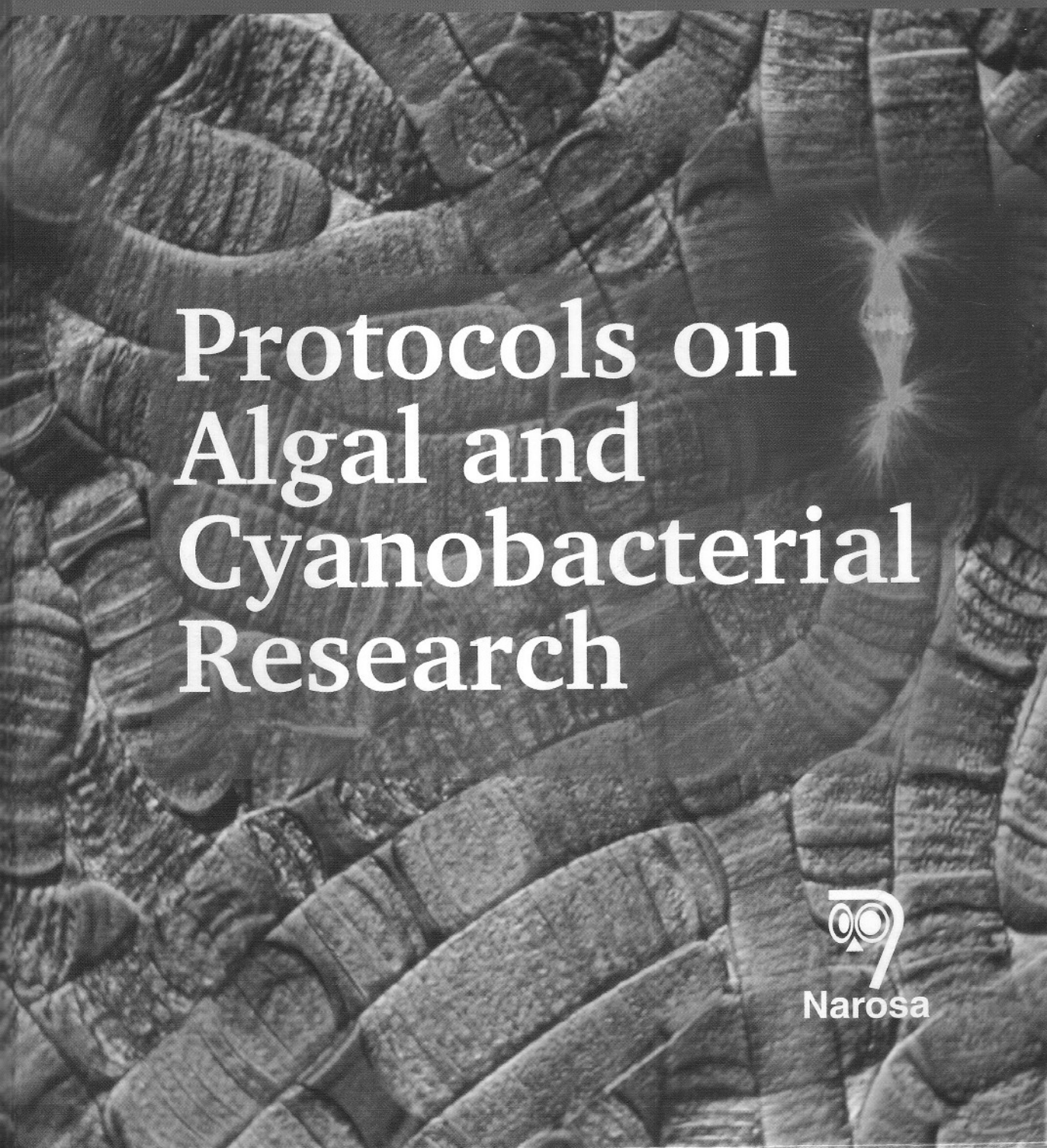
Editors
Suvendra Nath Bagchi • Diethelm Kleiner • Prasanna Mohanty



