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Long-term Photosynthetic *Chlorella* Cultivation in Recycled Medium for Sustainable Lipid Production

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ABSTRACT Effective recycling of spent medium is crucial for scaling up photosynthetic microalgal cultivation and achieving sustainable biofuel production. In this study, we investigated the long-term cultivation of *Chlorella* sp. KR-1 in a 1-L bubble-column photobioreactor over a 49-day period using recycled culture medium. Neutral lipid content, predominantly triacylglycerols—a promising feedstock for next-generation biofuels—was quantified as fatty acid methyl esters (FAMEs). Control cultures were grown in fresh N8 medium, while the experimental groups were grown in recycled medium supplemented with full N8 nutrients. Photosynthetic cell growth remained stable during the first three cycles, with final biomass concentrations of 3.07 ± 0.16 and 3.08 ± 0.17 g/L in the second and third cycles, respectively, slightly exceeding the control (2.96 ± 0.09 g/L). In later cycles (four to seven), biomass production gradually declined, stabilizing between 2.57 and 2.69 g/L. FAME productivity peaked at 135 mg FAMEs/L/day during the second and third cycles, representing a 12% increase over that of the control, before declining to 88 mg FAMEs/L/day by the seventh cycle. These findings suggest that recycling the culture medium, with appropriate management of inhibitory byproducts, can sustain microalgal cultivation with minimal reductions in biomass and lipid productivity.

Key words *Chlorella*(클로렐라), Biofuel(바이오연료), Neutral lipids(중성지질), Medium Recycling(배지 재사용), Photosynthetic cultivation(광합성 배양)

1. Introduction

Microalgae are among the smallest organisms, exhibiting a photosynthetic efficiency that exceeds that of C3 and C4 plants by 5-7%.[1,2] These microorganisms can produce a wide array of metabolites, including carbohydrates, proteins, lipids, antioxidant pigments, and bioactive compounds, with their production profiles influenced by cultivation conditions.[3,4] Moreover, microalgae play a crucial role in reducing atmospheric carbon dioxide (CO_2) by converting it into organic compounds through photosynthesis.[5] With significantly higher biomass production per unit area than terrestrial plants, microalgae are gained recognition as a promising feedstock for next-generation biofuels.[6,7] Under stress conditions, microalgae synthesize neutral lipids, primarily triacylglycerols, which consist of three fatty acids esterified to a glycerol backbone, serving as their

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main energy storage molecules. These triacylglycerols can be converted into biodiesel, green diesel, and aviation fuels through catalytic conversion processes.

The versatility of microalgae allows their application across diverse industries, including agriculture (fertilizers and animal feed), food, medicine, and transportation fuels, contributing to a steadily expanding global market.[8,9] Enhancing the economic feasibility of micro-algal mass culture systems is critical for industrial biofuel production, especially as market demands grow.[10,11] However, the production costs of microalgae-derived biofuels remain high compared to conventional fuels, with approximately 30% of these costs attributed to cultivation and harvesting processes.[5,12] Developing more economical and energy-efficient methods for microalgal biomass production is thus imperative.

Unlike terrestrial plants, microalgae are aquatic organisms, with water constituting the largest volume in their growth systems.[13,14] While photobioreactors are commonly used for large-scale cultivation, optimization of these systems remains underdeveloped, leading to high unit costs due to energy-intensive processes like harvesting, drying, lipid extraction, and purification. Addressing these challenges necessitates research into reusing microalgal culture medium.[15] Spent medium often contains residual polysaccharides and bacteria, which can inhibit growth and introduce contamination during subsequent cultivation cycles.[16]

Given the intensifying global droughts linked to climate change, sustainable management of water resources in microalgal cultivation has become increa-singly important. As aquatic photosynthetic micro-organisms, microalgae utilize water as a medium for nutrient absorption and biochemical reactions. Water, a fundamental component of all living organisms, is critical for their survival. Without recycling, water footprint of microalgae-derived biodiesel is estimated at approximately 3.7 m^3/kg, but recycling harvested water can reduce this footprint by up to 84.1%.[17]

However, using spent medium for further cultivation requires addressing challenges such as contamination from residual bacteria and the accumulation of growth-inhibiting organic compounds. Extracellular organic substances secreted by microalgae can cause oxidative damage and suppress cell activity, negatively affecting subsequent cultivation cycles.[18,19] While symbiotic bacteria can sometime benefit microalgal growth by providing cofactors like vitamin B_{12} or by reducing dissolved oxygen,[20] they can also complete for essential nutrients like nitrogen and phosphorus, posing additional challenges.

