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Sustainable Lutein Production from *Chlorella sorokiniana* NIES-2168 by Using Aquaculture Wastewater with Two-Stage Cultivation Strategies

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Abstract: Unnecessary discharge of nutrient-rich aquaculture wastewater is a significant threat to the environment, exacerbating massive resource wasting worldwide. Microalgae-based circular economy strategies utilize atmospheric CO_2 and aquaculture wastewater nutrients and convert them into valuable compounds. Lutein, a natural pigment, is a nutritional supplement for eye protection, anti-cancer and anti-inflammatory effects, and other health benefits. It is widely utilized in the food and pharmaceutical industries. The primary purpose of this study is to reuse aquaculture wastewater to grow microalgae and optimize conditions to achieve a high yield of lutein as well as the removal of nutrients from wastewater. When cultured in 1.0× BG11 nutrient-added aquaculture wastewater and aerated using 2% CO_2, the biomass concentration and lutein content of *Chlorella sorokiniana* NIES-2168 increased to 1.78 g L^{-1} and 7.43 mg g^{-1}, respectively. A two-stage culture strategy further increased the lutein content and yield of microalgae. The highest lutein content of 13.95 mg g^{-1} and lutein productivity of 3.63 mg L^{-1} d^{-1} in the second stage aligned with other phototrophic microalgae currently used for lutein production. *C. sorokiniana* NIES-2168 also showed exceptional nitrogen and phosphorus removal efficiency, with nitrate and phosphate removal rates reaching 96.07% and 96.75% during the two-stage culture process.

Keywords: aquaculture wastewater; microalgae; lutein; two-stage

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1. Introduction

With increasing consumer health awareness, natural products accompanied by health benefits and nutraceuticals have been sought after by consumers recently [1]. Carotenoids are a class of natural pigments with bioactive properties and health-promoting effects on various life forms [2]. Specific carotenoids have positive health effects on heart disease, cancer, vision protection, obesity, the immune system, and cognitive function [3].

Lutein, as an important carotenoid, is widely available across diverse plants and microalgae [4]. It acts as an accessory pigment in chloroplasts, playing an indispensable part in light harvesting, energy transfer, and protecting photosystems from oxidative damage during high light stress [5,6]. In humans, lutein is present in the macular region of the retina with strong antioxidant activity, protecting the eyes from blue light damage [6]. Several studies have indicated that lutein has benefits in the prevention and treatment of eye diseases such as cataracts, glaucoma, and AMD (age-related macular degeneration) [7]. Furthermore, lutein has also been shown clinically to have anti-tumor and anti-inflammatory benefits [8–11]. Thus, it has been developed as a nutraceutical with wide use in the food and pharmaceutical fields. The global market for lutein in 2022 was USD 324 million and is predicted to reach USD 491 million by 2029. The compound annual growth rate

(CAGR) of the lutein market from 2023 to 2029 will be 6.1% (Lutein Market: Global Industry Analysis and Forecast (2023–2029) (maximizemarketresearch.com). Currently, the only commercial supply of lutein comes from the petals of marigold flowers [5]. However, as a land plant, marigold harvesting is limited by seasonality and relies heavily on skilled labor for petal processing [5]. Recently, microalgae have attracted increasing attention as potential alternative options for lutein production [1,2,12].

Microalgae are the most diverse microorganisms globally, with outstanding photosynthetic efficiency and growth rates 5–10 times faster than those of terrestrial plants [13]. Microalgae are CO_2-starved biorefineries, storing 1.58 kg of CO_2 per kg of microalgae [5] and generating diverse valuable bioproducts, including lipids, protein, carbohydrates, polyunsaturated fatty acids, and pigments [14–17]. Presently, high-value-added compounds from microalgae are a promising market as nutritional supplements, such as astaxanthin from *Haematococcus pluvialis* [18], docosahexaenoic acid (DHA) from *Schizochytrium* sp. [19], and β-carotene from *Dunaliella salina* [20]. Microalgae are adaptable to the environment and can grow in fresh water, brackish water, seawater, and even extreme environments (such as extreme temperatures and saline environments) [1]. As a result, microalgae-based lutein production does not need to compete with conventional agriculture for arable land or water. It also provides additional CO_2 absorption benefits, increasing its sustainability. Numerous species, such as *Chlorella* sp. [6,21,22], *Scenedesmus* sp. [23], and *Acutodesmus* sp. [24], have been employed for the production of lutein, as they have higher lutein contents (3–17 mg g^{-1}) than marigolds (1.7–5.7 mg g^{-1}) [2]. Moreover, microalgae's growth is not restricted by seasonal changes, and microalgal biomass can be harvested throughout the year [25].

Today, the circular economy (CE) has emerged as a promising approach to addressing the intensive consumption of finite energy and resources accompanied by the need to reduce pollutant emissions. The circular economy aims to reduce material use and waste generation by recycling and reusing [26]. Microalgal cultivation requires a huge amount of water and nutrients. Therefore, the application of nutrient-rich wastewater to microalgal cultivation will limit the dependence on water resources and also serve as a green approach to recycling the nutrients from wastewater. Nutrients, including nitrogen and phosphorus, in wastewater can be absorbed by microalgae and converted into valuable biomass [27]. There have been studies on microalgae cultured in diverse wastewater systems, including cocktail wastewater [28], aquaculture wastewater [29], piggery wastewater [30], and industrial wastewater [31].

