J Appl Biotechnol Rep. 2021 Mar;8(1):37-40

doi 10.30491/JABR.2020.237342.1246

Journal of
Applied Biotechnology
Reports

Original Article

Improvement of Growth of *Chlamydomonas reinhardtii* in CO_2 – Stepwisely Aerating Condition

Akihito Nakanishi[1,2*], Yuri Sakihama[3], Nanami Ozawa[2]

[1]Graduate School of Bionics, Tokyo University of Technology, Tokyo, Japan
[2]School of Bioscience and Biotechnology, Tokyo University of Technology, Tokyo, Japan
[3]Tokyo University of Technology, Tokyo, Japan

Corresponding Author: Akihito Nakanishi, PhD, Assistant Professor, Graduate School of Bionics, Tokyo University of Technology, School of Bioscience and Biotechnology, Tokyo University of Technology, 1404-1 Katakuramachi, Hachioji, Tokyo, 192-0982, Japan. Tel: +81-42-637-2427, Email: nakanishiah@stf.teu.ac.jp

Received June 29, 2020; **Accepted** August 18, 2020; **Online Published** March 8, 2021

Abstract

Introduction: *Chlamydomonas reinhardtii* produces lipid and carbohydrate as an industrially useful bioproducts with supply of CO_2 as a carbon source. The CO_2 supplying system, especially aeration rate through the photobioreactor (PBR), should be controlled to enhance cell proliferation.

Materials and Methods: After fixing CO_2 concentration as 0.8%, the aeration rate was controlled to increase stepwisely by 10 mL.min^{-1}, 20 mL.min^{-1}, or 40 mL.min^{-1} beginning at 10 mL/min^{-1} to a maximum of 50 mL.min^{-1} after the pH > 6.5. To show the effect of CO_2-supply, the broth condition and the cell-component of lipid, carbohydrate, and protein was evaluated.

Results: The CO_2 supplying condition increasing by 10 mL.min^{-1} stepwisely when over pH 6.5 in 100 mL of broth led rapid cell proliferation reached a plateau 2 days earlier than in other conditions. On the other hand, the cell components incubated under 10 mL.min^{-1} stepwise condition showed no difference among the other conditions.

Conclusions: Cell proliferation was improved by optimized stepwise CO_2 aeration rates versus cell concentration in broth, and cell components were not changed even with improved cell proliferation. According to the results, it could be possible to improve material productivity by increasing biomass productivity.

Keywords: *Chlamydomonas reinhardtii*, Cell Proliferation, CO_2 Supply

Citation: Nakanishi A, Sakihama Y, Ozawa N. Improvement of growth of *chlamydomonas reinhardtii* in co_2 – stepwisely aerating condition. J Appl Biotechnol Rep. 2021;8(1):37-40. doi:10.30491/JABR.2020.237342.1246

Introduction

The green algae *Chlamydomonas* demonstrate a greater ability to fix carbon dioxide than other terrestrial plant species.[1] Recently, the production of lipids and carbohydrates was industrially expanded using *Chlamydomonas* due to its increased concentration of lipids and carbohydrates in cells.[2-6] In our study, *Chlamydomonas reinhardtii* was used as the model strain of *Chlamydomonas*, and the material productivity of *C. reinhardtii* was researched.[7-9] Material productivity is calculated by the mathematical product of biomass productivity and lipid and carbohydrate contents; to increase material productivity, the biomass productivity and control of lipid and carbohydrate contents in the cells must be enhanced.[3,8] Biomass productivity is a parameter that shows the production of cells per day by g·L^{-1}·d^{-1}, where g is the dry cell weight.[8,10] Biomass productivity is related to cell proliferation,[11,12] which is controlled by several factors that include carbon sources,[11] medium components (e.g., nitrogen source and metal ion contents),[3,11,13] temperature,[14] light intensity.[15] CO_2 gas was used as a carbon source for an autotrophic culture of *C. reinhardtii*, and the CO_2 concentration and aeration rate through the photobioreactor (PBR) are important factors of cell proliferation. The amount of CO_2 provided by the carbon source is dependent on the cell concentration and conditions in the broth; therefore, the supply of CO_2 through the PBR should be increased stepwise as cell growth increases. The aim of this study is to enhance the material productivity of *C. reinhardtii*. This study increased the aeration rate of CO_2 stepwise, evaluated cell components, and found that cell proliferation was improved.

