

An improved method for genomic DNA extraction from cyanobacteria

Shailendra P. Singh · Rajesh P. Rastogi ·
Donat-P. Häder · Rajeshwar P. Sinha

Received: 17 May 2010 / Accepted: 16 September 2010 / Published online: 29 September 2010
© Springer Science+Business Media B.V. 2010

Abstract We present an improved method for genomic DNA extraction from cyanobacteria by updating the earlier method from our group (Sinha et al. 2001) that does not require lysozyme treatment or sonication to lyse the cells. This method use lysis buffer to lyse the cells and also skips the initial treatments to remove the exopolysaccharides or to break the clumps. To test the efficacy of the method DNA was extracted from the freshwater cyanobacteria *Anabaena variabilis* PCC 7937, *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 6301 and *Rivularia* sp. HKAR-4 (Accession number: FJ939128). The spectrophotometric and gel electrophoresis analysis revealed high yield and high quality of genomic DNA extracted by this method. Furthermore, the RAPD resulted in the amplification of unidentified genomic regions of various lengths; however, rDNA amplification gave only one band of 1.5 kb in all studied cyanobacteria. Thymine dimer detection study revealed that thymine dimers are induced only by UV-B radiation in *A. variabilis* PCC 7937 and there is no effect of PAR and UV-A on its genome.

Collectively, all these findings put forward the applicability of this method in different studies and purposes.

Keywords Cyanobacteria · Genomic DNA · PCR · RAPD · rDNA · Thymine dimer

Introduction

Cyanobacteria are immense source of natural products of industrial and pharmaceutical importance having antiviral, antibacterial, antifungal, anti-inflammatory, proteinase-inhibiting, immunosuppressant, and anticancer activities (Rastogi and Sinha 2009). In addition, some of them also accumulate polyhydroxyalkanoates, which can be used as a substitute for non-biodegradable petrochemical-based plastics (Abed et al. 2009). Cyanobacteria from marine and freshwater habitats also produce a range of cyanotoxins which may have ecological role as allelochemical and can be potentially used for the development of new class of algacides, herbicides and insecticides (Berry et al. 2008). These organisms are a source of natural sunscreens such as mycosporine-like amino acids and scytonemin (Rastogi et al. 2010a, b) and are also considered as a source of biofuels; i.e. alcohols and hydrogen (Angermayr et al. 2009). Recently developed metagenomic approach will further add new compounds and genes from cyanobacteria that can not be cultured under laboratory conditions. However, use of this approach and further biotechnological exploitation of cyanobacteria requires the extraction of good quality of genomic DNA as a primary step that should be inexpensive, simple, efficient and safe for the workers in general and environment in particular.

The extraction of genomic DNA from cyanobacteria becomes complicated as these organisms have multilayered

S. P. Singh · R. P. Rastogi · D.-P. Häder
Department of Biology, Friedrich-Alexander University
Erlangen-Nuremberg, Staudtstrasse 5, 91058 Erlangen, Germany

R. P. Rastogi · R. P. Sinha (✉)
Laboratory of Photobiology and Molecular Microbiology,
Centre of Advanced Study in Botany, Banaras Hindu University,
Varanasi 221005, India
e-mail: r.p.sinha@gmx.net

Present Address:

S. P. Singh
MSU-DOE Plant Research Laboratory, Department
of Biochemistry and Molecular Biology, Michigan State
University, 322 Plant Biology Building, East Lansing,
MI 48824-1312, USA