The aquaculture industry has grown rapidly in recent years due to the significant increase in global demand for aquatic products. It is estimated that the future total fish supply will increase to 186 million tons in 2030, with aquaculture contributing to this growth [32]. However, with the increase in aquatic product output and continuous expansion of aquaculture, a massive amount of aquaculture wastewater will be discharged into the surrounding environment directly. This will cause serious environmental pollution and a waste of valuable resources. By utilizing aquaculture wastewater for microalgae culturing, microalgae can absorb nutrients from the wastewater, reducing the impact of direct discharge on the surrounding environment. Moreover, the freshwater required for microalgae culture is greatly reduced.

Conditions for microalgal growth and metabolite accumulation do not always align, so two-stage culture strategies have been developed to attain high yields of valuable compounds in microalgae. Presently, the two-stage culture strategy has been widely employed in microalgae to generate lipids, high-commercial-value pigments, and important polyunsaturated fatty acids [33]. Several studies have applied a two-stage culture approach to the production of lutein. For example, Li et al. [23] adopted a two-stage culture strategy to cultivate *Scenedesmus* FSP3 in photoautotrophic mode, applying salt stress and high light stress in the second stage to acquire a maximal lutein productivity of 2.30 mg L^{-1} d^{-1}. Moreover, Chen et al. [34] used a two-stage mixotrophic/photoautotrophic culture of *Chlorella sorokiniana* MB-1 to achieve a maximum lutein yield of 7.62 mg L d^{-1} in the second

stage. However, the use of a two-stage culture approach to microalgae for lutein production in wastewater has not been documented. This study integrated wastewater treatment with lutein production to enhance microalgae-based lutein production for cost-effectiveness and environmental benefit.

This study aimed to reuse aquaculture wastewater to grow lutein-rich microalgae and attain a high production of lutein by optimizing the culture conditions and strategy. The specific objectives of this study were: (1) to explore appropriate nutritional and aeration conditions for microalgae's cultivation in wastewater, and (2) to explore a new culture strategy to achieve the efficient production of lutein.

2. Materials and Methods

2.1. Microalgae Species and Cultivation

The microalgal strain employed in this study was *Chlorella sorokiniana* NIES-2168, provided by Shen Xiaofei (Anhui Normal University, Wuhu, China). *C. sorokiniana* NIES-2168 was pre-cultured using BG-11 medium in 0.5 L modified aerated flasks under continuous fluorescent light with a light intensity of 120 μmol m^{-2} s^{-1}, then aerated with air at 0.2 L min^{-1}. The temperature was kept at 26 ± 1 °C throughout the culture process.

2.2. Source of Aquaculture Wastewater and Nutrient Analysis

The aquaculture wastewater (AW) utilized in this study was obtained from Shanghai Guang Ming Fishery Co. Ltd. (Shanghai, China) The AW was obtained from ponds where longcat fish were farmed, using brackish water, with a consistent daily discharge of a certain amount of water. The AW was first filtered through a 1000 mesh filter to exclude large, insoluble particles, then filtered through a mixed cellulose ester membrane (BD, pore size 0.45 mm). The isolated wastewater was stored in a refrigerator at 4 °C for subsequent experiments.

For nutrient assessment of the aquaculture wastewater, three parallel samples were obtained from AW for measurements. The pH of the AW was determined using a PB-10 (Sartorius, Goettingen, Germany) pH meter. ASCIONIX SSM 2500 hand-held salinometer (WAHENYIDA, Beijing, China) was used to characterize the salinity of the AW. To determine the total nitrogen and total phosphorus content, the samples were digested at 120 °C first and then subjected to analysis using a Smartchem 200 fully automatic intermittent chemical analyzer (AMS, Caserta, Italy). To determine nitrate-N (NO_3^--N), nitrite-N (NO_2^--N), ammonia-N (NH_4^+-N), and phosphate (PO_4^{3-}-P), each sample was first centrifuged at 1157× *g* for 5 min, and the obtained supernatant was analyzed using a Smartchem 200 fully automatic intermittent chemical analyzer (AMS, Caserta, Italy). The chemical oxygen demand (COD) was identified using a DRB-200 COD quick-analysis apparatus (HachChina, Shanghai, China)). The metal element (K, Na, Ca, Mg, Zn, Cu, Mo, Mn, Co, and Fe) content was analyzed using AVIO 200 ICP-OES (Perkin Elmer, Waltham, Massachusetts). The difference between the initial and final concentrations of nutrients was utilized to calculate the nutrient removal rate from the wastewater.

2.3. Experimental Design to Optimize Nutrient Addition Concentration

The growth and biomass accumulation of microalgae were increased by supplementing extra nutrients in the AW. Six different concentration groups (0, 0.2×, 0.4×, 0.6×, 0.8×, and 1.0×) were established, and BG11 culture medium was added as the control group to assess the optimal concentration of nutrients. The nutrients were identical to the components of BG11.

All groups were grown under the same conditions: continuous white light irradiation for 24 h, light intensity of 120 μmol m^{-2} s^{-1}, temperature maintained at 26 ± 1 °C, and aerated with air (0.2 L min^{-1}).

2.4. Experimental Design for Optimization of CO_2 Aeration

The aeration conditions of microalgae grown in AW + 1.0× nutrients were optimized. Varying concentrations of CO_2 were introduced into the AW, with a concentration gradient of 0.03%, 2%, 5%, 10%, and 20%, using an aeration rate of 0.2 L min^{-1}. The culture was maintained at 26 ± 1 °C with continuous white light irradiation. The light intensity was 120 μ mol m^{-2} s^{-1}.

2.5. Two-Stage Culture of Microalgae

A two-stage culture approach was employed to further elevate the lutein levels and productivity of *C. sorokiniana* NIES-2168. In the first stage, microalgae were grown under conditions conducive to biomass production, while in the second stage, stress conditions were employed to promote lutein accumulation. The specific procedures were as follows:

Prior to the start of the two-stage approach, *C. sorokiniana* NIES-2168 was pre-cultured in BG11 medium. When the microalgae reached the logarithmic growth stage, the algal cells were collected and re-suspended for inoculation.

