

Incubation of Cyanobacteria under Dark, Anaerobic Conditions and Quantification of the Excreted Organic Acids by HPLC

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[Abstract] Succinate and lactate are commodity chemicals used for producing bioplastics. Recently, it was found that such organic acids are excreted from cells of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 under dark, anaerobic conditions. To conduct the dark, anaerobic incubation, cells were concentrated within a vial that was then sealed with a butyl rubber cap, following which N₂ gas was introduced into the vial. The organic acids produced were quantified by high-performance liquid chromatography via post-labeling with bromothymol blue as a pH indicator. After separation by ion-exclusion chromatography, the organic acids were identified by comparing their retention time with that of standard solutions. These procedures allow researchers to quantify the organic acids produced by microorganisms, contributing to knowledge about the biology and biotechnology of cyanobacteria.

Keywords: Anaerobic condition, Cyanobacteria, HPLC, Lactate, Organic acids, Succinate, *Synechocystis* sp.

[Background] Cyanobacteria are a group of bacteria that perform oxygenic photosynthesis. *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) is a non-nitrogen-fixing, unicellular cyanobacterium that is commonly used for basic and applied research studies. *Synechocystis* 6803 cells are able to excrete organic acids, such as succinate and lactate, under both dark and anaerobic conditions (Osanai *et al.*, 2015). Genetic manipulation and modification of the incubation conditions, such as addition of potassium or NaHCO₃, have succeeded in increasing the levels of organic acids excreted from *Synechocystis* 6803 cells (Osanai *et al.*, 2015; Hasunuma *et al.*, 2016; Iijima *et al.*, 2016; Ueda *et al.*, 2016). Since succinate and lactate are commodity chemicals used to make various materials, such as bioplastics, their bio-based production is a desirable way to reduce the environmental burden. Herein, we describe the methods of *Synechocystis* 6803 incubation under dark, anaerobic conditions, and quantification of the organic acids by HPLC via post-labeling with bromothymol blue.

Materials and Reagents

1. 50 ml disposable polypropylene tubes
2. Test tubes for cultivation (Iwaki, catalog number: TEST30NP)
3. 20 ml headspace vials (GL Sciences, catalog number: 1030-46026)

4. 22 G x 1" needle (Terumo Medical, catalog number: NN-2225R)
5. 22 G x 1 ½" needle (Terumo, catalog number: NN-2238S)
6. Aluminum foil
7. SupraPure hydrophilic PVDF syringe filters (Recenttec, catalog number: R7-PVDF033S022I)
8. Target DP vials (Thermo Fisher Scientific, Thermo Scientific™, catalog number: C4000-1)
9. Screw caps (GL Sciences, catalog number: 1030-45261)
10. 300 µl target polyspring inserts (Thermo Fisher Scientific, Thermo Scientific™, catalog number: C4010-S630)
11. Disposable syringes (Terumo, catalog number: SS-10LZ)
12. Open-top-style aluminum caps with butyl rubber septa (Systech, catalog number: 98552)
13. MF-Millipore membrane filters (Merck Millipore, catalog number: HAWP03700)
14. Cyanobacterium *Synechocystis* sp. PCC 6803
15. Ammonium chloride (NH₄Cl) (Wako Pure Chemical Industries, catalog number: 017-02995)
16. 60% perchloric acid (HClO₄) (Wako Pure Chemical Industries, catalog number: 160-05755)
17. Trichloroacetic acid
18. Succinate
19. Lactate
20. Citric acid (Nacalai Tesque, catalog number: 09109-85)
21. Acetate
22. Ferric ammonium citrate
23. Na₂EDTA (Nacalai Tesque, catalog number: 15111-45)
24. Sodium nitrate (NaNO₃) (Wako Pure Chemical Industries, catalog number: 195-02545)
25. Potassium phosphate dibasic (K₂HPO₄) (Wako Pure Chemical Industries, catalog number: 164-04295)
26. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (Wako Pure Chemical Industries, catalog number: 131-00405)
27. Calcium chloride dihydrate (CaCl₂·2H₂O) (Wako Pure Chemical Industries, catalog number: 031-00435)
28. Sodium carbonate (Na₂CO₃) (Nacalai Tesque, catalog number: 31311-25)
29. Boric acid (H₃BO₃) (Wako Pure Chemical Industries, catalog number: 021-02195)
30. Manganese chloride tetrahydrate (MnCl₂·4H₂O) (Wako Pure Chemical Industries, catalog number: 139-00722)
31. Zinc sulfate heptahydrate (ZnSO₄·7H₂O) (Wako Pure Chemical Industries, catalog number: 264-00402)
32. Pentahydrate copper sulphate (CuSO₄·5H₂O) (Wako Pure Chemical Industries, catalog number: 039-04412)
33. Sodium molybdate dehydrate (Na₂MoO₄·2H₂O) (Wako Pure Chemical Industries, catalog number: 196-02472)
34. Sulfuric acid (H₂SO₄) (Wako Pure Chemical Industries, catalog number: 199-15995)