cell wall and sheath outside the cell wall. Thus, lysis of the cell is the determining step in efficient extraction of the genomic DNA. Different methods which could be either physical or enzymatic or a combination of both have been proposed that can be used to disrupt the cells (Wu et al. 2000). Several methods have been given that advocate the use of lysozyme for cell lysis (Margheri et al. 1999; Wu et al. 2000; Saha et al. 2005; Morin et al. 2010). However, most of the filamentous cyanobacteria are often surrounded by a sheath which acts as a barrier for the enzymatic action of lysozyme on the cell wall (Philippis and Vincenzini 1998). Alternatively, sonication of samples was used to disrupt the cells (Sinha et al. 2001; Srivastava et al. 2007), however, sonicators generate sound waves that are outside the normal range of hearing and can cause hearing damage. It is also time taking and there is also chance of contamination of samples with sonication when working with several samples, additionally, DNA molecules are always susceptible to fragmentation with sonication. A xanthogenate nucleic acid extraction method, which does not require enzymatic or mechanical cell disruption, was given for cyanobacteria by Tillett and Neilan (2000); however, this method has number of its own limitations as mentioned by the authors. In this study we present a method for genomic DNA extraction from cyanobacteria that avoid both enzymatic and mechanical disruption of the cells by updating the earlier given method from our group (Sinha et al. 2001). The potentiality of improved method was tested by spectrophotometric analysis, gel electrophoresis, RAPD, 16 s rDNA amplification and thymine dimer detection by dot-blot assay.

Materials and methods

Experimental organisms and growth conditions

Cyanobacteria used in this study were *Anabaena variabilis* PCC 7937 (Fig. 1a), *Anabaena* sp. PCC 7120, *Synechocystis*

sp. PCC 6803 (Fig. 1b), *Synechococcus* sp. PCC 6301 and *Rivularia* sp. HKAR-4 (Fig. 1c). *Anabaena variabilis* PCC 7937, *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301 were obtained from the Pasteur culture collection (Institute Pasteur, France) while *Rivularia* sp. HKAR-4 was isolated from hot water (40–43°C) spring of Rajgir (25°2' 0" North, 85° 25' 0" East, Bihar, India) and was identified by 16 s rDNA phylogeny (Accession number: FJ939128; unpublished data). All cyanobacteria were routinely grown in an autoclaved BG11 medium (Stanier et al. 1971) in a culture room at a temperature of $20 \pm 2^\circ\text{C}$ and continuous fluorescence lamp illumination of $55.2 \pm 9.2 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. DNA was extracted from 50 ml of exponentially growing cultures of *Anabaena* sp. PCC 7120, *Synechococcus* sp. PCC 6301, *Synechocystis* sp. PCC 6803, *Rivularia* sp. HKAR-4 and *Anabaena variabilis* PCC 7937 having 0.34, 0.60, 0.87, 0.45 and 0.22 mg/ml protein concentrations, respectively.

DNA extraction

The DNA was extracted from 50 ml of liquid cultures of above mentioned five cyanobacteria. Cells were harvested by centrifugation at $3,000 \times g$ for 5 min (Centrifuge 5,702, Eppendorf, Hamburg, Germany) and pellets were transferred to 2 ml nuclease-free Eppendorf tubes. Thereafter, 400 μl of lysis buffer (Urea 4 M; Tris-HCl 0.2 M, pH 7.4; NaCl 20 mM and EDTA 0.2 M) and 50 μl Proteinase K (stock solution of 20 mg/ml) was added to the pellet and mixed immediately by pipetting. This mixture was incubated for 1 h at 55°C and mixed by pipetting every 10–15 min. Thereafter, 1 ml of prewarmed (55°C) DNA extraction buffer (CTAB 3%; NaCl 1.4 M; EDTA 20 mM; Tris-HCl 0.1 M, pH 8.0; Sarkosyl 1% and Mercaptoethanol 1%) was added, mixed gently by inverting the tubes and incubated at 55°C for another 1 h. During incubation Eppendorf tubes were 4–5 times gently inverted every 10 min to mix the solution. After this step mixture was divided into two fractions in 2 ml Eppendorf tubes for

