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Extraction and Molybdenum Blue-based Quantification of Total Phosphate and Polyphosphate in *Parachlorella*

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[Abstract] Inorganic phosphorus is a non-renewable resource and an essential element for life on Earth. Organisms such as algae, protists, and animals can store phosphate (Pi) through uptake of Pi as polyphosphate (poly-P), which is a linear polymer of orthophosphate residues linked by high-energy phosphoanhydride bonds. Here, we describe procedures for extraction of total phosphate and poly-P from *Parachlorella* cells and quantification of orthophosphate based on molybdenum blue assay. The present method may be applicable for other microalgae.
Keywords: Alga, *Chlorella*, *Parachlorella*, Phosphorus, Polyphosphate, Molybdenum blue reaction

[Background] Biological phosphorus recovery is a particularly attractive form of nutrient recycling. Algae can accumulate phosphate (Pi), and Pi-enriched algal biomass can be used as biofertilizer (Solovchenko *et al*., 2016). In a previous study, Ota *et al*. (2016) revealed the relationship between electron dense bodies and poly-P dynamics under sulfur-deficient (-S) conditions in *Parachlorella kessleri*. *Parachlorella* is a genus of green algae in the class Trebouxiophyceae, characterized by a rigid cell wall and an asexual, non-motile life cycle. The protocol presented here allows extraction of total Pi and polyphosphate (poly-P) from *Chlorella* and quantification of inorganic phosphorus based on molybdenum blue reaction, which is a standard method used to quantify orthophosphate. The theoretical background of the molybdenum blue reaction was reviewed previously by Nagul *et al*. (2015).

Materials and Reagents

1. Pipette tips for 10 µl, 200 µl and 1,000 µl (Labcon, catalog numbers: 1161-965, 1065-960, 1168-960)
2. 15-ml conical centrifuge tubes (FUKAEKASEI and WATSON, catalog number: 1332-015S)
3. 2-ml microtubes (SARSTEDT, catalog number: 72.695.500)
4. Aluminum foil (Mitsubishi Aluminum, 0.012 mm thick)
5. 96-well microplates, non-treated surface (Asahi Glass, catalog number: 1860-096)
6. Microplate seal (qPCR seal) (4titude, catalog number: 4ti-0560)
7. *Parachlorella kessleri* (National Institute for Environmental Studies, catalog number: NIES-2152)
8. TAP medium (without agar; see http://mcc.nies.go.jp/02medium.html)

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9. Sodium hypochlorite (available chlorine, min. 5.0%) (Wako Pure Chemical Industries, catalog number: 197-02206)
10. Glass beads, acid-washed 425-600 µm (Sigma-Aldrich, catalog number: G8772)
11. Ethanol (99.5% v/v) (Wako Pure Chemical Industries, catalog number: 057-00456)
12. Potassium peroxodisulfate ($K_2S_2O_8$) (Kishida Chemical, catalog number: 310-63931)
13. Antimony potassium tartrate trihydrate ($C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$) (Alfa Aesar, catalog number: A13766)
14. L-Ascorbic acid (Wako Pure Chemical Industries, catalog number: 012-04802)
15. Hexaammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) (Wako Pure Chemical Industries, catalog number: 016-06902)
16. Phosphate ion standard solution (NaH_2PO_4 in water) (Wako Pure Chemical Industries, catalog number: 168-17461)
17. Sulfuric acid (Wako Pure Chemical Industries, catalog number: 195-04706)
18. Ammonium molybdate tetrahydrate solution (see Recipes)

Equipment

1. Micro-spatula (AS ONE, catalog number: 6-524-06)
2. Pipettes for 10 µl, 200 µl and 1,000 µl (Eppendorf, model: Research® plus)
3. Microtube mixer (TOMY SEIKO, model: MT-360)
4. Autoclave (TOMY SEIKO, model: LSX-300)
5. Centrifuge, swing rotor (TOMY SEIKO, model: LC-121)
6. Refrigerated microcentrifuge (TOMY SEIKO, model: MX-300)
7. Microplate reader (BioTek Instruments, model: EPOCH)

Procedure

Note: See Figure 1 for an overview from sampling to molybdenum blue reaction.

