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# Effects of dissolved inorganic carbon concentrations on the stable growth of Ethiopian microalga *Limnospira fusiformis* under semi-continuous cultivation

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**Abstract**

*Limnospira fusiformis* LC-30 is a promising Ethiopian cyanobacterium for sustainable biomass production owing to its high growth potential and protein content. Dissolved inorganic carbon (DIC) is a key factor affecting both productivity and cultivation cost in *Spirulina*-related cyanobacterial production; however, the DIC concentration required to maintain stable biomass production during semi-continuous cultivation remains unclear. In this study, *L. fusiformis* LC-30 was cultivated at four DIC concentrations (0.02, 0.05, 0.10, and 0.20 M) to evaluate their effects on growth and biomass productivity. The highest dry weight and biomass productivity were obtained at 0.20 M DIC, reaching 1.14 ± 0.06 g $L^{-1}$ and 0.23 ± 0.01 g $L^{-1}$ $d^{-1}$, respectively, and were significantly higher than those under lower DIC conditions. However, *L. fusiformis* LC-30 maintained 65–74% of the maximum biomass productivity under 0.02–0.10 M DIC, demonstrating its ability to sustain substantial biomass production even with reduced inorganic carbon supply. Protein content remained high and stable at approximately 55–60% of dry weight, indicating that reduced DIC availability did not markedly compromise biomass quality. Among the tested conditions, 0.20 M DIC resulted in the highest biomass and protein productivity. Nevertheless, *L. fusiformis* LC-30 maintained substantial biomass productivity and a high protein content under reduced DIC conditions, highlighting the potential to reduce inorganic carbon input in semi-continuous cultivation.

**Keywords**

Biochemical characteristics, Biomass productivity, Dissolved inorganic carbon, *Limnospira fusiformis*, Semi-continuous cultivation

## 1. Introduction

With the increasing global demand for sustainable protein sources, microalgae have attracted attention owing to their high nutritional values, rapid biomass productivity, and diverse applications in food, feed, pharmaceuticals, and biofuels [1]. Several microalgal species are already commercially utilized as dietary supplements, with their increasing demand in the health food sector [2, 3]. *Arthrospira* spp., commonly marketed under the trade name *Spirulina*, is one of the most widely recognized microalgae and is valued as a nutritional supplement due to its high protein content (approximately 60–70% of dry weight) as well as its abundance of essential amino acids, vitamins, and antioxidant compounds [4]. Recent taxonomic studies have also improved our understanding of commercially important *Spirulina*-related cyanobacteria, facilitating the identification and characterization of promising production strains. In particular, species classified within the genus *Arthrospira* have been proposed for reassignment to the genus *Limnospira* [5]. Due to their ability to grow in strong alkaline environments (pH 9–10), where the growth of many other microorganisms is suppressed, *Spirulina* cultivation is less susceptible to contamination making it particularly suitable for large-scale outdoor cultivation [6–10].

Because of these cultivation advantages, further improvements in biomass productivity are required to enhance the economic feasibility and scalability of commercial production. Therefore, identification of highly productive strains remains essential for maximizing cultivation efficiency and biomass yield. Among these, *Limnospira fusiformis* LC-30, an Ethiopian strain isolated from Lake Chitu, has been reported to exhibit a high specific growth rate (1.2–1.6 $d^{-1}$) and a biomass yield of approximately 3 g $L^{-1}$ [11]. Furthermore, the LC-30 strain exhibits minimal photoinhibition and maintains stable growth at high irradiance conditions [12],

highlighting its potential for large-scale commercial outdoor cultivation. In order to understand the productivity potential of such strains, optimization of key cultivation parameters is essential. Among these, the availability of dissolved inorganic carbon (DIC) is particularly important because carbon supply directly influences photosynthetic activity and biomass production.

The high productivity of *Spirulina* is supported by photosynthetic carbon fixation, for which DIC serves as the major carbon source. Since microalgae grow by assimilating DIC and fixing it into organic matter, DIC supply conditions directly affect the rate of carbon fixation, cell growth, and biomass accumulation [13, 14]. Consequently, maintaining an adequate DIC supply is essential for achieving high productivity in commercial cultivation systems. The standard media for *Spirulina* cultivation, such as Zarrouk medium and SOT medium [15, 16], contains approximately 0.20 M sodium bicarbonate ($NaHCO_3$), which provides sufficient carbon supply while maintaining alkaline conditions. However, bicarbonate salts account for a large portion of medium costs [17], making efficient DIC management an important consideration for industrial-scale cultivation. This is particularly relevant when alternative nutrient sources such as anaerobic digestion effluent (ADE) are used. ADE contains nutrients and trace elements that can support microalgal growth; however, its DIC concentration is generally much lower than that of standard *Spirulina* media [18–21]. Therefore, clarifying the response of *Spirulina*-related cyanobacteria to reduced DIC availability is necessary for the development of cost-effective cultivation strategies using low-DIC nutrient sources.

Accordingly, the effects of DIC concentration on the growth and physiological characteristics of *Spirulina* have been investigated in previous studies. *Spirulina platensis* grown in repeated batch culture exhibited a specific growth rate of 0.8–0.9 $d^{-1}$ even at

0.04 M $NaHCO_3$ [22]. Similarly, *Limnospira platensis* maintained a high growth rate under a DIC concentration of 0.05 M [23]. However, these studies mainly focused on growth performance, and the effects of DIC concentration on stable biomass production and cellular characteristics during long-term semi-continuous cultivation remain insufficiently understood. Clarifying these responses is essential for evaluating how DIC availability influences both cultivation performance and biomass composition during prolonged operation.

*Spirulina* is a filamentous cyanobacterium characterized by its spiral-shaped trichomes, and its morphology and cellular characteristics have been reported to change depending on cultivation conditions [24, 25]. Such morphological changes may reflect differences in cultivation environment and growth conditions [26], and nutritional factors, particularly bicarbonate availability in the culture medium, have also been suggested to influence these characters [27]. As DIC is directly involved in photosynthetic carbon assimilation and biomass formation, variations in DIC availability may affect filament development and cell size. In addition to morphological traits, biochemical characteristics such as protein content and phycocyanin accumulation have also been reported to vary depending on DIC concentration and other cultivation conditions. Protein and phycocyanin are major valuable components of *Spirulina* biomass, and their contents can be influenced by cultivation conditions, including carbon and nutrient availability [28, 29]. For example, bicarbonate supply affected biomass accumulation and protein production in *Spirulina platensis* [28], while $NaHCO_3$ addition increased protein content to 64.20 ± 4.18% w/w and enhanced phycocyanin accumulation [29]. Collectively, these studies indicate that DIC availability influences growth, biomass productivity, biochemical and morphological characteristics in *Spirulina*. However, most

investigations have been conducted under batch cultivation conditions, and no study has systematically evaluated the growth, biomass productivity, protein productivity, and morphological characteristics under different DIC concentrations during semi-continuous cultivation. Furthermore, the DIC requirements of the high-productivity strain *L. fusiformis* have been established, and it remains unclear to what extent stable productivity can be maintained under reduced DIC conditions.

