

## Quantitative Determination of Poly- $\beta$ -hydroxybutyrate in *Synechocystis* sp. PCC 6803

Yvonne Zilliges<sup>1, \*</sup> and Ramon Damrow<sup>2</sup>

<sup>1</sup>Freie Universität Berlin, Institute of Experimental Physics/Biophysics and Photosynthesis, Arnimallee 14, Berlin, Germany; <sup>2</sup>Humboldt-Universität zu Berlin, Institute of Biology/Biochemistry of Plants, Chausseest. 117, Berlin, Germany

\*For correspondence: [yvonne.zilliges@fu-berlin.de](mailto:yvonne.zilliges@fu-berlin.de)

**[Abstract]** Cyanobacteria synthesize a variety of chemically-different, high-value biopolymers such as glycogen (polyglucose), poly- $\beta$ -hydroxybutyrate (PHB), cyanophycin (polyamide of arginine and aspartic acid) and volutin (polyphosphate) under excess conditions. Especially under unbalanced C to N ratios, glycogen and in some cyanobacterial genera also PHB are massively accumulated in the progression of the general nitrogen stress response. Several different technologies have been established for *in situ* and *in vitro* PHB analysis from different microbial sources. In this protocol, a rapid and reliable spectrophotometric method is described for PHB quantification in the cyanobacterium *Synechocystis* sp. PCC 6803 upon nitrogen deprivation as described in (Damrow *et al.*, 2016).

**Keywords:** Cyanobacteria, *Synechocystis*, PHB, Nitrogen deprivation

**[Background]** Non-diazotrophic cyanobacteria such as *Synechocystis* sp. PCC 6803 respond to the lack of combined nitrogen sources by bleaching, a process known as chlorosis (Allen and Smith, 1969). This acclimation response is characterized by four major structural and morphological changes: (i) a massive accumulation of electron-dense glycogen inclusions (approx. 40 nm in diameter) between the thylakoid layers accompanied by (ii) the degradation of the phycobilisome antenna complexes, (iii) the disassembling of the thylakoid membrane layers including a reduction by number and packing density, and (iv) the formation of distinct electron-transparent PHB granules (approx. 400-500 nm in diameter) (Damrow *et al.*, 2016). The physiological function of cyanobacterial PHB metabolism, synthesized just in a few species, is quite opaque due to the absence of both catabolic enzymes and evident phenotype of PHB-deficient mutants (Beck *et al.*, 2012; van der Woude *et al.*, 2014; Damrow *et al.*, 2016; Namakoshi *et al.*, 2016).

Facing the world's trash and global warming crisis, the demands for durable, recyclable, biodegradable, and synthetic-alternative plastics such as PHB is enormous and focus attention to cyanobacterial producers (Asada *et al.*, 1999; Ansari and Fatma, 2016). Various different techniques are published for the analysis of PHB molecules (for updated review see [Godbole, 2016]). We are presenting a combination of hydrolytic degradation of PHB to 3-hydroxybutyrate (3-HB) in alkaline regime, and a coupled colorimetric enzymatic assay. Here the coupling with a phenazine methosulphate-p-iodonitrotetrazolium violet (PMS-INT) system directs the enzymatic redox reaction of both NADH oxidation and 3-HB reduction by the 3-hydroxybutyrate dehydrogenase (HBDH) and thus precludes an

interfering backward reaction. This rapid spectrophotometric quantification of PHB just needs very simple lab equipment, is not much time-consuming, and is yet both reliable and reproducible.