This study aims to establish a method for sequential microalgal cultivation using recycled medium, addressing growth inhibition and contamination issues associated with medium reuse. The specific objectives of this research are to (1) sustain the growth and lipid pro-duction of *Chlorella* sp. KR-1 in recycled medium, (2) assess bacterial contamination and the presence of soluble organic substances in the spent medium, and (3) identify the factors contributing to growth inhibition and reduced lipid accumulation during recycling. Ulti-mately, this research seeks to lower the production costs of microalgae-based biofuels, making them a more economically viable and environmentally friendly alternative to fossil fuels.

2. Materials and Methods

2.1 Strain, Medium, and Flask cultivation

Chlorella sp. KR-1 was obtained from the Korea Institute of Energy Research, Daejeon, Korea. The strain was cultivated photoautotrophically in a 250-mL Erlenmeyer (working volume: 150 mL) sealed with a porous silicon stopper, using a modified N8 medium.[21] The composition of the N8 medium was as follows: KNO_3 5.0 mM, KH_2PO_4 5.44 mM, Na_2HPO_4 1.83 mM, $MgSO_4 \cdot 7H_2O$ 0.20 mM, $CaCl_2$ 0.12 mM, FeNaEDTA

0.03 mM, $ZnSO_4 \cdot 7H_2O$ 0.01 mM, $MnCl_2 \cdot 4H_2O$ 0.07 mM, $CuSO_4$ 0.07 mM, and $Al_2(SO_4)_3 \cdot 18H_2O$ 0.01 mM. The medium pH was adjusted to 6.5 and filter-sterilized using a 0.2 μm membrane prior to use. The flask cultures were incubated in a shaking incubator at 150 rpm and 30°C under continuous illumination of 70-80 μmol/m^2/s for 14 days.

2.2 Photobioreactor Cultivation and Recycling of Culture Medium

The main cultivation of *Chlorella* sp. KR-1 was conducted in a 1-L Pyrex glass bubble-column photobioreactor (length: 35 cm; inner diameter: 3.7 cm; working volume: 500 mL) at 30°C for seven days, with each experiment performed in duplicate. Illumination was provided continuously using three cool-white light-emitting diodes at a light intensity was 180-200 μmol/m^2/s. The initial inoculum density was adjusted to an optical density (OD) of 0.1 at 660 nm. The photobioreactor was continuously supplied with 5% CO_2 in air (v/v) at 0.6 vvm from the bottom. To prevent contamination, the gas mixture was filtered through a 0.2 um PTFE venting filter. Samples (4 mL) were taken every 24 hours to monitor biomass concentration and nitrate removal rates. Experiment conditions were based on protocols established protocols established by Praveenkumar *et al.* (2024)[21] and Jung *et al.* (2023).[22]

For medium recycling, the spent culture medium was supplemented with nutrients based on four strategies: (1) full N8 nutrient supplementation, (2) major components only (nitrogen and phosphorous), (3) major + minor components (magnesium [Mg], calcium [Ca], and iron [Fe]), and (4) major + trace elements (all components except minor).

The culture medium was reused for seven consecutive photobioreactor cultivation cycles. At the end of each cycle, 90% of the cultures was harvested by centrifugation at 3,000 rpm for 20 minutes at 4°C (Combi-514R, Hanil Scientific Inc., Korea) and reused for subsequent cycles. The remaining 10% was inoculated with fresh cultures grown separately in fresh N8 medium.