35. Cobalt dinitrate hexahydrate, $\text{Co}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ (Wako Pure Chemical Industries, catalog number: 031-03752)
36. HEPES (Dojindo Molecular Technologies, catalog number: 342-01375)
37. Potassium hydroxide (KOH) (Wako Pure Chemical Industries, catalog number: 168-21815)
38. Bromothymol blue (BTB) (Wako Pure Chemical Industries, catalog number: 027-03052)
39. Ethanol (Wako Pure Chemical Industries, catalog number: 057-00451)
40. Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Wako Pure Chemical Industries, catalog number: 196-02835)
41. Succinic acid (Wako Pure Chemical Industries, catalog number: 190-04332)
42. DL-Lactic acid lithium salt (MP Biomedicals, catalog number: 100824)
43. Sodium acetate (Wako Pure Chemical Industries, catalog number: 190-01071)
44. Modified BG-11 liquid medium (see Recipes)
45. Mobile phase (3 mM HClO_4) (see Recipes)
46. Reaction solution (0.2 mM BTB in 15 mM Na_2HPO_4) (see Recipes)
47. 500 mM succinate solution (see Recipes)
48. 500 mM lactate solution (see Recipes)
49. 500 mM citrate solution (see Recipes)
50. 500 mM acetate solution (see Recipes)
51. 1 M acetate solution (see Recipes)

Equipment

1. Cultivation chamber (TOMY Digital Biology, model: CLE-303)
2. A gas mixer (KOFLOC, model: RK120XM)
3. An air pump (Yasunaga, model: LP-30A)
4. Spectrophotometer (Shimadzu, model: UV-2700)
5. High-speed refrigerated microcentrifuge (TOMY Digital Biology, model: MX-305)
6. N_2 generator (Sanyo Denshi, model: SN4-4)
7. Rotary shaker (Nissin, model: NX-20D)
8. Freeze dryer (Tokyo Rikaikai, EYELA, model: FDU-2200)
9. Ultrasonic cleaning machine (SND, model: US-108)
10. Autoclave
11. The HPLC system (JASCO International, model: LC-2000Plus) was composed of the following equipment:
 - a. Photodiode array (PDA) detector (JASCO International, model: MD-2018Plus)
 - b. Intelligent HPLC pump (JASCO International, model: PU-2080Plus)
 - c. Line degasser (JASCO International, model: DG-2080-53)
 - d. Ternary gradient unit (JASCO International, model: LG-2080-02)
 - e. Intelligent column oven (JASCO International, model: CO-2065Plus)

- f. Intelligent autosampler (JASCO International, model: AS-2057Plus)
- g. Ion-exclusion column (300 x 8.0 mm) (GL Sciences, model: RSpak KC-811, catalog number: 5055-13509) and guard column (10 x 4.6 mm) (GL Sciences, model: GPC KF-G, catalog number: 5055-13150)
- h. Reaction coil unit (JASCO International, model: RU-2080-51)

