



Article

Enhanced Biomass, Paramylon, and Lipids Production by Non-Axenic Cultivation of *Euglena gracilis* in Anaerobically Digested Livestock Wastewater

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Abstract

Wastewater-based microalgal cultivation enables coupling environmental remediation with the production of sustainable, value-added biomass. In this study, *Euglena gracilis* was cultivated under non-axenic conditions in a 2% anaerobically digested livestock wastewater (LSWW)-based medium to enhance biomass accumulation, paramylon storage, and biodiesel precursor production, while simultaneously removing residual nitrogen and phosphorus. The LSWW medium was strongly phosphate-limited relative to ammoniacal nitrogen (N:P mass ratio ~39:1), which constrained growth. Adjustment of the N:P ratio to ~10:1 by NaH₂PO₄ supplementation, together with MgSO₄·7H₂O addition, significantly enhanced biomass production, whereas trace metals and CaCl₂ provided minimal benefit. Cultivation at an initial pH of 3 resulted in substantially higher biomass accumulation than at pH 7 under xenic conditions. Under these optimized conditions, total phosphate and ammonia were efficiently removed, decreasing from 5.27 to 0.009 mg/L (99.8%) and from 57.40 to 2.11 mg/L (96.3%), respectively. Although paramylon accumulation was low in LSWW alone (~4% dry weight), short-term ethanol supplementation (0.095%, v/v, 24 h) enhanced paramylon content to ~20% dry weight. Subsequent anaerobic treatment further enhanced lipid conversion, increasing fatty acid methyl ester (FAME) content to ~45% dry weight. Collectively, low-pH non-axenic cultivation of *E. gracilis* in LSWW, combined with minimal nutrient supplementation, provides an integrated platform for enhanced biomass, paramylon, and biodiesel precursor production with efficient nutrient removal.

Keywords: livestock wastewater; *Euglena gracilis*; low pH; paramylon; biodiesel production



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1. Introduction

Wastewater from the livestock industry has become a serious environmental problem because of the global growth of animal agriculture [1]. In particular, large-scale livestock operations generate large amounts of wastewater that contains high concentrations of organic matter, odorous compounds, pathogenic microorganisms, and nutrients, and discharge of this waste without adequate treatment can lead to soil contamination and deterioration of water quality [2–4]. This has led to increased interest in the development of technologies that can decrease the environmental burdens of these waste streams and simultaneously convert this waste into usable energy and other valuable resources. Biogas production

systems that utilize livestock wastewater under anaerobic conditions are a promising option for the sustainable valorization of these organic wastes [5], and this has led to the evolution of industrial models that integrate waste management, energy production, and the recycling of agricultural resources [6]. Although biogas production using livestock wastewater is regarded as a sustainable technology that contributes to the valorization of organic waste and energy generation [7], there are concerns regarding the actual operation of the production processes. If nitrogen, phosphorus, pathogenic microorganisms, antibiotic residues, heavy metals, and other contaminants remain in the effluent and in the digestate produced by anaerobic degradation, they can accumulate in agricultural soils and nearby water bodies [8]. This can lead to eutrophication, contamination by pathogens, and ecological disruption of rivers, lakes, and groundwater.

To address these challenges and increase the use of livestock wastewater for production of biogas, it is essential to develop an integrated, economically feasible, and sustainable biological treatment system that removes eutrophic substances (nitrogen and phosphorus) from the digestate and produces value-added products, such as biodiesel. Advanced livestock wastewater treatment technologies that use microalgae are a possible solution, because microalgae can efficiently remove eutrophic substances by photosynthesis, and the remaining biomass can potentially be utilized as biofuels, feed, or fertilizers. Microalgal treatment of wastewater thus has the potential to provide sustainable water purification and resource recovery [9,10].

Euglena gracilis is a microalga that can produce a range of high value-added metabolites, including paramylon starch and carotenoids [11,12]. Anaerobic metabolic pathways can convert paramylon into wax esters [13], and these esters can then be refined and catalytically upgraded to yield a drop-in fuel with physicochemical properties comparable to those of conventional fossil-based jet fuel [14]. Most studies of *Euglena* have used artificial culture media or various types of treated wastewater [15], but most cultivation strategies have employed strictly axenic conditions [16]. However, for practical industrial implementation, it is necessary to develop technologies that allow stable cultivation of *Euglena* under non-sterile (xenic) conditions. It is therefore necessary to develop operational conditions and management strategies that enable the sustainable growth of *Euglena* in wastewater while minimizing the impact of competition and contamination by exogenous microorganisms [17].

In this study, we cultivated *E. gracilis* in anaerobically digested livestock wastewater (LSWW), and then optimized the pH and the concentrations of phosphorus and other nutrients during this process. We then investigated whether ethanol supplementation could enhance as a supplementary carbon source to enhance paramylon accumulation [18], and monitored changes in fatty acid composition caused by the anaerobic conversion of paramylon into wax ester precursors. We also determined decreases in total ammonia nitrogen (TN) and total phosphate (TP) in anaerobically digested livestock wastewater during cultivation to assess the potential efficacy of this method for bioprocessing of wastewater.

2. Material and Methods

2.1. Treatment of Livestock Wastewater

Anaerobically digested livestock wastewater was obtained from an anaerobic digestion facility located in Yangsan, Gyeongsangnam-do, Republic of Korea. The raw sample was first passed through a fine sieve (170 mesh, pore size 0.1 mm) to eliminate coarse particles, and then subjected to centrifugation at 5000 rpm for 5 min using a large-capacity centrifuge (2236R, Labogene) to separate solids. The resulting supernatant was stored at -20°C until use. When needed for experiments, it was thawed at room temperature, and subsequently diluted 50-fold with *Euglena* culture to prepare the LSWW medium.

2.2. Cultivation of *Euglena gracilis*

Euglena gracilis UTEX 367 (hereafter, *Euglena*) was obtained from the UTEX Culture Collection of Algae at the University of Texas at Austin (USA). The growth medium was prepared by supplementing a 50-fold diluted LSWW solution with magnesium, calcium, phosphate, and a trace-metal mix, and the effect of each additive on cell growth was determined. The supplemental stock solutions were added at the following rates: 4 mL/L magnesium stock solution containing 100 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4 mL/L phosphate stock solution containing 100 g/L NaH_2PO_4 ; 1 mL/L metal stock solution containing 50 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 20 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1 g/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$; and 4 mL/L calcium stock solution containing 50 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. To evaluate the effects of other organic and inorganic constituents on cell growth, an LSWW-free synthetic medium was prepared as a reference. In this control medium, NH_4Cl was used as the ammonia source and was added to match the ammonia level of the 50-fold diluted LSWW. All other components had the same concentrations as in the LSWW medium.