2.3 Analytical Methods

Biomass concentration was determined by measuring the OD at 660 nm using a UV/Vis spectrophotometer (Optizen 3220UV, Mecasys, Korea), with calibration against dry cell weight. The dry weight of the microalgal biomass was measured using the suspended solids method.[22]

Biovolume ratios of bacteria and *Chlorella* were determined using a Multisizer™ 4 Coulter counter (Beckman Coulter, USA), based on the spherical sizes of *Chlorella* sp. KR-1 cells (1.8-8 μm in diameter) and bacterial cells (1.15-1.8 μm in diameter) as observed under a light microscope (Axio Imager.A2, Carl Zeiss, Germany; Kim *et al.* (2016)[23]). pH was measured using an HM-30R pH meter (DKK-TOA, Japan) and light intensity was recorded using an LI-250A quantum photometer (Li-Cor Inc., USA).

Microalgal cultures were centrifuged at 3,000 rpm for 10 minutes at 4°C (Combi-514R, Hanil Scientific Inc., Korea) and filtered through a 0.45 μm membrane filter (16555K, Minisart® syringe filter, Sartorius Stedim Biotech, USA). Nitrate concentration and soluble chemical oxygen demand (SCOD) were analyzed using assay kits (D5030-11 for nitrate and COD-L for COD; Humas Co., Korea).

Neutral lipid content in the microalgal biomass was quantified as measuring fatty acid methyl esters (FAMEs) using a direct transesterification method followed by gas chromatography (GC; Agilent 7890, Agilent Technologies, USA). Detailed analytical procedures were based on Cho *et al.* (2011)[24] and Jung *et al.* (2023).[22]

2.4 Statistical Analyses

Experimental data were analyzed with a two-way

analysis of variance (ANOVA) with the "agricolae' package in RStudio (R version 4.2.1). A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Cultivation of *Chlorella* with Fresh Medium

The growth curve of *Chlorella* sp. KR-1 over seven days of photosynthetic cultivation exhibited distinct growth phases (Fig. 1A). During the first three days, rapid exponential growth was observed, indicating optimal nutrient and light conditions. However, after this period, the growth rate transitioned to a linear phase, likely due to nutrient depletion—particularly nitrate—and limitations in light availability, which began to constrain further biomass accumulation. By the end day seven, the culture reached a biomass concentration of 3.05 ± 0.08 g/L, aligning well with previous reports for *Chlorella* sp. KR-1 under similar conditions.[22]

The depletion of nitrate by day three underscores its critical role as a key nutrient driving the exponential growth phase (Fig. 1B). Once nitrate levels were exhausted, the growth rate declined, indicating that nitrogen limitation was the primary factor slowing photosynthetic biomass production. Despite this limitation, the culture's pH remained stable throughout the experiment, ranging 6.92 to 7.83, which is conducive to maintaining a favorable environment for photosynthesis.

SCOD levels, which reflect the presence of extracellular organic matter, gradually increased during cultivation (Fig. 1C). Initial SCOD levels were low (17.1 ± 2.0 mg/L) but rose to 75.4 ± 4.2 mg/L by day three, coinciding with the exponential growth phase. After the culture transitioned to the linear growth phase, SCOD levels increased more sharply, reaching 380.8 ± 17.8 mg/L by day seven. This sharp rise in SCOD is likely attributable to the secretion of organic compounds, a