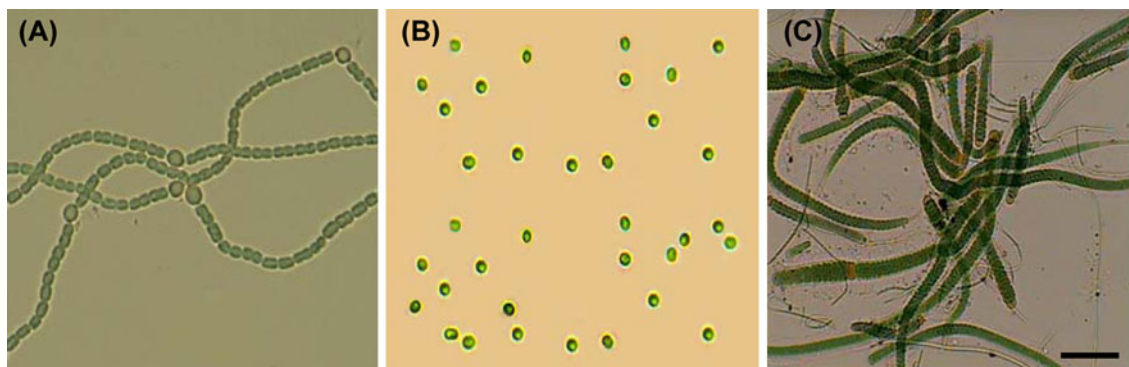


Fig. 1 Microphotographs of *Anabaena variabilis* PCC 7937 (a), *Synechocystis* sp. PCC 6803 (b) and *Rivularia* sp. HKAR-4 (c). Bar = 50 μm

Fig. 2 A flow-chart diagram showing the steps involved in extraction of genomic DNA from cyanobacteria



each cyanobacterium, allowed to cool and 2 vol. of chloroform: isoamyl alcohol (24:1 v/v) was added to it and mixed by gentle inversion until white emulsion appeared. After centrifugation at 10,000×g for 5 min (Centrifuge 5,424, Eppendorf), 500 µl from upper water phase was transferred to a new Eppendorf tube and 2 vol. of 100% ethanol and 0.1 vol. of 3 M sodium acetate (pH 5.2) was added to it, mixed once by inversion and transferred to −20°C for 1 h. After 1 h incubation suspensions were centrifuged at 10,000×g for 3 min and supernatant were discarded. Thereafter, pellets were washed once by 70% ice-cold ethanol (500 µl) and after evaporating the residual ethanol dry pellets were redissolved in 50 µl of sterile water. Finally, the extracted genomic DNA from each cyanobacterium (two fractions) was pooled in one Eppendorf tube. A flow-chart of the steps involved in DNA extraction is depicted in Fig. 2.

Spectrophotometric analysis and agarose-gel electrophoresis

The genomic DNA concentration was quantified by spectrophotometer (UV-2550, Shimadzu Scientific Instruments, Inc., Riverwood Drive, Columbia, MD USA). The

absorption at 260 nm gives the concentration of DNA (1 O.D. at 260 nm equals 50 µg/ml dsDNA). The purity of genomic DNA was checked by the ratio between absorption at 260 and 280 nm. The DNA was considered pure if the ratio between 260 and 280 nm was between 1.8 and 2.0. A ratio below 1.6 is typical for protein contamination while the ratio above 2.0 is characteristic of RNA contamination. The integrity of the extracted genomic DNA was tested by 0.8% agarose-gel electrophoresis.

Random amplified polymorphic DNA (RAPD) and 16S rDNA amplification

The template quality of extracted genomic DNA was tested by RAPD and 16S rDNA amplification by polymerase chain reaction (PCR). The sequence of primer used for RAPD was 5'-AGCGGCCATT-3' (Kumar et al. 2004) while forward and reverse primer sequences for 16S rDNA amplification were For_5'-GAGTT(CT)GATCCTGGCTCAGGA-3' and Rev_5'-TCCAGCCGCACCTTCCAGTA-3', respectively. Reactions were performed in 800 µl microcentrifuge tubes containing template DNA, primers, PCR buffer, dNTP mixture and heat stable *Taq* DNA polymerase (Takara Bio Inc., Japan). PCR reactions were performed using a