## **Materials and Reagents**

1. Pipette tips
2. 1.5 ml centrifuge tubes (Eppendorf® Safe-Lock microcentrifuge tubes) (Eppendorf, catalog number: 0030120086)
3. 15 ml centrifuge tubes (TubeSpin® Bioreactor 15) (TPP Techno Plastic Products, catalog number: 87015)
4. 50 ml centrifuge tubes (TubeSpin® Bioreactor 50) (TPP Techno Plastic Products, catalog number: 87050)
5. *Synechocystis* sp. PCC 6803 cells (Pasteur culture collection of cyanobacteria) (Institut Pasteur, catalog number: PCC 6803)
6. Crushed ice
7. Bi-distilled water
8. 0.5 N sodium hydroxide (NaOH) (Carl Roth, catalog number: 9356.1)
9. 1 N hydrochloric acid (HCl-ROTIPURAN® 37%) (Carl Roth, catalog number: X942.1)
10. 50 mM Tris-hydrochloride, pH 8.5 (PUFFERAN®  $\geq$  99%) (Carl Roth, catalog number: 9090.3)
11. 20 mM  $\beta$ -Nicotinamide adenine dinucleotide (NADH/H<sup>+</sup>), reduced disodium salt (Sigma-Aldrich, catalog number: N9785)  
*Note: This product has been discontinued.*
12. 1 mM  $\beta$ -Nicotinamide adenine dinucleotide (NAD<sup>+</sup>), reduced disodium salt (Sigma-Aldrich, catalog number: N0632)
13. 5 mM phenazine methosulfate (PMS) (Sigma-Aldrich, catalog number: P9625)
14. 5 mM *p*-Iodonitrotetrazolium violet (INT) (Sigma-Aldrich, catalog number: I8377)
15. 1 mM (*R*)-3-hydroxybutyric (*R*-3-HB) (Sigma-Aldrich, catalog number: 54920) with a molar mass of 104.10 g/mol
16. 15 U/ml 3-hydroxybutyrate dehydrogenase (3-HBDH) grade II from *Rhodobacter spheroides* (Roche Diagnostics, catalog number: 10127833001)
17. BG11 stock solution '+N'; autoclaved (use 1:100) (see Recipes)
  - a. Sodium nitrate (NaNO<sub>3</sub>  $\geq$  99%, p.a., ACS, ISO) (Carl Roth, catalog number: A136.1)
  - b. Calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O  $\geq$  99%, p.a., ACS) (Carl Roth, catalog number: 5239.1)
  - c. Citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>  $\geq$  99.5%, p.a., ACS, anhydrous) (Carl Roth, catalog number: X863.1)
  - d. Magnesium sulfate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O  $\geq$  99%, p.a., ACS) (Carl Roth, catalog number: P027.1)
  - e. Ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA  $\geq$  99%, p.a., ACS) (Carl Roth, catalog number: 8043.2)

18. BG11<sub>0</sub> stock solution '-N'; autoclaved (use 1:100) (see Recipes)
  - a. Calcium chloride (CaCl<sub>2</sub>·2H<sub>2</sub>O ≥ 99%, p.a., ACS) (Carl Roth, catalog number: 5239.1)
  - b. Citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> ≥ 99.5%, p.a., ACS, anhydrous) (Carl Roth, catalog number: X863.1)
  - c. Magnesium sulfate (MgSO<sub>4</sub>·7H<sub>2</sub>O ≥ 99%, p.a., ACS) (Carl Roth, catalog number: P027.1)
  - d. Ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA ≥ 99%, p.a., ACS) (Carl Roth, catalog number: 8043.2)
19. Trace Metal Mix for BG11; sterile filtrated (use 1:1,000) (see Recipes)
  - a. Boric acid (H<sub>3</sub>BO<sub>3</sub>) (≥ 99.8%, p.a., ACS, ISO) (Carl Roth, catalog number: 6943.2)
  - b. Manganese(II) chloride tetrahydrate (MnCl<sub>2</sub>·4H<sub>2</sub>O ≥ 99%, p.a.) (Carl Roth, catalog number: T881.3)
  - c. Zinc sulfate heptahydrate (ZnSO<sub>4</sub>·7H<sub>2</sub>O ≥ 97%, extra pure) (Carl Roth, catalog number: 7316.1)
  - d. Sodium molybdate dihydrate (Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O), ≥ 99.5% (Sigma-Aldrich, catalog number: M1651)
  - e. Cupric(II) sulfate pentahydrate (CuSO<sub>4</sub>·5H<sub>2</sub>O ≥ 99%, Ph.Eur., BP) (Carl Roth, catalog number: P025.1)
  - f. Cobalt(II) nitrate hexahydrate (Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O) (Sigma-Aldrich, catalog number: 203106)
20. Extra solutions for BG11; sterile filtrated (use 1:1,000) (see Recipes)
  - a. Ammonium iron(III) citrate (Carl Roth, catalog number: 9366.1)
  - b. Potassium phosphate dibasic (K<sub>2</sub>HPO<sub>4</sub> ≥ 98%, Ph.Eur., BP) (Carl Roth, catalog number: T875.1)
  - c. Sodium carbonate carbonate monohydrate (Na<sub>2</sub>CO<sub>3</sub>·H<sub>2</sub>O ≥ 99.5%, p.a., ACS) (Carl Roth, catalog number: 2597.2)
21. Buffer solution for BG11; autoclaved (use 1:200) (see Recipes)