The initial pH of the medium was adjusted to 3.8 using a 7% (v/v) HCl solution. For the 'neutral pH' (control condition), no HCl was added and the culture was initiated at pH 7. The pH was not adjusted during cultivation. All pH measurements were performed using a SevenDirect SD20 pH meter (Mettler Toledo Co., Ltd., Greifensee, Switzerland).

Cultivation of *Euglena* was conducted in a cylindrical column photobioreactor with a working volume of 2 L. A hydrophobic syringe filter was installed at the bottom to enable continuous aeration with air containing 3% CO_2 . The culture temperature was maintained at 26 °C, and continuous illumination was provided by a flat LED panel at an incident photon flux density of 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. All experiments were performed under xenic conditions.

To compare the effects of supplemental medium components and initial pH on *Euglena* growth in the LSWW medium, cell counting (cells/mL) and dry cell weight (DCW) were monitored. Cell density (cells/mL) was quantified by hemacytometer-based microscopic counting of *Euglena gracilis* cells under light microscopy. Dry cell weight (DCW) was determined by passing the cell suspension through a 0.45 μm cellulose nitrate membrane filter (Whatman, Clifton, NJ, USA) that was pre-dried and pre-weighed. The retained cells were rinsed with deionized water, dried in an oven at 80 °C for 24 h, and DCW was then determined [19].

2.3. Measurement of Total NH_3 and Total PO_4

To evaluate the removal of nutrients in LSWW during *Euglena* cultivation, the concentrations of total ammonia (T- NH_3) and total phosphate (T- PO_4) were measured at two-day intervals. Collected samples were centrifuged at $4000 \times g$ for 5 min at 25 °C, and the supernatants were used for analysis. T- NH_3 was quantified using an NH_3 analysis kit (HS- $\text{NH}_3(\text{N})$ -L, Humas, Daejeon, Republic of Korea), in which a 0.5 mL supernatant sample was sequentially added to the kit reagents, followed by spectrophotometry (HS-2300PLUS spectrophotometer (Humas, Republic of Korea)). T- PO_4 was analyzed using a PO_4 kit (HS- $\text{PO}_4(\text{P})$ -L, Humas, Republic of Korea), in which a 5 mL of supernatant sample was mixed with reagents and incubated for 10 min, followed by spectrophotometry (HS-2300PLUS, Humas, Republic of Korea).

2.4. Determination of Paramylon

Paramylon content was determined using a gravimetric method [20]. Briefly, suspended solids were first removed from the *Euglena* culture by passing the broth through a fine sieve (250 mesh, pore size 0.063 mm), followed by lyophilization of the filtrate. A 50 mg

sample of the freeze-dried cell powder was suspended in 5 mL of acetone to remove chlorophyll, and the sample was then sonicated for 30 min. The suspension was then subjected to a second round of acetone treatment and sonication under the same conditions, followed by centrifugation at 4000 rpm for 5 min. After discarding the supernatant, the pellet was resuspended in 1% (*w/v*) sodium dodecyl sulfate (SDS) to remove non-paramylon components and then heated at 100 °C for 30 min. The sample was cooled to room temperature, subjected to a second SDS treatment under identical conditions, and then washed twice with distilled water. The final pellet was transferred to a 1.5 mL microtube, centrifuged at 4000 rpm for 5 min, and the supernatant was then discarded. The remaining precipitate was dried at 80 °C overnight and weighed. The paramylon content (%) was calculated as a percentage of DCW. All measurements were performed in triplicate and results are presented as means with standard deviations.

2.5. Anaerobic Digestion

Because anaerobic treatment can increase the production of fatty acid methyl esters (FAMEs) by *Euglena*, anaerobic digestion of the cell culture was performed following an ethanol pretreatment step. Briefly, 95% (*v/v*) ethanol was added to the *Euglena* culture to obtain a final concentration of 0.095% (*v/v*). After 1 day, the culture was transferred to a 2 L vessel, which was completely filled to eliminate headspace, tightly capped, and sealed with tape to prevent the ingress of atmospheric oxygen. The sealed vessel was then placed in a dark room for 3 days to enable dark digestion under anaerobic conditions.

2.6. Lipid Analysis

Total lipid content was analyzed using a modified Folch method [21]. A freeze-dried sample (10 mg) was placed in a screw-cap tube, and 2 mL of a chloroform:methanol mixture (2:1, *v/v*) was added, followed by agitation for 20 min. Subsequently, 1 mL of an internal standard solution (C_{19:0}, Sigma, Livonia, MI, USA; 0.5 mg/mL), 1 mL of methanol, and 300 µL of a sulfuric acid (H₂SO₄) solution were added, and the mixture was incubated at 100 °C for 20 min. After cooling to room temperature, 1 mL of deionized water was added, and the mixture was centrifuged at 896 × *g* for 10 min to separate the organic phase. The organic layer was collected, passed through a 0.2 µm regenerated cellulose membrane syringe filter (Sartorius Stedim Biotech, Göttingen, Germany), and subjected to analysis. The fatty acid methyl ester (FAME) composition was determined by gas chromatography (GC; HP6890, Agilent, Santa Clara, CA, USA) with a flame ionization detector (FID) and an HP-INNOWAX polyethylene glycol capillary column (HP 19091N-213, Agilent, Santa Clara, CA, USA).

Each FAME peak was identified by comparing its retention time with that of an internal standard (F.A.M.E. MIX C8–C24, Supelco, Bellefonte, PA, USA), and quantified by determining its peak area relative to the internal standard. The FAME yield (%) was calculated as:

$$(\text{total FAME weight/biomass weight}) \times 100\%$$

3. Results and Discussion

3.1. Effect of Supplemental Phosphate on Growth of *Euglena* in LSWW Medium

We first cultivated *E. gracilis* in a basal medium containing 2% LSWW with and without phosphate supplementation (0.4 mL/L phosphate stock solution containing 100 g/L NaH₂PO₄) (Figure 1). Growth (Cell concentration) was clearly greater in the medium with phosphate supplementation. In particular, the P-supplemented group had about 2.0-fold greater growth on day 2, 1.6-fold greater growth on day 4, and 1.7-fold greater growth on

day 8. Based on these initial experiments, we used 0.334 mM NaH_2PO_4 supplementation of the 2% LSWW medium in subsequent experiments.