Materials and Methods

Culturing Conditions

Chlamydomonas reinhardtii strain C-9: NIES-2235 was cultivated under phototrophic conditions in Modified Bold 6N (MB6N) medium, as described in previous studies.[3,16] The cells were cultured with a light intensity of 50 µmol photons·m^{-2}·s^{-1} (white fluorescent lamps) at 23°C. The cell suspension was adjusted to an initial OD_{750} = 0.01 with cells cultured in 100 mL MB6N medium before nitrogen starvation. During pre-cultivation, the aeration rate of 0.8% CO_2 was 10 mL.min^{-1}. The aeration rate of 0.8% CO_2 was increased

stepwise by 10 mL.min^{-1}, 20 mL.min^{-1}, or 40 mL.min^{-1} beginning at 10 mL.min^{-1} to a maximum of 50 mL.min^{-1} after the pH > 6.5. The aeration rate was evaluated and controlled with a flowmeter (Model RK1200 series, KOFLOC, Kyoto, Japan).

Evaluation of Broth

The cell numbers were counted with a disposable cell counting plate (WATSON Bio Lab, Tokyo, Japan).[11] The nitrate concentration and pH were measured after centrifugation of broth at 5000 × *g* for 1 minute. To measure the nitrate concentration, the absorbance of the supernatant was diluted 50-fold with distilled water and was measured at Abs_{220} using an appropriate calibration curve.[3,17] To measure pH, the supernatant was placed on a digital pH meter (LAQUAtwin, Horiba, Kyoto, Japan).[18]

Evaluation of Cells

The total lipid content and fatty acid composition were evaluated with gas chromatography with a flame ionization detector (GC/FID) system using previously described analytical methods.[3] Cells were collected by centrifugation at 5000 × *g* for 1 minute, washed with distilled water twice, and dried using a vacuum. The dried cells were fractured with 0.5 mm glass beads using a bead crusher (μT-12, Taitec, Saitama, Japan) at 23°C. The total lipids were esterized and extracted by a fatty acid methylation kit (FAME; Nacalai Tesque, Kyoto, Japan). The FAMEs were identified and quantified using GC-2025 (Shimadzu, Kyoto, Japan) equipped with a DB-23 capillary column (60 m, 0.25 mm internal diameter, 0.15 μm film thickness; Agilent Technologies, Palo Alto, CA) with nitrogen as the carrier gas at a flow rate of 2.3 mL.min^{-1}. The injector, ion source, and interface source temperatures were set at 230, 230, and 250°C, respectively. The oven temperature was initially set at 50°C for 1 min, then increased from 50 to 175°C at a rate of 25°C·min^{-1}, then increased from 175 to 230°C at a rate of 4°C·min^{-1}, and finally held at 230°C for 5 min. Rapeseed oil (Merck KGaA, Darmstadt, Germany) was utilized as a quantitative standard, and heptadecanoic acid (Sigma-Aldrich Co., St. Louis, MO) was used as an internal standard. The total carbohydrate content was evaluated with the anthrone-sulfuric acid method as described previously.[3] The total protein content was revealed with a bicinchoninic acid (BCA) protein assay kit (Takara Bio, Shiga, Japan).

Results and Discussion

Flow-through of the appropriate CO_2 supply into the *C. reinhardtii* broth limited waste CO_2 and supported efficient cell proliferation. In this study, the aerating rate of 0.8% CO_2 was increased stepwise into the PBR with progressing cell growth. Aeration rates of CO_2 greater than are necessary could result in waste due to the release of CO_2 from the PBR; pH increased as CO_2 dissolved in the broth decreased, which was used by *C. reinhardtii* as a carbon source.[19] In this study, the aeration rate of 0.8% CO_2 was increased into the PBR stepwise when the pH was greater than 6.5, and the maximum aeration rate was 50 mL.min^{-1} (Figure 1). The volume of broth in the PBR decreased each time a sample was taken, which resulted in a slightly increased volume per volume per minute (vvm) when the aeration rate of CO_2 did not actually increase into the PBR.

