

Extraction and Quantification of Alkanes in *Cyanobacteria*

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[Abstract] Many species of cyanobacteria accumulate alkanes in their cells. It has been reported that these cyanobacteria accumulate mainly 15 long carbon chain alkane, pentadecane (C₁₅H₃₂), or/and 17 long chain alkane, heptadecane (C₁₇H₃₆). Here we describe a protocol of our laboratory for extraction and quantification of cyanobacterial intracellular pentadecane and heptadecane. We have confirmed this protocol was applicable to at least three kinds of cyanobacteria, nitrogen-fixing filamentous cyanobacterium *Anabaena* sp. PCC7120, non-diazotrophic unicellular cyanobacterium *Synechococcus elongatus* PCC7942 and halotolerant unicellular cyanobacterium *Aphanothece halophytica*.

Materials and Reagents

1. Sampling tubes (1.5 ml) (Ina-optika corporation, Ina Optica, catalog number: CF-0150)
2. Sampling tubes (50 ml) (Corning, Falcon®, catalog number: 352070)
3. Glass vial (Shimadzu Scientific Instruments, catalog number: GLC4010-17)
4. Capillary column (15 m, 0.25 mm internal diameter) (Restek Corporation, model: Rtx-1MS)
5. Cyanobacteria (*Anabaena* sp. PCC7120, *Synechococcus elongatus* PCC7942 and *Aphanothece halophytica*)
6. Sodium nitrate (NaNO₃) (Sigma-Aldrich, catalog number: 28-3440-5)
Note: Currently, it is "Sigma-Aldrich, catalog number: 28-3440".
7. Potassium phosphate dibasic (K₂HPO₄) (Sigma-Aldrich, catalog number: 24-5240-5)
Note: Currently, it is "Sigma-Aldrich, catalog number: 24-5240".
8. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (KANTO KAGAKU, catalog number: 25034-00)
9. Calcium chloride dihydrate (CaCl₂·2H₂O) (Sigma-Aldrich, catalog number: 05-0590-5)
Note: Currently, it is "Sigma-Aldrich, catalog number: 05-0590".
10. Citric acid (KATAYAMA CHEMICAL, catalog number: 05-4930)
Note: Currently, it is "Sigma-Aldrich, catalog number: 05-4930".
11. Ferric ammonium citrate (Wako Pure Chemical Industries, Siyaku, catalog number: 097-00835)

12. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na₂) (Sigma-Aldrich, catalog number: 09-1420-5)
13. Sodium carbonate (Na₂CO₃) (Sigma-Aldrich, catalog number: 28-2180-5)
14. Boric acid (H₃BO₃) (KATAYAMA CHEMICAL, catalog number: 03-2900)
Note: Currently, it is "Sigma-Aldrich, catalog number: 03-2900".
15. Manganese(II) chloride (MnCl₂) (KANTO KAGAKU, catalog number: 25061-00)
16. Zinc sulfate heptahydrate (ZnSO₄·7H₂O) (KATAYAMA CHEMICAL, catalog number: 37-0550)
Note: Currently, it is "Sigma-Aldrich, catalog number: 37-0550".
17. Sodium molybdate dihydrate (Na₂MoO₄·2H₂O) (KATAYAMA CHEMICAL, catalog number: 28-3400)
Note: Currently, it is "Sigma-Aldrich, catalog number: 28-3400".
18. Copper(II) sulfate pentahydrate (CuSO₄·5H₂O) (KATAYAMA CHEMICAL, catalog number: 05-6220)
Note: Currently, it is "Sigma-Aldrich, catalog number: 05-6220".
19. Cobalt(II) nitrate hexahydrate [Co(NO₃)₂·6H₂O] (KATAYAMA CHEMICAL, catalog number: 05-5230)
Note: Currently, it is "Sigma-Aldrich, catalog number: 05-5230".
20. Sodium chloride (NaCl) (KANTO KAGAKU, catalog number: 37144-00)
21. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 24-4290-5)
22. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (KANTO KAGAKU, catalog number: 25009-00)
23. Methanol (KANTO KAGAKU, catalog number: 25183-70)
24. Pentadecane (TCI America, catalog number: S0287)
25. Heptadecane (TCI America, catalog number: S0289)
26. BG-11 medium for *Anabaena* sp. PCC7120 and *Synechococcus elongatus* PCC7942 (Stanier *et al.*, 1971) (see Recipes)
27. BG-11 medium plus Turk Island salt solution (0.5 M NaCl) for *Aphanothece halophytica* (Hibino *et al.*, 1999) (see Recipes)