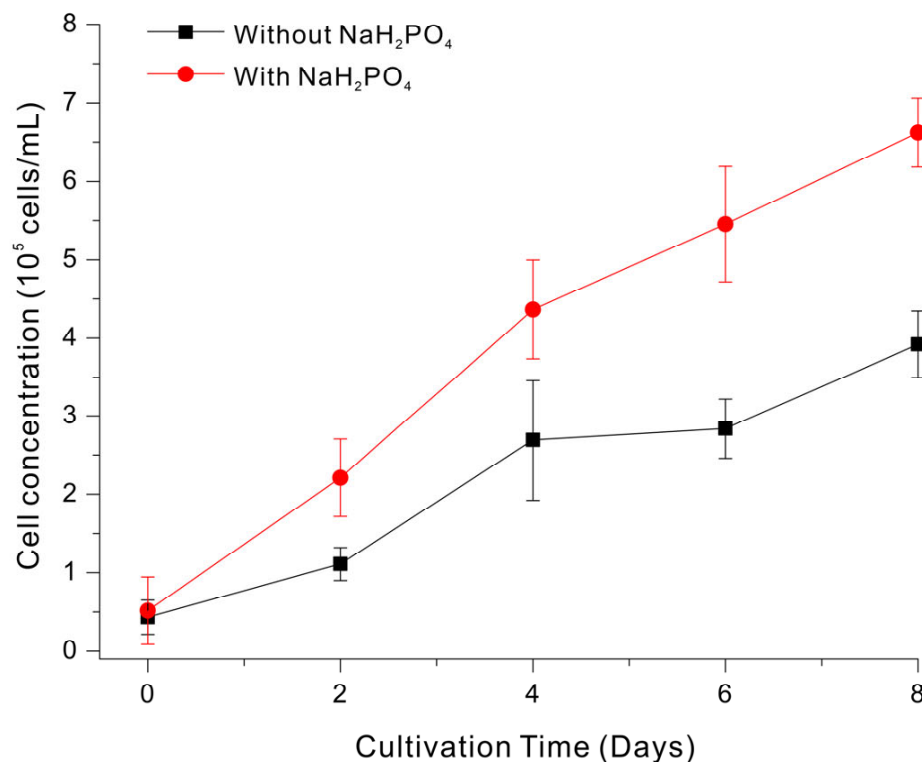


Figure 1. Growth of *Euglena* in 2% LSWW with and without supplemental NaH_2PO_4 .

In LSWW, ammonia nitrogen generally occurs at a higher concentration than phosphate [22]. Similarly, our results demonstrated that phosphate limited *Euglena* growth during cultivation in medium with LSWW, even though there was some growth without phosphate supplementation. Previous studies that used wastewater as a culture medium showed that an imbalanced nitrogen-to-phosphorus (N/P) ratio inhibited growth of the photosynthetic microalgae *Chlorella*, and also proposed adjusting the wastewater N/P ratio as a necessary operational strategy [23].

3.2. Effect of Supplemental Trace Metals and Magnesium on Growth of *Euglena* in LSWW Medium

A trace-metal stock solution containing Zn, Mn, Fe, Cu, and Mo is commonly used to support the cultivation of many microalgae. In particular, Zn is a cofactor for numerous metalloenzymes, such as carbonic anhydrase, which functions as a CO_2 shuttle and is important in photosynthesis [24]. Mn is an essential constituent of the photosystem II oxygen-evolving complex that drives water oxidation and electron transfer [25]. Fe is essential for chlorophyll metabolism and the photosynthetic and respiratory electron transport chains that rely upon cytochromes, ferredoxins, and Fe-S proteins [26,27]. Cu functions in plastocyanin-mediated electron transfer and in antioxidative defense systems (e.g., Cu/Zn-superoxide dismutase) that mitigate photooxidative stress [28]. Mo is a metal cofactor in key nitrogen assimilation reactions, most notably nitrate reductase and nitrogenase-associated processes, that sustain photosynthesis, maintain redox homeostasis, and support growth of phototrophic microorganisms [29]. Anaerobically digested LSWW generally contains these and other trace metals, but they are generally not in a bioavailable form [30].

We therefore determined whether supplementation of the LSWW medium with trace metals affected cell growth. Magnesium (Mg^{2+}) is considered a macronutrient in microalgal cultivation because it is required at a higher level than trace metals. Mg^{2+} is the central ion

in chlorophyll, provides Mg-ATP for numerous phosphorylation reactions, and is needed in relatively large amounts for ribosome stabilization, enzyme activation, and ionic/osmotic balance [31]. Accordingly, we separately evaluated the effect of supplemental Mg.

Figure 2 shows cell growth under four different conditions: (i) no supplementation ($-MgSO_4$, $-Metals$); (ii) $MgSO_4$ alone ($+MgSO_4$, $-Metals$); (iii) trace metals alone ($-MgSO_4$, $+Metal$); and (iv) combined supplementation ($+MgSO_4$, $+Metals$). Overall, growth was best with combined supplementation, closely followed by Mg supplementation alone; the medium with combined supplementation had greater growth on days 6 to 14. Growth without Mg was much lower, regardless of the presence of trace metals. Notably, the trace-metal mixture only had a positive effect when $MgSO_4$ was present. This suggests that Mg^{2+} supplementation alleviated the primary growth constraint, and supplementation with trace metals further improved growth when supplemental Mg^{2+} was present. Based on these results, we performed subsequent experiments using supplemental phosphate (as described above) and supplemental $MgSO_4$ and trace metals to support *Euglena* growth in LSWW medium.