1 M HEPES buffer (pH 8.0) (PUFFERAN<sup>®</sup> ≥ 99.5%) (Carl Roth, catalog number: 9105.4)
22. BG11 ('+N') medium (see Recipes)
23. BG11<sub>0</sub> ('-N') medium (see Recipes)

## **Equipment**

1. Wide-neck 500 ml Erlenmeyer flasks (Carl Roth, DURAN<sup>®</sup>, catalog number: C150.1)
2. Personal protective equipment (PPE)

*Note: These should be worn at all times when dealing with concentrated acids and alkali. See Note 3.*

  - a. Safety glasses (Sekuroka<sup>®</sup>-safety glasses EN166) (Carl Roth, catalog number: Y254.1)
  - b. Lab coat (Sekuroka<sup>®</sup>-lab coats) (Carl Roth, catalog number: T413.1)
  - c. Gloves (Rotiprotect<sup>®</sup>-nitrile evo) (Carl Roth, catalog number: CPX7.1)
3. Pipette set (Rainin Pipet-Lite LTS Starter Kit L-STARTXLS+) (Mettler-Toledo International, catalog number: 17014406)

4. Ice bucket
5. Laminar flow bench (Thermo Fisher Scientific, Thermo Scientific™, model: MSC-Advantage™ Class II, catalog number: 51028225)
6. Analytical balance (Mettler-Toledo International, model: XS105)
7. Centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Biofuge™ Primo R, catalog number: 75005440)
8. Light meter (LI-COR, model: LI-189)
9. Incubator (Infors, model: Multitron Pro, 'Algae Special')
10. UV-Vis spectrophotometer (Analytic Jena, model: Specord® 50 PLUS)
11. Bench pH/mV/°C meter (pH 1,000 L, pHenomenal®) (VWR, catalog number: 662-1422)
12. Thermomixer (Biometra, model: ThermoShaker TS1, catalog number: 846-051-500)
13. Vortex shaker (VWR, Peqlab, model: peqTWIST)
14. Autoclave (Systec, model: Systec VX-150)

## **Procedure**

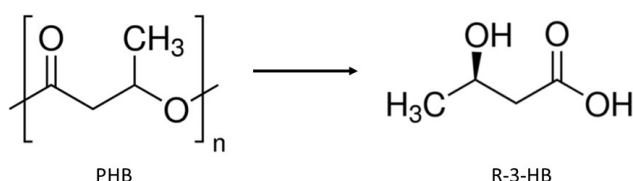
1. Induction of PHB biosynthesis in *Synechocystis* sp. PCC 6803 upon nitrogen deprivation
  - a. For nitrogen step down experiments as described in (Grundel *et al.*, 2012; Damrow *et al.*, 2016), an axenic pre-culture of *Synechocystis* sp. PCC 6803 (2 x 250 ml) grows in Erlenmeyer flasks to an OD<sub>750</sub> of 0.5 at 28 °C under continuous illumination with white light of 80  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$  in BG11 medium (see Recipes) containing 20 mM HEPES buffer (pH 8.0) and 17.6 mM sodium nitrate as the nitrogen source.
  - b. For nitrogen deprivation, cells (500 ml) are pelleted by centrifugation at 4,000 x g for 10 min at room temperature and washed twice with 500 ml BG11<sub>0</sub> (see Recipes), which lacks sodium nitrate '-N' (Stanier *et al.*, 1971).
  - c. The final cell suspension is split (2 x 250 ml) and both cultures '-N' and '+N' are supplemented with sodium acetate (final concentration 10 mM) as described by (Hein *et al.*, 1998). Sodium nitrate is only added to the control '+N' sample (final concentration 17.6 mM).
  - d. The cultivation upon nitrogen deprivation is—likewise to the pre-culture cultivation—implemented in Erlenmeyer flasks at 28 °C under continuous shaking (80 rpm; Multitron Pro 'Algae Special') and continuous illumination using white light (80  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ ) for seven days.
2. Quantification of dry weight
  - a. 10 ml of each cell culture is pelleted by centrifugation (4,000 x g, 10 min), washed twice with 10 ml sterile water and finally concentrated up to 1 ml and transferred into a 1.5 ml reaction tube which has to be pre-weighted before.
  - b. After another centrifugation step (10,000 x g, 3 min) the supernatant is entirely removed and the remaining cell pellet is dried completely at 80 °C in a thermomixer overnight (open cap).

