacylglycerol acyltransferase and increase the fatty acid acyl-CoA intracellular content, and then convert fatty acid acyl-CoA into triglyceride [24]. Accordingly, microalgae under low nitrogen concentration could increase the total FAMEs. Conversely, the higher $(NH_4)_2SO_4$ concentration increased microalgal cell growth but decreased FAMEs production.

The FAMEs productivity decreased when the $(NH_4)_2SO_4$ concentration increased from 0.2 to 0.8 g/L. A higher FAMEs content (>35%) and productivity (>0.14) were obtained when $(NH_4)_2SO_4$ concentration in the medium was from 0 to 0.4 g/L, owing to the supply of excessive nitrogen sources in the culture medium, which lead to a decreased microalgal FAMEs productivity and biomass productivity [22]. This is due to nitrogen source oversupply, which alters the nitrogen limiting factor, resulting in triggering FAMEs accumulation in microalgal cells to prevent nitrogen depletion, resulting in cell growth cessation. However, the maximal FAMEs content obtained was approximately 52% when the cells were grown without $(NH_4)_2SO_4$ and only adding 1.2 g/L (0 g/L) of biomass concentration. These results suggest that, to achieve the optimal production efficacy for microalgae, a compromise is necessary between the increase in growth and the FAMEs content. Therefore, the optimal $(NH_4)_2SO_4$ concentration for cell growth and FAMEs accumulation of *Chlorella* sp. G3H3-1-2 was considered to be 0.2 g/L among the tested conditions.