Protocols on Algal and Cyanobacterial Research

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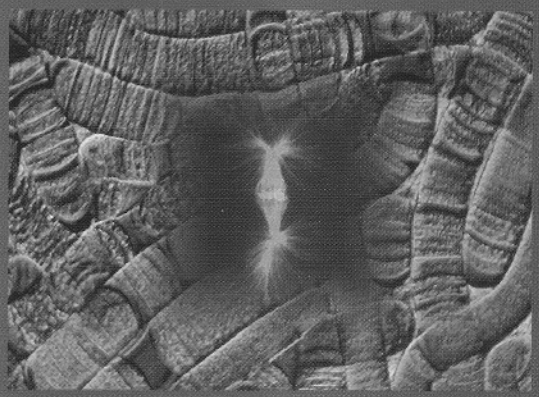

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Protocols on Algal and Cyanobacterial Research is an up-to-date and consolidated compilation of ready-to-use protocols embodied in twenty three chapters and grouped in four sections, highlighting methods related to preparation and characterization of components, assessment of biological activities of toxins, and biotechnology utilizing algae and cyanobacteria as research material.

In the first section aspects of sub-cellular fractionation and estimation of photosynthetically active particles, phycobilisomes , carotenoids, lipids, DNA, RNA, proteins and peptides are discussed. The second section is devoted to the analysis of physiological processes namely transport and assimilation of ammonia, phosphate, nitrate and water (solutes), and primary photochemical activity and CO_2 fixation. A variety of toxins of planktonic algae affect the ecosystem balance and human/animal life and the third section illustrates the procedures for their determination. Since microalgae can sense and respond to toxic anthropogenic compounds like heavy metals, the application of various indicatory/sequestering bioassays is the highlight of the fourth section.

KEY FEATURE

- Protocols conceived and standardized by experts

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Chapter 11

Assay Methods for Nitrate Assimilatory Enzymes in *Spirulina (Arthospira)*

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Summary

Spirulina, a non-nitrogen fixing, nitrate utilizing photo-autotrophic organism, is very popular as a food supplement due to its high protein content. But the mechanism by which this filamentous cyanobacterium regulates its nitrogen metabolism to make such a large amount of protein remains to be studied. The present chapter deals with some of the methods for biochemical and molecular genetic studies in *Spirulina*, including enzyme assays for nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase (GOGAT), which constitute the enzymes of primary nitrate assimilation in this organism. In addition, a DNA isolation protocol specifically adapted for *Spirulina* and used for the PCR cloning of nitrate reductase gene is also provided.

Key Words: Glutamate synthase; glutamine synthetase; methods; nitrate reductase; nitrite reductase; *Spirulina platensis.*

1. Introduction

Spirulina (Arthrospira) is an intriguing microorganism with tremendous biotechnological importance. Its cellular protein content is among the highest known in the living world (60-70 % by dry wt.), making it a popular nutritional supplement in the international market ***(1)***. However, the mechanism by which this filamentous cyanobacterium steers its nitrogen metabolism to make such a large amount of protein is unclear, except that it is a nitrate utilizing, non-nitrogen fixing, photosynthetic organism, comparable to higher plants in this respect.

Although nitrogen fixation in cyanobacteria is very well studied, nitrate assimilation in non - nitrogen fixing cyanobacteria has not received much attention. In general, nitrate assimilation involves nitrate transport into the cell followed by its reduction to ammonium by