In the first stage, *C. sorokiniana* NIES-2168 was inoculated into AW + 1.0× nutrients at an initial density of 0.1 g L^{-1} supplemented with 2% CO_2 at an aeration rate of 0.2 L min^{-1}. The culture was incubated under continuous illumination with white LED light at an intensity of 120 μmol m^{-2} s^{-1}, and the temperature was maintained at 26 ± 1 °C.

When *C. sorokiniana* NIES-2168 reached its plateau stage and the biomass concentration reached approximately 1.8–2.0 g L^{-1}, the microalgae were transferred to the second stage of culture. In this stage, the culture medium was unchanged, and the light source was converted to blue LED at the same intensity (120 μmol m^{-2} s^{-1}). The microalgae were grown with air (0.2 L min^{-1}), and the temperature was maintained at 26 ± 1 °C. The total incubation time was 12 days.

2.6. Microalgal Growth and Biomass Production

The biomass concentration of *C. sorokiniana* NIES-2168 was determined according to the optical density of microalgae culture samples at 680 nm. The equation for the biomass concentration of *C. sorokiniana* NIES-2168 and OD_{680} was established via linear regression, as shown in Equation (1):

Daily, 5 mL of the culture was diluted to a suitable concentration, and its optical density at 680 nm was assessed using a UV1000 UV-Vis spectrophotometer (Techcomp, Shanghai, China). Then, the dry cell weight per liter of *C. sorokiniana* NIES-2168 was calculated according to Formula (1), R^2 = 0.9974.

$$Biomass\ concentration(g\ L^{-1}) = 0.2136 \times OD_{680} \quad (1)$$

The biomass productivity was calculated using Formula (2).

$$Biomass\ bioproductivity\ (g\ L^{-1}d^{-1}) = \frac{C_2 - C_1}{T_2 - T_1}$$

where C_2 and C_1 are the biomass concentrations of microalgae on T_2 and T_1 (days), respectively.

2.7. Extraction and Determination of Lutein

The extraction of lutein from the microalgae was conducted following a protocol established by Xie et al. [35], with some modifications. A sample of 10 mg lyophilized microalgal biomass was weighed and placed into a centrifuge tube, and an appropriate amount of glass beads 0.2 mm in diameter was added and mixed with 1 mL of 60% (*w*/*w*) KOH aqueous solution. The lyophilized algal powder was crushed in a grinder at 65 Hz for 10 min, and the mixture was then placed in a water bath at 40 °C for 40 min. After the mixture was cooled to room temperature, the lutein was extracted using anhydrous ether; the cells were crushed (65 Hz, 3 min) and centrifuged (3214× *g*, 2 min); and the supernatant was removed. Extraction was repeated until the supernatant was nearly

colorless. All extracts were pooled and dried under nitrogen and re-solubilized with 3 mL of chromatographic-grade acetone.

Following extraction, the lutein content was determined using HPLC (Agilent 1260 Infinity II, Waldbronn, Germany). The chromatographic column utilized was the Eclipse XDB-C18 (4.6 mm × 150 mm × 5 μm). The binary mobile phase consisted of phase A: methanol, acetonitrile, and water (8:1:1, *v*/*v*/*v*) and phase B: methanol and acetonitrile (4:6, *v*/*v*). The extracts were eluted using a flow rate of 0.8 mL min^{-1}, and lutein was detected via absorbance at the wavelength range of 220–750 nm. The lutein content was precisely determined at the peak area at the maximal absorbance (447 nm).

2.8. Statistical Analysis

All experiments were conducted in triplicate. The statistical results were processed using IBM SPSS software (version 26) and are presented as mean ± standard deviation (SD). One-way ANOVA was used to determine the presence of significant differences between the mean values based on the assumption of normality and the assumption of chi-square. $p < 0.05$ indicated a significant difference. The LSD test was employed to construct multiple comparisons between the mean values of the biomass productivity of result 2 and the lutein content of result 3.

3. Results

3.1. Chemical Composition of Aquaculture Wastewater

To use aquaculture wastewater recycling for the growth of microalgae, the main chemical composition of the wastewater must first be clarified. As outlined in Table 1, the aquaculture wastewater was slightly alkaline, with a pH of 7.71 (Table 1), suitable for the growth of microalgae. However, the nutrients in the aquaculture wastewater were insufficient for the growth of *C. sorokiniana* NIES-2168, with total nitrogen and total phosphorus contents of only 5.68 mg L^{-1} and 0.21 mg L^{-1} (Table 1), respectively. Aquaculture wastewater typically has essential metal elements such as K, Ca, Na, Mg, and Zn, but some trace elements such as Cu, Co, Mn, and Fe, which are also essential for the growth of microalgae, were undetected. (Table 1). Therefore, the aquaculture wastewater had to be supplemented with appropriate concentrations of nutrient salts to satisfy the growth conditions of microalgae.

Table 1. Characteristics of the aquaculture wastewater.