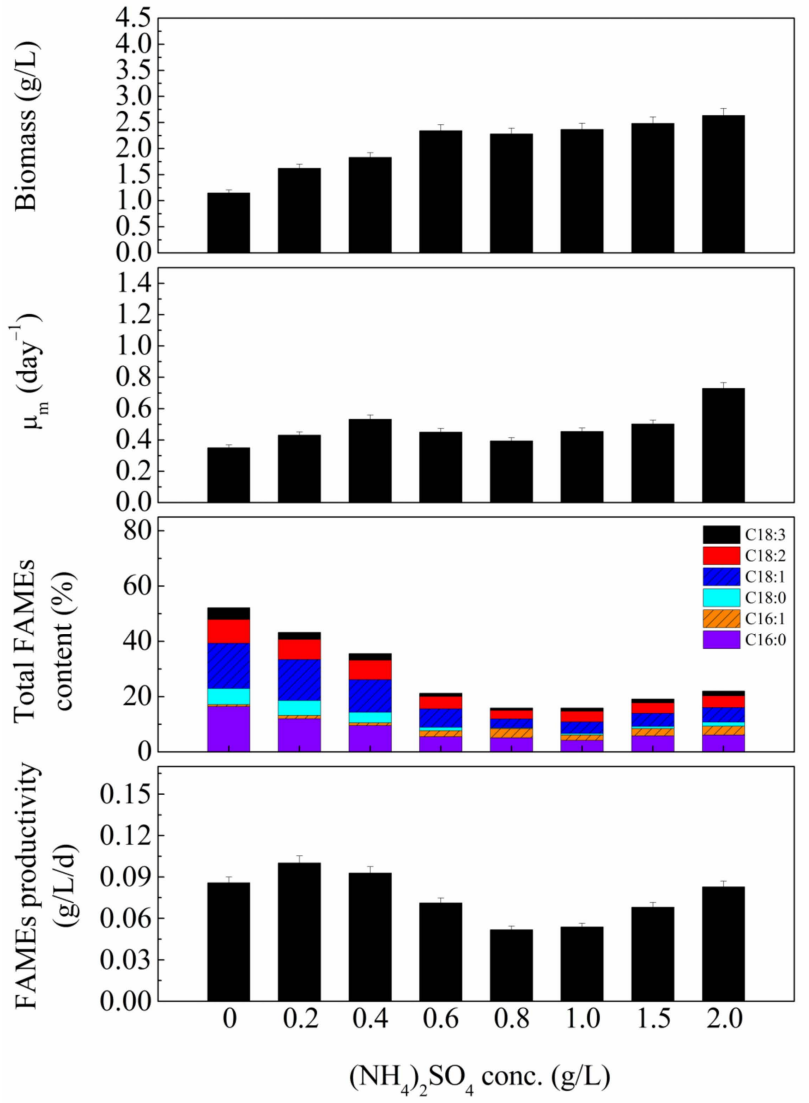

Figure 5. *Chlorella* sp. G3H3-1-2 biomass growth and total FAMEs performances at various $(NH_4)_2SO_4$ concentrations. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; carbon sources: sucrose 1.0 g/L; various $(NH_4)_2SO_4$ concentrations; aeration: 10% CO_2; n = 3. Note: 0 g/L $(NH_4)_2SO_4$: autotrophic cultivation; 0.2–2.0 g/L $(NH_4)_2SO_4$: mixotrophic cultivation.

3.3. Carbon and Nitrogen Sources Concentration Effect

Various previous test results indicate that glucose [18], glycerol [25] and sucrose [5] were suitable carbon sources for microalgae cultivation. In order to choose the most appropriate combinations of different carbon and nitrogen source ratios for cell growth and FAMEs accumulation of *Chlorella* sp. G3H3-1-2, the three carbon sources at 1 g/L combined with $(NH_4)_2SO_4$ at concentrations of 0.2, 0.4 and 0.6 g/L were compared (Figure 6). As shown in Figure 6, the biomass performance obtained from using glucose, glycerol and sucrose as the carbon source was insignificantly different when cultured with $(NH_4)_2SO_4$ as the nitrogen source. However, the results show that the highest specific growth rate of 1.08 d^{-1} was obtained under the glycerol culture condition as carbon source and $(NH_4)_2SO_4$ 0.6 g/L as the nitrogen source. Our result was similar to the report from Bhatnagar et al., which reveals that the microalgal growth was slightly enhanced when using glycerol as the carbon source and suggesting that glycerol is a useful carbon source for microalgal growth [26]. Glucose is the preferred choice for the high biomass and FAMEs productivities of microalgae in the current studies [27]. The high price of glucose could constitute about 80% of the medium cost and restrict its commercial scale development [7].

As shown in Figure 6, compared with these three carbon sources, a higher total FAMEs content (around 49%) under the culture condition of 0.2 g/L $(NH_4)_2SO_4$ as nitrogen source was obtained when sucrose was the carbon source. The highest total FAMEs productivity (around 0.13 g/L/d) under the culture condition of 0.2 g/L $(NH_4)_2SO_4$ as nitrogen source was obtained when glycerol was the carbon source. The results reported above indicate that the higher total FAMEs content (>40%) with glucose, glycerol or sucrose as the carbon source was obtained using $(NH_4)_2SO_4$ 0.2 g/L as the nitrogen source. This may be attributed to *Chlorella* being more adaptable to grow in the culture condition of nitrogen limitation [28].