- c. The dry weight of the cells (~5 mg) is determined by the weight of the 'cell tubes' subtracted from the dead weight of the tube (Table 1).

**Table 1. Determined dry weights**

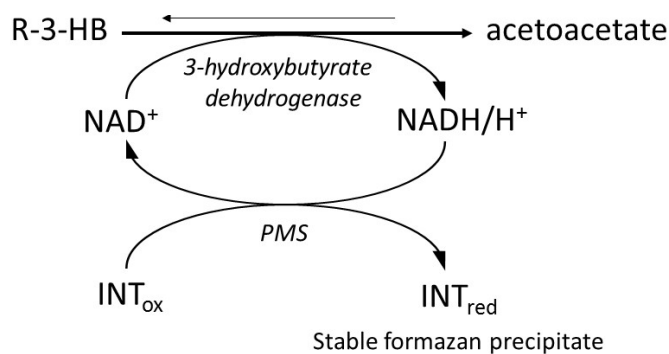
Sample	Dead weight of the tube [g]	Weight of the 'cell tube' [g]	Dry weight (DW) of the cells [mg]
'+N', control	1.0526	1.0580	5.4
'-N'	1.0583	1.0622	3.9

3. Extraction and de-polymerization of PHB to (*R*)-3-hydroxybutyric acid (Figure 1) by alkaline hydrolysis.



**Figure 1. Hydrolytic degradation of poly- $\beta$ -hydroxybutyrate (PHB) to (*R*)-3-hydroxybutyric acid (R-3-HB)**

- a. 0.5 N NaOH (< 5 mg DW→300  $\mu$ l 0.5 N NaOH; > 5 mg DW→400  $\mu$ l 0.5 N NaOH) are added to the dried cell pellet from step 2 ('+N' and '-N', separately) and incubated at 85 °C for 1 h by continuously shaking in a thermomixer and vigorously vortexing from time to time both to break the cells and to hydrolyze PHB into its monomer (*R*)-3-hydroxybutyrate (R-3-HB) as described by (Yu and Marchessault, 2000; Cui *et al.*, 2006).
- b. After cooling on ice, the samples ('+N' and '-N', separately) are neutralized by the addition of 1 N HCl in a ratio of four volumes 0.5 N NaOH (e.g., 300  $\mu$ l) to one volume 1 N HCl (e.g., 100  $\mu$ l). The samples ('+N' and '-N', separately) are thoroughly mixed by vortexing.
- c. Afterwards the R-3-HB containing fraction (supernatant) of each sample ('+N' and '-N', separately) is separated from the cell debris (pellet) by centrifugation (1 min, 4,500  $\times$  g, room temperature). The R-3-HB containing fraction (supernatant) is transferred to a new 1.5 ml reaction tube for each sample ('+N' and '-N', separately).
- d. For further quantitative analysis, the R-3-HB containing samples ('+N' and '-N', separately) are diluted in ratio of 1:10 dilution with bi-distilled water.
4. Spectrophotometric quantification of R-3-HB monomers
- a. The R-3-HB content in each sample ('+N' and '-N', separately) is finally spectrophotometrically quantified via an enzymatic assay. In a redox reaction, R-3-HB is oxidized to acetoacetate while NAD<sup>+</sup> is reduced to NADH/H<sup>+</sup> catalyzed in a 1:1 stoichiometry by 3-hydroxybutyrate dehydrogenase (HBDH) (Figure 2).