The broth condition of *C. reinhardtii* was evaluated measuring the cell number (concentration), pH, and nitrate concentration (Figure 2). On the first day of the culture,

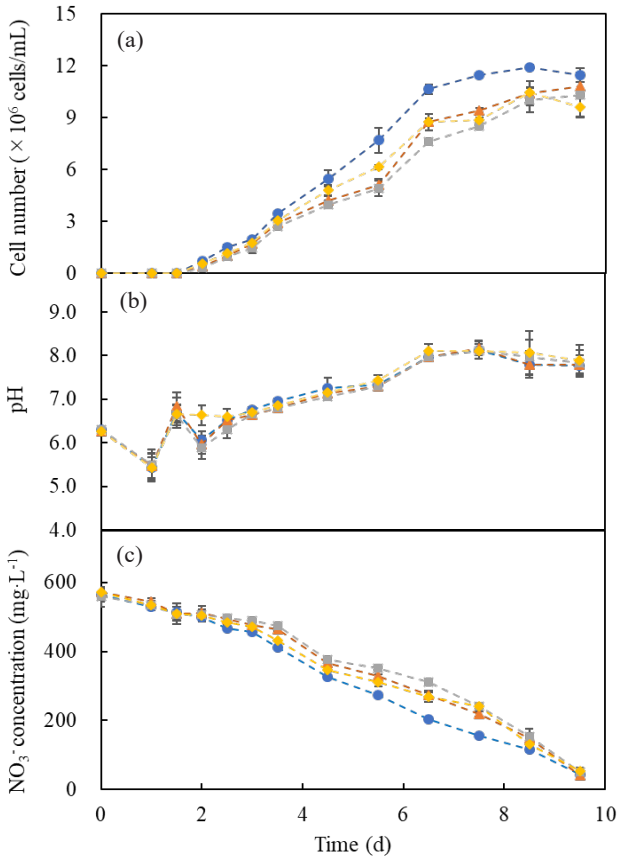

Figure 2. Broth Condition of *Chlamydomonas reinhardtii* in Each Culturing System. Time-course profiles of (a) cell number, (b) pH, and (c) nitrate concentration are shown as blue circles (10 mL.min^{-1} stepwise), orange triangle (20 mL.min^{-1} stepwise), gray square (40 mL.min^{-1} stepwise), and yellow diamond (constant). Error bars indicate the standard deviation of three replicate experiments.

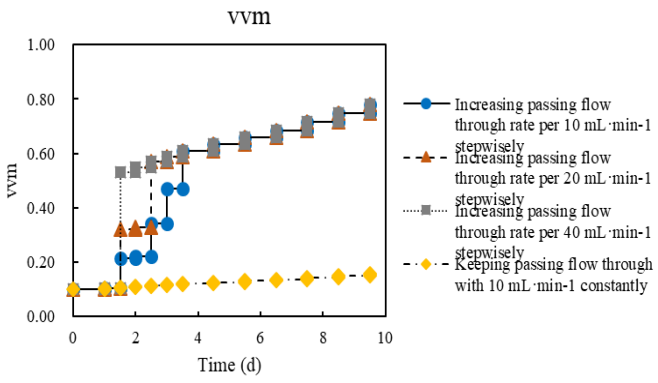

Figure 1. Shift of Volume Per Volume Per Minute (vvm) Versus Time in Each Culturing System. The shift of vvm in each culturing system is shown as follows: blue circle (10 mL.min^{-1} stepwise), orange triangle (20 mL.min^{-1} stepwise), gray square (40 mL.min^{-1} stepwise), and yellow diamond (constant). The gradual increase of vvm when the passing flow-through rate was not increased represented the decreasing volume of culture due to culture sampling while maintaining the flow-through rate.