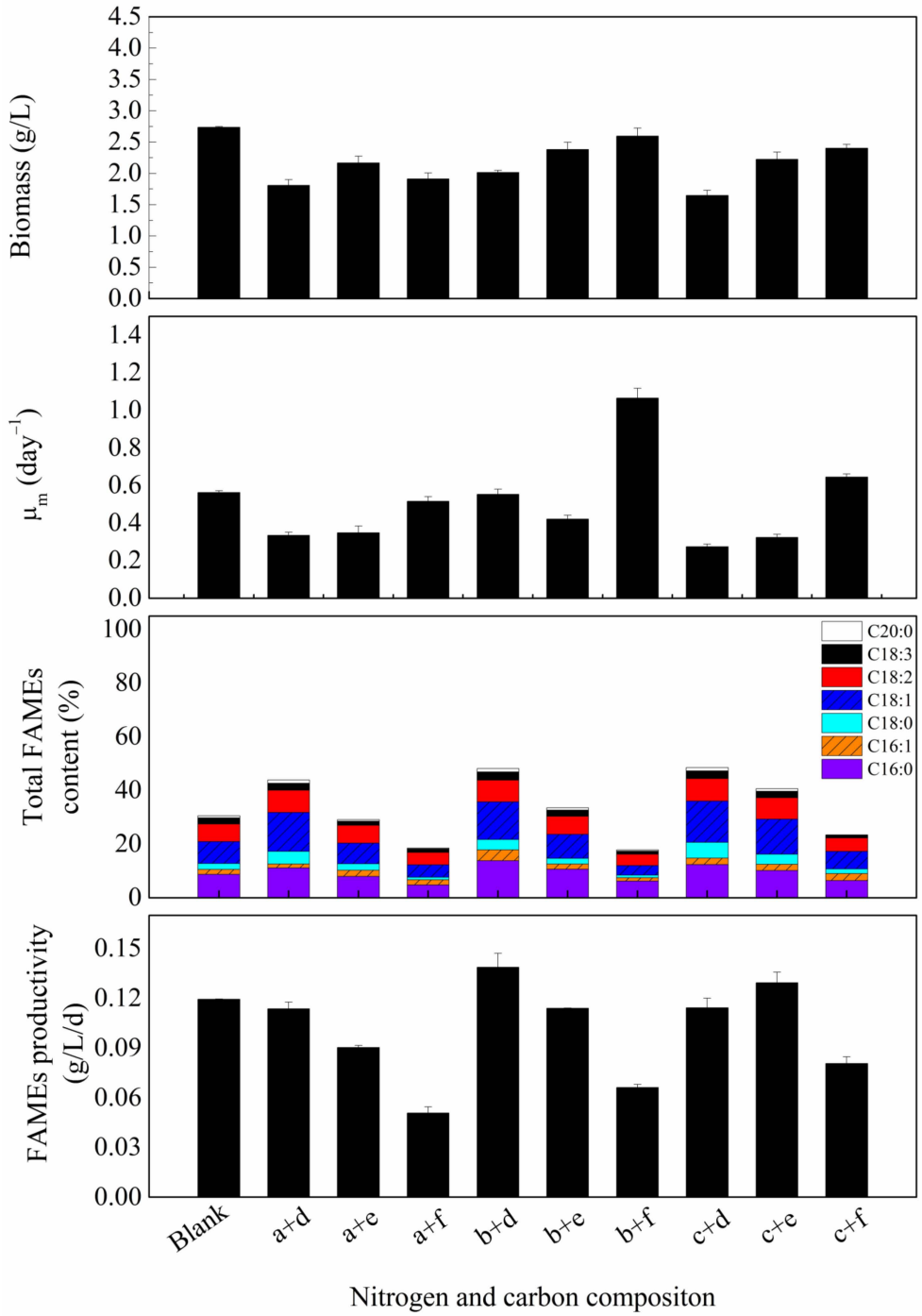

Figure 6. *Chlorella* sp. G3H3-1-2 biomass growth and total FAMEs performances at various carbon and nitrogen sources. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; aeration: 10% CO_2; n = 3. Note: carbon sources 1.0 g/L: (**a**) glucose; (**b**) glycerol; (**c**) sucrose. Nitrogen concentration: (**d**) 0.2 g/L; (**e**) 0.4 g/L; (**f**) 0.6 g/L.