Items	Mean ± SD
pH	7.71 ± 0.05
TN (mg L^{-1})	5.68 ± 0.053
TP (mg L^{-1})	0.21 ± 0.008
Salinity %	0.14
COD (mg L^{-1})	270 ± 25
NH_4^+-N (mg L^{-1})	0.697 ± 0.051
NO_2^--N (mg L^{-1})	0.055 ± 0.006
NO_3^--N (mg L^{-1})	3.168 ± 0.281
K (mg L^{-1})	36.41 ± 0.23
Na (mg L^{-1})	504.6 ± 3.6
Ca (mg L^{-1})	73.45 ± 0.05
Mg (mg L^{-1})	67.98 ± 0.12
Zn (mg L^{-1})	0.018
Cu (mg L^{-1})	_
Co (mg L^{-1})	_
Mn (mg L^{-1})	_
Mo (mg L^{-1})	_
Fe (mg L^{-1})	_

3.2. *C. sorokiniana* NIES-2168 Cultured in Aquaculture Wastewater with Nutrient Addition

To enhance the growth and biomass concentration of *C. sorokiniana* NIES-2168, different ratios (0, 0.2, 0.4, 0.6, 0.8, and 1×) of BG11 nutrients were supplemented to the aquaculture wastewater used for the growth of *C. sorokiniana* NIES-2168. BG11 medium and raw aquaculture wastewater (AW) were used as controls.

The addition of nutrients promoted the growth of *C. sorokiniana* NIES-2168 in aquaculture wastewater. As illustrated in Figure 1, with increasing concentrations of additional nutrients (0, 0.2, 0.4, 0.6, 0.8, and 1.0×), the biomass concentration and productivity of *C. sorokiniana* NIES-2168 increased significantly. The biomass concentrations on day 10 were 0.29 g L^{-1}, 0.53 g L^{-1}, 0.58 g L^{-1}, 0.68 g L^{-1}, 0.72 g L^{-1}, and 0.82 g L^{-1} (Figure 1A), respectively, and the biomass productivity levels were 0.029 g L^{-1} d^{-1}, 0.053 g L^{-1} d^{-1}, 0.058 g L^{-1} d^{-1}, 0.068 g L^{-1} d^{-1}, 0.072 g L^{-1} d^{-1}, and 0.082 g L^{-1} d^{-1}, respectively (Figure 1B). The maximum biomass concentration (0.82 g L^{-1}) and productivity (0.082 g L^{-1} d^{-1}) were identified in the group with 1.0× nutrients added to the wastewater (AW + 1.0× nutrients) (Figure 1A,B), 2.85-fold higher than microalgae growing without extra nutrients.

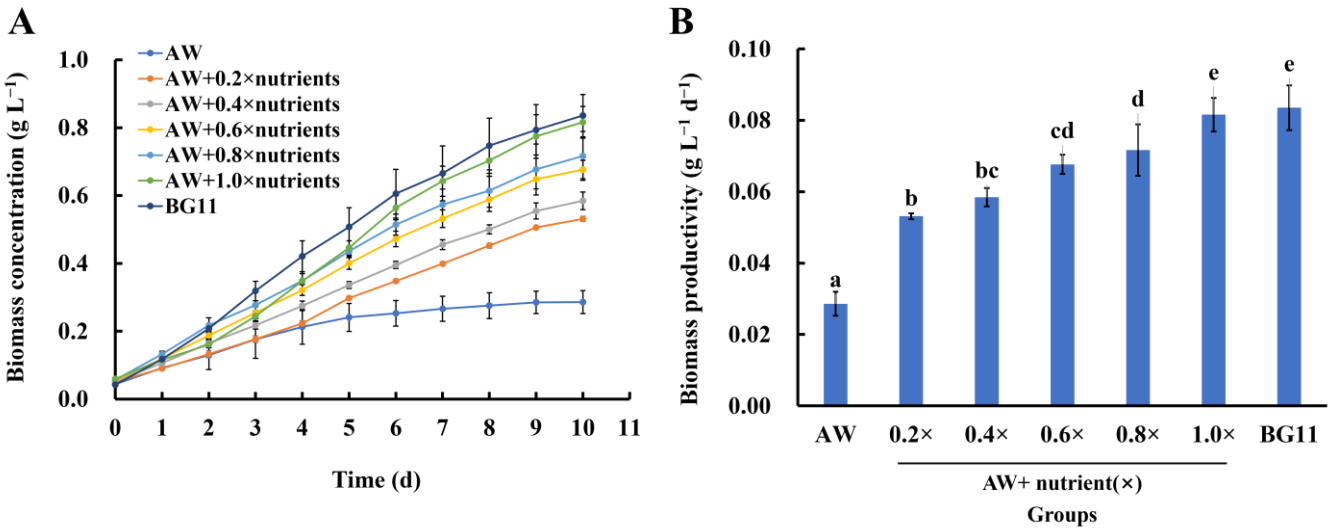

Figure 1. Growth (**A**) and average biomass productivity (**B**) of *C. sorokiniana* NIES-2168 cultured in different groups. Different letters (a, b, c, d, and e) indicate significant differences between groups ($p < 0.05$), $n = 3$.

Notably, *C. sorokiniana* NIES-2168 cultured in the AW + 1.0× nutrients group showed no significant difference in average biomass productivity between the AW + 1.0× nutrients group (0.082 g L^{-1} d^{-1}) and BG11 group (0.084 g L^{-1} d^{-1}) during the 10 days of culture (Figure 1B). This result suggests that aquaculture wastewater can be a complete substitute for freshwater culture of microalgae, with biomass production almost equivalent to standard BG11 medium (Figure 1B).

3.3. Increasing CO_2 Concentration for Higher Biomass Productivity and Lutein Content

In the above experiments, the optimal nutrient conditions (AW + 1.0× nutrients) for the growth of *C. sorokiniana* NIES-2168 in aquaculture wastewater were determined. CO_2 concentration represents a key factor for the growth and metabolism of autotrophic microalgae, and in this study, we investigated the possible effects of different CO_2 concentrations (0.03, 2, 5, 10, and 20%) on microalgal biomass production and lutein accumulation.