Table 1. Ratio of Cell Component After Cultivation on Cell Concentration Over 1.0 × 10^7 cells·mL^{-1}

Content (%: Each Component Weight Per Dry Cell Weight)	Carbohydrate	Lipid	Protein
Increasing aeration rate per 10 $mL.min^{-1}$ stepwise (6.5 d)	32.7 ±5.9	19.3 ±3.5	15.5±3.4
Increasing aeration rate per 20 $mL.min^{-1}$ stepwise (8.5 d)	33.3 ±3.0	20.9 ±1.1	16.4 ±2.4
Increasing aeration rate per 40 $mL.min^{-1}$ stepwise (8.5 d)	34.8 ±0.6	17.8 ±1.8	15.5 ±1.0
Keeping aeration rate constant at 10 $mL.min^{-1}$ (8.5 d)	34.9 ±0.8	18.6 ±0.1	16.2 ±2.1

Values are the averages of three replicated experiments, ±SD.

Table 2. Fatty Acid Composition of *C. reinhardtii* on Cell Concentration Over 1.0 × 10^7 cells·mL^{-1}

Lipid Ratio (%: Each Component Weight Per Dry Cell Weight)	Myristic Acid (C14:0)	Palmitic Acid (C16:0)	Stearic Acid (C18:0)	Oleic Acid (C18:1)	Linoleic Acid (C18:2)	Linolenic Acid (C18:3)
Increasing aeration rate per 10 $mL.min^{-1}$ stepwise (6.5 d)	52.5 ±4.3	14.5 ±2.1	17.8 ±2.6	3.1 ±3.8	5.7 ±1.3	6.5 ±2.8
Increasing aeration rate per 20 $mL.min^{-1}$ stepwise (8.5 d)	55.2 ±5.1	11.3 ±1.3	16.5 ±0.5	1.6 ±0.6	8.1 ±1.7	7.2 ±2.5
Increasing aeration rate per 40 $mL.min^{-1}$ stepwise (8.5 d)	54.6 ±5.4	11.8 ±0.8	21.4 ±2.7	1.7 ±0.7	4.7 ±0.4	5.8 ±1.3
Keeping aeration rate constant at 10 $mL.min^{-1}$ (8.5 d)	53.8 ±1.2	11.8 ±0.2	20.9 ±0.9	2.9 ±2.4	4.2 ±1.7	6.4 ±0.7

Values are the averages of three replicated experiments, ±SD.

the cell number did not considerably increase with slightly decreasing nitrate concentrations, and the pH decreased from 6.3–6.4 to 5.4–5.5 in all CO_2-aerating conditions. The decreased pH could be caused by the overaeration of CO_2 into the broth with lower cell concentrations. Although the cell number did not show substantial increases in all CO_2-aerating conditions after 1.5 d, the pH increased to 6.6–6.8, which indicated that the CO_2 dissolved in the broth decreased. Therefore, the result showed that the CO_2 fixation by *C. reinhardtii* could be activated for later growth. Thereafter in all CO_2-aerating conditions, the cell number plateaued almost 1.0×10^7 cells·mL^{-1} in the PBR system. The time to reach the plateau in the increasing 10 $mL.min^{-1}$ condition was faster than the other conditions. The fastest growth in the PBR system would be supported by an appropriate CO_2 supply to reach the plateau. Conversely, there were few differences in the 20 and 40 $mL.min^{-1}$ conditions as compared to the steady 10 $mL.min^{-1}$ condition. In *Chlamydomonas* sp., the growth could be repressed with the oversupply of CO_2 into the PBR with lower cell concentration, which indicates that too much CO_2 would negatively impact cell proliferation in 20 and 40 $mL.min^{-1}$ conditions. To determine the optimal vvm to cell concentration, units of vvm, which were calculated by dividing vvm by cell number, were evaluated using data from the early culturing stage (48–72 hours) for total culturing (data not shown). The unit was 1.0–1.5 $\times10^{-7}$ vvm·$cells^{-1}$·mL^{-1} in the increasing 10 $mL.min^{-1}$ condition; alternatively, the units were 1.9– 2.6 $\times10^{-7}$ vvm·$cells^{-1}$·mL^{-1} and 3.4–5.5 $\times10^{-7}$ vvm·$cells^{-1}$·mL^{-1} in the 20 and 40 $mL.min^{-1}$ conditions, respectively. The units in the 20 and 40 $mL.min^{-1}$ conditions were higher than the unit in the increasing 10 $mL.min^{-1}$ condition, which showed the possibility of an oversupply of CO_2. Under constant 10 $mL.min^{-1}$ condition, the units were 1.7 $\times10^{-7}$ vvm·$cells^{-1}$·mL^{-1} at 48 h and 0.6–0.9 $\times10^{-7}$ vvm·$cells^{-1}$·mL^{-1} between 60–72 hours, which could represent an oversupply of CO_2 at 48 h and insufficiency of CO_2 between 60–72 hours.