Therefore, this study aimed to evaluate the effect of DIC concentration on growth characteristics, biomass productivity, protein content, protein productivity and morphological characteristics in *L. fusiformis* LC-30. Specifically, semi-continuous cultivation was conducted under four DIC concentration conditions (0.02, 0.05, 0.10, and 0.20 M). This study provides fundamental insights into the growth response of *L. fusiformis* under reduced DIC conditions and contributes to the development of cultivation strategies with lower inorganic carbon inputs.

## 2. Materials and Methods

### 2.1 Stock and preculture preparation

The microalgae *Limnospira fusiformis* LC-30 used in this study was originally isolated from Lake Chitu, located in the Federal Democratic Republic of Ethiopia [11]. The strain used in this study was provided by Injibara University, Ethiopia, and imported into Japan in compliance with the applicable Access and Benefit-Sharing (ABS) regulations under a permit (reference No. EBI 71/3232/2014) issued by the Ethiopian Biodiversity Institute of the Federal Democratic Republic of Ethiopia on March 29, 2022. The algal stock was maintained in modified *Spirulina* Ogawa Terui (SOT) medium, with composition (mg $L^{-1}$) as follows: $Na_2CO_3$ (10,599), $NaHCO_3$ (8401), $K_2HPO_4$ (500),

144 $NaNO_3$ (2500), $Na_2SO_4$ (815), NaCl (1000), $MgSO_4 \cdot 7H_2O$ (200), $CaCl_2$ (30), $FeSO_4 \cdot$
145 $7H_2O$ (10), $Na_2EDTA \cdot 2H_2O$ (80), $H_3BO_3$ (2.86), $MnSO_4 \cdot 5H_2O$ (2.17), $ZnSO_4 \cdot 7H_2O$
146 (0.222), $CuSO_4 \cdot 5H_2O$ (0.079), and $Na_2MoO_4 \cdot 2H_2O$ (0.021). The pH of the medium was
147 adjusted to 10, which is optimal for the growth of *L. fusiformis* LC-30, by adding $NaHCO_3$
148 and $Na_2CO_3$ [30]. The total DIC concentration was adjusted to 0.20 M, as defined in the
149 standard SOT medium.
150 Preculture cultivation was carried out in two stages. In the first stage, cells from
151 stock were cultured in 1 L glass Erlenmeyer medium bottles and maintained at light
152 intensity 600 μmol photons $m^{-2}$ $s^{-1}$ with a 12h L:12h D photoperiod and aeration rate 0.25
153 L $min^{-1}$ at 30°C. In the second stage, cells in the logarithmic phase from the first-stage
154 culture were re-inoculated into 2 L medium bottles with increased aeration rate (0.50 L
155 $min^{-1}$) under similar conditions as described above.
156 Throughout the stock culture, preculture, and semi-continuous cultivation
157 procedures, all culture vessels, glassware, and other culture apparatus were autoclaved at
158 121°C for 20 min using an autoclave (SX-500, TOMY SEIKO CO., LTD., Japan). The
159 modified SOT medium was also autoclaved under the same conditions. All culture
160 handling was performed aseptically. During cultivation, air was supplied through a sterile
161 0.2-μm air filter to prevent microbial contamination.
162
163 **2.2 Semi-continuous cultivation**
164 The cells in the logarithmic phase of the second preculture stage were used in
165 the semi-continuous cultivation experiments. In the experiment, batch cultivation was
166 conducted from the start of cultivation until day 5 (0-5 days), followed by semi-

continuous cultivation for the remainder of the cultivation period (6-59 days). In this study, the four different DIC concentrations of 0.02, 0.05, 0.10, and 0.20 M were used. The molar ratio of $NaHCO_3$ to $Na_2CO_3$ was maintained at 1:1, and the total DIC concentration was adjusted by varying their amounts in the SOT medium. The cultures were carried out in 1.5 L glass column reactors with an effective working volume of 1.2 L. The precultural cells were washed with DIC-free SOT medium twice before inoculation into the 1.2 L column reactors containing sterilized SOT medium. The initial optical density at 750 nm ($OD_{750}$) of the culture was adjusted to 0.08. Cultures were irradiated with a white fluorescent light (600 μmol photons $m^{-2}$ $s^{-1}$; 12h L:12h D photoperiod) and maintained in a water bath at 30°C with an aeration rate of 0.50 L $min^{-1}$. A dilution rate of 20% (240 mL $d^{-1}$) was applied, and the cultivations were continued until cells reached the stationary phase. Sampling was collected every 2 days at the end of the dark period.

**2.3 Analytical parameters**

Optical density at 620 nm and 678 nm ($OD_{620}$ and $OD_{678}$) were measured using a UV–visible spectrophotometer (UV-530, JASCO, Japan). The pH was measured using a pH meter (MJ-7200, SATOTECH, Japan).

The dry cell weight (DW) was calculated by pre-combusting the glass fiber filters (GF/F, Whatman, USA) at 550°C for 2 h and measuring the pre-weight (LIBROR AEG-220, Shimadzu, Japan). The filtered cells were washed three times with 2 mL of 20% $NH_4HCO_3$ to remove salts and were later oven-dried (DG82, Yamato, Japan) at 60°C for at least 24 h before measurement.

The cell length (μm) was measured by taking photos using an inverted microscope (Axioskop-2 Plus Carl Zeiss, Germany) equipped with a digital camera (EOS Kiss X7i, Canon, Japan). Then, analyses were performed using Image J software

(National Institute of Health, USA). To determine the size distribution of the cells, 20 cells were measured in each sample.

To assess bacterial contamination in the cultures, bacterial cells were counted using a hemocytometer (Suntory Glass Limited, Japan) under an inverted microscope (Axioskop-2 Plus Carl Zeiss, Germany). Samples were fixed with glutaraldehyde (final concentration 1%) for at least 24 h, stained with 5% Loeffler's methylene blue, and enumerated microscopically at 400× magnification.

DIC concentration was determined by using a total organic carbon analyzer machine (TOC-L, Shimadzu). The filtered samples were sealed in glass ampoules and were stored at −80°C in the dark until measurement.

Protein content was determined by the Lowry method [31]. The dried cells (200 µL, 0.5% w/v in 100 mM phosphate buffer, pH 7) were treated with 1.0 mL of 1 N NaOH, mixed, and then dried again. Later, the dried cells were resuspended by adding 3.0 mL ultrapure water. A 0.5 mL aliquot was further mixed with 2.5 mL of alkaline copper solution and was incubated for ≥10 min, followed by the addition of 0.25 mL of half-diluted Folin Ciocalteu reagent with incubation at room temperature for 30 min. The absorbance was measured at 750 nm using a benchtop spectrophotometer (DR3900, HACH, USA).