the sequential action of the enzymes nitrate reductase (NR) and nitrite reductase (NiR). Ammonium ions are then incorporated into amino acids mainly by the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. These enzymes and their genes are well studied in higher plants algae and fungi ***(2,3)***. However, the enzymes of nitrate assimilation in *Spirulina* are relatively unknown ***(1)***, except the partial purification and characterization of NiR ***(4)***. The genes encoding for the uptake and assimilation of nitrate and nitrite are usually clustered in most cyanobacteria and constitute an operon. Nitrate/nitrite is transported inside the cells by the ABC - type upake transporters encoded by four genes *nrtABCD* or *nrtP*. The operon consists of *nirA* for nitrite reductase followed by *nrtABCD* and *narB* encoding nitrate reductase ***(5,6)***. The genetic and biomolecular characterization of the N – assimilating genes have been done in a few cyanobacteria, whose cDNA/genomic sequences are available. It is generally considered that nitrate reductase and other proteins in the operon are substrate-inducible in nitrogen fixing cyanobacteria and constitutively expressed in non-nitrogen fixing cyanobacteria. However, our studies in *Spirulina* have shown that nitrate reductase activity is lowered by withdrawal of nitrate from the media and induced by its reintroduction ***(7)***. As dinitrogen fixation became the main focus of research on cyanobacterial nitrogen metabolism over many decades, much of the physiology, biochemistry and molecular genetics of nitrate assimilation in *Spirulina* and other such organisms have remained relatively underexplored. *Spirulina* could also be an attractive natural source of abundant, stable ***(8)*** and thermotolerant nitrate assimilatory enzymes (Lochab et al., unpublished data). Therefore, methods to study them would be of significant interest.

The present chapter deals with the various methods for biochemical, and molecular genetic studies in *Spirulina*. They include enzyme assays for some of the enzymes of nitrogen metabolism in this organism i.e., nitrate reductase, nitrite reductase glutamine synthetase and glutamate synthase. In addition, a DNA isolation protocol specifically adapted and used for *Spirulina* is also provided. It was used for carrying out the PCR cloning of nitrate transporter gene for further molecular characterization.

2. Materials

1. Strain: *Spirulina* (*Arthrospira) platensis* strain PCC 7345.
2. Medium: BG11 medium: For 1 L medium dissolve 1.5 g $NaNO_3$, 0.04 g $K_2HPO_4.3H_2O$, 0.075 g $MgSO_4.7H_2O$, 0.036 g $CaCl_2.2H_2O$, 0.006 g citric acid, 0.006 g ferric ammonium citrate, 0.001 g Na_2EDTA and 0.04 g Na_2CO_3 in distilled water and to this add 1 mL of A5 + Co trace metal mix with the following composition (per L): 2.86 g H_3BO_3, 1.81 g $MnCl_2$, 0.222 g $ZnSO_4$, 0.390 g Na_2MoO_4, 0.079 g $CuSO_4.5\ H_2O$ and 0.0494 g. $Co(NO_3)_2.6\ H_2O$ (*see* **Note 1**).
3. Medium: ASN–III: For 1 L dissolve 25 g NaCl, 2.0 g $MgCl_2.6H_2O$, 0.5 g KCl, 0.75 g $NaNO_3$, 0.02 g $K_2HPO_4.3H_2O$, 3.5 g $MgSO_4.7H_2O$, 0.5 g $CaCl_2.2H_2O$, 0.003 g citric acid, 0.003 g ferric ammonium citrate, 0.0005 g Na_2EDTA and 0.04 g Na_2CO_3 (*see* **Note 1**).
4. Working medium: 1:1 (v/v) mixture of BG11 and ASN–III media.