Equipment

1. Glass conical flask (500 ml size)
2. Shaker (TAITEC CORPORATION, model: NR-3)
3. Thermostatic chamber [Temperature was set to 30 °C (±0.1 °C) by the regulator (Panasonic, catalog number: MCU-201 CPH2)]
4. Fluorescent lamp (light quality: natural white, total luminous flux: 2,950 lm) (Yodobashi Camera Co., TOSHIBA, model: FL40SN)
5. Spectrometer (Shimadzu Scientific Instruments, model: UV-160A)
6. Centrifuge (for 50 ml tubes) (KUBOTA Corporation, catalog number: 5911)

7. Centrifuge (for 1.5 ml tubes) (KUBOTA Corporation, catalog number: 3700)
8. Vacuum evaporator (TAITEC CORPORATION, model: Vc-15s)
9. Sonicator (TAITEC CORPORATION, model: VP-5s)
10. Fridge
11. GC-MS (Shimadzu Scientific Instruments, catalog number: GCMS-QP2010)
12. Helium gas

Software

1. GCMSsolution software version 2.40 (Shimadzu Scientific Instruments, catalog number: 225-13067-91)

Procedure

A. Growth of cyanobacteria

Anabaena sp. PCC7120 and *Synechococcus elongatus* PCC7942 were grown in liquid BG-11 media in glass conical flasks at 30 °C under constant white fluorescent light at approximately 40 $\mu\text{E}/\text{m}^2/\text{s}$ in thermostatic chamber. The cultures were shaken at 105 rpm. In the case of *Aphanothece halophytica*, BG-11 medium plus Turk Island salt solution (0.5 M NaCl) was used instead of BG-11 medium. The growth of cyanobacterial cells was monitored by measuring the absorbance at 730 nm with a spectrophotometer.

Notes:

- a. Other conditions such as abiotic stress condition (nitrogen starvation, high salinity, etc.) were applicable (Kageyama *et al.*, 2015).
- b. Culture volume should be less than 200 ml in 500 ml size flask.

B. Harvesting cells

1. Cyanobacterial cells grown exponentially were precipitated by centrifugation at 4,300 x g for 15 min at 4 °C from 50 ml cultures ($\text{OD}_{730\text{nm}} = \sim 0.6$).
2. After discarding media by decantation, the cells were suspended in the remaining media (~1 ml).
3. Cell suspensions were transferred to preweighed 1.5 ml sampling tubes.
4. Cells were precipitated by centrifugation at 22,000 x g for 10 min at 4 °C, then media were removed completely.
5. Cells were stored at -80 °C before use.

C. Extraction of alkanes

1. Frozen cells were dried up using a vacuum evaporator.
2. Dry cell weight was measured.

Note: Usually ~20 mg of dried cells was obtained.

3. Methanol was added to dried cells.

Note: We added 1 ml of Methanol/20 mg of dried cells.

4. Cells were disrupted by sonication (output: 7, total on time: 6 min).

Notes:

- a. *Samples were on ice during sonication treatment with 12 burst of 30 sec followed by intervals of 30 sec for cooling.*
- b. *Be careful that the methanol extract does not scatter during sonication treatment.*

5. Samples were incubated at 4 °C for overnight.

6. Cell debris was precipitated by centrifugation at 22,000 x g for 10 min at 25 °C, then the supernatants were transferred into new 1.5 ml tubes. This methanol extract contained alkanes.

Note: After preparing the extracts, we subjected extracts to GC-MS analysis immediately.

D. Detection of alkanes by GC-MS

1. 500 µl of methanol extracts contain alkanes were transferred into glass vials designed for GC-MS.
2. Samples were subjected to GC-MS. The following is a gas chromatography method for alkanes (pentadecane and heptadecane) using a capillary column RESTEK Rtx-1MS (15 m, 0.25 mm internal diameter):
 - a. Helium was used as the carrier gas at the flow rate of 1.3 ml min⁻¹.
 - b. 8 µl of samples were injected using a 1:100 split* ratio of helium carrier gas to column at 300 °C of inlet temperature.
 - c. Initial oven temperature of 100 °C for 3 min.
 - d. Ramp to 320 °C at a rate of 20 °C/min.