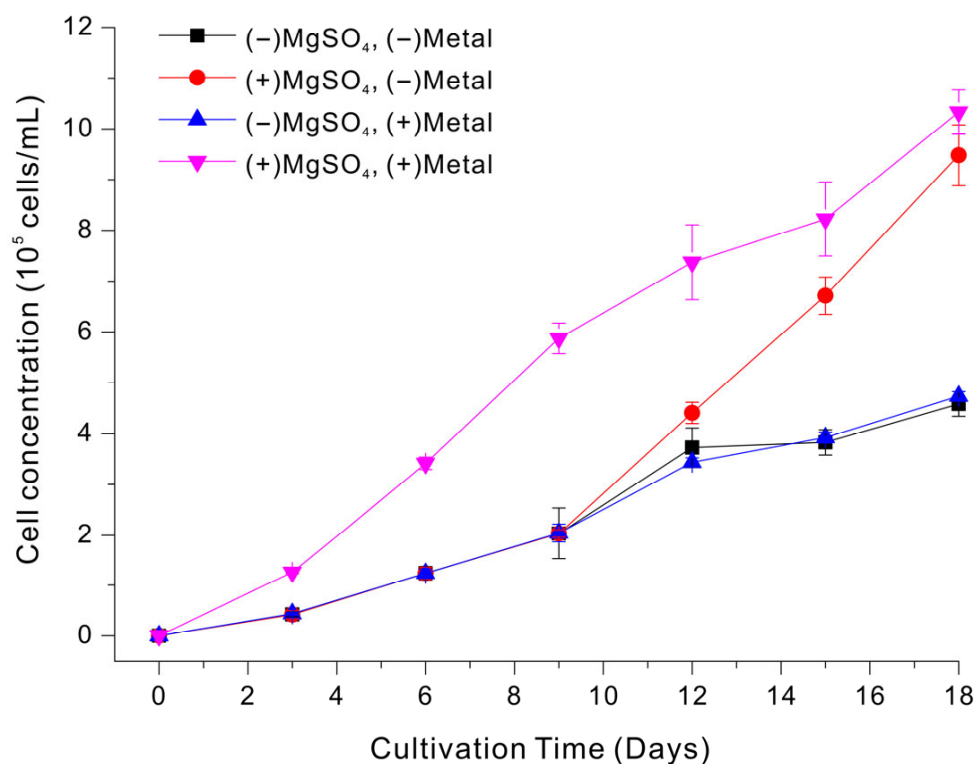


Figure 2. Growth of *Euglena* in 2% LSWW with and without supplemental magnesium and trace metals.

3.3. Effect of Supplemental Calcium on Growth of *Euglena* in LSWW Medium

Ca^{2+} is also considered a macronutrient in microalgal culture media because it is required in large amounts to support core cellular functions, although it also functions as an enzyme cofactor [32]. In particular, Ca^{2+} contributes to membrane and cell-surface stability by neutralizing negative charges on phospholipids and extracellular polymers, and this helps maintain cell integrity, and regulates cell aggregation and adhesion [33]. Ca^{2+} also acts as an intracellular second messenger that regulates key physiological processes, including the activities of photosynthetic and metabolic enzymes, ion transport, stress responses, and cell-cycle progression [34]. Although anaerobically digested LSWW often contains substantial calcium, the proportion of soluble and bioavailable Ca^{2+} can vary widely due to precipitation and phase partitioning [35].

We therefore compared the growth of *Euglena* in LSWW medium with and without supplementation with 0.20 g/L CaCl_2 (Figure 3). Overall, the two growth curves were very similar, and most data points had overlapping error bars, indicating that supplemental CaCl_2 had no clear or consistent benefit. The final cell concentration (day 18) was essentially the same for both treatments ($\sim 1.1\text{--}1.15 \times 10^6$ cells/mL). Taken together, these results suggest that Ca^{2+} was not a limiting factor under the tested conditions, likely because the wastewater already contained sufficient bioavailable Ca^{2+} . Consistently, previous studies of other microalgal cultivation systems reported that the Ca^{2+} in wastewater was sufficient to support cell growth, and that supplementation was not necessary [36].

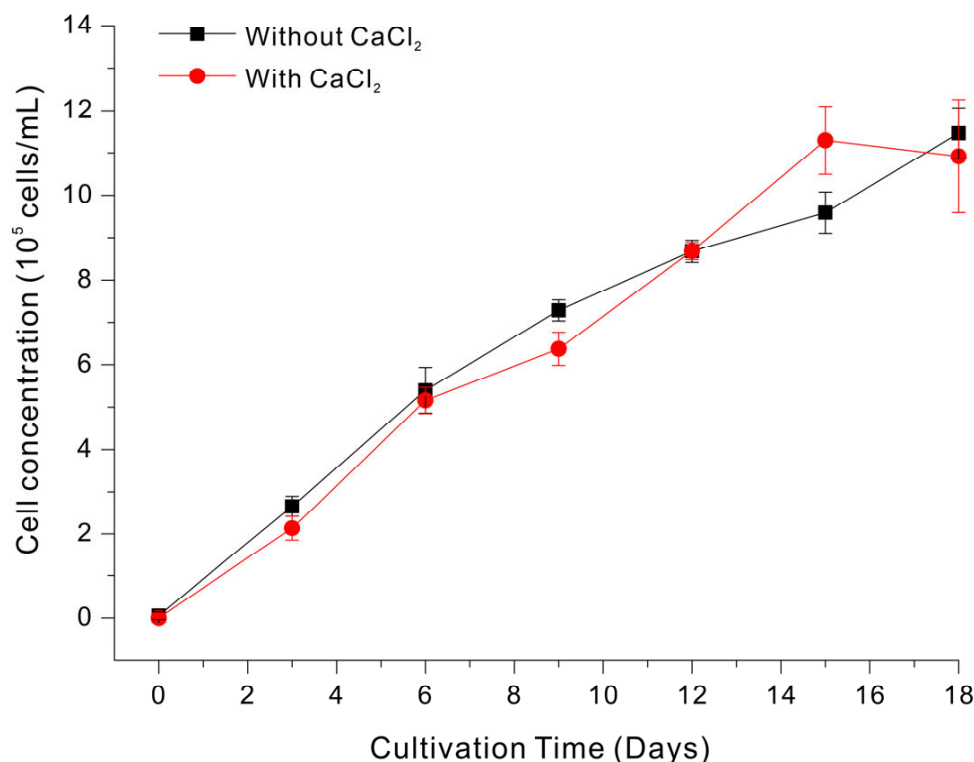


Figure 3. Growth of *Euglena* in 2% LSWW with and without supplemental CaCl_2 .

3.4. Effect of Initial pH on Growth of *Euglena* in LSWW Medium

pH is another key variable that affects the growth of microalgae due to its effect on carbon availability, chemical speciation and bioavailability of nutrients, and core physiological functions, such as enzyme activity and membrane transport [37]. The pH also affects transmembrane electrochemical gradients and nutrient uptake kinetics, and the activities of enzymes involved in photosynthesis and respiration [37]. Although *Euglena*, as a photosynthetic species, typically has maximal growth rate near pH 6 to 7, it has notable acid tolerance and can survive when cultivated at pH ~ 3 to 4 [38,39]. Importantly, a low pH can also inhibit the growth of contaminating organisms [40], because acidic conditions are generally unfavorable for the growth of non-acidophilic bacteria and fungi [41]. Therefore, we examined the effect of pH on growth of *Euglena* in LSWW medium with previously optimized levels of phosphate, Mg, and trace metals.