**Figure 2. Coupled enzymatic assay of R-3-HB oxidation by the 3-hydroxybutyrate dehydrogenase and simultaneous reoxidation of NADH/H<sup>+</sup> to NAD<sup>+</sup> by the PMS-INT system forming a stable color precipitate**

- b. In order to prevent a backward reaction (reduction of acetoacetate to R-3-HB; oxidation of NADH/H<sup>+</sup> to NAD<sup>+</sup>), the NADH/H<sup>+</sup> synthesis is directly coupled to a phenazinemethosulphate-p-iodonitrotetrazolium violet (PMS-INT) colorimetric assay as described by (Lim and Buttery, 1977; Hinman and Blass, 1981). Here, the electron is transferred in a 1:1 stoichiometry from NADH/H<sup>+</sup> via PMS to INT. The reduced form of INT is a stable formazan, which absorbs light at a wavelength of 500 nm (or 505 nm) (Lim and Buttery, 1977) (Figure 2).
- c. The level of R-3-HB is proportional both to the level of synthesized NADH/H<sup>+</sup> and of formazan (Figure 2).
- 100  $\mu$ l of the diluted sample from step 3 or 100  $\mu$ l 1 mM standard ((*R*)-3-hydroxybutyric) is added into a cuvette (1 ml assay volume) containing (triplicates for each sample):
- 730  $\mu$ l 50 mM Tris-HCl buffer, pH 8.5 (optimum pH for oxidation reaction)
- 50  $\mu$ l 1 mM NAD<sup>+</sup>
- 10  $\mu$ l 0.5 mM PMS
- 100  $\mu$ l 0.05 mM INT
- d. The reaction is monitored at 25 °C as the change ( $\Delta E = E_2 - E_1$ ) in A<sub>500</sub> (or 505 nm) on spectrophotometer before ( $E_1$ ) and ten min after ( $E_2$ ) initiation with 10  $\mu$ l 15 U/ml HBDH according to the protocol as described in (Hinman and Blass, 1981) (Table 2). Tris-HCl buffer, pH 8.5 (50 mM) is used as a blank.

**Table 2. Absorption change at 500 nm (or 505 nm) for nitrogen-deprived samples**

Sample	<i>E1</i>	<i>E2</i>	$\Delta E$
'+N', negative control (triplicates)	0.062	0.136	0.074
	0.062	0.140	0.078
	0.065	0.139	0.074
'-N' (triplicates)	0.065	0.829	0.764
	0.064	0.792	0.728
	0.068	0.814	0.746
R-3-HB standard, positive control (triplicates)	0.056	1.416	1.360
	0.056	1.352	1.296
	0.060	1.522	1.462

- e. The R-3-HB (and thus PHB) level is calculated via the calibration series of six different NADH/H<sup>+</sup> concentrations ranging from 0, (0.1), 0.25, 0.5, 0.75, and 1.0 mM (triplicates for each concentration) in a PMS-INT formazan reaction. Tris-HCl buffer, pH 8.5 (50 mM) is used as a blank. Here, the  $\Delta E$  (Table 3) is determined at 25 °C as the change in A<sub>500</sub> (or 505 nm) on spectrophotometer before (*E1*) and three min after (*E2*) addition of 100  $\mu$ l respective NADH/H<sup>+</sup> standard solution to a cuvette containing (triplicates for each sample):
- 730  $\mu$ l 50 mM Tris-HCl buffer, pH 8.5
  - 60  $\mu$ l bi-distilled water
  - 10  $\mu$ l 0.5 mM PMS
  - 100  $\mu$ l 0.05 mM INT