5. Washing solution: 10 m*M* $NaHCO_3/Na_2CO_3$ buffer pH 10.5.
6. Extraction buffer without DTT, PMSF: 10 m*M* $Na_2CO_3/NaHCO_3$ buffer pH 10.5, 1 m*M* $MgCl_2$ and 20% glycerol, store at 4 °C.
7. Extraction buffer: Add 1 m*M* DTT and 100 m*M* PMSF (from a stock in methanol/isopropanol) before use.
8. Bradford's reagent: 100 mg of Commasie brilliant blue G – 250 in 50 mL of 95% ethanol. Add 100 mL of 85% o-phosphoric acid (v/v). Dissolve overnight and make the final volume to one L with distilled water.
9. 0.3 *M* $NaHCO_3$ solution.
10. NR Assay stock solutions: 100 m*M* $NaHCO_3/Na_2CO_3$ buffer pH 10.5, 0.4 *M* KNO_3, 50 m*M* methylviologen, and freshly prepared 60 m*M* $Na_2S_2O_4$ in $NaHCO_3$.
11. NiR Assay stock solutions: 200 m*M* K_2HPO_4 buffer pH 7.5, 5 m*M* KNO_2, 50 m*M* methylviologen, and freshly prepared 200 m*M*. $Na_2S_2O_4$ in $NaHCO_3$ solution.
12. Coloring reagent: 1:1 (v/v) mixture of 1 % sulfanilamide (3 *N* HCl) and 0.01 % *N*1-naphthylethylenediamine dihydrochloride (in distilled water).
13. GS Assay stock solutions: 100 m*M* Immidazol or Tris buffer pH 7.0, 60 m*M* sodium ATP pH 7.0, 1 *M* sodium glutamate pH 7.0, 1.8 *M* $MgCl_2$, and 1 *M* NH_4Cl.
14. 5.89 μ*M* (800 μg/mL) KH_2PO_4 stock solution.
15. 2.02 *M* ammonium molybdate reagent in 5 N H_2SO_4.
16. 6 m*M* 1- Amino–2-naphthol-4-sulphonic acid (ANSA) in 15% sodium bisulphite.
17. GOGAT Assay stock solutions: 200 m*M* K_2HPO_4 buffer pH 7.5, 50 m*M* 2-oxoglutarate, 50 m*M* glutamine, 100 m*M* aminooxyacetate, 50 m*M* methylviologen, and freshly prepared 150 m*M*. $Na_2S_2O_4$ in $NaHCO_3$ solution.
18. Amino Acid standards: 1.5 m*M* aqueous stock solutions.
19. OPA solution (54 mg o-phthaldialdehyde, 1.0 mL distilled methanol, 9.0 mL 400 m*M* borate buffer pH 9.5 and 0.2 mL 2-mercaptoethanol).
20. Mobile phase: 20 m*M* Na-PO_4 buffer pH 6.8 and methanol (64:36, v/v).
21. DNA isolation buffer: 100 m*M* Tris-HCl pH 8, 50 m*M* Na_2EDTA and 100 m*M* NaCl.
22. TE buffer: 10 m*M* Tris-HCl pH 8 and 1 m*M* Na_2EDTA.
23. TES buffer: 50 m*M* Tris HCl pH 8, 5 m*M* Na_2EDTA and 50 m*M* NaCl.
24. TAE buffer: 40 m*M* Tris–acetate pH 8.0 and 1 m*M* EDTA.
25. 70% ethanol (v/v).
26. 10% *N*-Lauroylsarcosine (sarkosyl).
27. 10% SDS (w/v).
28. 50 mg/mL Lysozyme, prepare fresh.
29. 20 mg/mL Proteinase K in double distilled water.
30. 10 mg/mL RNase A.
31. Tris saturated phenol pH 8.0.
32. Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v).
33. Chloroform: Isoamyl alcohol (24:1, v/v).
34. 5 *M* NaCl.
35. Isopropanol.
36. 1 μg/mL Ethidium bromide.
37. Agarose for DNA electrophoresis.

38. Custom Primers: F 5'-GGGGAATTCAATGTTGCCCTCACAGTTCC-3'
R 5'-CCCCTGGAGCTTTATTCGCTTCCCAGC-3'.
39. Restriction enzyme: Bsp1431 ((MBI Fermentas).
40. PCR Amplification kit (25 m*M* $MgCl_2$, 10x reaction buffer, 10 m*M* dNTP and 1 U *Taq* DNA polymerase (MBI Fermentas).
41. DNA ladder.
42. UV-Visible Spectrophotometer (Hitachi U-2800).
43. Thermal Cycler (MJ Research PTC –100).
44. Refrigerated High speed Centrifuge (Remi CPR – 30).
45. Sonicator (Sonics vibracell 20 Khz).
46. Incubator Shaker (Kühner).
47. HPLC (Shimadzu).
48. µC18 column 150 × 4 mm; particle size, 10 µm.