Software

1. ChromNAV software (Ver. 1.14)

Procedure

1. Cultivation under light, aerobic (a) and incubation under dark, anaerobic conditions (b)
 - a. The glucose-tolerant strain (designated as GT) of *Synechocystis* sp. PCC 6803, isolated by Williams (1988), is cultivated in modified BG-11 medium (see Recipes), consisting of BG-11_o liquid medium (Rippka, 1988) supplemented with 5 mM NH₄Cl (buffered with 20 mM HEPES-KOH, pH 7.8). In our study, the GT-I strain was used (Kanesaki *et al.*, 2012). The GT cells are cultivated in liquid modified BG-11 medium under air containing 1% (v/v) CO₂, using a gas mixer (RK120XM, KOFLOC) and an air pump (LP-30A, Yasunaga) at the flow rate of 30-50 ml/min, in a cultivation chamber (CLE-303, TOMY). The temperature is kept at 30 °C and white light is supplied continuously at 50-70 μmol photons·m⁻² sec⁻¹. Cell densities are measured at A₇₃₀ using the Shimadzu UV-2700 spectrophotometer. Cells cultivated in 70 ml of modified BG-11 medium (started from A₇₃₀ = 0.4) for 3 days under, light, aerobic condition. The light, aerobic cultivation affects the levels of succinate and lactate, and optimization of culture conditions is required for each laboratory.
 - b. The cell are collected by centrifugation at 5,600 x g for 2 min and concentrated into 10 ml of HEPES buffer (20 mM HEPES-KOH, pH 7.8) or modified BG-11 to reach an A₇₃₀ of 20 in a vial (Figure 1). The vial is sealed with open-top-style aluminum caps with butyl rubber septa, and two needles (Needle 22 G x 1" and Needle 22 G x 1 ½") are stabbed through a septum. The 22 G x 1 ½" needle is connected to an N₂ gas generator to introduce N₂ gas for 30-60 min in order to generate the anaerobic condition.
 - c. After introduction of the N₂ gas, the needles are removed and the vial is wrapped with aluminum foil. The cells are incubated at 30 °C with shaking on a rotary shaker at 160 rpm for 1-5 days. For 1 day, succinate and lactate were less excreted from the cells. Succinate and lactate levels increased during long incubation, however, the levels reached maximum at 3 or 4 days, and started to decrease at 5 days (Osanai *et al.*, 2015).



Figure 1. A 20 ml vial for anaerobic incubation. Cells were concentrated into 10 ml of buffer or medium to reach an A_{730} of 20, and the vial was then sealed with a butyl rubber cap. Two syringes were inserted into the cap, and N_2 gas was introduced through one of two syringes for 30-60 min. After introduction of the N_2 gas, the syringes were removed and the vial was wrapped in aluminum foil to maintain a dark condition.

2. Sample preparation for HPLC analysis
 - a. After incubation, the aluminum cap with butyl rubber is removed with an opener. The cell cultures are centrifuged at $5,600 \times g$ for 2 min. Using a SupraPure sterile syringe filter, the supernatants are filtered to remove debris.
 - b. Then, 1 ml of supernatant is transferred into a 2 ml plastic tube. The supernatants are placed in a flask and dried at $-80 \text{ }^\circ\text{C}$ with an FDU-2200 freeze dryer for 1 day. The freeze-dried samples are resolved with 100 μl of 3 mM perchloric acid (HClO_4) (since HClO_4 is highly corrosive, it should be handled with care) and centrifuged at $5,600 \times g$ for 2 min.
 - c. Then, transfer 100 μl of the supernatant into a Target DP vial using a glass insert. Set it in the autosampler for HPLC analysis.
 - d. In case of samples containing large amount of proteins, freeze-dried samples should be resolved in 97 μl of 3 mM HClO_4 and 3 μl of 100% trichloroacetic acid. Mix thoroughly and then centrifuge the samples at $20,400 \times g$ for 10 min. Use the supernatant for HPLC analysis.
 - e. To prepare standards, mix 100 μl of 500 mM succinate solution, 160 μl of 500 mM lactate solution, 100 μl of 500 mM citrate solution, 200 μl of 1 M acetate solution (see Recipes), and 440 μl of dH_2O to make up a total 1 ml of organic acids standard. This solution was named STD1. The final concentration of each organic acid in STD1 is 80 mM lactate, 50 mM succinate, 50 mM citrate, and 200 mM acetate.