**Table 3. Absorption change at 500 nm (or 505 nm) for NADH calibration series**

NADH/H <sup>+</sup> [mM]	<i>E1</i>	<i>E2</i>	$\Delta E$
0 (triplicates)	0.063	0.092	0.029
	0.063	0.093	0.029
	0.059	0.089	0.030
0.25 (triplicates)	0.065	0.360	0.295
	0.065	0.358	0.293
	0.062	0.356	0.294
0.5 (triplicates)	0.065	0.678	0.613
	0.066	0.691	0.625
	0.070	0.686	0.616
0.75 (triplicates)	0.066	1.025	0.959
	0.066	1.020	0.954
	0.069	1.028	0.959
1.0 (triplicates)	0.071	1.340	1.269
	0.073	1.280	1.207
	0.066	1.299	1.233



## Data analysis

### 1. NADH/H<sup>+</sup> standard curve

The calibration curve is plotted as a function of respective absorbance ( $\Delta E$  value) versus known concentration of NADH/H<sup>+</sup> [mM]. The fitting curve and parameters are obtained by linear regression analysis. The linear regression coefficient should be in the range of  $0.97 \leq R^2 \leq 1$  (Figure 3).

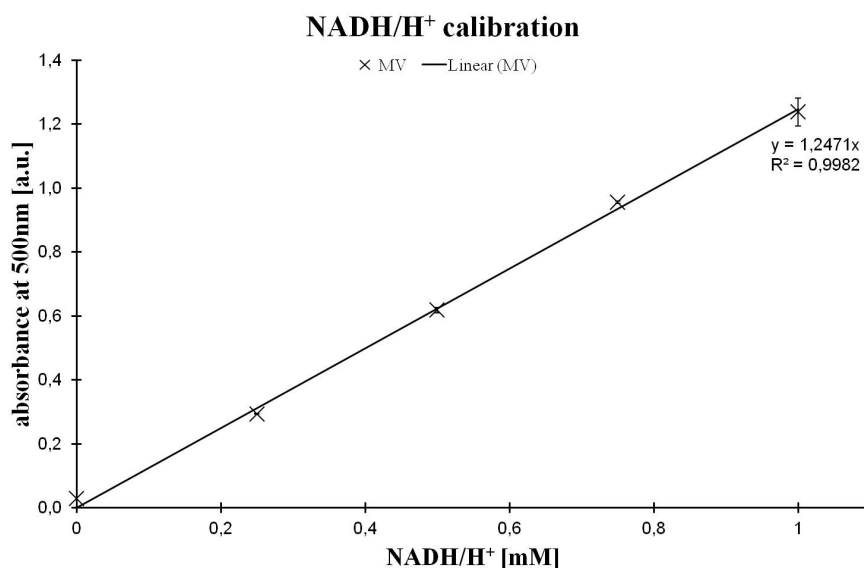


Figure 3. NADH/H<sup>+</sup> calibration curve

### 2. R-3-HB standard control reaction

Assuming a complete chemical conversion (1:1 stoichiometry), the respective absorbance of control reaction (0.1 mM R-3-HB) corresponds to 0.1 mM NADH/H<sup>+</sup> (see NADH/H<sup>+</sup> standard curve, Figure 3).

### 3. PHB level quantification related to the dry weight

The converted NADH/H<sup>+</sup> and thus R-3-HB concentrations are calculated from the slope function of the linear regression analysis for each absorbance ( $\Delta E$  value). Including all dilution and concentration factors into the equations, the average R-3-HB amount of all three replicates is related to the determined dry weight (Table 4). Whereas in the control samples '+N' no R-3-HB is detectable, the average R-3-HB (and thus PHB) content of the nitrogen-deprived cells of *Synechocystis* sp. PCC 6803 corresponds to 6-10% of the dry weight (Damrow *et al.*, 2016).