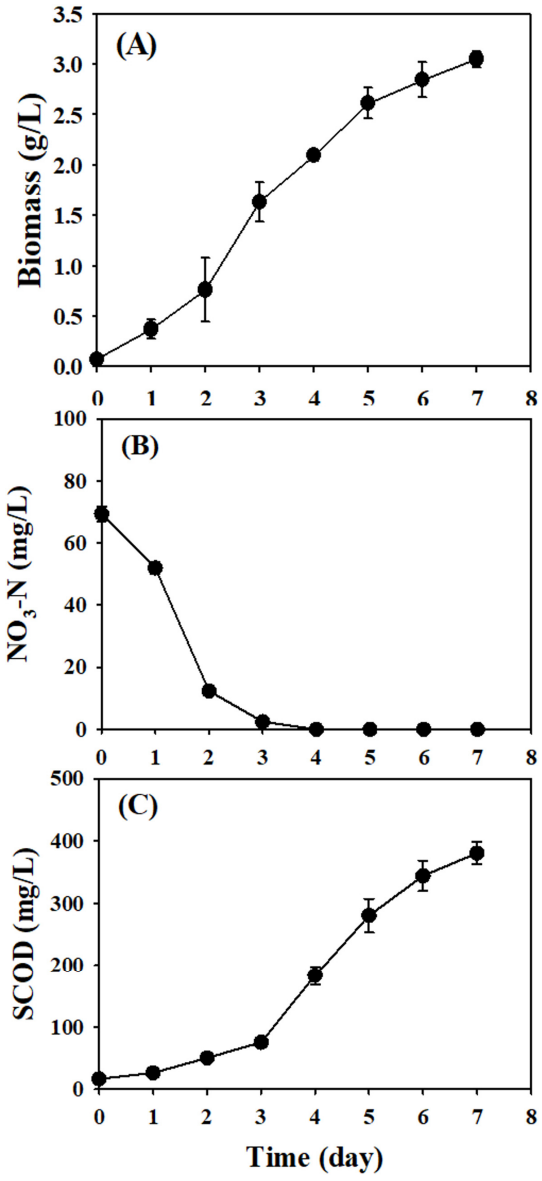

Fig. 1. Time-course profiles of biomass concentration (A), nitrate level (B), and soluble chemical oxygen demand (SCOD) (C) during the seven-day cultivation of *Chlorella* sp. KR-1 in photobioreactors

phenomenon often associated with nutrient limitation and cellular oxidative stress in photoautotrophic cultures.[19]

3.2 Recycling of Culture Medium

The performance of *Chlorella* sp. KR-1 in recycled culture medium was assessed under four nutrient supplementation strategies: full N8 nutrients, major

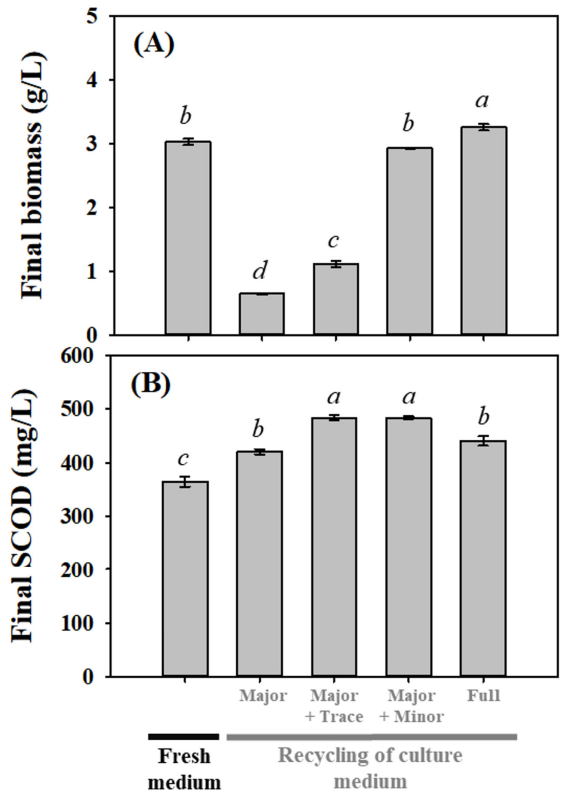

Fig. 2. Final biomass production (A) and soluble chemical oxygen demand (SCOD) (B) after seven days of cultivation of *Chlorella* sp. KR-1 in photobioreactors. Four nutrient supplementation strategies were evaluated: full N8 nutrient supplementation, major components only (nitrogen and phosphorus), major plus minor components (magnesium, calcium, and iron), and major plus trace elements (other components). The error bars represent the mean and standard deviations (n = 4), and different italic letters indicate the significance of the differences ($p < 0.05$)

components (nitrogen and phosphorus), major + minor components, and major + trace elements (Fig. 2A). The highest biomass yield, 3.25 ± 0.05 g/L, was achieved with full N8 nutrient supplementation, exceeding the control yield obtained with fresh N8 medium (3.03 ± 0.05 g/L). This result underscores the importance of complete nutrient replenishment for maintaining high biomass productivity in recycled media.