3.4. pH Effect

Culture pH is one of the main controlling factors influencing microalgae cultivation. Generally, most microalgal species prefer to grow under neutral pH [29]. Therefore, searching for a suitable microalga strain that could withstand alkaline pH for biodiesel production is needed. Figure 7 shows the different initial pH levels in the culture medium on pH change, *Chlorella* sp. G3H3-1-2 biomass concentration and nitrogen consumption. Using glucose as an additional carbon source, the biomass concentration was increased from 0.4 to 2.2 g/L when the pH increased gradually from 6.0 to 9.0. However, the biomass concentration was slightly decreased when the pH was continuously increased to more than 9.0. Similarly, in terms of NH_4^+-N consumption, besides the pH 7 test group, the consumption trend presentation is approximately similar and leveled off at the next day. Microalgae can grow over a wide pH range (pH 6.0–11.0), but the most suitable pH was dependent on the species [10]. Figure 8 shows that the highest specific *Chlorella* sp. G3H3-1-2 growth rate was increased when the pH increased from 6.0 to 8.0 with glucose as an additional carbon source. The highest specific growth rate was reduced when the pH was continuously increased beyond 9.0. The highest total FAMEs content reached 59% at pH 10.0, while the highest FAMEs productivity (0.16 g/L/d) occurred at pH 9.0, due primarily to the higher biomass production at pH 9.0 (2.2 g/L). FAMEs productivity was calculated as the biomass productivity and FAMEs content result. Therefore, the best performances for both biomass and FAMEs production for *Chlorella* sp. G3H3-1-2 may be obtained at pH 9.0–10.0. This shows that the pH significantly affects the *Chlorella* sp. G3H3-1-2 growth and FAME concentration under mixotrophic conditions.

The results reported above indicate that greater total FAMEs content and FAMEs productivity were obtained with glucose as the carbon source under the culture condition of pH 9.0 to 11.0, owing to the highest total FAMEs content (59%) being obtained at pH 10.0 when using glucose 1.0 g/L and $(NH_4)_2SO_4$ 0.2 g/L as the carbon and nitrogen source, respectively. High CO_2 culture medium concentration may cause the pH to decline significantly, then decrease the carbonic anhydrase extracellular enzyme activity and inhibit microalgal growth [30]. The optimal initial pH for FAMEs production of *Chlorella* sp. G3H3-1-2 was selected as 10.0. However, the previous studies were less focused on alkaline pH and temperature tolerance for the biomass and FAMEs production in microalgae. Table 2 summarizes the performances of the oleaginous microalgae under various culture conditions. Only a few microalgae species have been evaluated under high pH and high temperature for FAMEs production. Bartley et al. [31] revealed that pH values from 8 to 9 may be conducive to increasing algae production. The final biomass of 1.75 g/L with a FAMEs content of 59% was achieved for *Chlorella* sp. G3H3-1-2 in this study, which were the highest values compared with other reported oleaginous *Chlorella* species. In some cases, the pH value in the cultivation condition could be regulated by the NH_4^+-N and CO_2 concentration [32]. Hence, increasing the pH in the cultivation could improve CO_2 utilization and absorbability by the microalgae [33]. The appropriate microalgae strain can withstand pH changes in the culture medium and variable CO_2 concentration in the flue gases and also have high FAMEs production efficiency when flue gases are used for microalgal culture for applications in industrial production in the future [29].