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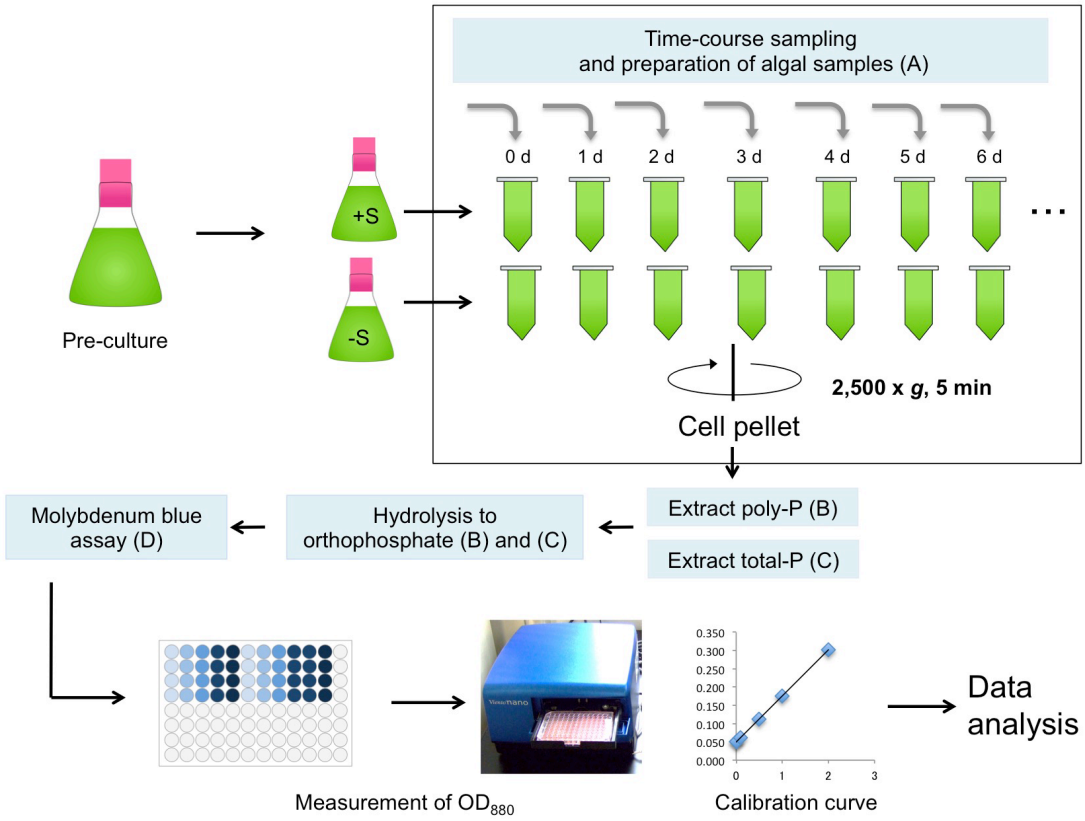

Figure 1. Overview of the phosphate assay in *Chlorella*. +S, sulfur-replete medium (control culture); -S, sulfur-depleted medium (experimental culture; for details, see Ota *et al*., 2016).

A. Sampling and preparing algal samples

1. Take 10 ml of algal culture [OD_{595}: 1-2 in TAP medium (Ota *et al*., 2016)] in a 15-ml conical centrifuge tube. Centrifuge for 5 min at 2,500 *x g* at room temperature with the swing rotor. Discard the supernatant. At this point, additional samples should be taken for dry weight and/or cell number measurement (see Data analysis below).
2. Resuspend the pellet with 2 ml distilled water and transfer to a new 2-ml microtube. The suspended samples can be divided into halves: 1 ml for poly-P assay and 1 ml for total-P assay.
3. Centrifuge again at room temperature for 5 min at 2,500 *x g*. Discard the supernatant. At this point, the pellet can be stored in a freezer at -20 °C for further analysis.

B. Extraction of poly-P

1. Add 1 ml of > 5% sodium hypochlorite to the cell pellet.
2. Add 2-3 microspatulas of glass beads (approximately 50 mg) and mix vigorously using a microtube mixer for 10-15 min at 4 °C in a cold room. Centrifuge for 2 min at 14,000 *x g* at 4 °C. Remove the supernatant.
3. Add 1 ml of sodium hypochlorite to the cell pellet. Centrifuge for 2 min at 14,000 *x g* at 4 °C. Remove the supernatant. Repeat step 3.

   Note: The high chain length of polyphosphate is virtually insoluble in alkaline sodium

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hypochlorite (Sutherland and Wilkinson, 1971).

4. Add 100 µl of distilled water and incubate for 5 min at room temperature. Centrifuge for 2 min at 14,000 *x g* at 4 °C. Collect the supernatant (a).
5. Add 100 µl of distilled water and incubate for 5 min. Centrifuge for 2 min at 14,000 *x g* at 4 °C. Collect the supernatant (b).
6. Add 1.8 ml of ethanol to the supernatant (a + b) and centrifuge for 10 min at 14,000 *x g* at 4 °C. In this step, poly-Ps are precipitated as a white pellet at the bottom of the test tube.
7. Remove the supernatant carefully, add 500 µl of distilled water, and mix vigorously (poly-P solution).
   Note: Amount of distilled water may be adjusted from 50 to 500 µl (OD_{880} > 0.3; see Data analysis below).
8. Add 100 µl of 4% (w/v) potassium persulfate to the poly-P solution.
9. For hydrolysis to orthophosphate, autoclave the poly-P solution at 121 °C for 20 min without fast exhaust option to avoid loss of samples. Leave the cap open and cover the microtube with aluminum foil when autoclaving.
10. The autoclaved samples are now ready to use for molybdenum blue assay (see Procedure D).