**Table 4. Determination of PHB content per dry weight**

Sample	Dry weight of the cells [mg] <sup>1</sup>	$\Delta E^2$	NADH/H <sup>+</sup> [mM] <sup>3</sup>	R-3-HB [mM] <sup>4</sup>	R-3-HB [mM] <sup>5</sup>	R-3-HB [ $\mu$ g/ml] <sup>6</sup>	% (w/w) R-3-HB
'+N', control	5.4	0.074	0.059	0.059	0.032	3.29	0.61
		0.078	0.063	0.063	0.033	3.47	0.64
		0.074	0.059	0.059	0.032	3.29	0.61
'-N'	3.9	0.764	0.613	0.613	0.245	25.51	6.54
		0.728	0.584	0.584	0.234	24.31	6.23
		0.746	0.583	0.583	0.239	24.91	6.39

<sup>1</sup>From Table 1

<sup>2</sup>From Table 2

<sup>3</sup>Concentration in the assay as calculated from the slope function in Figure 3

<sup>4</sup>Concentration in the assay according to the 1:1 stoichiometry

<sup>5</sup>Concentration in the cell suspension regarding the dilution factor (1:10) in step 3d and concentration factor in step 3a which is calculated as the quotient of cell suspension volume (10 ml) and sum of NaOH (300 or 400  $\mu$ l) and HCl (100 or 133  $\mu$ l) volumes

<sup>6</sup>Concentration in the cell suspension

## Notes

1. All cell passages and media are implemented under sterile conditions using a laminar flow bench and an autoclave. The intensity of photosynthetically active radiation (PAR) is measured in  $\mu\text{E m}^{-2} \text{sec}^{-1}$  ( $\mu\text{mol photons per m}^2 \text{ and sec}$ ) using a LI-189 light meter.
2. The dried cell pellets are stored at 4 °C for at least three weeks.
3. For safety reasons, adjust to protective measures and tight-closing tubes when handling acids and bases. For safety reasons, assure that the hot samples are cooled down before HCl addition. The supernatant containing R-3-HB monomers could be stored at -20 °C for at least three weeks.
4. The PMS-INT reaction is light-sensitive. The samples are handled in darkness.

## Recipes

*Note: Unless otherwise indicated, bi-distilled water was used as a solvent in solutions.*

1. BG11 stock solution '+N'; autoclaved (use 1:100)  
17.65 M NaNO<sub>3</sub>  
0.18 M CaCl<sub>2</sub>·2H<sub>2</sub>O  
0.0031 M citric acid  
0.304 M MgSO<sub>4</sub>·7H<sub>2</sub>O  
0.0034 M EDTA
2. BG11 stock solution '-N'; autoclaved (use 1:100)  
0.18 M CaCl<sub>2</sub>·2H<sub>2</sub>O  
0.0031 M citric acid  
0.304 M MgSO<sub>4</sub>·7H<sub>2</sub>O  
0.0034 M EDTA

3. Trace Metal Mix for BG11; sterile filtrated (use 1:1,000)
  - 1.43 g/L H<sub>3</sub>BO<sub>3</sub>
  - 0.09 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O
  - 0.11 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O
  - 0.195 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O
  - 0.0395 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O
  - 0.0247 g/L Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O
4. Extra solutions for BG11; sterile filtrated (use 1:1,000)
  - 23 mM ferric ammonium citrate
  - 172 mM K<sub>2</sub>HPO<sub>4</sub>
  - 188 mM Na<sub>2</sub>CO<sub>3</sub>·H<sub>2</sub>O
5. Buffer solution for BG11; autoclaved (use 1:200)
  - 1 M HEPES buffer (pH 8.0)
6. BG11 ('+N')
  - 10 ml 100x BG11 (+N) stock solution
  - 1 ml Trace Metal Mix
  - 1 ml 23 mM ferric ammonium citrate
  - 1 ml 172 mM potassium phosphate dibasic
  - 1 ml 188 mM sodium carbonate
  - 5 ml 1 M HEPES buffer (pH 8.0)
  - Add up to 1 L with deionized water
7. BG11<sub>0</sub> ('-N')
  - 10 ml 100x BG11 (-N) stock solution diminished by sodium nitrate
  - 1 ml Trace Metal Mix
  - 1 ml 23 mM ferric ammonium citrate
  - 1 ml 172 mM potassium phosphate dibasic
  - 1 ml 188 mM sodium carbonate
  - 5 ml 1 M HEPES buffer (pH 8.0)
  - Add up to 1 L with deionized water

## **Acknowledgments**

We particularly acknowledge Wolfgang Lockau for his commitment and valuable discussions on this topic and for his sustained support of our research. We gratefully thank Gisa Baumert (Humboldt-Universität zu Berlin, Germany) for her excellent technical assistance. We thank the Deutsche Forschungsgemeinschaft (DFG) for financial support (to YZ) in the framework of the Collaborative Research Center 1078 on 'Protonation Dynamics in Protein Function' (SFB1078, project A4/Holger Dau).