In contrast, when only major nutrients (nitrogen and phosphorous) or major + trace elements were supplemented, biomass yields dropped significantly to 0.65 ± 0.01 and 1.11 ± 0.05 g/L, respectively. This indicates the critical impact of deficiencies in minor nutrients, specifically Mg, Ca, and Fe, on microalgal growth. Supplementation with major + minor components, however, achieved biomass yields comparable to the control, highlighting the essential role of minor nutrients in sustaining photosynthetic growth in recycled media.

Mg serves as a central component of the chlorophyll molecule, which is critical for light harvesting during photosynthesis, while Ca maintains the structural integrity of photosystem complexes. Fe is essential for photosynthetic electron transport, chlorophyll biosynthesis, and nitrogen assimilation, all of which are vital processes for cellular metabolism and optimal microalgal growth.[5,6,8]

SCOD levels were elevated across all recycling conditions (420-484 mg/L) compared to the control (365 ± 9 mg/L) (Fig. 2B), indicating the accumulation of organic matter in the recycled media. Despite the increased SCOD levels, microalgal growth was not inhibited in cultures supplemented with full N8 nutrients or major + minor components. This suggests that *Chlorella* sp. KR-1 exhibits a high tolerance for elevated concentrations of organic compounds in recycled media, provided that essential nutrients are adequately replenished.

3.3 Repeated Cultivations with Recycling of Spent Medium

To assess the long-term feasibility of recycling spent culture medium, the photobioreactor was operated across seven consecutive cycles (49 days), with the medium replenished with full N8 nutrients at the start of each cycle (Fig. 3). During the first three cultivation cycles, consistent biomass production rates of 0.40-0.41 g cell/L/day were observed over the initial four days. However, as the number of cycles increased from four to seven, biomass production rates during the same period declined to 0.30-0.38 g cell/day. Despite this, the final biomass concentrations in the second (3.07 ± 0.16 g/L) and third (3.08 ±

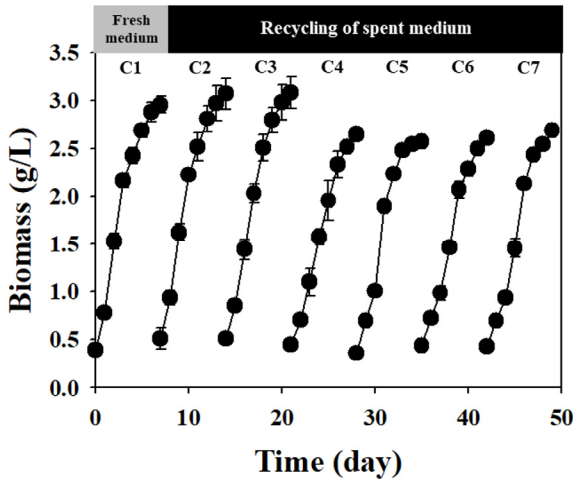

Fig. 3. Time-course profiles of cell growth of *Chlorella* sp. KR-1 during sequential cultivation in fresh and recycling media over 49 days using photobioreactors