**2.4 Calculations**

Biomass productivity (g $L^{-1}$ $d^{-1}$) during the steady-state period was calculated based on the equation (1) [32, 33].

$$\text{Biomass productivity} = 0.20 \times S_{DW} \quad (1)$$

where 0.20 is the dilution rate ($d^{-1}$), and $S_{DW}$ is the steady-state dry weight (g $L^{-1}$).

The dry cell weight of bacteria (g $L^{-1}$) was calculated based on a previous study

[34].

$$\text{Dry cell weight} = C_d \times C_w \quad (2)$$

where $C_d$ is the cell density (cells $mL^{-1}$) and $C_w$ is the dry weight per cell (0.30 pg $cell^{-1}$).

Phycocyanin concentration (mg $L^{-1}$) was estimated from the optical density using the absorbance at 620 nm and 678 nm as described in a previous study [35].

$$\text{Phycocyanin} = (138.5*A620)-(35.49*A678) \quad (3)$$

where, A is the absorbance of optical density at 620 and 678 nm.

Data are presented as the mean ± standard deviation (SD) of measurements obtained on at least three different days during the steady-state period (53–59 days) of semi-continuous cultivation.

Differences among the four DIC conditions were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test, using Statcel 3 add-in software (OMS Publishing Inc., Saitama, Japan). Differences were considered statistically significant at $p < 0.01$. Each DIC condition was tested in a single reactor; therefore, measurements obtained on different days were treated as temporal replicates representing the steady-state performance of each culture rather than as independent reactor-level replicates. Although reactor-specific effects cannot be completely excluded, all reactors were operated simultaneously under the same controlled temperature, light, and aeration conditions. The analysis was nevertheless useful for identifying differences that were consistently maintained among the DIC conditions during the steady-state period. Therefore, the statistical results represent treatment-associated differences observed within this cultivation experiment and should be interpreted within the scope of the experimental design.

## 3. Results and Discussion

### 3.1 pH and DIC dynamics of *L. fusiformis* under different DIC concentrations

The growth performance of *L. fusiformis* was assessed throughout the long-term cultivation period. This cultivation period was divided into two parts; batch (0-5 days) and semi- continuous cultivation throughout (6-59 days). In the batch cultivation, the pH of the cultures reached around 11 by day 4 in all DIC conditions (0.02, 0.05, 0.10, and 0.20 M; Fig. 1a). The pH dynamics observed during the batch cultivation phase can be attributed to photosynthetic activity and bicarbonate utilization as reported in previous studies [29, 36]. During photosynthesis, bicarbonate ions are converted to $CO_2$ by carbonic anhydrase, and $OH^-$ ions are released into the medium, resulting in an increase in pH. This shows that increasing bicarbonate supply can promote photosynthetic carbon fixation and biomass accumulation in *L. fusiformis*. During the semi-continuous cultivation period of *L. fusiformis*, the pH was maintained within the range of 10–11 (Fig. 1a). The average pH during the steady-state phase (days 53–59) reached 10.70 ± 0.05, 10.93 ± 0.14, 10.73 ± 0.12, and 10.53 ± 0.05 in 0.02, 0.05, 0.10, and 0.20 M DIC conditions, respectively (Fig. 1a). Previous studies have reported that the bicarbonate/carbonate system contributes to maintaining alkaline pH in cyanobacterial cultures and that higher DIC concentrations generally enhance carbonate buffering capacity [28, 37]. The observed increase and stable pH in this study were consistent with this general physiological response. It is known that the cells utilize carbon in $HCO_3^-$ form, and pH above 10 contributes to a greater proportion of DIC as $HCO_3^-$ and $CO_3^{2-}$ [38]. The bicarbonate/carbonate system acts as a chemical buffer with higher DIC concentrations enhancing resistance to pH fluctuations, while lower DIC concentrations exhibit reduced buffering capacity [39]. Therefore, stable external pH conditions help

maintain carbonate equilibrium and consistent inorganic carbon availability through regulation of the $HCO_3^-/CO_3^{2-}$ ratio, thereby supporting sustained photosynthetic activity during *L. fusiformis* cultivation. The DIC conditions 0.02–0.05 M were likely attributable to weaker buffering capacity [39], which may have caused temporal instability in $HCO_3^-$ availability. In contrast, higher DIC concentrations (0.10–0.20 M) likely provided stronger buffering effects, resulting in more stable pH conditions and carbon supply.

However, during the steady state, it was noticed that the DIC concentrations decreased by only 2–6% as compared with the added medium (0.02-0.20M), and no notable depletion was observed under any condition (Fig. 1b). Thus, the improved growth under different DIC concentration can be due to the differences in pH buffering capacity and the resulting stability of $HCO_3^-$ supply under alkaline conditions, rather than differences in the total amount of DIC consumed during cultivation. For example, it has been previously reported that even under a $NaHCO_3$ concentration of 0.3 M, the DIC concentration decreased from 3600 mg $L^{-1}$ (0.3 M) to approximately 2531 mg $L^{-1}$ (0.21 M) after 18 days of cultivation, corresponding to a utilization efficiency of about 26.7% [28]. These results suggest that the differences in growth behavior described in the following section were related more closely to differences in external pH stability and the resulting stability of $HCO_3^-$ availability than to the total amount of DIC consumed.

### 3.2 Cell dry weight and productivity of *L. fusiformis* in semi-continuous cultivation

The cell dry weight and biomass productivity showed clear differences during the steady-state phase (53–59 days) among all the DIC concentration conditions (Fig. 2). The cell dry weight of *L. fusiformis* reached 0.73 ± 0.05, 0.79 ± 0.04, 0.87 ± 0.06, and 1.14 ± 0.06 g $L^{-1}$under DIC concentrations 0.02, 0.05, 0.10, and 0.20 M, respectively (Fig.

2). This study showed that increase in DIC concentrations results in greater biomass accumulation in *L. fusiformis*. It is possible that at higher DIC concentrations (0.10–0.20 M), photosynthesis-induced increases in pH were likely moderated by the carbonate buffering system, resulting in a more stable $HCO_3^-/CO_3^{2-}$ equilibrium throughout cultivation. The increase in cell dry weight was consistent with a previously reported study, where maximum cell dry weight (2.24 g $L^{-1}$) was obtained under a high DIC condition of 0.3 M $NaHCO_3$ in *Spirulina platensis* [28]. However, the carbonate buffering capacity was possibly insufficient under lower DIC concentration (0.02–0.05 M), in this study leading to larger pH fluctuations and potentially limiting the supply of $HCO_3^-$. In contrast, the weaker buffering capacity at lower DIC concentrations (0.02–0.05 M) may have resulted in less stable inorganic carbon availability. This difference may have affected carbon uptake during the long-term cultivation of *L. fusiformis* and contributed to the lower biomass accumulation under the lower DIC conditions. In semi-continuous cultivation, stable external conditions, including medium pH and inorganic carbon availability, are important for supporting biomass formation during repeated cycles of dilution and regrowth [6].