Controlling the material contents in the cells is important to improve material productivity, which is represented by the mathematical product of biomass productivity and the content in the cells.[3] However, even when the rate of cell proliferation is improved, the material productivity does not considerably change cell composition.[11] The ratio of carbohydrates, lipids, and proteins in the cell composition and the ratio of total fatty acids were evaluated in each aerating condition when the cell concentration reached 1.0×10^7 cells·mL^{-1} (Table 1 and Table 2). Although the ratios of cell components and total fatty acids in green algae are substantially changed by environmental changes, including CO_2 concentration,[20] there were few differences in each aerating condition. Therefore, biomass productivity could be improved by increased cell proliferation with balanced rates of cell components and fatty acids in the increasing 10 $mL.min^{-1}$ condition, resulting in enhanced material productivity.

Conclusions

In this study, cell proliferation and cell components at a cell concentration of 1.0×10^7 cells·mL^{-1} in broth were evaluated by stepwise increases of the CO_2 aeration rate through the PBR. These results revealed that cell proliferation was improved by optimized stepwise CO_2 aeration rates versus cell concentration in broth, and cell components were not changed even with improved cell proliferation. According to the results, it could be possible to improve material productivity by increasing biomass productivity.

Author's Contributions

The research was supervised by AN and US. The manuscript writing and its finalization were carried out by AN and US. All experiments were manipulated by AN, YS, and NO.

Conflict of Interest Disclosures

Authors declare that they have no conflicts of interest.

Acknowledgments

The authors appreciated Prof. Nishino T, Prof. Matsui T, Prof. Yano K, and Senior Assistant Prof. Yoshida W for technical support, and Nihon Morita Yakusyo Co., Ltd. for research funding. We would like to thank Editage (www.editage.com) for English language editing.