The growth curves at pH 3 and pH 7 clearly show growth was better at pH 3 (Figure 4). At the end of the experiment (day 18), the cell concentration was approximately $1.1\text{--}1.2 \times 10^6$ cells/mL at pH 3 but was only $\sim 5.4 \times 10^5$ cells/mL at pH 7. Notably, microscopic observations of the cultures grown at pH 7 consistently revealed an abundance of diverse bacteria in addition to *Euglena*. This contamination may explain the decreased growth at a higher pH, because contaminating species can rapidly consume available nutri-

ents (including micronutrients and growth factors), modify the culture microenvironment via respiration and production of metabolites, and increase the turbidity of the growth medium, thereby decreasing light availability and photosynthesis [42,43]. Collectively, these results indicate that growth of *Euglena* at an initial pH 3.0 provides a practical operational advantage for growth in LSWW medium because it suppresses bacterial growth and enables dominance of *Euglena*. This interpretation is consistent with prior studies of *Euglena* which used acidic operating conditions (pH ~3–4.5) to suppress competing microorganisms and maintain *Euglena* dominance in mixed microbial systems [44]. Our subsequent experiments therefore utilized an ‘optimized’ LSWW medium that contained supplemental phosphate, magnesium, and trace metals at pH 3.

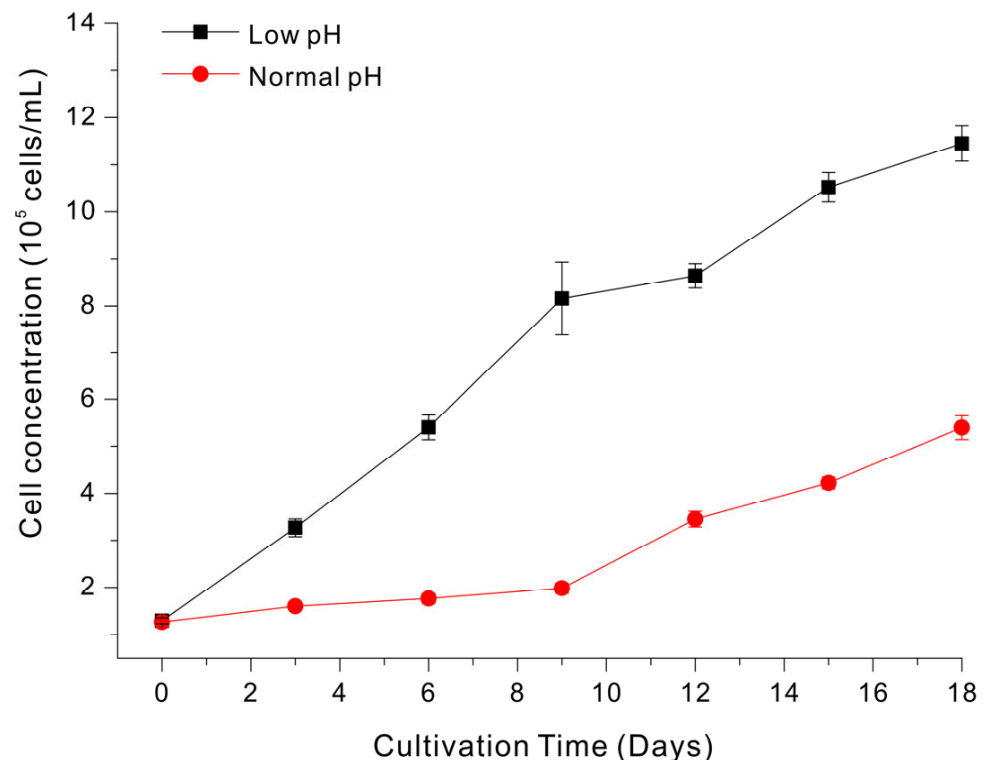


Figure 4. Growth of *Euglena* in 2% LSWW at pH 3 and pH 7.

3.5. Evaluation of LSWW as an Alternative Carbon Source for *Euglena*

We then evaluated LSWW as an alternative carbon source for *Euglena* by cultivating cells in three different media: (i) the ‘optimized’ LSWW medium (described above); (ii) an LSWW-free synthetic (control) medium formulated to match the ammonia level of the ‘optimized’ LSWW medium using NH_4Cl and the same supplements; and (iii) an acetate-supplemented medium prepared by adding 0.8 g/L sodium acetate to the LSWW-free synthetic medium (Figure 5). The results show that the ‘optimized’ LSWW medium supported robust accumulation of biomass, which increased from ~0.08 g/L (at inoculation) to ~0.97 g/L by day 20. In contrast, the LSWW-free medium led to only modest growth, and reached a maximum of ~0.33 g/L on day 16, followed by a slight decline, indicating that matching the ammonia supplement alone was insufficient to achieve the high biomass achieved in LSWW. Notably, supplementation of the LSWW-free medium with acetate markedly increased the production of biomass, and led to a final biomass of ~1.10 g/L at day 20. These results suggest that LSWW medium provides additional growth-promoting substances that are not in the LSWW-free synthetic medium, and that a major component of this benefit may be attributable to readily available organic carbon, which enables mixotrophic growth [45]. In addition, because *Euglena* growth in the acetate-supplemented LSWW-free

medium was similar to that in the ‘optimized’ LSWW medium, it seems likely that LSWW contains a comparable level of available organic carbon.