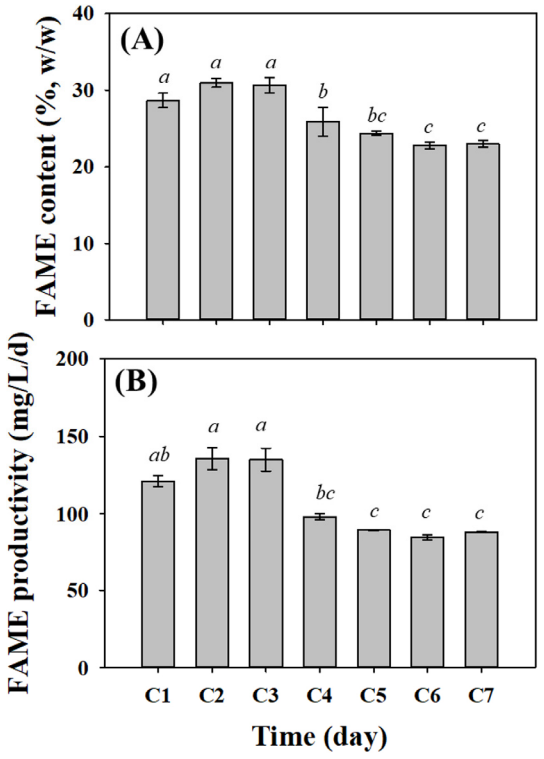

Fig. 4. Changes in fatty acid methyl ester (FAME) content (A) and productivity (B) of *Chlorella* sp. KR-1 during sequential cultivations in fresh and recycling medium for 49 days using photobioreactors. Error bars represent the mean and standard deviations ($n = 4$), and different italic letters indicate the significance of the differences ($p < 0.05$).

0.17 g/L) cycles slightly exceeded the control (2.96 ± 0.09 g/L). This indicates that the recycled medium maintained sufficient quality to support microalgal growth for at least three cycles.

From the fourth to the seventh cycle, final biomass concentrations gradually declined, ranging from 2.57 to 2.69 g/L likely due to the accumulation of inhibitory byproducts such as organic metabolites in the recycled medium. These byproducts can create oxidative stress or disrupt cellular processes, negatively impacting biomass production.[18,19]

Similarly, FAME content remained stable through the first three cycles (28.6 to 30.9%) but gradually decreased to 23.0 ± 0.4% by the seventh cycle (Fig. 4A). FAME productivity peaked during the second and third cycles at 135 mg FAMEs/L/day, representing a 12% increase over the control (121 mg FAMEs/L/day). However, by the seventh cycle, FAME productivity declined to 88 mg FAMEs/L/day (Fig. 4B). These results suggest that the recycling spent medium can sustain high biomass and FAME productivity for up to three cycles. Beyond this point, further declines in performance necessitate optimization measures, such as more precise nutrient supplementation or periodic addition of fresh medium, to maintain productivity over extended cultivation cycles.

Environmental stress, as documented in several studies, can either enhance or inhibit lipid production in microalgae.[25,26] For example, Zhang *et al.* (2016)[16] observed that the total lipid content of *Nannochloropsis oceanica* grown in recycled culture medium was significantly lower compared to cultures grown in fresh medium. Similarly, the lipid content decreased steadily with each recycling round, suggesting that inhibitory effects from reused medium can negatively impact lipid accumulation.

3.4 Soluble SCOD and Co-existing Bacteria

To investigate the factors contributing to the decline in biomass production during later cultivation cycles, SCOD levels and the biovolume ratio of bacteria to

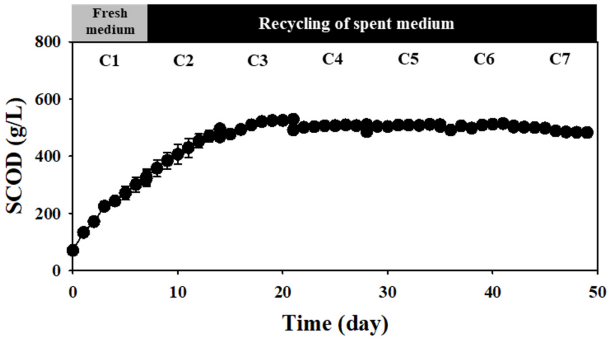

Fig. 5. Time-course profiles of soluble chemical oxygen demand (SCOD) concentration during sequential cultivations of *Chlorella* sp. KR-1 in fresh and recycling media over 49 days using photobioreactors