References

1. Wang B, Li Y, Wu N, Lan CQ. CO(2) bio-mitigation using microalgae. Appl Microbiol Biotechnol. 2008;79(5):707-718. doi:10.1007/s00253-008-1518-y.
2. Jang S, Kong F, Lee J, et al. CrABCA2 facilitates triacylglycerol accumulation in *Chlamydomonas reinhardtii* under nitrogen starvation. Mol Cells. 2020;43(1):48-57. doi:10.14348/molcells.2019.0262.
3. Ho SH, Nakanishi A, Kato Y, et al. Dynamic metabolic profiling together with transcription analysis reveals salinity-induced starch-to-lipid biosynthesis in alga *Chlamydomonas* sp. JSC4. Sci Rep. 2017;7:45471. doi:10.1038/srep45471.
4. Nguyen MT, Choi SP, Lee J, Lee JH, Sim SJ. Hydrothermal acid pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. J Microbiol Biotechnol. 2009;19(2):161-166. doi:10.4014/jmb.0810.578.
5. Karpagam R, Preeti R, Ashokkumar B, Varalakshmi P. Enhancement of lipid production and fatty acid profiling in *Chlamydomonas reinhardtii*, CC1010 for biodiesel production. Ecotoxicol Environ Saf. 2015;121:253-257. doi:10.1016/j.ecoenv.2015.03.015.
6. Toyoshima M, Sato N. Optimization of triacylglycerol and starch production in *Chlamydomonas debaryana* NIES-2212 with regard to light intensity and CO2 concentration. Microbiology (Reading). 2018;164(3):359-368. doi:10.1099/mic.0.000603.
7. Cakmak T, Angun P, Ozkan AD, Cakmak Z, Olmez TT, Tekinay T. Nitrogen and sulfur deprivation differentiate lipid accumulation targets of *Chlamydomonas reinhardtii*. Bioengineered. 2012;3(6):343-346. doi:10.4161/bioe.21427.
8. Daniela Morales-Sánchez, Peter SC. Schulze, Viswanath Kiron, René H Wijffels. Production of carbohydrates, lipids and polyunsaturated fatty acids (PUFA) by the polar marine microalga Chlamydomonas malina RCC2488, Algal Research 2020; 50:102016. doi: 10.1016/j.algal.2020.102016.
9. Horst BG, Stewart EM, Nazarian AA, Marletta MA. Characterization of a carbon monoxide-activated soluble guanylate cyclase from *Chlamydomonas reinhardtii*. Biochemistry. 2019;58(17):2250-2259. doi:10.1021/acs.biochem.9b00190.
10. Tan CH, Show PL, Ling TC, et al. Exploring the potency of integrating semi-batch operation into lipid yield performance of *Chlamydomonas* sp. Tai-03. Bioresour Technol. 2019;285:121331. doi:10.1016/j.biortech.2019.121331.
11. Nakanishi A, Aikawa S, Ho SH, et al. Development of lipid productivities under different CO2 conditions of marine microalgae *Chlamydomonas* sp. JSC4. Bioresour Technol. 2014;152:247-252. doi:10.1016/j.biortech.2013.11.009.
12. Shin SE, Koh HG, Kang NK, et al. Isolation, phenotypic characterization and genome wide analysis of a *Chlamydomonas reinhardtii* strain naturally modified under laboratory conditions: towards enhanced microalgal biomass and lipid production for biofuels. Biotechnol Biofuels. 2017;10:308. doi:10.1186/s13068-017-1000-0.
13. Mosulén S, Domínguez MJ, Vigara J, Vílchez C, Guiraum A, Vega JM. Metal toxicity in *Chlamydomonas reinhardtii*. Effect on sulfate and nitrate assimilation. Biomol Eng. 2003;20(4-6):199-203. doi:10.1016/s1389-0344(03)00053-4.
14. Abbas S, Saeed S, Ammar M, Aman S, Shakeel SN. Effect of high temperature on activities and lipid production in mutants of *Chlamydomonas reinhardtii*. Int J Agric Biol. 2018;20(6):1331-1338. doi:10.17957/ijab/15.0634.
15. Ho SH, Nakanishi A, Ye X, et al. Dynamic metabolic profiling of the marine microalga *Chlamydomonas* sp. JSC4 and enhancing its oil production by optimizing light intensity. Biotechnol Biofuels. 2015;8:48. doi:10.1186/s13068-015-0226-y.
16. Berges JA, Franklin DJ, Harrison PJ. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. J Phycol. 2001;37(6):1138-1145. doi:10.1046/j.1529-8817.2001.01052.x.
17. Ho SH, Nakanishi A, Ye X, et al. Optimizing biodiesel production in marine *Chlamydomonas* sp. JSC4 through metabolic profiling and an innovative salinity-gradient strategy. Biotechnol Biofuels. 2014;7:97. doi:10.1186/1754-6834-7-97.
18. Salas-Herrera G, González-Morales S, Benavides-Mendoza A, Castañeda-Facio AO, Fernández-Luqueño F, Robledo-Olivo A. Impact of microalgae culture conditions over the capacity of copper nanoparticle biosynthesis. J Appl Phycol. 2019;31(4):2437-2447. doi:10.1007/s10811-019-1747-8.
19. Frick R, Junker B. Indirect methods for characterization of carbon dioxide levels in fermentation broth. J Biosci Bioeng. 1999;87(3):344-351. doi:10.1016/s1389-1723(99)80043-7.
20. Tang D, Han W, Li P, Miao X, Zhong J. CO2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO2 levels. Bioresour Technol. 2011;102(3):3071-3076. doi:10.1016/j.biortech.2010.10.047.