3. Methods

3.1. Strain and Culture Conditions

1. Strain: Axenic *Spirulina (Arthospira) platensis* strain PCC 7345 was obtained from the Pasteur Cyanobacterial Collection (PCC) of the Institüt Pasteur, Paris.
2. Transfer 10 % inoculum from previous batch to the working medium.
3. Grow under continuous shaking (125 rpm) and continuous illumination with 1–2 fluorescent tubes (Osram Lumilux 18W, 6500K) at 27±1°C.
4. Periodically transfer once cultures attain stationary phase (*see* **Note 2**).

3.2. Enzyme Assays

3.2.1. Preparation of Extracts for Enzyme Assays

1. Centrifuge and harvest the cells 11,600g for 15 min and at 4°C.
2. Re-suspend the pellet in equal volume of ice-cold extraction buffer without DTT, PMSF and repeat step 1.
3. Weigh the centrifuge tube without and then with the pellet and consider difference as fresh weight of the pellet.
4. Alternatively, scoop the pellet onto a pre-weighed weighing boat and find difference in weight (*see* **Note 3**).
5. Sonicate the cell suspension for 7 min with pulses of 5 s ON and 10 s OFF at 35 % amplitude 20 kHz frequency.
6. Centrifuge at 12,600*g* for 30 min at 4°C to prepare crude extract.
7. Transfer clear supernatant to pre–cooled microfuge tubes and keep in ice bath for immediate use or freeze at –20 °C for later use.

3.2.2. Protein Estimation

Use Bradford's reagent and method to determine protein concentration ***(9)***.

3.2.3. Determination of NR Activity ***(10,11)***

1. In sequence, add reaction components (**Table 1.**) and extract equivalent to 200 µg of protein (total volume 400 µL). Make three sets of the reactions.
2. Start reaction by adding $Na_2S_2O_4$ (*see* **Note 4**).
3. Incubate at 25 °C for 20 min.
4. Stop the reaction by vigorous agitation (vortex) of the mixture till the blue colour disappears completely.
5. Add 0.6 mL of coloring reagent and incubate for 15 min at room temperature.
6. Read the absorbance at 540 nm.
7. Always freshly prepare standard curve of KNO_2 (1 µmol stock) in range of 20-100 nmol.

Table 1. The reaction mixture along with the concentration and volume of each reagent for blank and test for nitrate reductase assay.

Name of the component (stock conc.)	Blank (µL)	Test (µL)
$NaHCO_3 - Na_2CO_3$ Buffer	200	200
KNO_3	20	20
Methylviologen	x	32
Crude extract	50	50
Distilled water	110	78
Sodium dithionite	20	20
Total volume	400	400

One U enzyme activity is defined as 1 nmol nitrite formed per min (IU) or per h. Sp. activity is expressed per mg protein. Each experiment is repeated thrice with different biological samples (using internal triplicates in each experiment) and the mean data is plotted along with standard errors. Standard deviation is used to represent the error bars when only one of the two types of replicates is available, either internal replicates or independent biological replicates.

3.2.4. Determination of NiR Activity

1. In sequence add the reaction components (**Table 2.**) and extract equivalent to 200-250 µg of protein (total volume 400 µL). Make two sets of the reactions.
2. Start reaction by addition of dithionite.
3. Stop after incubation at 25°C for 5 min by vigorous shaking till the blue color completely disappears. Vortex if color appears again.
4. Prepare blanks in two sets: B, lacked KNO_2, while O, lacked methylviologen and $Na_2S_2O_4$.
5. Record absorbance (A_{540}) of the test (T) and O against B and nitrite consumed can be calculated from above standard curve.

The activity was expressed as per hr per mg protein, and represented as mean of duplicates samples. Each such experiment is repeated thrice with different biological replicates and the mean data is plotted alongwith standard error bars.

Table 2. The reaction mixture along with the concentration and volume of each reagent for blank and test for nitrite reductase assay.