PeQLab thermocycler (PeQLab Biotechnologie, Erlangen, Germany). The genomic DNA thus obtained was used in 50 μl PCR reaction mixture containing 100 ng genomic DNA, 1 μl (50 pmol/ μl) of each forward and reverse primers for 16S rDNA amplification and single primer mentioned above for RAPD, 5 μl 10 \times PCR buffer, 5 μl dNTP mixture, 0.2 μl *Taq* DNA polymerase and sterile water to a final volume of 50 μl . The PCR cycles were: 1 cycle of denaturation at 95°C for 5 min, 33 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 16S rDNA and 36°C for RAPD for 1 min and extension at 72°C for 1 min 36 s for 16S rDNA and 2 min for RAPD, 1 cycle of final extension at 72°C for 15 min and a final hold at 4°C. The PCR products were analyzed by agarose-gel electrophoresis.

Thymine dimer induction and detection

For the induction of thymine dimer cultures of *A. variabilis* PCC 7937 were irradiated in a plastic box to simulated solar radiation (Sol 1,200 W mercury lamp, no. 0383, Dr. Hönle GmbH, Martinsried, Germany) from the height of 94 cm for 24 h (Fig. 3a). The samples were covered with 395 nm (Ultraplan, UV Opak Digefra, Munich, Germany), 320 nm (Folex PR Montagefolie 320 nm Art. Nr. 10155099, Folex, Dreieich, Germany) or 295 nm (Ultraplan, Digefra) cut-off filters to get the PAR (photosynthetically active radiation; 400–700 nm) only or PAR + UV-A (ultraviolet-A; 315–400 nm) or PAR + UV-A + UV-B (ultraviolet-B; 280–315 nm) radiation regimes. The irradiances effectively received by the samples were UV-B: 0.31 Wm^{-2} ; UV-A: 25.70 Wm^{-2} ; PAR: 118.06 Wm^{-2} based on the transmission characteristics of the cut-off filters (Fig. 3b). After irradiation genomic DNA was extracted immediately as mentioned above. The detection of thymine dimer was essentially performed according to Sinha et al. (2001) with some modifications. The blot paper (GB002, Schleicher and Schuell, Dassel, Germany) and the nylon membrane (Roti-[®] Nylon Plus, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were placed on a slot or dot blot manifold (Minifold I, Schleicher and Schuell). Thereafter 500 ng DNA samples were transferred to the membrane and washed once with TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The membrane was incubated for 1 h at 80°C to immobilize the DNA. Afterwards, the membrane was incubated for 1 h in PBS-T [phosphate buffer saline: 0.14 M NaCl, 3.4 mM KCl, 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4 and 0.1% (v/v) Tween 20] with 5% (w/v) skimmed milk powder to block the non-specific sites. Thereafter, the membrane was incubated with the 400 μl primary antibody (anti-thymine dimer KTM53, Kamiya Biomedicals, Seattle, USA; diluted 1:100 in PBS-T) in 10 ml of PBST + 5% (w/v) milk powder for 2 h at room temperature with continuous shaking and then washed (3 \times 10 min) with PBS-T. Subsequently, the