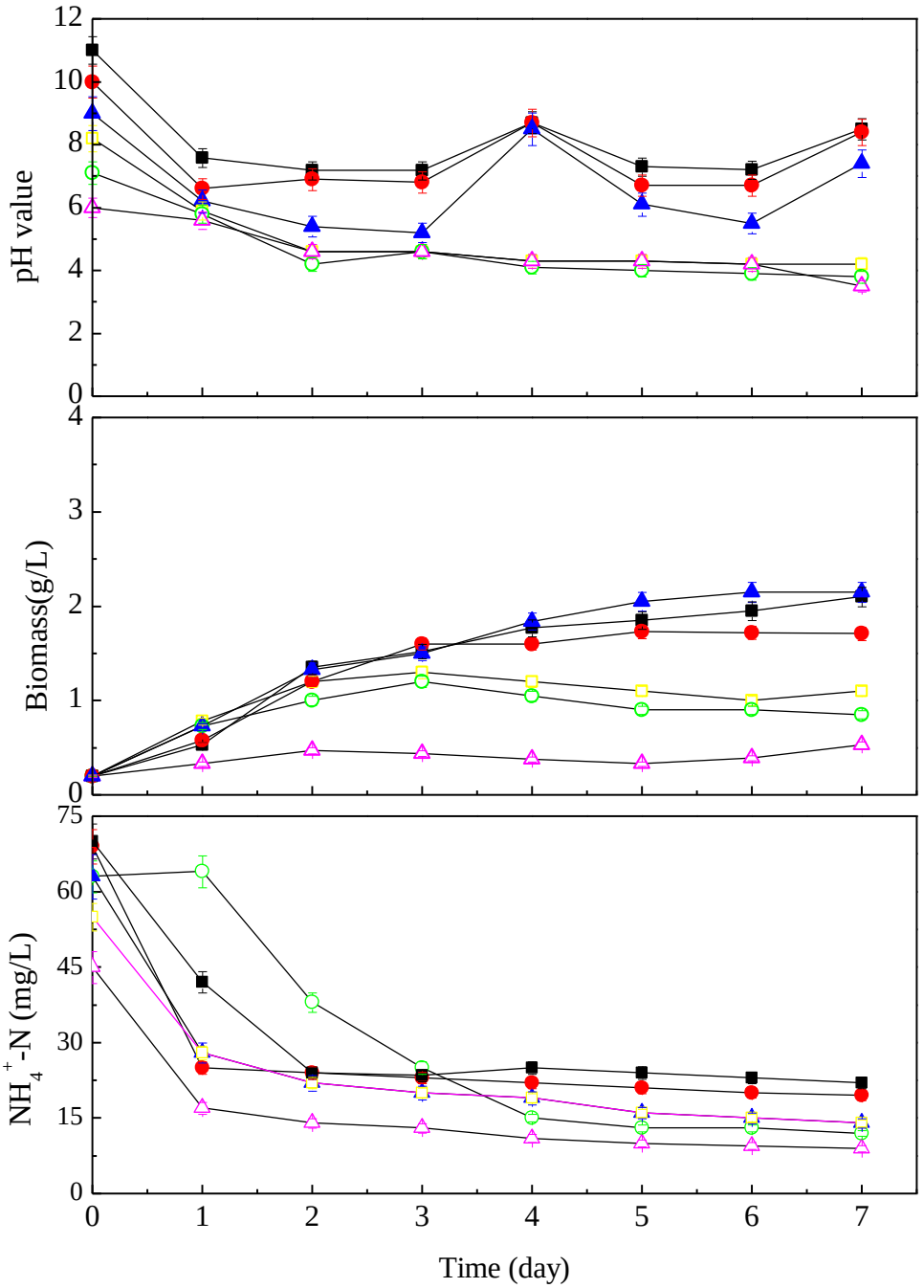

Figure 7. *Chlorella* sp. G3H3-1-2 biomass and NH_4^+-N variation at various pH values. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; nitrogen sources: $(NH_4)_2SO_4$ 0.2 g/L; carbon sources: glucose 1.0 g/L; aeration: 10% CO_2; n = 3. Note: pH value: pH value: (△) pH 6; (○) pH 7; (○) pH 8; (▲) pH 9; (●) pH 10; (■) pH 11.