The highest biomass productivity (0.23 ± 0.01 g $L^{-1}$ $d^{-1}$) was obtained at a DIC concentration of 0.20 M during the steady-state phase (Fig. 3). In contrast, biomass productivities at DIC concentrations of 0.02, 0.05, and 0.10 M were 0.15 ± 0.01, 0.16 ± 0.01, and 0.17 ± 0.01 g $L^{-1}$ $d^{-1}$, respectively (Fig. 3). It was found that retained 65–74% of the biomass productivity was maintained under DIC concentration 0.02–0.10 M as compared to the highest DIC condition (0.20 M). In semi-continuous cultivation, relatively stable culture conditions are maintained through periodic medium renewal; therefore, stable growth can be achieved even under relatively low inorganic carbon

concentrations. Thus, this study indicates that *L. fusiformis* can maintain biomass productivity of about 0.15 g $L^{-1}$ $d^{-1}$ even under relatively low DIC conditions. The biomass productivity of *L. fusiformis* obtained in this study, in semi-continuous cultivation with different culture medium was almost similar to the previously reported studies (Table 1). For example, semi-continuous cultivation of *Spirulina platensis* in Zarrouk medium reported biomass productivity approximately 0.20 g $L^{-1}$ $d^{-1}$ [40], while another study reached range 0.28–0.297 g $L^{-1}$ $d^{-1}$ under semi-continuous cultivation, where *Spirulina platensis* LEB-52 was cultivated with only Zarrouk medium and Zarrouk medium supplemented with 20% ADE [41] (Table 1). Another study reported higher biomass concentration and productivity 2.82 g $L^{-1}$ and 0.50 g $L^{-1}$ $d^{-1}$, respectively, where *L. fusiformis* LC-30 was cultured at a high initial biomass density (approximately 2.6 g $L^{-1}$) with high light intensity of 1400 µmol $m^{-2}$ $s^{-1}$ under semi-continuous cultivation [12](Table 1). These differences may be attributable not only to variations in medium composition, but also the initial biomass density and culture conditions. However, this study maintained 65–74% biomass productivity under the lower DIC conditions of 0.02–0.10 M, suggesting that *L. fusiformis* can adapt to semi-continuous cultivation under reduced inorganic carbon availability. However, future studies should be conducted to improve the biomass productivity of *L. fusiformis* by combining DIC concentration with high initial cell density and light intensity to clarify their respective contributions and interactions.

### 3.3 Morphology

The morphological variations in the trichomes of *L. fusiformis* under different DIC concentrations were also observed. Both trichome length and the degree of coiling

varied among the DIC conditions, ranging from tightly coiled structures to more loosely arranged forms (Fig. 4). The highest trichome length obtained was 177.52 ± 32.24 and 202.16 ± 36.87 μm under 0.10 and 0.20 M DIC concentrations, respectively. In contrast, trichome length under DIC concentrations 0.02 and 0.05 M reached 91.39 ± 20.81 and 117.06 ± 26.16 μm, respectively (Fig. 5a). Similarly, helix pitch showed a comparable increasing trend, with values of 14.35 ± 2.77 μm, 15.63 ± 2.23 μm, 28.50 ± 3.02 μm, and 34.74 ± 5.09 μm under DIC concentrations 0.02, 0.05, 0.10, and 0.20 M, respectively (Fig. 5b). Previous research reported that strain LC-30 exhibited trichome length and helix pitch of approximately 244 μm and 57 μm, respectively [11]. In another study, *Arthrospira fusiformis* isolated from Lake Chitu, Ethiopia, showed a strong positive correlation between $HCO_3^-$ concentration and trichome length [42]. This trend was similar to that observed in *Arthrospira platensis*, in which filament length increased with increasing $NaHCO_3$ concentration [27]. Similar ranges of trichome length (177–202 μm) and helix pitch (28–35 μm) in *L. fusiformis* was observed under high DIC conditions (0.10–0.20 M). However, the trichome length decreased to 91–117 μm under low-DIC conditions (0.02–0.05 M), which was lower than previously reported values [11]. This suggested that the morphology of *L. fusiformis* may change depending on carbon availability within the same strain. Thus, it can be said that increased bicarbonate supply may influence filament morphology of *L. fusiformis*.

### 3.4 Assessment of bacterial contamination

In addition to morphological changes, microbial contamination during semi-continuous cultivation was also evaluated. On day 59, the abundance of bacteria other than *L. fusiformis* reached $1.51 \times 10^7$, $1.61 \times 10^7$, $1.20 \times 10^7$, and $0.82 \times 10^7$ inds $mL^{-1}$

under the 0.02, 0.05, 0.10, and 0.20 M DIC conditions, respectively (Fig. 6a), showing a tendency to decrease with increasing DIC concentration. The estimated dry weight of these bacteria was 0.0045, 0.0048, 0.0036, and 0.0025 g $L^{-1}$ (Fig. 6b), corresponding to 0.2–0.6% of the total dry weight (0.73-1.14 g $L^{-1}$). In addition, microscopic observation of 1 mL of culture revealed no protozoa or zooplankton under any DIC condition. These results indicate that biological contamination in the semi-continuous cultivation of *L. fusiformis* had almost negligible impact on dry weight and productivity. Previous study [43] demonstrated that the addition of 160–200 mM $NaHCO_3$ inhibited the growth of protozoa in cultures of *Neochloris oleoabundans*. The decreasing trend in bacterial abundance observed with increasing DIC concentration in this study is broadly consistent with this suppressive effect, suggesting that higher DIC levels may contribute to reducing microbial contamination, even though its overall impact on biomass yield remained minor under the conditions tested here.

### 3.5 Cell composition

The highest protein content was observed at 0.10 M DIC concentration under steady-state conditions (53–59 days) (Fig. 7a), although no significant differences were observed among the DIC conditions. Protein content reached 59.11 ± 7.93, 58.49 ± 1.65, 60.17 ± 7.52, and 54.79 ± 2.12 % of DW at DIC concentrations of 0.02, 0.05, 0.10, and 0.20 M, respectively (Fig. 7a). The protein content ranged approximately from 55 to 60% of DW in all DIC conditions, which was consistent with the protein content reported in the previous studies for *Spirulina* (approximately 46–70% DW) [44]. Moreover, despite 59 days of *L. fusiformis* semi-continuous cultivation, no significant decrease in protein content was observed under any condition, and relatively stable values were maintained

385 across all DIC treatments in *L. fusiformis*. In long-term microalgal cultivation, the cellular
386 composition of microalgae is known to change depending on the variations in nutrient
387 availability and cultivation conditions [12, 45]. This suggests that *L. fusiformis* is capable
388 of maintaining a stable nutritional composition under long-term cultivation conditions.
389 The protein productivity, on the other hand, reached 0.086 ± 0.010, 0.092 ± 0.006, 0.105
390 ± 0.012, and 0.125 ± 0.008 g $L^{-1}$ $day^{-1}$ at DIC concentrations of 0.02, 0.05, 0.10, and 0.20
391 M, respectively (Fig. 7b). The increase in protein productivity observed in this study is
392 likely attributable to enhanced biomass accumulation under higher DIC conditions, rather
393 than changes the protein contents [46]. Therefore, these findings indicate that increased
394 DIC concentrations improve protein productivity mainly through enhanced biomass
395 accumulation, whereas the protein content of *L. fusiformis* remains comparatively stable
396 during long-term semi-continuous cultivation.