Name of the component (stock conc.)	Blank (µL)	0 time (µL)	Test (µL)
K-PO_4 buffer	150	150	150
KNO_2	x	10	10
Methylviologen	16	x	16
Crude extract	50	50	50
Distilled water	144	190	134
Sodium dithionite	40	x	40
Total volume	400	400	400

3.2.5. GS-Biosynthetic Assay (12)

1. Desalt the crude extract by passing through Sephadex G-25 column and collect the fractions with high enzyme activity.
2. In sequence add reaction components (**Table 3.**) and gel filtered extract equivalent to 20 µg of protein (total volume 800 µL). Make two sets of reactions.
3. Incubated for 15 min at 25 °C.
4. Add 50 µL ammonium molybdate reagent and 150 µL m*M* ANSA and mix thoroughly.
5. Continue incubation for 15 min and measure color developed (A_{680}) against blank sets.
6. In triplicate prepare serial dilutions by adding 20 µL (16 µg)/ 0.147 µ*M* to 100 µL (80 µg)/0.736 µM from KH_2PO_4 stock to the GS-Biosynthetic reaction mixture minus extract, and make volume upto 0.8 mL. Ensure using phosphate-free water.
7. Incubate at 25 °C for 15 min and add components as in step 4.
8. Record absorbance at 680 nm and prepare standard curve with error bars.

GS activity is represented as µg of phosphate formed from ATP per mL extract per h and sp. activity corrected for per mg protein. Each experiment should be repeated twice or thrice and the mean data should be plotted alongwith standard error bars.

Table 3. The reaction mixture along with the concentration and volume of each reagent for blank and test for glutamine synthetase assay.

Name of the component (stock conc.)	Blank (µL)	Test (µL)
Immidazole buffer	400	400
Sodium glutamate	80	80
$MgCl_2$	22	22
NH_4Cl	40	40
Distilled water	248	148
Sodium ATP	x	100
Crude extract	10	10
Total volume	800	800

3.2.6. GOGAT Assay (13)

1. Prepare 100 µL assay mixture as shown in **Table 4.** and ~75 µg of extract protein. Start reaction by adding dithionite- reduced methyl viologen.
2. Incubate for 15 min at 25°C and stop the reaction by vortexing until blue color disappears.
3. Derivatize the glutamate produced in the reaction and 20 µL amino acid standards by reacting with 100 µL OPA solution at room temperature for 90s (*see* **Note 6**).
4. Filter all solutions through 0.22 µm Millipore filters before applying to HPLC.
5. After purging with mobile phase, inject 20 µL into the HPLC column.
6. Set flow rate at 1.8 mL/min at room temperature.
7. Wash the column after every 30 injections with 20 mL of methanol:water (65:35, v/v) mixture followed by 20 mL mobile phase prior to the next injection.
8. Record absorbance of elute at 340 nm. Typical chromatogram is shown in **Fig. 1.**

Table 4. The reaction mixture along with the concentration and volume of each reagent for blank and test for GOGAT assay.

Name of the component (stock conc.)	Blank (µL)	Test (µL)
K-PO_4 buffer	25	25
2-Oxoglutarate	6	6
Glutamine	x	6
Aminooxyacetate	10	10
Methylviologen	8	8
Distilled water	26	20
Crude extract	15	15
Sodium dithionite	10	10
Total volume	100	100