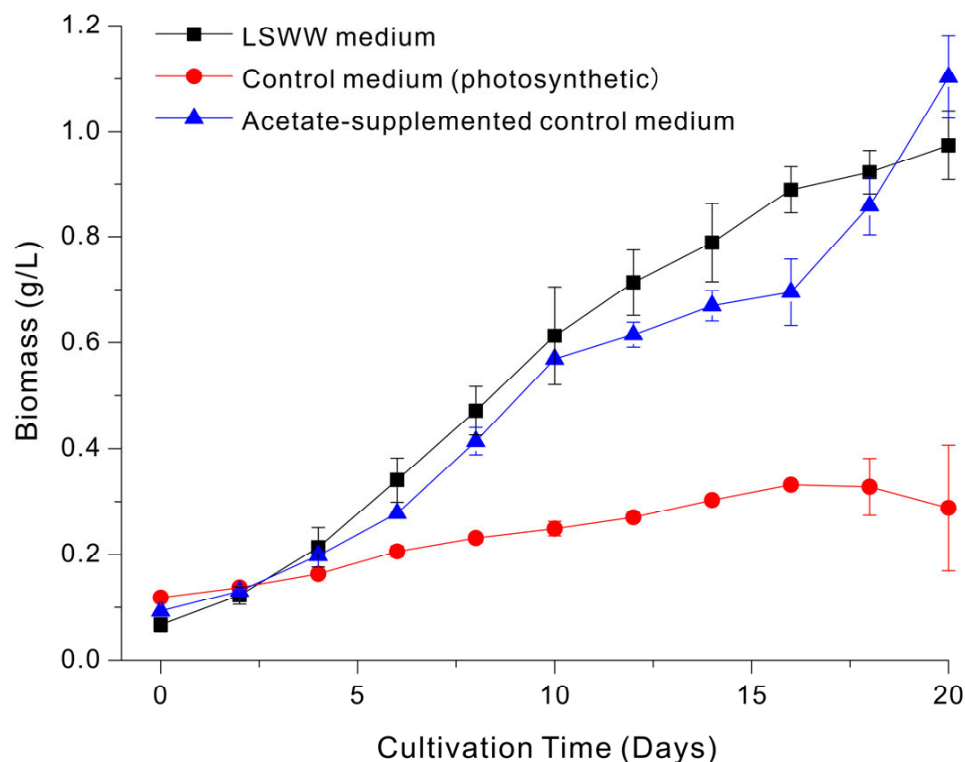


Figure 5. Growth of *Euglena* in ‘optimized’ LSWW medium, LSWW-free photosynthetic medium (control), and LSWW-free medium with supplemental acetate.

3.6. Nutrient Uptake by *Euglena* During Cultivation

Wastewaters from livestock- and food-processing often have high levels of ammoniacal nitrogen and phosphate, and these can cause deterioration of water quality due to eutrophication and algal blooms [46]. There is therefore a growing need for technologies that can efficiently remove nitrogen and phosphorus while producing valuable products. Thus, we evaluated the removal of nitrogen and phosphorus when *Euglena* was grown in the ‘optimized’ LSWW medium. In this ‘optimized’ LSWW medium, the initial concentration of total phosphate (T-PO₄), including supplemental phosphate, was 5.27 mg/L. The T-PO₄ level decreased gradually until day 6, and then declined sharply, with approximately 90% depletion at day 12 and 99.8% depletion at day 20 (Figure 6A). Measurement of total ammonia (T-NH₃) indicated a similar pattern, and there was 96.3% depletion at day 20 (Figure 6B).

Notably, these results indicated the residual percentage of phosphate was 0.19% and the residual percentage of ammonia was 7.2% at day 16 (Figure 6). Consistently, the growth of *Euglena* in the ‘optimized’ LSWW medium began to gradually decline at about this time (Figure 5). This suggests that nutrient depletion likely reduced biomass accumulation at about day 16. Similarly, growth of cells in LSWW without supplemental phosphate led to much slower growth than in phosphate-supplemented LSWW (Figure 1). Prior to phosphate supplementation, the LSWW medium had a phosphate concentration of 1.489 mg/L, markedly lower than the concentration of ammoniacal nitrogen (57.40 mg/L). This highly unbalanced initial N:P mass ratio (39:1) is insufficient for the sustainable growth of *Euglenas* [47]. Our adjustment of the N:P mass ratio to approximately 10:1 enabled efficient removal of ammonia and phosphate and greater accumulation of *Euglena* biomass (Figures 5 and 6). Consistent with these findings, previous studies that cultivated microal-

gae in wastewater also reported that adjusting the initial N:P mass ratio to approximately 10:1 led to increased biomass productivity [48].

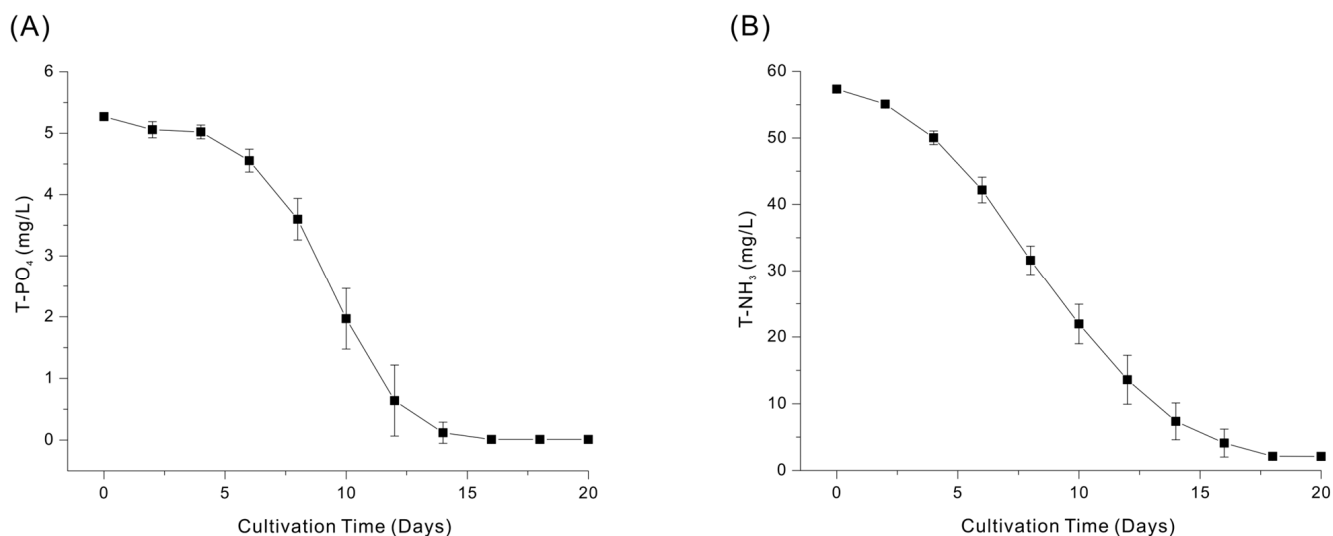


Figure 6. Removal of total phosphate (T-PO₄, (A)) and ammoniacal nitrogen (T-NH₃, (B)) during growth of *Euglena* in ‘optimized’ LSWW medium.

The nitrogen-to-phosphate ratio can vary substantially in different types of wastewater [49] and treatment conditions. Our findings suggest that when cultivating *Euglena* in LSWW, an appropriate supplementation of phosphate is needed to achieve stable growth and consistent process performance.

3.7. Paramylon Accumulation by Cells Grown in ‘Optimized’ LSWW Medium and Then Subjected to Anaerobiosis

Paramylon, a β -1,3-glucan storage polysaccharide that accumulates in *Euglena*, can be converted into hydrocarbons through established downstream upgrading processes, making it a promising renewable intermediate for the production of sustainable aviation fuel (SAF) [14]. The paramylon content in *Euglena* cultivated in ‘optimized’ LSWW was approximately 4%, but was ~17% in the LSWW-free photosynthetic medium (control) medium, although growth in this medium led to a lower biomass (Figure 7A). To compensate for the low paramylon accumulation in the ‘optimized’ LSWW under phototrophic conditions, we added 0.095% ethanol to the LSWW culture. After 24 h, the paramylon content was approximately 20%, nearly a five-fold increase. This suggests that supplementation with an exogenous organic carbon source (ethanol) redirected intracellular carbon allocation toward the accumulation of paramylon [12,18,50].