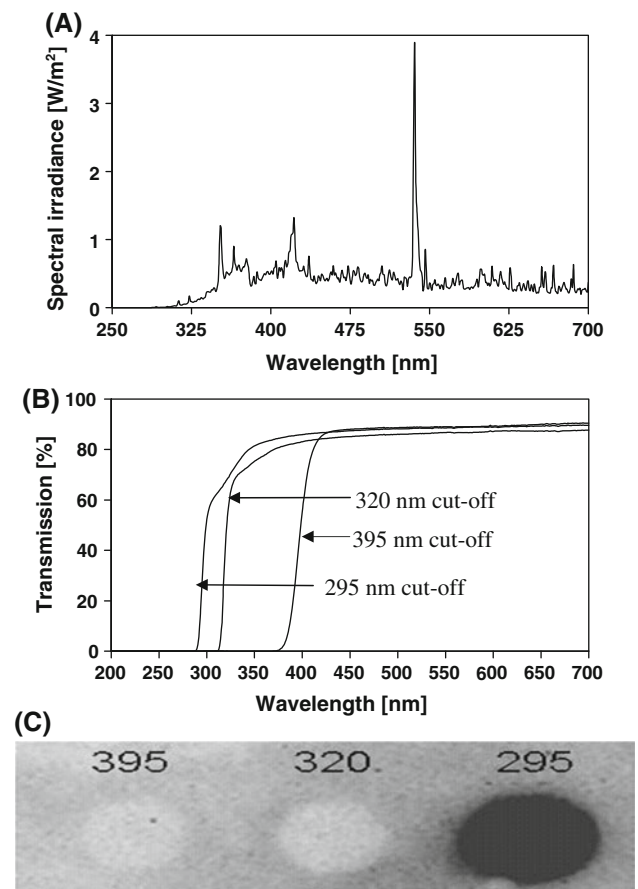


Fig. 3 Spectral characteristics of the light source (a), transmission spectra of 395, 320 and 295 nm cut-off filters used in thymine dimer induction experiments (b) and immunodot-blot showing induction of thymine dimers in 295 nm cut-off filter covered sample of *Anabaena variabilis* PCC 7937 (c)

membrane was incubated with 400 μl secondary antibody [anti-mouse IgG (Fab specific) peroxidase conjugate, Sigma, Saint Louis, Missouri, USA; diluted 1:100 in PBS-T] in 10 ml of PBST + 5% skimmed milk powder for overnight at room temperature with continuous shaking and then washed next morning with PBS-T (4 \times 10 min). Finally, the detection reagent (Renaissance, NEN Life Science Products, Cologne, Germany) was spread over the membrane and image was taken with Kodak digital science image station 440CF system (Kodak, Rochester, New York, USA).

Results and discussion

In the present investigation, an improved method for genomic DNA extraction from both sheathed and non-sheathed cyanobacteria was developed that does not require enzymatic and mechanical lysis of the cell (Sinha et al. 2001). The results from the spectrophotometric analysis suggested that the present method worked well

Table 1 The concentrations and quality of genomic DNA extracted from different cyanobacteria

Cyanobacteria	Concentration extracted DNA		260/280 Ratio
	µg/ml	µg/mg protein	
<i>Rivularia</i> sp. HKAR-4	478.5 ± 40.31	2.04 ± 0.17	1.93 ± 0.15
<i>Anabaena</i> sp. PCC 7120	362.5 ± 17.68	2.13 ± 0.10	1.84 ± 0.14
<i>Anabaena variabilis</i> PCC 7937	230 ± 28.28	2.09 ± 0.26	1.89 ± 0.07
<i>Synechocystis</i> sp. PCC 6803	433.5 ± 144.96	1.0 ± 0.33	1.84 ± 0.11
<i>Synechococcus</i> sp. PCC 6301	259.5 ± 57.28	0.90 ± 0.19	1.91 ± 0.07

Values are mean ± S.D. of three replicates

and was efficient to extract genomic DNA from all cyanobacteria giving high yield (Table 1). The 260/280 ratio also supported that the DNA extracted by this method was of high quality without any contamination of protein or RNA (Table 1). The spectrophotometric analysis tells only the quantity and quality in view of RNA or protein contamination, however, it does not tell about the integrity of the molecule. Therefore, agarose-gel electrophoresis was used to ensure the integrity of the genomic DNA extracted by this method. High molecular weight DNA was extracted using this method indicating that DNA molecule was not heavily fragmented (Fig. 4a). Thus, spectrophotometric and agarose-gel electrophoresis analyses showed that this method gives genomic DNA of high quantity as well as quality and can be further used for various purposes.