As depicted in Figure 2A, CO_2 supplementation has a strongly positive effect on the growth and biomass accumulation of *C. sorokiniana* NIES-2168. The biomass concentrations on day 7, cultured in AW + 1.0× nutrients aerated with different concentrations of CO_2 (0.03%, 2%, 5%, 10%, and 20%), were 0.74 g L^{-1}, 1.78 g L^{-1}, 1.74 g L^{-1}, 1.60 g L^{-1}, and 0.88 g L^{-1}, respectively. The growth of *C. sorokiniana* NIES-2168 was promoted to varying

degrees in all groups exposed to extra CO_2 compared to the 0.03% group. The maximum biomass concentration of *C. sorokiniana* NIES-2168 was obtained at 2% CO_2 (1.78 g L^{-1}) (Figure 2A), 2.4 fold higher than the 0.03% group (0.74 g L^{-1}) (Figure 2A). Notably, there was no significant difference in biomass concentration in the 2% CO_2 (1.78 g L^{-1}) or 5% CO_2 (1.74 g L^{-1}) groups, indicating that the range of 2–5% represents the most suitable CO_2 concentration for the growth of *C. sorokiniana* NIES-2168.

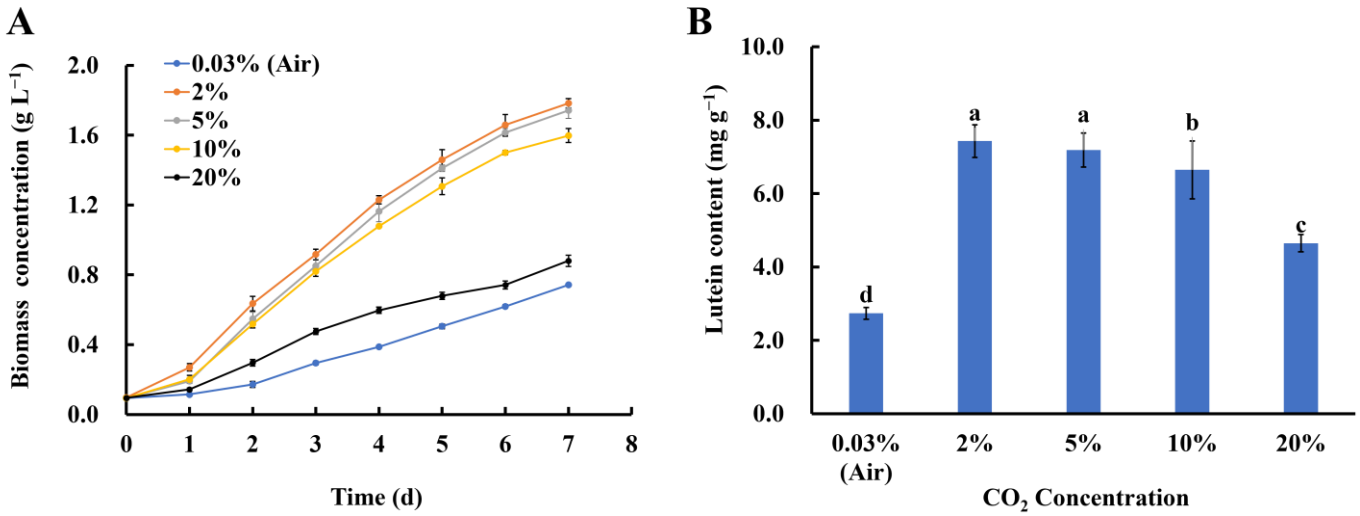

Figure 2. Biomass concentration (**A**) and lutein content (**B**) of *C. sorokiniana* NIES-2168 cultured in AW + 1.0× nutrients aerated with different concentrations of CO_2. Different letters (a, b, c and d) indicate significant differences between multiple groups ($p < 0.05$), $n = 3$.

As illustrated in Figure 2B, the increase in CO_2 concentration significantly promoted the accumulation of lutein in microalgae. The intracellular lutein content of microalgae increased from 2.74 mg g^{-1} to 7.43 mg g^{-1} on day 7 using CO_2 concentrations of 0.03% and 2%. It then decreased when the CO_2 concentration further increased to 20% (Figure 2B), with no significant difference in lutein content between the 5% CO_2 (7.19 mg g^{-1}) and 2% CO_2 (7.43 mg g^{-1}) groups, which is consistent with the growth trend of microalgae (Figure 2A). The maximum lutein content of *C. sorokiniana* NIES-2168 was also obtained at 2% CO_2 (7.43 mg g^{-1}) (Figure 2A), 2.7-fold higher than the 0.03% group (2.74 mg g^{-1}) (Figure 2A). Therefore, the optimal CO_2 concentration for culturing *C. sorokiniana* NIES-2168 in AW + 1.0× nutrients was found to be 2.0%, which obtained the maximum biomass productivity (0.28 g L^{-1} d^{-1}), the highest lutein content (7.43 mg g^{-1}), and the highest lutein productivity (1.89 mg L^{-1} d^{-1}).