**Note: Glass insert designed for split analysis should be used.*

E. Quantification of alkanes

For Quantification of pentadecane and heptadecane, we used authentic standards. These authentic standards of alkane were diluted with methanol, and then subjected to GC-MS to make standard curves. Standard curves were made by using the peak area calculated by GCMSsolution software. Retention time of pentadecane and heptadecane using the method described above was ~5.6 and ~7.0 min, respectively (Figure 1A). For identification of pentadecane and heptadecane in cyanobacterial extracts, their retention times of the peaks in chromatogram and fragmentation patterns of product peaks were compared with authentic standards (Figure 1 and Figure 2). As a representative data, chromatogram using *Anabaena* sp. PCC7120 extract and fragmentation pattern of the peak at 7 min were shown (Figure 2). For quantification of pentadecane and heptadecane in cyanobacteria, area of the target peak obtained by GC-MS was calculated by GCMSsolution software, and then concentration was determined using standard curve of

authentic standards. As shown in Figure 1C, standard curve of heptadecane was linear at the range of 0.005 - 0.5 mg/ml. A similar line was also obtained for pentadecane.

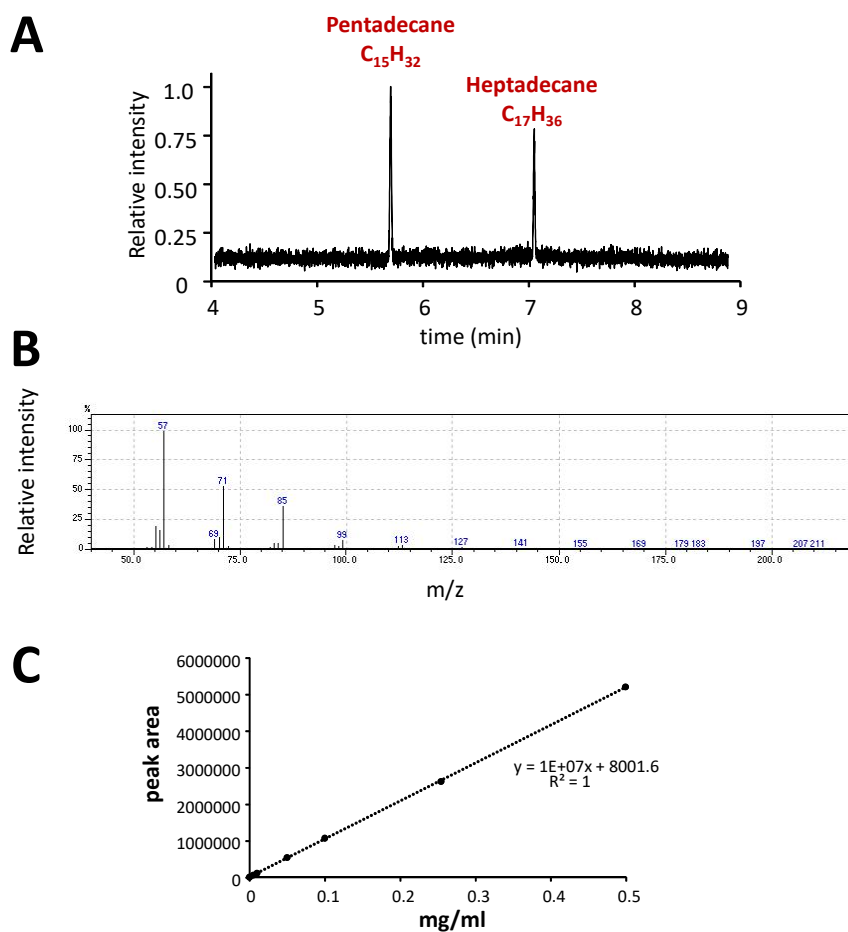


Figure 1. GC-MS profile and standard curve of authentic standards. Chromatogram of pentadecane and heptadecane (A), and fragmentation pattern (B) and standard curve (C) of heptadecane are shown.