The sonic shock treatment in an ultrasonic cleaner bath and homogenization using sterile glass beads on a vortex mixer was used for clump forming cyanobacteria to separate and break the filaments (Fiorea et al. 2000; Srivastava et al. 2007). However, our method does not require such treatments as we were able to extract genomic DNA efficiently from the *Rivularia* sp. HKAR-4 which forms massive clumps when grown in liquid medium. Mixing by pipetting during incubation with lysis buffer was enough to achieve the task. *Anabaena* and *Rivularia* are sheath containing cyanobacteria (Berrendero et al. 2008; Leak 1967). The sheath is known to act as a barrier for lysozyme; however, this method successfully extracted

genomic DNA from these cyanobacteria and thus overcoming the problem of cell lysis.

We also tested the template activity of the extracted DNA by using the RAPD and 16S rDNA amplification approach by PCR. The results from the RAPD assay showed amplification of different genomic regions of various lengths in *Rivularia* sp. HKAR-4, *Anabaena* sp. PCC 7120, *Anabaena variabilis* PCC 7937, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301, respectively (Fig. 4b). The results from the rDNA amplification showed amplification of a single DNA band (1.5 kb) from all cyanobacteria corresponding to rDNA (Fig. 4c). Thus, both the RAPD and 16S rDNA assay also supported the results from spectrophotometric analysis and suggested that genomic DNA was of good quality for their further use in PCR and RAPD analysis.

This method also skips the initial washing of the cells with washing buffer that has been used by several workers to remove the extracellular polysaccharides (Margheri et al. 1999; Fiorea et al. 2000; Srivastava et al. 2007; Morin et al. 2010). Sarkosyl (0.1%) has also been used to remove the lipid polysaccharide which was essential for cell lysis by lysozyme (Wu et al. 2000), however, all these additional steps will render DNA fragmentation by longer exposure to deoxyribonucleases and integrity of the genomic DNA will be compromised. Silica has been used for enhancing the cell lysis, but it may also cause shearing of the high molecular weight DNA (Saha et al. 2005).

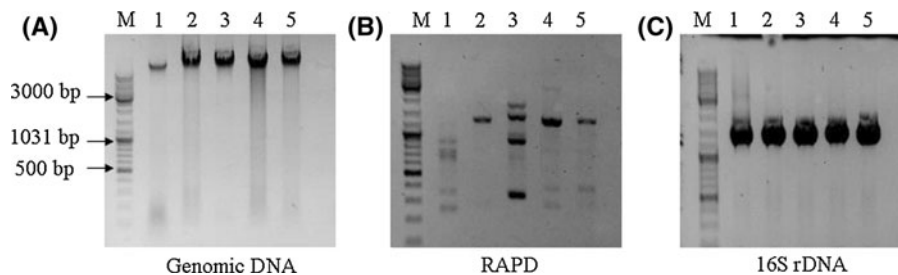


Fig. 4 Electrophoresis of extracted genomic DNA in 0.8 % (w/v) agarose gel (a), RAPD profile of genomic DNA (b) and 16S rDNA amplified by PCR from genomic DNA of different cyanobacteria (c). 1, *Rivularia* sp. HKAR-4; 2, *Anabaena* sp. PCC 7120; 3, *Anabaena*

variabilis PCC 7937; 4, *Synechocystis* sp. PCC 6803; 5, *Synechococcus* sp. PCC 6301; M, O' Gene Ruler DNA ladder mix (Fermentas, Germany) used as marker

In addition, we also tested the efficacy of this method in DNA damage study. To achieve this, cultures of *A. variabilis* PCC 7937 were exposed to PAR, PAR + UV-A or PAR + UV-A + UV-B radiation regime for 24 h and thereafter, genomic DNA was extracted from these samples and thymine dimers were detected using the method as given earlier (Sinha et al. 2001). The results from dot-blot assay revealed the efficiency of present method in DNA damage study and further it was found that thymine dimers are induced only by UV-B radiation in *A. variabilis* PCC 7937 and there was no effect of PAR and UV-A on structure of the genome (Fig. 3c).