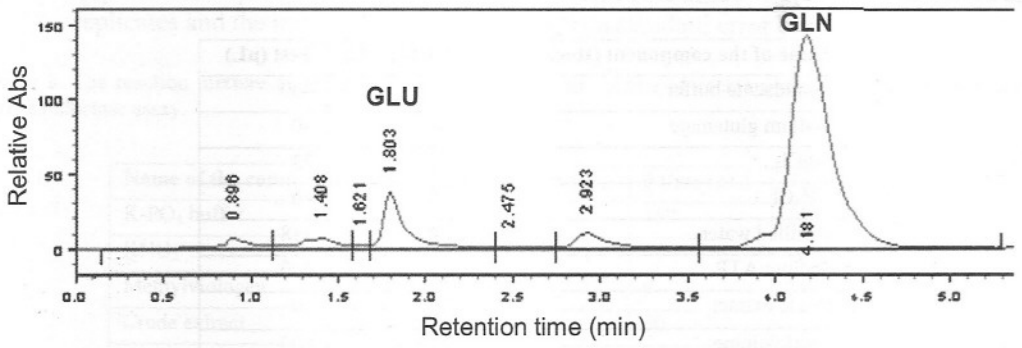

Fig. 1. HPLC Chromatogram of the Fd-GOGAT assay: The chromatogram was obtained after derivatization of glutamate and glutamine with OPA following the Fd-GOGAT reaction, with their peaks at respective retention times 1.803 and 4.181.

3.3. DNA Isolation

Given protocol is modified from Wu et al. *(14)*.

1. Centrifuge 250 mL culture at 11,600*g* and 4°C for 10 min
2. Thoroughly wash pellet with 10 m*M* $NaHCO_3/Na_2CO_3$ buffer.
3. Suspend in 10 mL of DNA isolation buffer and kept at room temperature for 10 min.
4. Add 0.1 mL Sarkosyl and incubate on a shaker at 37°C for 30-90 min. Repeat this step twice.
5. Centrifuge at 11,600*g* for 10 min, wash with 20 mL TES, and re-suspend in 2.5 mL of TES buffer.
6. Add lysozyme to final concentration of 0.5 mg/mL and incubate on a shaker at 37 °C for 30 min.
7. Add SDS to a final concentration of 1% (w/v). Mix gently by inverting the tubes and then keep at 60°C for 30 min.
8. Now supplement Proteinase K to final concentration 50 µg/mL and incubate at 37°C for 1 h. This step can be extended upto few hours or even overnight.
9. Add RNase A to reach concentration 20 µg/mL.
10. Extracted sequentially with: equal volume of Tris saturated phenol, equal volume of Phenol: Chloroform: Isoamyl alcohol (mixing at 37°C for 5-10 min or incubating the samples at 4°C overnight, both resulted in satisfactory yields), and then with equal volume of Chloroform: Isoamyl alcohol (repeat until the interphase between aqueous and organic phases disappears). The mixing of two layers should be done gently, slowly by inversion. Do not vortex. Centrifuge at 12,600*g* at 4°C and for 20 min.
11. Transfer top aqueous phase to a new 50 mL tube using a wide-bore pipette tip, and mix sequentially with 2/3 volume 5 *M* NaCl and then with 1 volume of isopropanol.
12. Centrifuge at 12,600*g* for 20 min at room temperature. Transfer the precipitated DNA with 1 mL pipette tips or disposable glass pipettes to 70% ethanol in 1.5 mL

eppendorf tubes. Invert the tubes several times centrifuge at 5000 RPM for 5 min in a microfuge to get DNA.

13. Discard supernatant, air dry the pellet for about 30 min under hood.
14. Dissolve in 50-200 µL autoclaved double distilled water or TE.
15. Electrophorese DNA samples in a 0.8% (w/v) agarose gel using TAE containing 1 µg/mL ethidium bromide, and observe on a UV-transilluminator. A typical gel is shown in **Fig. 2**.

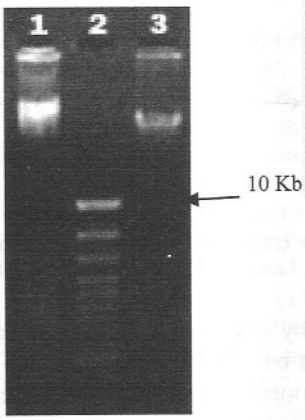

Fig. 2. Genomic DNA of *Spirulina* electrophoresed on a 0.8 % agarose gel. Lane 1: *Spirulina* DNA (1 µg), Lane 2: DNA Marker, Lane 3: *Spirulina* DNA (0.5 µg).