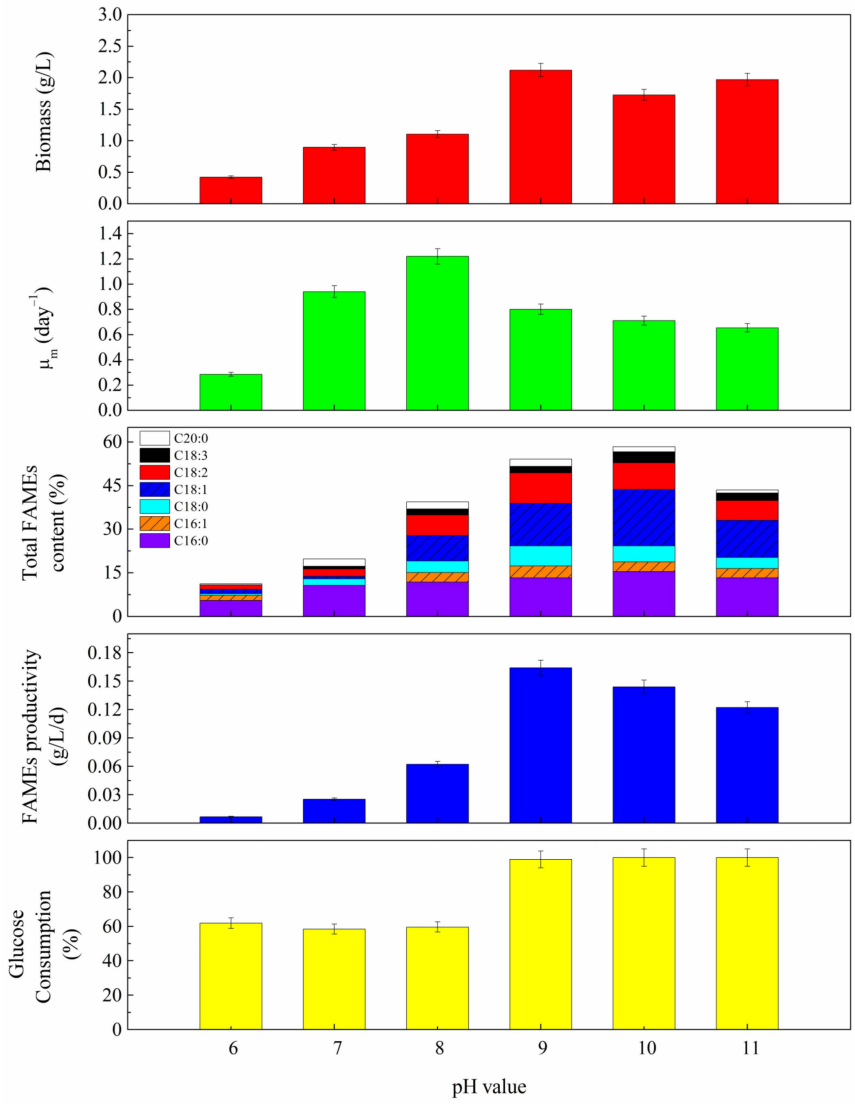

Figure 8. *Chlorella* sp. G3H3-1-2 biomass growth and total FAMEs performances at various pH values. Culture conditions: light/dark cycle: 24/0; light source: fluorescent; light intensity: 4300 lux; nitrogen sources: $(NH_4)_2SO_4$ 0.2 g/L; aeration: 10% CO_2; n = 3. Note: carbon sources: glucose 1.0 g/L.

Table 2. Biomass and lipid production comparisons from various microalgal species.

Strains	Growth Type	Carbon Source	Nitrogen Source	pH	Temp (°C)	Biomass Concentration (g/L)	Lipid Content (*w*/*w*, %)	Lipid Concentration (g/L)	Lipid Productivity (mg/L/day)	References
Marine *Chlorella* sp.	Mixotrophy	Glucose	$NaNO_3$	7.3	30	4.48	25.1	1.1237	112.4	[27]
Nannochloropsis sp.	Mixotrophy	Glucose	$NaNO_3$	7.3	30	5.87	25.3	1.4825	148.3	[27]
Chlorella vulgaris ESP-31	pH-stat photo-heterotrophy	Acetic acid	$NaNO_3$	7	25	2.13	49.7	0.986	70	[7]
Scenedesmus obliquus SA1	–	–	–	–	25	4.975	33.04	–	–	[34]
Monoraphidium sp. SB2	–	–	KNO_3	7.5	30	0.65	31.5	–	29.2	[35]
Stichococcus bacillaris	Inclined bubble column	CO_2	$NaNO_3$	7	23	4.27	32	–	81	[6]
Nannochloropsis oceanica DUT01	–	–	$NaNO_3$	8	25	1.4	33.9	0.6	31	[36]
Desmodesmus sp. F2	Autotrophy	CO_2	$NaNO_3$	7.6	35	3.32	64.13	–	263	[22]
Chlorella sp. Y8-1	Mixotrophy	Sucrose	urea	9	30	0.45	35.5	–	–	[5]
Picochlorum sp. BDUG100241	Mixotrophy	Sodium acetate	–	7	25	2.4	53.5	–	–	[37]
Chlorella sp.	Mixotrophy	Acetic acid	KNO_3	7	25	8.78 (protein content)	37.1	–	–	[25]
Thalassiosira pseudonana	Mixotrophy	Acetic acid	KNO_3	7	25	10.71 (protein content)	19.0	–	–	[25]
Chlorella sp. G3H3-1-2	Mixotrophy	Glucose	$(NH_4)_2SO_4$	10	40	1.75	59 (FAMEs)	49 (FAMEs)	144 (FAMEs)	This study

Note: Values from the study's results are not shown and labeled as "–" in the table.