Thus, the present improved method was found sufficient enough to extract genomic DNA from studied cyanobacteria without any initial washing step to remove the exopolysaccharides and enzymatic or mechanical cell lysis; however, further studies with several other cyanobacteria are needed to test this method for its broad applicability. The genomic DNA thus extracted by this method is of high quantity as well as quality and can further be used for various purposes.

Acknowledgments The authors gratefully thank M. Schuster for providing excellent technical support.

References

- Abed RMM, Dobretsov S, Sudesh K (2009) Applications of cyanobacteria in biotechnology. *J Appl Microbiol* 106:1–12
- Angermayr SA, Hellingwerf KJ, Lindblad P, de Mattos MJT (2009) Energy biotechnology with cyanobacteria. *Curr Opin Biotechnol* 20:257–263
- Berrendero E, Perona E, Mateo P (2008) Genetic and morphological characterization of *Rivularia* and *Calothrix* (Nostocales, Cyanobacteria) from running water. *Int J Syst Evol Microbiol* 58:447–460
- Berry JP, Gantar M, Perez MH, Berry G, Noriega FG (2008) Cyanobacterial toxins as allelochemicals with potential applications as algacides, herbicides and insecticides. *Mar Drugs* 6:117–146
- Fiorea MF, Moona DH, Tsaia SM, Leeb H, Trevorsb JT (2000) Miniprep DNA isolation from unicellular and filamentous cyanobacteria. *J Microbiol Meth* 39:159–169
- Kumar A, Tyagi MB, Jha PN (2004) Evidences showing ultraviolet-B radiation-induced damage of DNA in cyanobacteria and its detection by PCR assay. *Biochem Biophys Res Commun* 318:1025–1030
- Leak LV (1967) Fine structure of the mucilaginous sheath of *Anabaena* sp. *J Ultra Res* 21:61–74
- Margheri MC, Bosco M, Giovannetti L, Ventura S (1999) Assessment of the genetic diversity of halotolerant coccoid cyanobacteria using applied 16S rDNA restriction analysis. *FEMS Microbiol Lett* 173:9–16
- Morin N, Vallaeyts T, Hendrickx L, Natalie L, Wilmotte A (2010) An efficient DNA isolation protocol for filamentous cyanobacteria of the genus *Arthrospira*. *J Microbiol Meth* 80:148–154
- Philippis RD, Vincenzini M (1998) Exocellular polysaccharides from cyanobacteria and their possible applications. *FEMS Microbiol Rev* 22:151–175
- Rastogi RP, Sinha RP (2009) Biotechnological and industrial significance of cyanobacterial secondary metabolites. *Biotechnol Adv* 27:521–539
- Rastogi RP, Richa, Sinha RP, Singh SP, Häder D-P (2010a) Photoprotective compounds from marine organisms. *J Ind Microbiol Biotechnol* 37:537–558
- Rastogi RP, Richa, Singh SP, Häder D-P, Sinha RP (2010b) Mycosporine-like amino acids (MAAs) profile and their activity under PAR and UVR in a hot spring cyanobacterium *Scytonema* sp. HKAR-3. *Aus J Bot* 58:286–293
- Saha SK, Uma SL, Subramanian G (2005) An improved method for marine cyanobacterial DNA isolation. *World J Microbiol Biotechnol* 21:877–881
- Sinha RP, Dautz M, Häder D-P (2001) A simple and efficient method for the quantitative analysis of thymine dimers in cyanobacteria, phytoplankton and macroalgae. *Acta Protozool* 40:187–195
- Srivastava AK, Ara A, Bhargava P, Mishra Y, Rai SP, Rai LC (2007) A rapid and cost-effective method of genomic DNA isolation from cyanobacterial culture, mat and soil suitable for genomic fingerprinting and community analysis. *J Appl Phycol* 19:373–382
- Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chococcales). *Bact Rev* 35:171–205
- Tillett D, Neilan BA (2000) Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. *J Phycol* 36:251–258
- Wu X, Zarka A, Boussiba S (2000) A simplified protocol for preparing DNA from filamentous cyanobacteria. *Plant Mol Biol Rep* 18:385–392