3.4. PCR Amplification

The present protocol is used for amplification of *Spirulina* NrtP gene:

1. Carry out PCR in final volume 12.5 µL with 1.25 µL reaction buffer, 0.25 µL dNTP, 0.6 µL of each primer, 1 U of Taq DNA polymerase, and 1 µg of DNA.
2. Set instrument conditions: Initial denaturation temperature - 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min; annealing at 55 °C for 1 min; extension at 72°C for 1 min (back to denaturation); and final extension at 72°C for 10 min.
3. Electrophorese in a 1.2 % (w/v) agarose gel using TAE buffer containing 1 mg/mL ethidium bromide. Amplification of *NrtP2* product is shown in **Fig. 3**.

4. Notes

1. Autoclave citric acid, $K_2HPO_4.3H_2O$, Na_2CO_3, ferric ammonium citrate and EDTA solutions individually before addition into the media. The solutions should be mixed in the given proportion at room temperature to avoid precipitation. All the media stocks should be autoclaved and stored at 4°C.
2. Ensure that the cultures are free of any bacterial and fungal contamination. Check regularly for contamination on nutrient agar plates for 1–2 days for bacterial growth and 3–4 days for fungal growth.

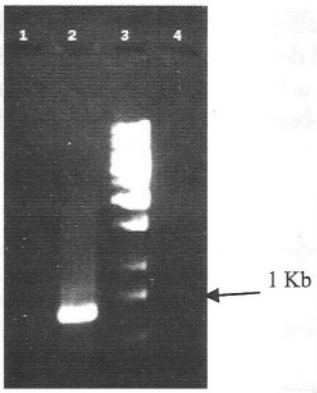

Fig. 3. PCR amplification of the nitrate transporter (nrtP) gene from *Spirulina* genomic DNA, run on a 1.5 % agarose gel. Lane 2: PCR product (NrtP2 ~ 900 bp), Lane 3: 1 Kb DNA ladder (MBI Fermentas).

3. For floccular cultures, harvesting by centrifugation may be difficult and cells can be collected by filtration. Care should be taken to recover entire mass from the filter by repeated washing, which can be scooped out using a sterile spatula.
4. Alternatively, 20-24 µL/mL toluene or alkyl trimethylammoniumbromide (50 µg/mL suspension can be used for permeabilization, after which vortex the tubes for 2 min. Such extracts can be used directly or id necessary are centrifuged. The crude extract should be thawed and kept in an ice bath. After use, the remaining extract can be frozen at -20 °C for later use. Avoid repeated freeze thawing, though.
5. Freshly prepare $Na_2S_2O_4$ in 0.5 mL microfuge tube leaving no air gap. The reaction mixture should turn intense blue indicating reduction of dithionite.
6. OPA solution and o-phthaldialdehyde should be kept in dark and at 0-4°C. OPA solution should be allowed to age for at least 24 h before use, and its strength is maintained by adding 20 µL 2-mercaptoethanol every 3-4 d.
7. Sometimes chaotropic salt like NaI, grinding in liquid N_2, sonication, grinding in a blender, and incubation at 50°C for 30-60 min, facilitate breakage of *Spirulina* cells. The solution should appear viscous and sticky when cells are completely lysed. Prolonged incubation at lower temperatures could also help gentle lysis. The protocols are given elsewhere.
8. Assess the purity of DNA by the ratio A_{260}/A_{280}. The ratio should be 1.8-1.9. If it is slightly higher it indicates RNA and/or nucleotide contamination, and if lower it indicates protein contamination. The quantity of DNA was assessed as per the formula: Concentration(X µg/mL) = (A_{260} × 50) / dilution factor, where A_{260} of 1.0 indicates 50 µg/mL of ds DNA measured in a quartz cuvette of 1 cm pathlength.
9. Further confirmation of the quality of DNA as a substrate for enzymatic reactions may be carried out by restriction digestion and/or PCR. The DNA isolated by the method described here has been successfully used for restriction digestion and cloning.

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