- f. Dilute STD1 to make a range of concentrations for drawing the analytical curves.
3. HPLC analysis

Quantification of the organic acids is performed by HPLC using post-labeling with BTB (Figure 2). Use 3 mM HClO₄ solution as the mobile phase and 0.2 mM BTB in 15 mM Na₂HPO₄ solution as the reaction solution. The mobile phase and reaction solution are degassed for approximately 20 min using the ultrasonic cleaning machine and the aspirator. HPLC measurement is performed with the following conditions:

 - a. Intelligent HPLC pump (Mobile phase): the flow rate is set at 0.7 ml/min. The pump is connected to the line degasser and the ternary gradient unit, and further connected to the intelligent column oven. Before sample injection, equilibrate the columns with mobile phase at same flow rate for one hour.
 - b. Intelligent HPLC pump (Reaction solution): the flow rate is set at 1.2 ml/min.
 - c. Intelligent column oven: the column temperature is 60 °C.
 - d. Intelligent autosampler: the sampler temperature is 4 °C and the injection volume is 20 µl. To clean up the injection needle, use HClO₄ solution. Flush three times with HClO₄ before sample injection.
 - e. Reaction coil unit was used for mixing mobile phase including samples and BTB solution.
 - f. PDA: single beam photometer with deuterium (D₂) and halogen (W) lamps; single data analysis is performed for 42 min using scans in the wavelength range of 200-900 nm. Before starting the HPLC analysis, wait for approximately 60 min until the flow is stable (baseline without drift and noise).

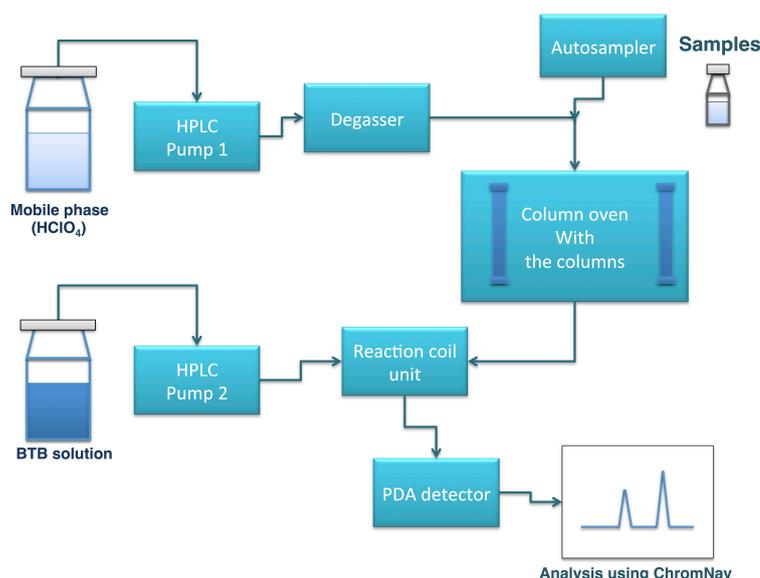


Figure 2. Schematic diagram of HPLC analysis with bromothymol blue post-labeling. Two HPLC pumps are used for mobile phase and BTB solution, respectively. Samples are injected with autosampler and divided by size exclusion columns, followed by mixed with BTB solution. Organic acids are detected by PDA detector and analyzed by ChromNAV software.