397 The phycocyanin concentration under steady state conditions were estimated to
398 be 96.78 ± 6.24, 108.84 ± 4.72, 110.65 ± 13.89, and 118.14 ± 17.73 mg $L^{-1}$ at DIC
399 concentrations 0.02, 0.05, 0.10, and 0.20 M, respectively. The estimated phycocyanin
400 content under the DIC concentration 0.02, 0.05, and 0.10 M conditions was 18%, 8%, and
401 6% lower, respectively, than under the 0.20 M concentration. DIC concentrations were
402 adjusted through the addition of bicarbonate/carbonate salts, resulting in parallel
403 increases in the salinity of the culture medium. Therefore, DIC concentration and salinity
404 were closely associated in the present study. The salinity of the culture medium, in this
405 study, increased with increasing DIC concentration (0.02, 0.05, 0.10 and 0.20 M) reaching
406 5, 8, 13, and 23‰, respectively. Previous studies have also reported that increasing DIC
407 concentrations can enhance the production of phycobiliproteins, particularly phycocyanin
408 [29, 47]. In addition, salinity is also known to influence the synthesis of phycobiliproteins,

and several studies have reported that increasing salinity in the culture medium can enhance phycocyanin production in *Spirulina* [48, 49]. Therefore, the increase in phycocyanin content observed in this study may have resulted from the combined effects of increased DIC and the associated rise in medium salinity. However, due to the simultaneous change in DIC concentration and salinity, the contribution of each factor was not clearly distinguished in this study. Thus, DIC concentration and salinity should be controlled independently to more precisely evaluate their respective effects on the physiology and pigment synthesis of *L. fusiformis*. In addition, more precise analytical methods are required to accurately quantify and evaluate the phycocyanin productivity in *L. fusiformis*. Furthermore, future studies are needed to evaluate whether the findings obtained in this study can be applied to ADE-based or other waste-derived media which could not only reduce medium costs but also possibly improve morphology, protein content, and phycocyanin accumulation in *L. fusiformis*.

## 4. Conclusion

In this study, the enhanced biomass concentration and productivity of *Limnospira fusiformis* LC-30 were achieved at 0.20 M DIC, which may be attributed to improved pH stability and sustained bicarbonate availability mediated by the carbonate buffering system during long-term semi-continuous cultivation. Despite differences in biomass productivity, the protein content remained relatively stable across all DIC conditions, indicating that biomass quality was largely maintained under reduced inorganic carbon availability. Notably, cultures cultivated at lower DIC conditions (0.02–0.10 M) retained 65–74% of the biomass productivity observed under the 0.20 M DIC demonstrating a high degree of physiological adaptability to carbon-limited conditions.

These findings highlight the capacity of *L. fusiformis* LC-30 to sustain productive semi-continuous cultivation under reduced inorganic carbon inputs and suggest that substantial reductions in DIC supplementation may be feasible without severe losses in biomass performance. Consequently, this study provides economical and resource-efficient cultivation systems utilizing low-DIC nutrient sources, including anaerobic digestion effluents, for sustainable cyanobacterial biomass production.

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**Author contributions**

**Yuka Uedani:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft; **Anupreet Kaur Chowdhary**: Conceptualization, Methodology, Writing – Review & Editing; **Mutsumi Sekine**: Conceptualization, Validation, Methodology, Formal analysis, Writing – Review & Editing; **Kanae Hayashi**: Methodology, Investigation, Writing – Review & Editing; **Woldie Adugna Ayirkm**: Writing – Review & Editing; **Banchiamlak Getnet Admasu**: Writing – Review & Editing; **Tatsuki Toda**: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Funding acquisition, Project administration.

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**Data availability**: All data generated or analyzed during this study are included in this published article.

**Declarations**

**Competing interests:** The authors report no commercial or proprietary interest in any product or concept discussed in this article.

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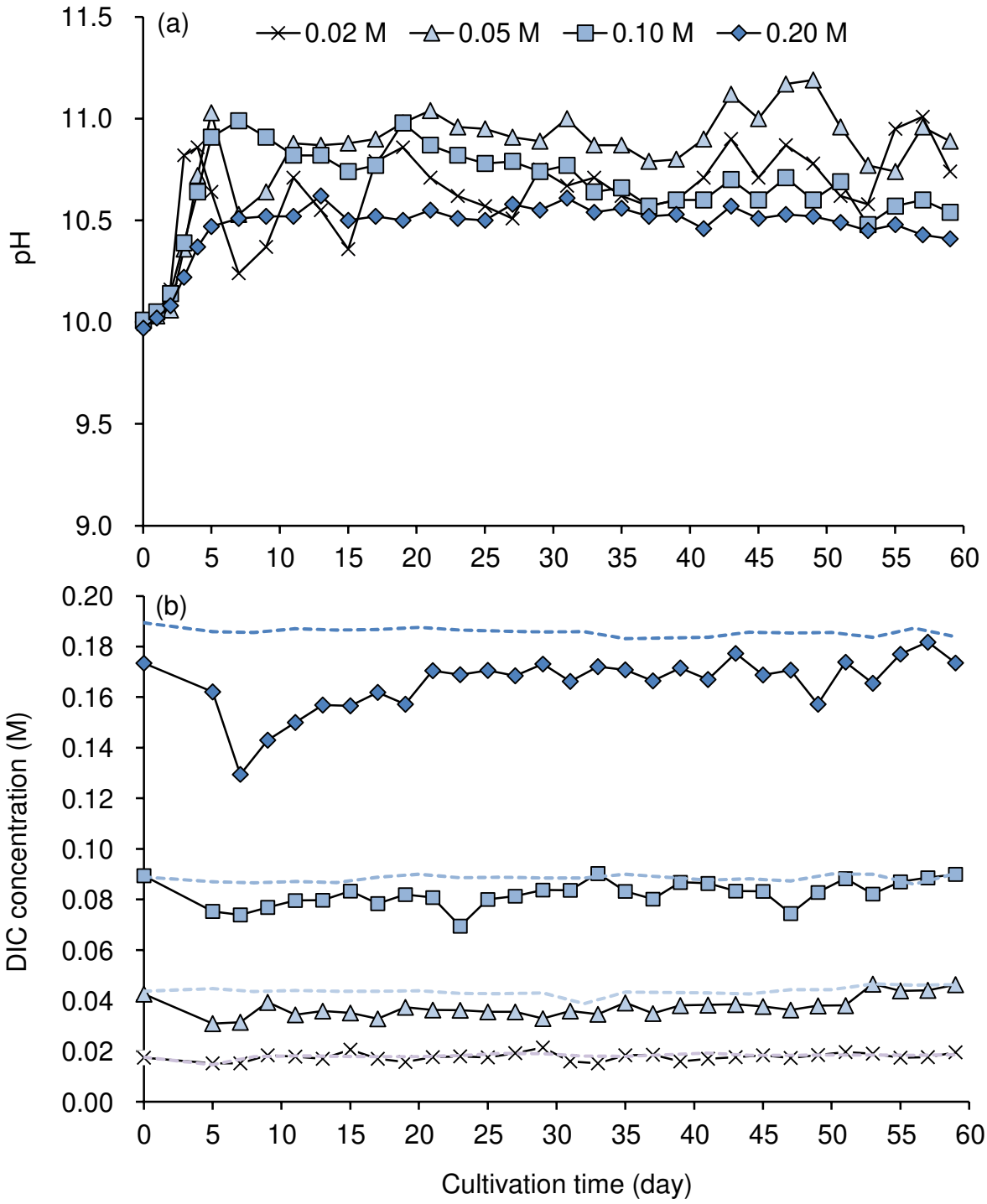