3.4. Lutein Production of C. sorokiniana NIES-2168 under Two-Stage Cultivation Strategies with Blue Light

Light wavelength is a key abiotic factor modulating the development and pigment accumulation of microalgae [36]. Blue light, with a wavelength range of 420 to 520 nm, promotes lutein accumulation. In a study by Li et al. [15], a lutein content of 9.58 mg g^{-1} was observed in *Chlorella* sp. AE10 cultured under blue light, which was 1.63-fold higher than under white light. However, the growth of *Chlorella* sp. AE10 was inhibited under blue light, and the biomass concentration under blue light was 2.65 g L^{-1} at day 4, lower than the 3.57 g L^{-1} observed under white light. In another study, Zhao et al. [37] found that the highest lutein content (2.95 mg g^{-1}) for *Chlamydomonas* sp. JSC4 was obtained under blue LEDs, while the biomass productivity (728 ± 19 mg L^{-1} d^{-1}) of JSC4 was highest under white light conditions. As indicated in previous studies, blue light may be favorable for lutein accumulation in microalgae, but not for microalgal biomass accumulation. Therefore, to enhance the lutein content and productivity, we used a two-stage culture strategy to grow *C. sorokiniana* NIES-2168 with aquaculture wastewater. In the first stage, the

optimized conditions from the above experiments were adopted. *C. sorokiniana* NIES-2168 was cultured in AW + 1.0× nutrients with 2% CO_2 under white LED to attain the optimal growth. When the microalgae reached the plateau stage, cells were transferred to the second stage and incubated with blue LED to accumulate lutein.

As depicted in Figure 3A, in the first stage, the microalgae rapidly accumulated biomass in AW + 1.0× nutrient medium with 2% CO_2. After 6 days of incubation, the biomass concentration reached 1.80 g L^{-1}. The cells were then transferred to the second stage, where microalgae were cultured under blue light for another six days under an unchanged light intensity of 120 μ mol m^{-2} s^{-1}. The growth of *C. sorokiniana* NIES-2168 was delayed for the following two days in the second stage, then grew slowly. The lutein content in microalgae gradually accumulated under blue light, and the maximum lutein content and productivity were obtained on the 12th day of growth. These were 13.95 mg g^{-1} and 3.63 mg L^{-1} d^{-1}, respectively. The lutein content and productivity of *C. sorokiniana* NIES-2168 were increased using the two-stage culture strategy.

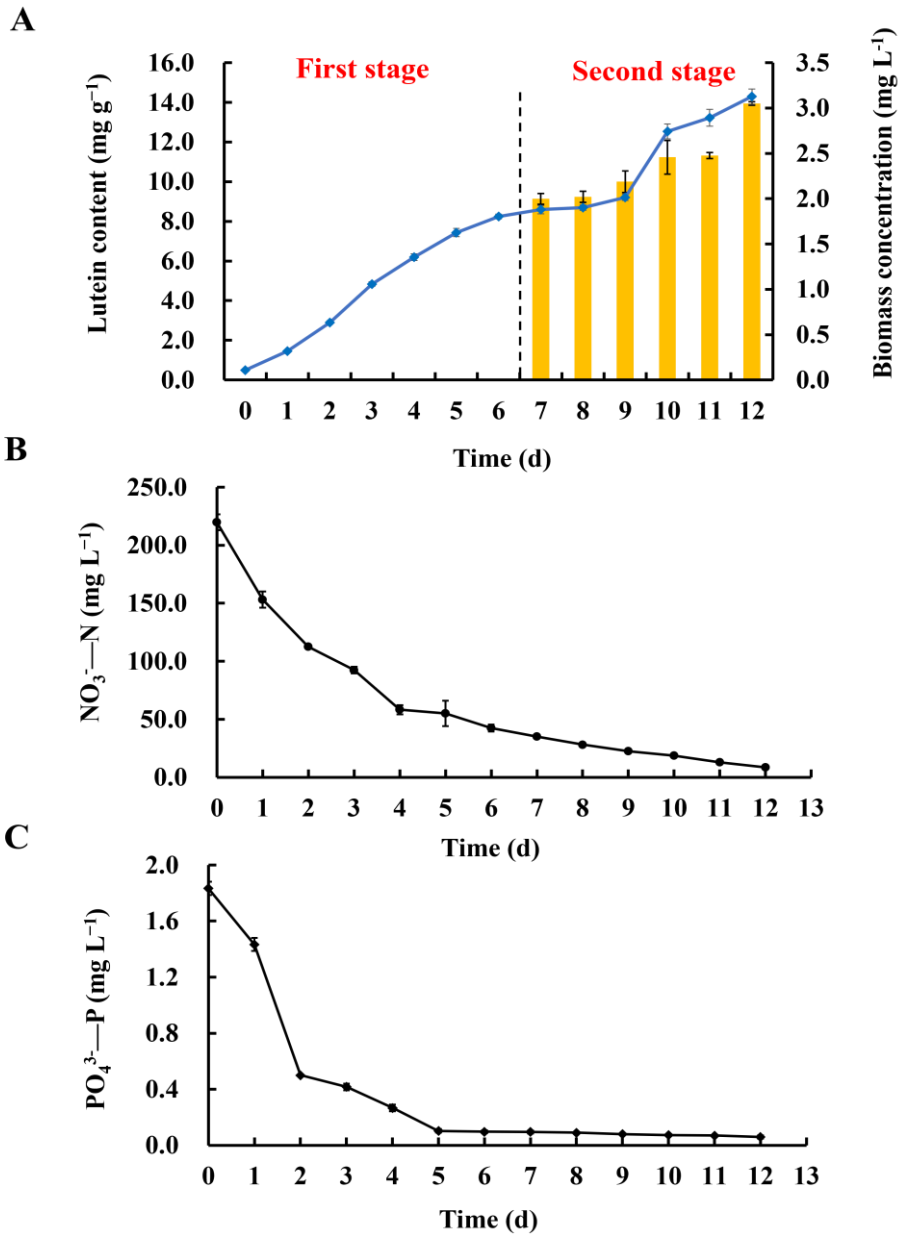

Figure 3. Growth and lutein content (**A**) of *C. sorokiniana* NIES-2168; consumption of nitrate (**B**) and phosphate (**C**) under two-stage culture conditions, $n = 3$.