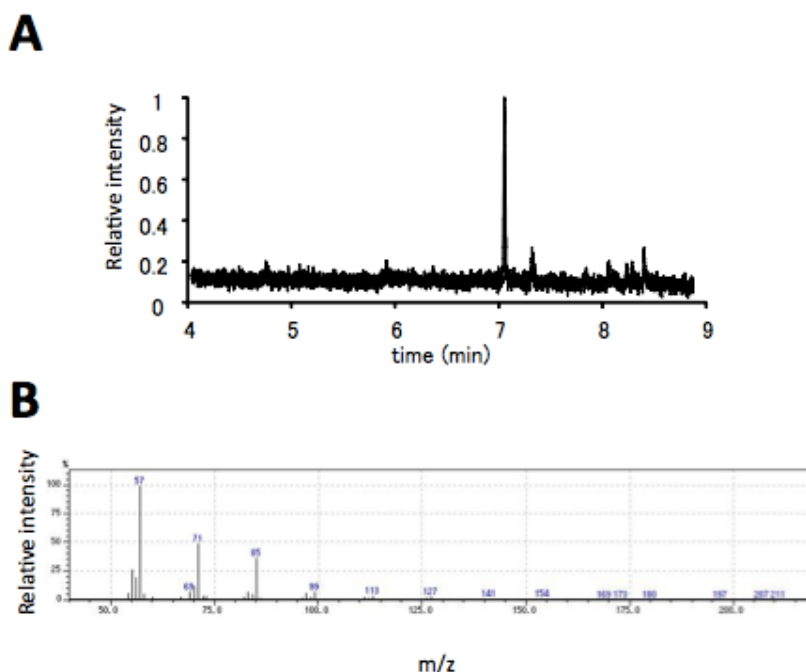


Figure 2. GC-MS profile of methanol extract of *Anabaena* sp. PCC7120. A. Chromatogram indicated the main peak appeared at 7.0 min. B. Fragmentation pattern of the main peak at 7 min was similar to that of heptadecane authentic standard (see Figure 1B).

Recipes

1. BG-11 medium for *Anabaena* sp. PCC7120 and *Synechococcus elongatus* PCC7942 (Stanier *et al.*, 1971)

Preparation of stock solutions (These solutions were autoclaved.)

Stock #1: 6.27 g of K_2HPO_4 /200 ml H_2O

Stock #2: 15.0 g of $MgSO_4 \cdot 7H_2O$ /200 ml H_2O

Stock #3: 7.20 g of $CaCl_2 \cdot 2H_2O$ /200 ml H_2O

Stock #4: 4.00 g of Na_2CO_3 / 200 ml H_2O

Stock #5: 1.20 g of citric acid + 1.20 g of Ferric ammonium citrate + 0.20 g of EDTA 2Na/200 ml H_2O

Stock #6: 572 mg of H_3BO_3 + 362 mg of $MnCl_2$ + 44.4 mg of $ZnSO_4 \cdot 7H_2O$ + 78.0 mg of $Na_2MoO_4 \cdot 2H_2O$ + 15.8 mg of $CuSO_4 \cdot 5H_2O$ + 9.9 mg of $Co(NO_3)_2 \cdot 6H_2O$ /200 ml H_2O

For preparation of BG-11 medium, firstly 1.5 g of $NaNO_3$ was dissolved in H_2O (~700 ml). Then the stock solutions above were added 1 ml each. Final volume was adjusted to 1 L with H_2O . This medium was distributed into flasks and autoclaved for culturing cyanobacteria, *Anabaena* sp. PCC7120 and *Synechococcus elongatus* PCC7942.

2. BG-11 medium plus Turk Island salt solution (0.5 M NaCl) for *Aphanothece halophytica* (Hibino *et al.*, 1999)

For preparation of BG-11 medium plus Turk Island salt solution (0.5 M NaCl), 28.17 g of NaCl, 0.67 g of KCl, 6.92 g of MgSO₄·7H₂O, 5.50 g of MgCl₂·6H₂O and 1.47 g of CaCl₂·2H₂O were added before adjusted to 1 L with H₂O. It is noted that *Aphanothece halophytica* can grow in a wide range of salinity from 0.25 to 3.0 M NaCl.

Acknowledgments

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