**Fig. 1** Time course of pH (a) and DIC concentration (b) in the semi-continuous cultivations of *Limnospira fusiformis* LC-30 under different DIC concentrations. Dotted lines show initial DIC concentration of medium

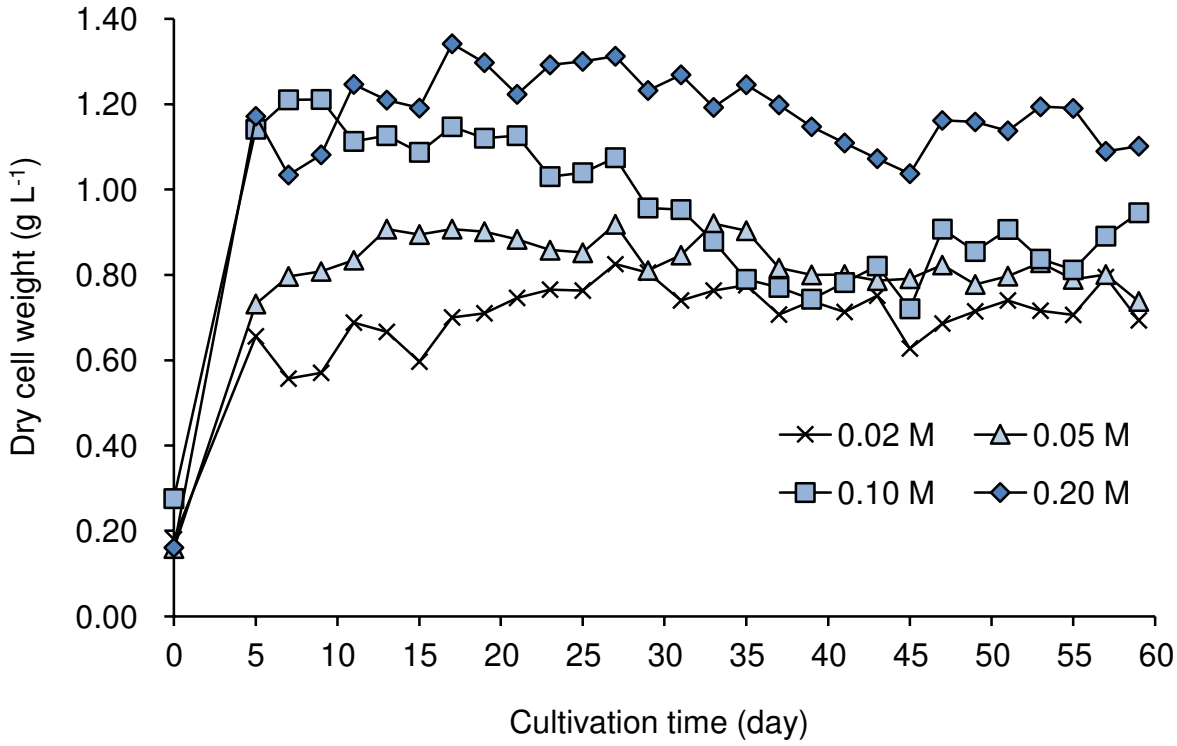



**Fig. 2** Time course of dry cell weight in the semi-continuous cultivations of semi-continuous *Limnospira fusiformis* LC-30 cultivation

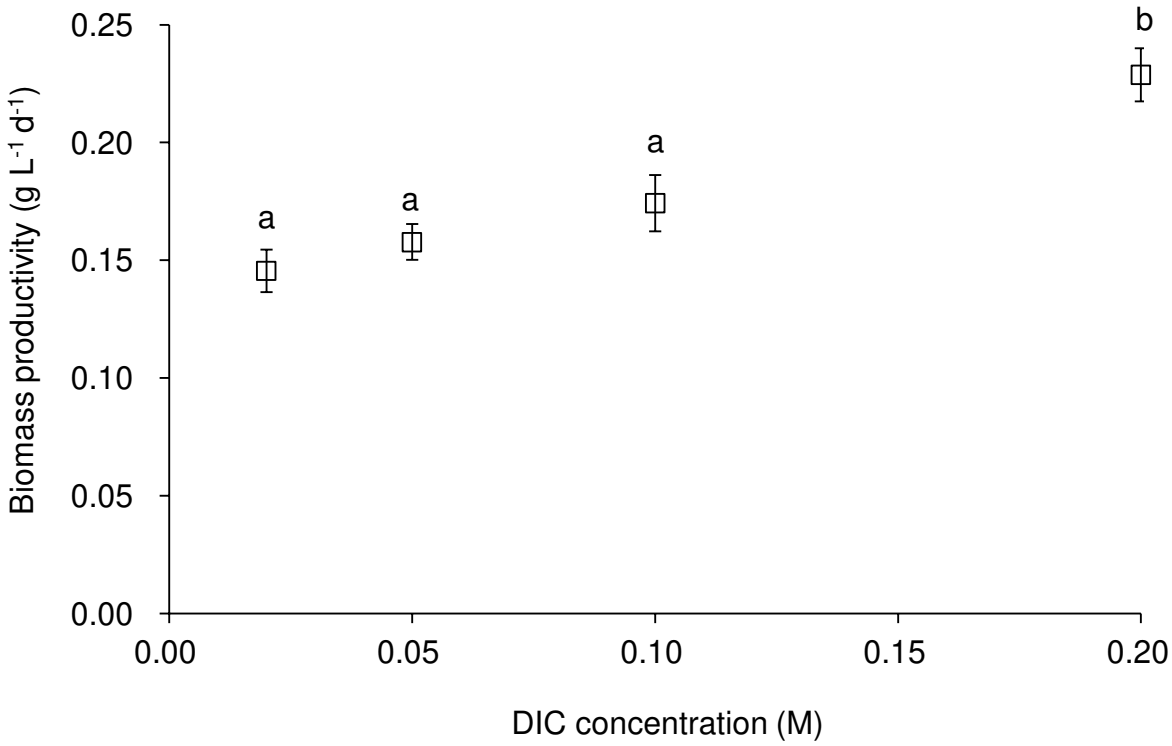



**Fig. 3** Amount of biomass productivity in steady state (days 53-59) of semi-continuous *Limnospira fusiformis* LC-30 cultivation. Error bars indicate standard deviations. Different letters indicate statistically significant differences (One-way ANOVA, Tukey-Kramer, $p<0.01$).