We also monitored the dynamic depletion process of nitrate and phosphate content in aquaculture wastewater using this two-stage model. Nitrate (Figure 3B) was rapidly consumed during the first 4 days of the *C. sorokiniana* NIES-2168 culture in wastewater, and nitrate removal was almost complete at the conclusion of the 12-day incubation period, with a NO_3^--N removal rate of 96.07%. The phosphate content (Figure 3C) decreased significantly on day 2 of the *C. sorokiniana* NIES-2168 culture, and the phosphate was almost completely consumed during the first stage, with a removal rate of PO_4^{3-}-P of 96.75% during the entire two-stage incubation cycle. The NO_3^--N content and PO_4^{3-}-P content were only 8.65 mg L^{-1} and 0.06 mg L^{-1} on the 12th day of culture, respectively. The nutrients in the aquaculture wastewater were largely consumed by the microalgae. The cultivation of microalgae is conducive to the comprehensive treatment of wastewater, aligned with the concept of the green economy.

4. Discussion

Aquaculture wastewater can be used as a water source for growing *C. sorokiniana* NIES-2168, but in our study, microalgal growth was limited due to the low nutrient content (Figure 1A), especially NH_4^+-N (0.697 mg L^{-1}), NO_3^--N (3.168 mg L^{-1}), and TP (0.2 mg L^{-1}), in aquaculture wastewater (Table 1). In a previous study [38], the contents of TN, NO_3^--N, and TP in aquaculture wastewater were 18.5 mg L^{-1}, 18.1 mg L^{-1}, and 17.5 mg L^{-1}, respectively. In another study [39], the concentrations of NO_3^--N and PO_4^{3-}-P in wastewater were 0.67 mg L^{-1} and 8.82 mg L^{-1}, respectively. The characteristics of wastewater generated from aquaculture farms vary significantly across different studies. Therefore, the optimization of nutrient concentration in wastewater culture systems is crucial for biomass production in microalgae. Our study demonstrates that the growth of microalgae was promoted in aquaculture wastewater supplemented with 1.0× nutrients, and the biomass concentration on day 10 was 2.85 times that found in the group lacking extra nutrients. A similar phenomenon has been identified in previous reports. The biomass concentration of *Chlorella* sp. GD was increased when extra nutrients were added to the aquaculture wastewater. The maximum biomass concentration and productivity of *Chlorella* sp. GD were obtained in the group supplemented with 1× nutrients to aquaculture wastewater; they increased by 2.38 times and 2.58 times, respectively [27]. In another study, the highest biomass concentration of *Chlorella sorokiniana* MB-1-M12 was obtained in 75% SCW (shrimp culture wastewater) supplemented with 100% BG11 medium [40]. Therefore, in relatively nutrient-poor wastewater, the addition of an appropriate amount of extra nutrients to the wastewater or supplementation with a certain amount of medium is a substantial benefit for the biomass production of microalgae. Notably, in our study, there was no significant difference in the biomass concentration and productivity of *C. sorokiniana* NIES-2168 grown by adding 1.0× BG11 nutrients to aquaculture wastewater over 10 days aerated with air (0.82 g L^{-1}, 0.082 g L^{-1} d^{-1}) (Figure 1A,B) compared to the standard BG11 medium (0.84 g L^{-1}, 0.084 g L^{-1} d^{-1}) (Figure 1A,B). This suggests that aquaculture wastewater can completely replace freshwater-cultured microalgae for biomass production. This has the potential to save a significant amount of freshwater, which can alleviate freshwater scarcity in China.

CO_2 concentration is a critical factor influencing the cell growth and metabolism of autotrophic microalgae. A limited supply of CO_2 inhibits microalgal biomass productivity, while a high CO_2 concentration leads to cytotoxicity due to the reduced pH of the culture [41]. In this study, *C. sorokiniana* NIES-2168 grew well in AW + 1.0× nutrients at CO_2 concentrations between 2% and 20% (Figure 2A), with adequate CO_2 tolerance. The increase in CO_2 concentration promoted the growth and lutein accumulation of microalgae. The maximum biomass concentration and lutein content were both obtained at a 2% CO_2 concentration, which were 1.78 g L^{-1} and 7.43 mg^{-1}, respectively (Figure 2A,B). The maximum biomass productivity of *C. sorokiniana* NIES-2168 cultured in AW + 1.0× nutrients at 2% CO_2 was 0.28 g L^{-1} d^{-1}, a 3.1-fold increase compared to incubation with air (0.09 g L^{-1} d^{-1}). A similar phenomenon was observed by Kuo et al. [27]: When *Chlorella*

sp. GD was cultured with 2% CO_2 in aquaculture wastewater, the biomass productivity of the 7-day culture was 0.076 g L^{-1} d^{-1}, which was fourfold higher than that of the culture with air (0.309 g L^{-1} d^{-1}). However, in another study [39], *Chlorella sorokiniana* and *Ankistrodesmus falcatus* showed low biomass productivity in wastewater without additional CO_2 supplementation, obtaining maximum biomass productivity levels of 0.16 g L^{-1} d^{-1} and 0.20 g L^{-1} d^{-1}, respectively. These results suggest that CO_2 concentration is essential for the biomass production of microalgae.