Data analysis

Typical chromatograms measured by HPLC with BTB post-labeling are shown in Figure 3. Organic acid peaks are quantified using the absorbance at 445 nm. Use the ChromNAV program to quantify the peak areas of each organic acid. Draw standard curves for each organic acid by marking with a dot of known concentration samples. Calculate the concentrations of organic acids in the samples by using the following standard curves (Figure 4).

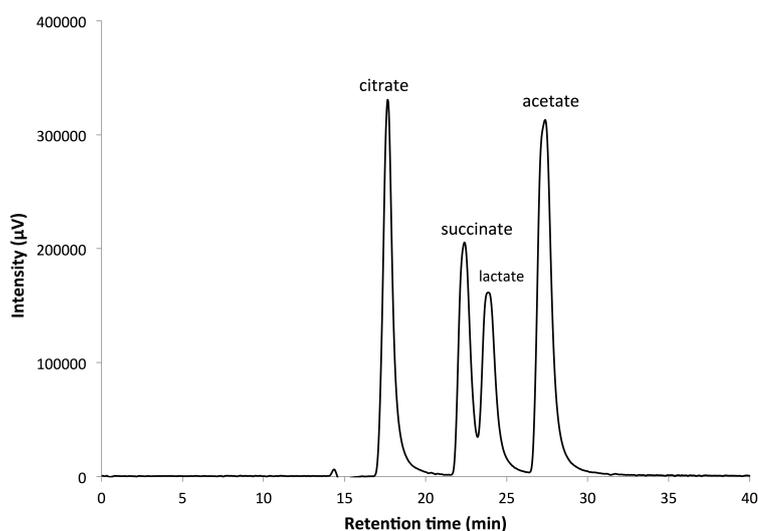


Figure 3. Typical chromatograms measured by HPLC with bromothymol blue post-labeling. The peak intensity was calculated from the absorbance at wavelength 445 nm. The retention times for each organic acid were as follows: citrate 17.55 min, succinate 22.36 min, lactate 23.80 min, and acetate 27.34 min.

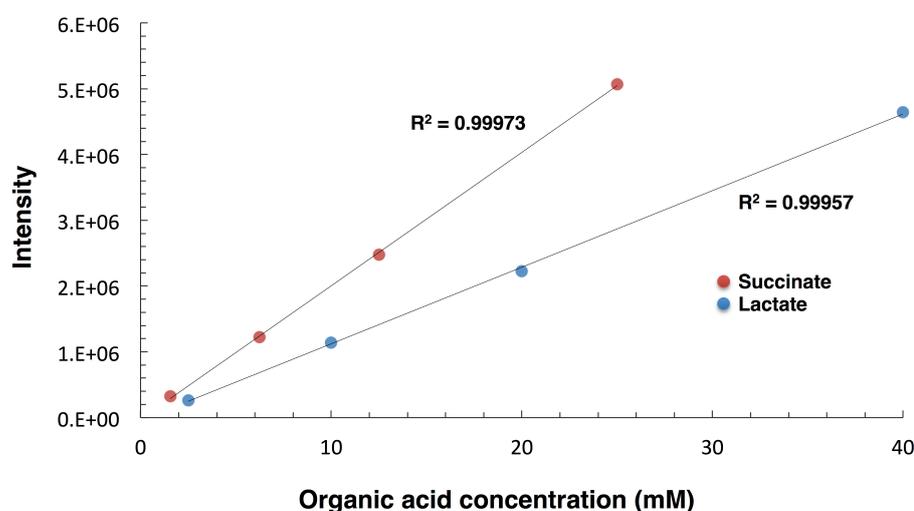


Figure 4. Standard curves for succinate (orange) and lactate (blue). The intensity values were calculated from the chromatograms by integrating the peak areas of each organic acid.