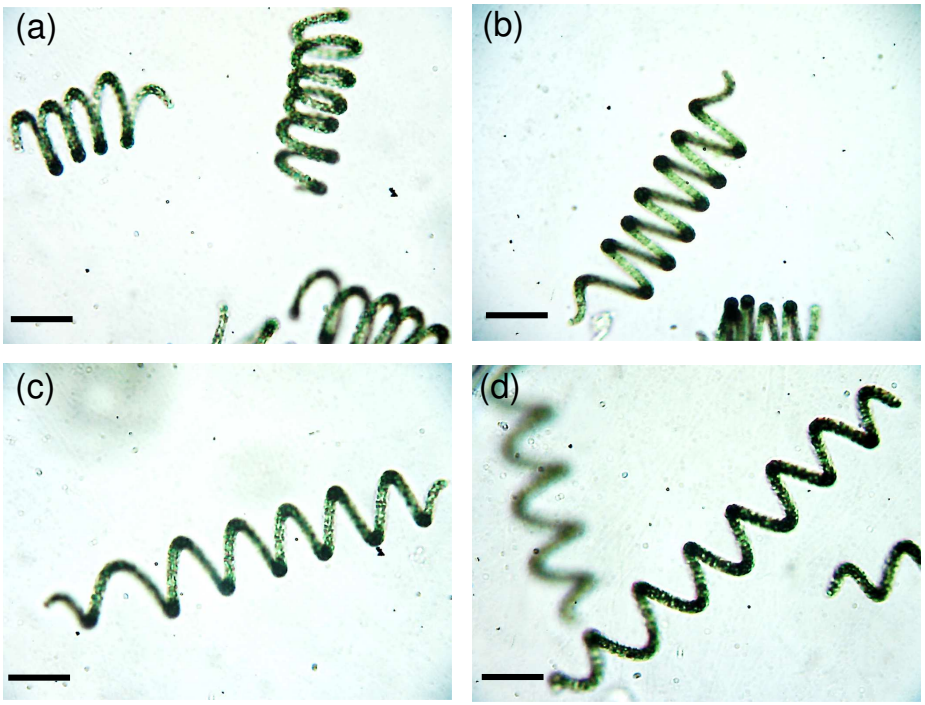



**Fig. 4** Cell morphology of *Limnospira fusiformis* LC-30 cultivated under the different DIC concentrations (M): 0.02 (a), 0.05 (b), 0.10 (c), 0.20 (d). The scale bar represents 50 μm

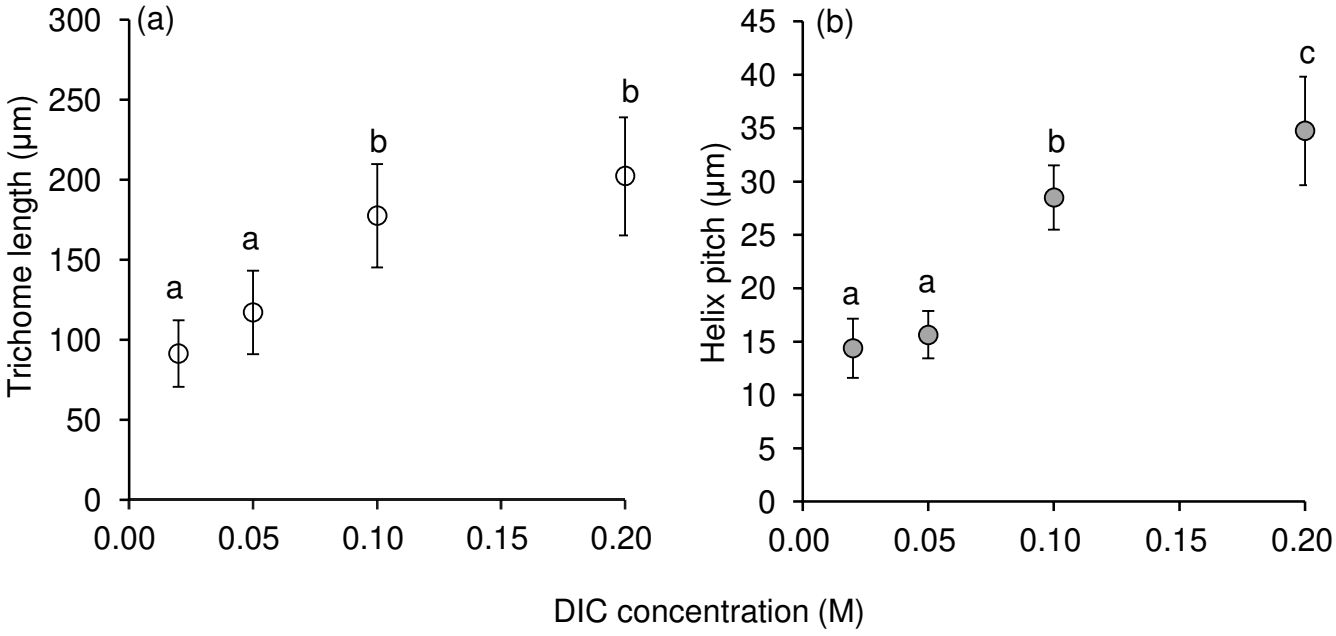



**Fig. 5** Trichome length (a) and helix pitch (b) in steady state (days 53-59) of semi-continuous *Limnospira fusiformis* LC-30 cultivation. Error bars indicate standard deviations. Different letters indicate statistically significant differences (One-way ANOVA, Tukey-Kramer, $p<0.01$)