Fatty acids produced by microalgae can be converted into biodiesel in the form of FAMES (fatty acid methyl esters) through trans-esterification. This is an alternative to conventional diesel, and can also be utilized as a feedstock for value-added chemicals, such as lubricants and surfactants [51,52]. We therefore measured the total FAME content (percent dry weight) of cells cultivated in ‘optimized’ LSWW and in conventional *Euglena* medium. Interestingly, cells grown under different conditions produced about 33 to 35% FAMES by dry weight, despite substantial difference in paramylon production (Figure 7B). Growth of cells in the ‘optimized’ LSWW culture with supplemental ethanol led to a slight decrease in the FAME content to ~30% after 24 h, but a subsequent 3-day anaerobic treatment to induce wax ester conversion increased the FAME content to ~45%, resulting in a fatty acid productivity of 0.0224 g/L/day. This suggests that the paramylon accumulated via ethanol supplementation was subsequently consumed during anaerobic fermentation as a carbon precursor for the production of wax ester-derived FAMES [13].

Euglena experiences inhibition of mitochondrial oxidative respiration under oxygen-limited anaerobic conditions, which restricts the reoxidation of NADH and disrupts intracellular redox balance. To overcome this metabolic constraint, *Euglena* degrades its primary storage carbohydrate, paramylon (β -1,3-glucan), thereby supplying carbon skeletons and reducing equivalents through glycolysis. Paramylon-derived glucose is converted to pyruvate, which is subsequently channeled into acetyl-CoA formation under anaerobic conditions. This acetyl-CoA is then redirected into a malonyl-CoA-independent fatty acid biosynthetic pathway, and the synthesized fatty acids are esterified with alcohols to accumulate as wax esters [53]. In this pathway, fatty acid chain elongation is limited, leading to the preferential accumulation of short- to medium-chain saturated fatty acids such as C_{13:0} and C_{14:0} rather than long-chain fatty acids [54,55]. As a result, anaerobiosis-driven conversion of paramylon into wax esters is manifested as increased C_{13:0} and C_{14:0} contents in the FAME profile (see Section 3.8).

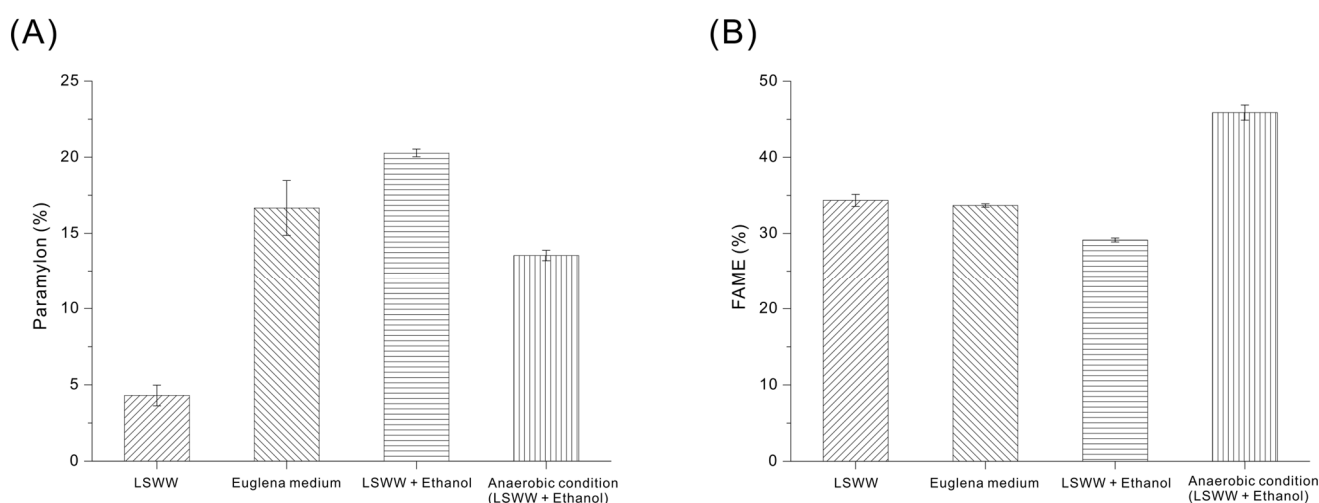


Figure 7. Production of paramylon (A) and total FAMES (B) by *Euglena* under different cultivation conditions. Numbers indicate percent dry weight. The data were analyzed using one-way ANOVA, followed by Duncan's test ($p < 0.05$). Lowercase letters indicate statistically significant differences among groups.

3.8. Fatty Acid Profile of *Euglena* Cultivated in 'Optimized' LSWW and Modification by Anaerobiosis

We then compared the fatty acid composition of cells cultivated in 'optimized' LSWW with ethanol with that of cells grown in the synthetic control medium. These two growth conditions led to similar overall FAME profiles, in that C_{14:0}, C_{16:0}, and C_{18:0} were the major saturated fatty acids and C_{18:1} and C_{20:4} were the major unsaturated fatty acids (Table 1). These results demonstrate that a LSWW medium can function as an alternative cultivation platform for the production of FAMES, even though the growth medium had significant effects on the production of paramylon [56].

We then examined the effect of a subsequent 3-day anaerobiosis treatment on the FAME profile of *Euglena* cells (Table 2). The results indicated that anaerobiosis led to a marked shift in the FAME composition. Specifically, C_{13:0} increased substantially from ~3% before treatment to ~17% after treatment, and C_{14:0} increased from ~9% before treatment to ~17% after treatment, indicating that these short- to medium-chain saturated fatty acids became major components. In contrast, the long-chain saturated fatty acid C_{16:0} decreased from ~18% to ~12%, and the monounsaturated fatty acid C_{18:1} decreased from ~11% to ~5%. Thus, a 3-day anaerobiosis treatment altered the FAME composition from a profile dominated by longer-chain and unsaturated species toward one dominated by short- to medium-chain saturated fatty acids, such as C_{13:0} and C_{14:0}. Notably, saturated fatty acids

in the C₁₃ to C₁₄ range can be readily converted into short-chain alkanes within the jet-fuel boiling range after hydrogenation and deoxygenation [54,57]. Thus, cultivation of *Euglena* in 'optimized' LSWW followed by anaerobiosis may provide a promising feedstock for microalgae-derived SAF.