4. Conclusions

This research demonstrates the feasibility of using an indigenous microalga *Chlorella* sp. G3H3-1-2 grown under a mixotrophic culture for biodiesel production. The higher biomass production (1.8 g/L) and FAMEs productivity (0.1 g/L/d) were obtained using glucose (1 g/L) as the carbon source and $(NH_4)_2SO_4$ (0.2 g/L) as a nitrogen source in the absence of pH control. Remarkably, the optimized pH values (10) can further improve the biomass concentration (1.75 g/L), the highest specific growth rate (0.71 day^{-1}) and the total FAMEs content (59%). These results indicate that *Chlorella* sp. G3H3-1-2 has a greater tolerance to a high pH value and this property can be exploited for reducing the CO_2 concentration. Therefore, further studies on CO_2 sequestration through *Chlorella* sp. G3H3-1-2 will be applied to capture CO_2 from flue gas and simultaneously produce biodiesel from the microalgal lipids.

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Data Availability Statement: Not applicable.

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

Appendix A

The microalgal samples were isolated from seawater from the seacoast in Yunlin County, Taiwan (23.714173, 120.173138). Using BLAST software to align and compare the 18S rRNA gene sequences with other known microorganisms in the GenBank database revealed that this strain exhibited a close phylogenic relationship with the Chlorella family (Figure A1).

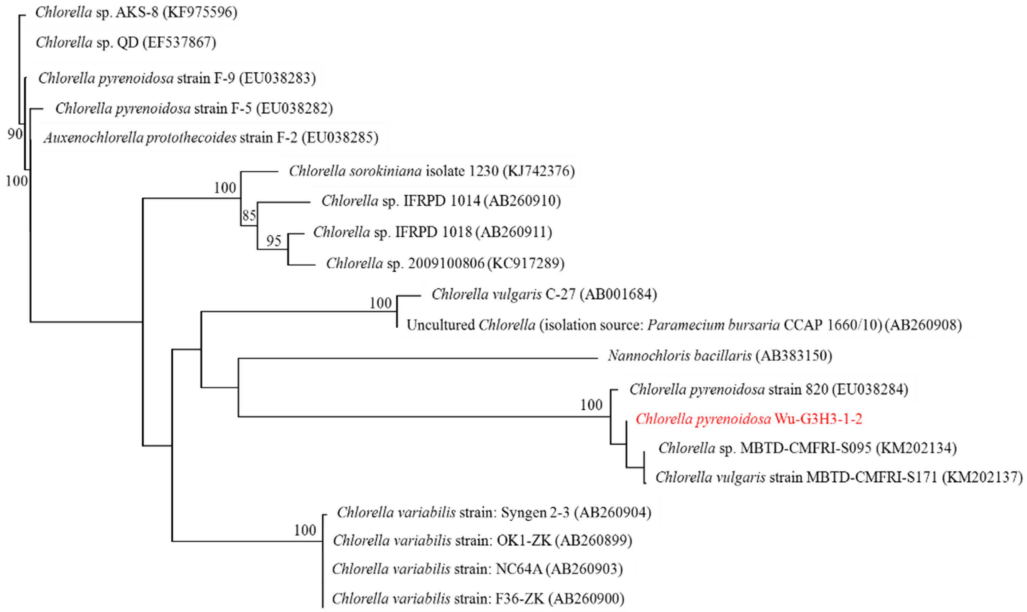

Figure A1. The phylogenetic tree of isolated microalgae.

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