## References

1. Allen, M. M. and Smith, A. J. (1969). [Nitrogen chlorosis in blue-green algae](#). *Arch Mikrobiol* 69(2): 114-120.
2. Ansari, S. and Fatma, T. (2016). [Cyanobacterial polyhydroxybutyrate \(PHB\): screening, optimization and characterization](#). *PLoS One* 11(6): e0158168.
3. Asada, Y., Miyake, M., Miyake, J., Kurane, R. and Tokiwa, Y. (1999). [Photosynthetic accumulation of poly-\(hydroxybutyrate\) by cyanobacteria--the metabolism and potential for CO<sub>2</sub> recycling](#). *Int J Biol Macromol* 25(1-3): 37-42.
4. Beck, C., Knoop, H., Axmann, I. M. and Steuer, R. (2012). [The diversity of cyanobacterial metabolism: genome analysis of multiple phototrophic microorganisms](#). *BMC Genomics* 13: 56.
5. Cui, Y., Barford, J. P. and Renneberg, R. (2006). [Determination of poly\(3-hydroxybutyrate\) using a combination of enzyme-based biosensor and alkaline hydrolysis](#). *Anal Sci* 22(10): 1323-1326.
6. Damrow, R., Maldener, I. and Zilliges, Y. (2016). [The multiple functions of common microbial carbon polymers, glycogen and PHB, during stress responses in the non-diazotrophic cyanobacterium \*Synechocystis\* sp. PCC 6803](#). *Front Microbiol* 7: 966.
7. Godbole, S. (2016). [Methods for identification, quantification and characterization of polyhydroxyalkanoates](#). *Int J Bioassays* 5(04).
8. Grundel, M., Scheunemann, R., Lockau, W. and Zilliges, Y. (2012). [Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium \*Synechocystis\* sp. PCC 6803](#). *Microbiology* 158(Pt 12): 3032-3043.
9. Hein, S., Tran, H. and Steinbuchel, A. (1998). [Synchocystis sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria](#). *Arch Microbiol* 170(3): 162-170.
10. Hinman, L. M. and Blass, J. P. (1981). [An NADH-linked spectrophotometric assay for pyruvate dehydrogenase complex in crude tissue homogenates](#). *J Biol Chem* 256(13): 6583-6586.
11. Lim, H. H. and Buttery, J. E. (1977). [Determination of ethanol in serum by an enzymatic PMS-INT colorimetric method](#). *Clin Chim Acta* 75(1): 9-12.
12. Namakoshi, K., Nakajima, T., Yoshikawa, K., Toya, Y. and Shimizu, H. (2016). [Combinatorial deletions of glgC and phaCE enhance ethanol production in \*Synechocystis\* sp. PCC 6803](#). *J Biotechnol* 239: 13-19.
13. Stanier, R. Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971). [Purification and properties of unicellular blue-green algae \(order \*Chroococcales\*\)](#). *Bacteriol Rev* 35(2): 171-205.
14. van der Woude, A. D., Angermayr, S. A., Puthan Veetil, V., Osnato, A. and Hellingwerf, K. J. (2014). [Carbon sink removal: Increased photosynthetic production of lactic acid by \*Synechocystis\* sp. PCC6803 in a glycogen storage mutant](#). *J Biotechnol* 184: 100-102.
15. Yu, G-e. and Marchessault, R. H. (2000). [Characterization of low molecular weight poly\( \$\beta\$ -hydroxybutyrate\)s from alkaline and acid hydrolysis](#). *Polymer* 41(3): 1087-1098.