C. Extraction of total P
   1. Resuspend the cell pellet from A3 with 1 ml of distilled water.
   2. Disrupt samples. Add 2-3 microspatulas of glass beads and mix vigorously using a microtube mixer for 10-15 min at 4 °C in a cold room.
   3. Add 200 µl of 4% (w/v) potassium persulfate to the sample.
   4. Autoclave the poly-P solution at 121 °C for 20 min without fast exhaust to avoid loss of samples. Leave the cap open and cover the microtube with aluminum foil when autoclaving.
   5. The autoclaved samples are now ready to use for molybdenum blue assay (see Procedure D).
      Note: The supernatant is used for assay. Normally, there is no need for centrifugation step.

D. Molybdenum blue reaction in a 96-well microplate
   1. Pipette 200 µl of diluted sample (196 µl of distilled water + 4 µl of poly-P/total-P samples) per well.
   2. Add 8 µl of ammonium molybdate tetrahydrate solution (see Recipes below).
   3. Add 2 µl of 7.2% (w/v) L-ascorbic acid solution.
   4. Seal with plate seal film and mix well (Invert the plate 3-5 times).
   5. Incubate for 20 min in the dark at room temperature. The mixture will turn blue in color if orthophosphate is present (Figure 2).

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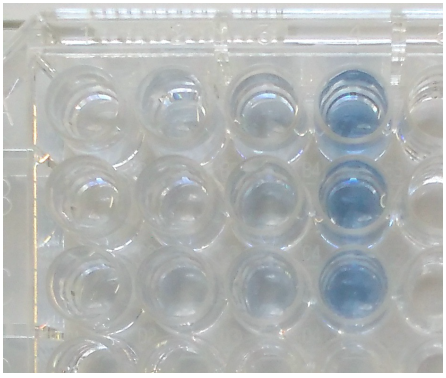

Figure 2. Molybdenum blue reaction in a 96-well microplate. This is an example of the phosphate ion standard dilution series (0, 0.5, 1, 2 mg/L from left to right, *n* = 3).

6. Measure the absorbance at 880 nm (OD_{880}) using a microplate reader.
   Note: If wavelength at 880 nm is not available, select an alternative wavelength near 880 nm.

Data analysis

A calibration curve is necessary for each experiment for calculation of corresponding absolute values. A phosphate ion standard solution is diluted with distilled water ranging from 0 to 2 mg/L (*e.g.*, 0, 0.1, 0.5, 1, 2 mg/L), where linearity between absorbance (OD_{880}) and Pi concentration is confirmed (Figure 3). Measure OD_{880} at one point per well, and a mean value is calculated from at least three replicates per sample ($n > 3$). A calculation example is as follows: The concentration rate in poly-P extraction is 5-fold (Procedure A), 1,000/(100 + 500) - fold (Procedure B), and 4/200 - fold (Procedure D), respectively. Then, the poly-P concentration (mg/L) is calculated as follows: the corresponding value = {(OD_{880} - 0.05)/0.13} x1/5 x 600/1,000 x 200/4. Dry weight and/or cell number should be measured for normalization when sampling cultures. These values are used for calculating the amount of Pi per dry weight or cell.

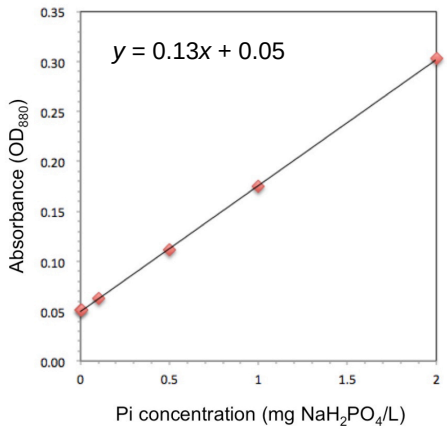

Figure 3. Example of a calibration curve

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Notes

1. Phosphate ion standard solution can be self-produced using sodium dihydrogenphosphate (NaH_2PO_4).
2. All stock solutions (potassium persulfate, ammonium molybdate tetrahydrate, L-ascorbic acid) should be prepared with distilled water (see also Recipes below).
3. Solutions are prepared at time of use. The stock solutions can be stored up to one month at 4 °C.
4. Samples should be diluted with distilled water, if OD_{880} is more than 0.3.

Recipes

1. Ammonium molybdate tetrahydrate solution (100 ml)
   1.2 g of hexaammonium heptamolybdate tetrahydrate
   4.8 mg of potassium antimonyl tartrate sesquihydrate
   32 ml of diluted sulfuric acid (sulfuric acid:distilled water = 1:1)
   Make up with distilled water to volume

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