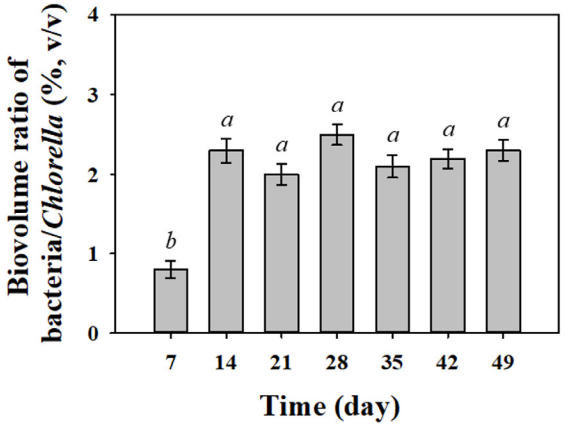

Fig. 6. Changes in the biovolume ratio of bacteria to *Chlorella* during sequential cultivations in fresh and recycling media over 49 days using photobioreactors. Error bars represent the mean and standard deviations (n = 4), and different italic letters indicate the significance of the differences ($p < 0.05$).

Chlorella cells were monitored. SCOD levels increased from 71 ± 8 mg/L at the start to 496 ± 8 mg/L by the second cultivation cycle and remained relatively stable (480 to 530 mg/L) through subsequent cycles (Fig. 5). This trend suggests that *Chlorella* sp. KR-1 may regulate the release of organic compounds as an adaptive response to changing environmental conditions.

The accumulation of SCOD likely contributed to growth inhibition in later cycles, as extracellular organic substances can interfere with nutrient absorption and lead to self-damage.[18] Previous studies have demonstrated that soluble organic products, such as carbohydrates, proteins, lipids, and organic acids, can inhibit microalgal growth. For example, in *Scenedesmus* sp. LX1, such byproducts have been shown to hinder cell proliferation under similar conditions.[19]

The biovolume ratio of bacteria to *Chlorella* remained relatively low, between 2.0% and 2.5% after the second cultivation cycle (Fig. 6). Co-existing bacteria can influence microalgal growth through both positive and negative mechanisms.[27] Several bacteria groups, including *Pseudomonas, Klebsiella, Rhizobium, Sinorhizobium*, and *Sphingomonas*, have been identified in association with *Chlorella* sp. KR-1 in photoautotrophic and mixotrophic cultures using 16S rRNA gene-fragment analysis.[23] These bacteria can support microalgal growth by reducing oxygen tension, which can inhibit photosynthesis, and by supplying essential nutrients like vitamins, amino acids and hormones.[28] However, excessive bacterial growth can negatively impact microalgae by competing for carbon and energy sources or reducing light penetration, which is crucial for photosynthesis.[29] Fig. 6 indicates that bacterial co-existence did not significantly inhibit the photosynthetic cultivation of *Chlorella* sp. KR-1 in recycled media during repeated cycles.

Overall, results from Figs. 5 and 6 suggest the decline in biomass and FAME production after the third cultivation cycle is primarily due to the accumulation of inhibitory organic byproducts rather than bacterial contamination. Further research to identify these byproducts and develop effective removal or mitigation strategies may enhance the efficiency and sustainability of long-term medium recycling for microalgal cultivation.

4. Conclusions

This study explored the effects of recycling culture medium on the photosynthetic cultivation of *Chlorella*

sp. KR-1, aiming to establish a sustainable and water-efficient sequential culture system. Biomass production (2.96-3.08 g/L) remained stable during the initial cycles, but progressively declined in cycles four to seven, with concentrations ranging from 2.57 to 2.69 g/L. FAME productivity peaked during cycles two and three at 135 mg FAMEs/L/day, representing a 12% increase compared to the control, but dropped to 88 mg FAMEs/L/day by the seventh cycle. The findings revealed that growth inhibition in later cycles was primarily caused by organic substances secreted by *Chlorella* sp. KR-1 rather than bacterial contamination. To support sustainable microalgal cultivation and enhance lipid productivity, it is essential to effectively address the accumulation of these organic compounds through appropriate treatment strategies.

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