Table 1. Fatty acid profile of *Euglena* grown in 'optimized' LSWW and synthetic medium.

Fatty Acid	LSWW (%)	Synthetic Medium (%)
Saturated		
C _{12:0}	0.63 ± 0.02 ^{mno}	0.62 ± 0.01 ^{mno}
C _{13:0}	1.33 ± 0.06 ^k	0.97 ± 0.01 ^{lm}
C _{14:0}	6.55 ± 0.23 ^f	10.39 ± 0.19 ^b
C _{16:0}	9.67 ± 0.10 ^d	9.25 ± 0.21 ^e
C _{17:0}	0.23 ± 0.00 ^p	
C _{18:0}	2.71 ± 0.05 ^j	2.45 ± 0.11 ^j
Unsaturated		
C _{15:1} ω5c	0.68 ± 0.37 ^{mn}	0.30 ± 0.33 ^{op}
C _{15:1} ω8c	0.24 ± 0.01 ^p	
C _{16:1} ω7c alcohol	1.17 ± 0.02 ^{kl}	0.74 ± 0.01 ^{mn}
C _{17:1} ω8c	0.69 ± 0.00 ^{mn}	
C _{18:1} ω5c	10.99 ± 0.19 ^a	10.00 ± 0.10 ^c
C _{20:4} ω6,9,12,15c	5.74 ± 0.28 ^h	6.14 ± 0.33 ^g
Branched-chain fatty acid		
C _{20:0} iso	1.13 ± 0.16 ^{kl}	0.49 ± 0.02 ^{nop}
C _{19:0} anteiso	0.15 ± 0.03 ^p	0.64 ± 0.04 ^{mno}
Other		
C _{16:0} N alcohol		3.40 ± 0.05 ⁱ

The data were analyzed using one-way ANOVA, followed by Duncan's test ($p < 0.05$). Lowercase letters indicate statistically significant differences among groups.

Table 2. Fatty acid profile of *Euglena* grown in LSWW and then subjected to anaerobiosis.

Fatty Acid	Before Anaerobiosis (%)	After Anaerobiosis (%)
Saturated		
C _{11:0}	0.15 ± 0.06 ^k	0.58 ± 0.21 ^k
C _{12:0}	1.12 ± 0.23 ^{jk}	2.90 ± 0.69 ^{hij}
C _{13:0}	3.36 ± 0.51 ^{ghi}	17.10 ± 2.89 ^a
C _{14:0}	8.84 ± 0.56 ^c	16.77 ± 1.77 ^a
C _{16:0}	17.64 ± 0.98 ^a	11.88 ± 0.95 ^b
C _{17:0}	0.40 ± 0.06 ^k	0.25 ± 0.04 ^k
C _{18:0}	1.83 ± 0.18 ^{ijk}	1.36 ± 0.01 ^{jk}
Unsaturated		
C _{14:1} ω5c	0.15 ± 0.01 ^k	0.16 ± 0.02 ^k
C _{16:1} ω7c alcohol	1.18 ± 0.27 ^{jk}	0.94 ± 0.13 ^k
C _{17:1} ω8c	0.29 ± 0.01 ^k	0.20 ± 0.01 ^k
C _{18:1} ω5c	0.56 ± 0.44 ^k	1.10 ± 0.52 ^{jk}
C _{18:1} ω9c	11.32 ± 0.76 ^b	4.90 ± 0.53 ^{efg}
C _{18:3} ω6c (6,9,12)		0.26 ± 0.05 ^k
C _{20:1} ω7c	1.69 ± 0.04 ^{ijk}	1.85 ± 0.11 ^{ijk}
C _{20:4} ω6,9,12,15c	5.96 ± 0.16 ^{de}	5.04 ± 0.01 ^{efg}
Branched-chain fatty acid		
C _{15:1} anteiso A	1.81 ± 0.04 ^{ijk}	
C _{19:0} anteiso		0.16 ± 0.01 ^k
Other		
C _{16:0} N alcohol	4.01 ± 0.35 ^{fgh}	5.13 ± 0.21 ^{ef}

The data were analyzed using one-way ANOVA, followed by Duncan's test ($p < 0.05$). Lowercase letters indicate statistically significant differences among groups.

4. Conclusions

In this study, we established optimal operational conditions for the stable cultivation of *Euglena* under non-sterile conditions using 2% LSWW by evaluating the removal of nitrogen and phosphorus and the production of biodiesel precursors. The results demonstrated that the LSWW had an imbalanced nutrient composition (a deficiency of phosphate and an excess of ammoniacal nitrogen), and that phosphate supplementation markedly improved cell growth and ammonia removal. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ supplementation was critical for obtaining high cell proliferation, but CaCl_2 supplementation only had a negligible effect. Moreover, acidification of the medium to pH 3 increased cell growth, and led to approximately double than at pH 7, likely due to acid-suppression of competing microorganisms. Finally, growth in the 'optimized' LSWW medium led to a similar level of biomass as growth in LSWW-free medium with acetate supplementation. This suggests that LSWW provides carbon and other essential nutrients needed for *Euglena* growth.

Our analysis of nutrient removal by cells grown in 'optimized' LSWW medium indicated that the total phosphate (T-PO_4) decreased from approximately 5.27 mg/L to 0.009 mg/L (99.8%) and the total ammonia (T-NH_3) decreased from 57.40 mg/L to 2.11 mg/L (96.3%). This confirmed that *Euglena* can provide sufficient water purification when cultivated in LSWW to produce biogas. In parallel, the fatty acid profiles were similar for cells grown in LSWW medium and synthetic medium. When ethanol was supplied as an auxiliary carbon source in the LSWW medium, the intracellular paramylon content increased by approximately 4- to 5-fold. When a subsequent anaerobic treatment was applied, the FAME content increased to ~45%, and there was a shift toward $\text{C}_{13:0}$ - and $\text{C}_{14:0}$ -dominant short- to medium-chain saturated fatty acids. This suggests that paramylon, which accumulates during the aerobic phase, can be metabolically converted into wax ester precursors under anaerobic conditions, potentially enabling the production of lipids with carbon-chain lengths and saturation levels suitable for the production of SAF.

Our *Euglena*-based cultivation system links the treatment of LSWW, recovery of renewable energy-related resources, and provision of SAF feedstock within a single process framework. This system therefore has the potential for development into a closed-loop industrial system that integrates the livestock, energy, and environmental sectors. However, further verification at a large-scale and long-term operational studies are required to validate the applicability and industrial scalability of this system.

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