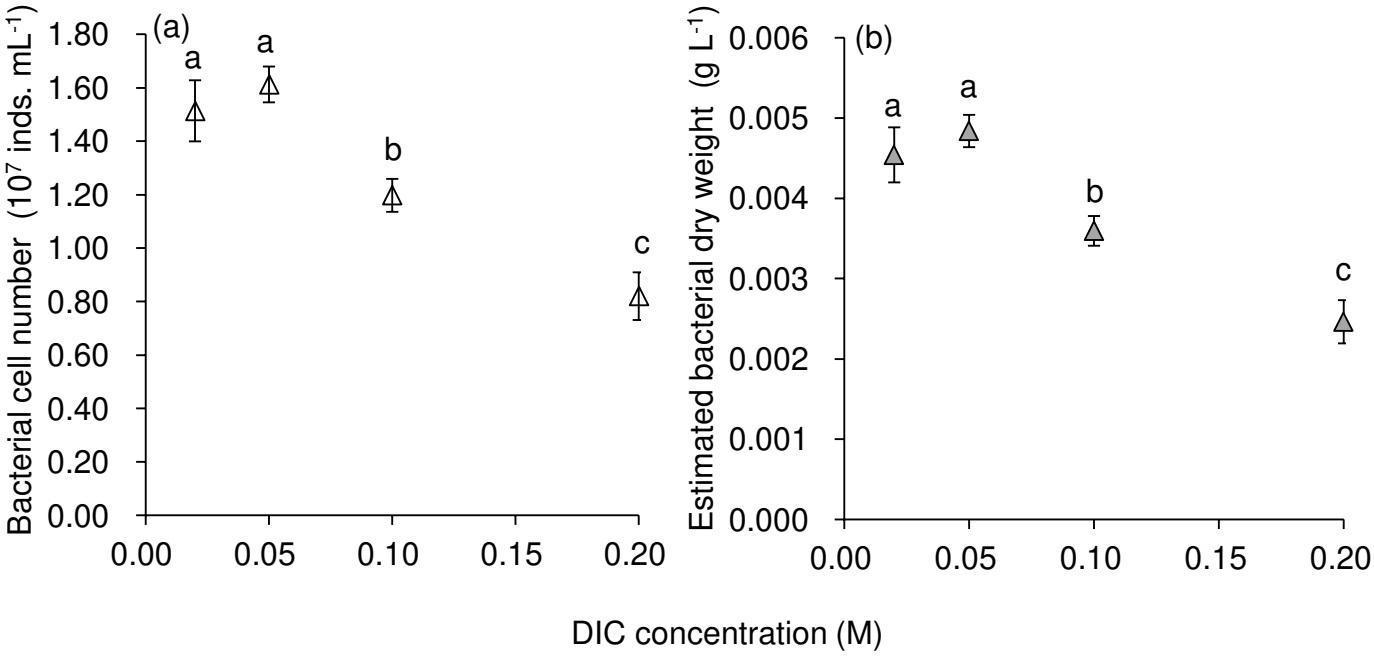



**Fig. 6** Cell number (a) and estimated bacterial dry weight (b) of bacteria contaminated into the culture at the end of experiment. Error bars indicate standard deviations. Different letters indicate statistically significant differences (One-way ANOVA, Tukey-Kramer, $p<0.01$)

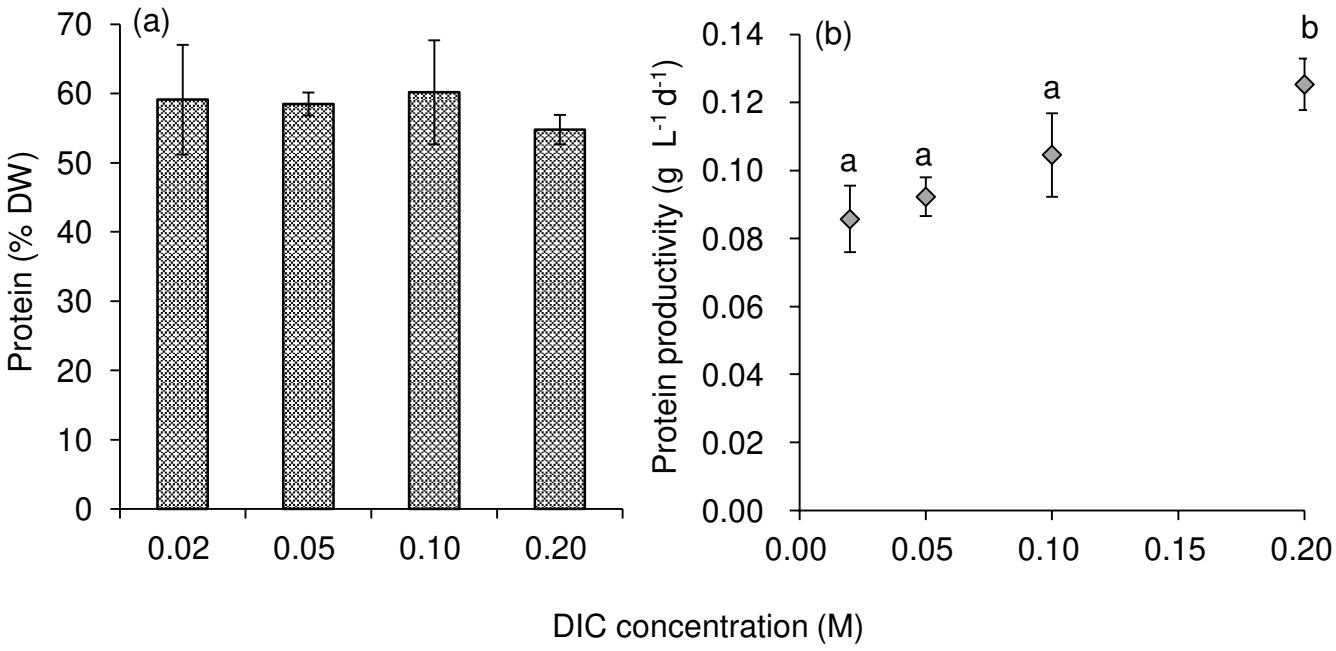



**Fig. 7** Protein content (a) and protein productivity (b) in steady state of semi-continuous *Limnospira fusiformis* LC-30 cultivation. Error bars indicate standard deviations. Different letters indicate statistically significant differences (One-way ANOVA, Tukey-Kramer, $p<0.01$)

**Table 1.** Comparison with previous studies under different DIC concentrations and cultivation conditions

| Species | Strain | Culture medium | Cultivation mode | DIC concentration (M) | Dry weight (g $L^{-1}$) | Biomass productivity (g $L^{-1}$ $d^{-1}$) | References |
|---|---|---|---|---|---|---|---|
| *Spirulina platensis* | - | Modified Jourdan | Batch | 0.048 | 0.85 | 0.09 | [29] |
| | | | | 0.095 | 1.01 | 0.12 | |
| | | | | 0.14 | 1.07 | 0.13 | |
| | | | | 0.19 | 1.29 | 0.17 | |
| *Spirulina platensis* | LEB-52 | Zarrouk + ADE(20%) | Semi-continuous | 0.033 | - | 0.280 | [41] |
| | | | | 0.063 | - | 0.280 | |
| | | Zarrouk | | 0.20 | - | 0.297 | |
| *Arthrospira platensis* | BP | Zarrouk | Semi-continuous | 0.20 | 0.92 | 0.20 | [40] |
| *Limnospira fusiformis* | LC-30 | Modified SOT | Semi-continuous | 0.20 | 2.82 | 0.50 | [12] |
| *Limnospira fusiformis* | LC-30 | Modified SOT | Semi-continuous | 0.02 | 0.73 | 0.15 | This study |
| | | | | 0.05 | 0.79 | 0.16 | |
| | | | | 0.10 | 0.87 | 0.17 | |
| | | | | 0.20 | 1.14 | 0.23 | |