According to Table 2, with increasing CO_2 concentration, a corresponding decrease in the final pH of the medium was observed. The final pH values in each group were 9.39, 7.81, 7.37, 7.03, and 6.50 with the increasing CO_2 concentration. The pH was higher in the wastewater medium aerated with air than that in the groups given CO_2, possibly because 0.03% CO_2 in the air did not react with the OH^- generated from the nitrate consumption process, causing a higher pH. The addition of extra CO_2 to react with OH^- could lower the pH to a certain extent, providing a favorable pH environment for the growth of microalgae. Additional CO_2 supply could increase the concentration of dissolved inorganic carbon (DIC) in the medium, allowing for more available carbon sources for the growth of microalgae. This explains why the growth of *C. sorokiniana* NIES-2168 was promoted when the CO_2 concentration was increased to 2–5%. However, when the CO_2 concentration exceeded 5%, a decrease in biomass concentration (Figure 2A) was observed, potentially related to the decreased pH of the culture. Once the pH is reduced to a certain level, it can be harmful to the growth of microalgae by reducing the activity of enzymes involved in photosynthesis, such as carbonic acid extracellular anhydrase [42]. The increase in lutein accumulation at higher CO_2 concentrations may be due to the fact that a high CO_2 concentration causes the photosynthetic apparatus (PSA) to transition from state I to state II, which has been suggested to improve the electron chain transport over photosystem I (PSI) to produce more ATP and support pH homeostasis within microalgae cells [43,44]. Accordingly, this results in an increase in the size of the PSI light-harvesting antenna, and lutein, a light-harvesting pigment bound to a natural protein at the L1 site, will be elevated as the antenna protein level increases [45].

Table 2. Biomass concentration and lutein accumulation of *C. sorokiniana* NIES-2168 cultured in different groups; initial and final pH of different cultures.

CO_2 Concentration (%)	Maximum Biomass Concentration (g L^{-1})	Maximum Lutein Content (mg g^{-1})	Lutein Productivity (mg L^{-1} d^{-1})	Initial pH	Final pH
0.03	0.74	2.74	0.29	8.56	9.39
2	1.78	7.43	1.89	8.53	7.81
5	1.74	7.19	1.79	8.53	7.37
10	1.60	6.65	1.52	8.57	7.03
20	0.88	4.65	0.58	8.55	6.50

Two-stage culture approaches have been widely applied to enhance biomass and lutein production in microalgae [23,25,37]. Usually, in the first stage, microalgae accumulate biomass rapidly, and when the maximum biomass concentration is reached, the cells are transitioned to the second stage to accumulate the desired compound. Blue light has been reported [15,37] to induce an increase in lutein content, but to inhibit the growth of microalgae. Therefore, blue light can be applied in the second stage to stimulate the accumulation of intracellular lutein in order to attain improved lutein productivity. As illustrated in Figure 3A, the two-stage culture mode further enhanced the lutein content and productivity of *C. sorokiniana* NIES-2168. The maximum lutein content and productivity were obtained in the second stage, on the 12th day of culture, at 13.95 mg g^{-1} and 3.63 mg L^{-1} d^{-1}, respectively (Figure 3A). The lutein content and productivity are very competitive among phototrophic microalgae currently used for lutein production. Xie et al. [35] reported that phototrophic cultivation of *Desmodesmus* sp. F51 resulted in a lutein content of 5.05 mg g^{-1} and lutein productivity of 3.56 mg^{-1} L^{-1} d^{-1}. Li et al. [23] reported

a two-stage culture of *Scenedesmus* sp. FSP3, achieving the highest lutein content and productivity of 6.45 mg g^{-1} and 3.36 mg L^{-1}, respectively. Vaquero et al. [46] demonstrated that the lutein content and productivity of *Coccomyxa onubensis* in the photoautotrophic mode were 4.5 mg g^{-1} and 1.22 mg L^{-1} d^{-1}, respectively. The lutein content we obtained was substantially higher than that reported in most of the related studies, which may be attributed to the promotion of lutein accumulation by blue light in *C. sorokiniana* NIES-2168. We integrated aquaculture wastewater for microalgae cultivation, but unlike the previous study by Chen et al. [40], no freshwater was used to dilute our wastewater. The only water source for culturing microalgae was aquaculture wastewater, enhancing the conservation of freshwater resources. Chen et al. [40] attained a maximal lutein content and productivity of 3.89 mg g^{-1} and 5.0 mg L^{-1} d^{-1} in *Chlorella sorokiniana* MB-1-M12 with aquaculture wastewater using a mixotrophic mode. The high lutein productivity was primarily due to the high biomass concentration of the microalgae with mixotrophic cultivation. However, given the potential contamination risks and complicated impurity challenges of heterotrophic or mixotrophic cultivation, the autotrophic production of lutein with microalgae appears to be commercially viable, with the additional benefit of limiting CO_2.

5. Conclusions

In this study, we reused aquaculture wastewater to grow *C. sorokiniana* NIES-2168 and achieved efficient lutein production via a two-stage cultivation approach. The maximum lutein content and productivity obtained were 13.95 mg g^{-1} and 3.63 mg L^{-1} d^{-1}, respectively, which was competitive across autotrophic microalgae in contemporary studies. The removal rates of nitrate and phosphate throughout the two-stage culture were 96.07% and 96.75%, respectively, indicating that the two-stage process of lutein production was environmentally friendly. *C. sorokiniana* NIES-2168 exhibited exceptional potential for both lutein production and wastewater treatment, indicating its potential as a strain to be used for the commercial production of lutein. In this study, aquaculture wastewater was utilized as the sole source of water, and no freshwater was employed for dilution, maximizing the conservation of freshwater resources. Compared to the current studies employing two-stage strategies to generate lutein, this study is the first to use a two-stage culture strategy to grow microalgae in wastewater, making lutein production more economically feasible. This study elucidates an environmentally friendly and cost-effective solution for commercializing microalgae-based lutein generation.

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