Notes

In case of samples containing large amount of proteins, freeze-dried samples should be resolved in 97 μ l of 3 mM HClO₄ and 3 μ l of 100% trichloroacetic acid. Mix thoroughly and then centrifuge the samples at 20,400 x g for 10 min. Use the supernatant for HPLC analysis.

Recipes

1. Modified BG-11 liquid medium (store at room temperature)
 - a. Solution 1 (store at 4 °C)
Weigh 0.6 g citric acid, 0.6 g ferric ammonium citrate, and 0.1 g Na₂EDTA, and add distilled water (dH₂O) to 200 ml
 - b. Solution 2 (store at 4 °C)
Weigh 30 g NaNO₃, 0.78 g K₂HPO₄, and 1.5 g MgSO₄·7H₂O, and add dH₂O to 1 L
 - c. Solution 3 (store at 4 °C)
Weigh 3.8 g CaCl₂·2H₂O and add dH₂O to 200 ml
 - d. Solution 4 (store at 4 °C)
Weigh 4 g Na₂CO₃ and add dH₂O to 200 ml
 - e. Solution 5 (store at 4 °C)
Weigh 2.86 g H₃BO₃, 1.81 g MnCl₂·4H₂O, 0.22 g ZnSO₄·7H₂O, 0.08 g CuSO₄·5H₂O, 0.021 g Na₂MoO₄, and 50 μ l H₂SO₄, and add dH₂O to 1 L (1*)
Weigh 0.0494 g Co(NO₃)₂·5H₂O and add to (1*)
 - f. 1 M HEPES-KOH, pH 7.8 (store at 4 °C)
Weigh 119.1 g HEPES and add dH₂O to approximately 400 ml
Adjust pH to 7.8 with 6 N KOH and add dH₂O to 500 ml
Autoclave (121 °C for 15 min) and store at 4 °C
 - g. K₂HPO₄ solution (store at 4 °C)
Weigh 0.39 g K₂HPO₄ and add dH₂O to 200 ml
 - h. MgSO₄·7H₂O solution (store at 4 °C)
Weigh 0.75 g MgSO₄·7H₂O and add dH₂O to 200 ml
Mix 20 ml 1 M HEPES-KOH (pH 7.8), 50 ml Solution 2, 2 ml Solution 3, 1 ml Solution 4, and 1 ml Solution 5, and add dH₂O to 1 L
Autoclave the mixture and store at room temperature
Before use, add 2 ml Solution 1 to 1 L mixture
2. Mobile phase (3 mM HClO₄)
Weigh 0.5 g 60% HClO₄ and add dH₂O to 1 L
Filter with the MF-Millipore membrane filter

3. Reaction solution (0.2 mM BTB in 15 mM Na₂HPO₄)
Weigh 125 mg BTB and add to 10 ml ethanol (1)
Weigh 5.3 g Na₂HPO₄·12H₂O and add to dH₂O (2)
Mix (1) and (2), and add dH₂O to 1 L
Filter the mixture with the MF-Millipore membrane filter
4. 500 mM succinate solution
Weigh 590.45 mg succinate and add to 10 ml dH₂O using a measuring flask
5. 500 mM lactate solution
Weigh 480.05 mg DL-lactate lithium salt and add to 10 ml dH₂O using a measuring flask
6. 500 mM citrate solution
Weigh 960.6 mg citrate and add dH₂O to 10 ml using a measuring flask
7. 1 M acetate solution
Weigh 820.3 mg sodium acetate and add dH₂O to 10 ml using a measuring flask

Acknowledgments

This protocol was adapted from our previous studies published by Ueda *et al.* (2016) and Iijima *et al.* (2016). This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by a grant to T.O. from ALCA (Project name 'Production of cyanobacterial succinate by the genetic engineering of transcriptional regulators and circadian clocks') from the Japan Science and Technology Agency, and by JSPS KAKENHI Grant-in-Aid for Scientific Research on Innovative Areas (Grant No. 16H06559).

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