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Research Paper

Isolation of fresh water Cyanobacterial DNA of north east India by modified Xanthogenate method

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Abstract

The genomic DNA of fresh water cyanobacteria collected from different ecological habitats of North East region of India* were hard to isolate and effectively with reproducible results *by Xanthogenate method of Tiellat and Nielan and CTAB method due to the presence of high polysaccharides but can be isolated by the modified xanthogenate method particularly by Xanthogenate-SDS-Phenol with modifications providing results in less time with more consistency. This method can isolate genomic DNA belonging to the three major families of cyanobacteria based on Desikachary viz. Oscillatoriaceae, Nostocacea and Rivulariaceae. Isolation of genomic DNA from purified cyanobacterial cultures obtained from National Facility for Fresh water cyanobacterial and Microalgal repository at Institute of Bioresources and Sustainable Development, Imphal, Manipur, India viz. Plectonema indica*, *Plectonema notatum, Limnothrix redekei, Anabaenopsis* sp., *Phormidium* sp *Nostoc* sp., *Calothrix* sp., *Tolypothrix* sp., *Anabaena spiroides*, *Wollea* sp., *Cylindrospermum* sp., *Nostoc commune*, *Rivularia* sp. and *Phormidium tenue were studied and its downstream applicability for doing PCR techniques like 16S rRNA and RAPD profiling were done.

Keywords: Cyanobacteria, DNA, north east India, xanthogenate.

Introduction

The cyanobacteria are a diverse, ecologically important, assemblage of photosynthetic prokaryotes that are believed to be responsible for the Earth's early oxygenic atmosphere [1]. They are, for the most part, obligate photoautotrophs and occur in a variety of morphological forms. They are dominant group of organisms in freshwater environments [2]. Certain genera of cyanobacteria can perform nitrogen fixation by a specialized cell type called heterocyst [3] [4] and apart from this they are symbiotic to taxonomically wide range of organisms viz. algae, plants, fungi, non-photosynthetic protistas and heterotrophic bacteria [5].

Cyanobacteria mostly produce copious amount of polysaccharides which makes difficult for cellular lysis and also interferes with nucleic acid [6][7]. Extraction of DNA from plant using metal xanthates was first introduced by Jhingan [8] where plant cell walls were disrupted by formation of water soluble polysaccharide xanthate formed with Potassium ethyl xanthogenate. The Xanthogenate-Sodium dodecyl

Sulphate (XS) nucleic acid isolation protocol from cyanobacteria was later developed by Tillett and Nielan [9] for rapid extraction keeping in view of removal of polysaccharides by using metal xanthates.

The XS nucleic acid isolation protocol developed by Tillett and Nielan was observed with less frequency of DNA extraction and less yield for different genera of fresh water cyanobacterial isolates obtained from different ecological habitats of North East region of India as polysaccharides content was found to be high in most of the non-heterocystous as well as heterocystous forms of cyanobacteria. The cyanobacterial total carbohydrates or polysaccharides content reported from North east India showed high content of carbohydrates viz. *Phormidium tenue* (Menegh.) Gomont (73.00 µg/ml), *Phormidium bohneri* Schmidle (42.00 µg/ml), *Phormidium fragile* (Meneghini) Gomont (36.00 µg/ml), *Plectonema nostocorum* Bornet ex Gomont (48.00 µg/ml), *Lyngbya truncicola* Ghose (56.00 µg/ml), *Anabaena naviculoides* Fristch (15.00 µg/ml), *Anabaena variabilis* Kutzing ex Born. et Flah (29.00 µg/ml), *Anabaena fuellebornii* Schmidle (29.00 µg/ml), *Nostoc spongiaeforme* Agardh ex Born. et Flah (22.00 µg/ml), *Microchaete uberrima* Carter, N. (27.00 µg/ml) at the log phase of their growth and more on later stationary phase [10] .

Apart from this, the original Xanthogenate (Potassium ethyl xanthogenate) method and CTAB did not include a tissue homogenization step, did not show reproducible banding patterns due to miniscule and inconsistent quantities of DNA extracted, or possibly due to inadequate purification [11]. Some constituents occurring widely in the freshwater environment, such as humic and fulvic acids, copurify with DNA and inhibit PCR and restriction digestion of DNA also [12]. The cyanobacterial genera like *Lyngbya, Anabaena, Nostoc, Calothrix, Spirulina, Wollea, Westellopsis, Oscillatoria, Phormidium, Plectonema Limnothrix* were hard to isolate using the original XS method. Modification like mechanical process using grinding by pestle and mortar was included even though original protocol does not recommend. It was found that XSP (Xanthogenate-SDS-Phenol) used for RNA isolation mentioned by Tillett and Nielan (2000) can also be used for DNA extraction with modification on the steps like mechanical lysis, incubation temperature and hours and also precipitation steps along with concentration of RNase. The modifications made show consistent DNA results as compared to the original protocol. Viewing less efficient techniques and isolation kits available specifically for isolation of genomic DNA of cyanobacteria, this method is applicable to broad range of genera with high efficiency and less time taking bearing high concentration and quality DNA.

Materials and Methods

Collection, isolation and identification of cyanobacteria

The cyanobacterial samples were collected from different ecological habitats of North east region of India with locations recorded using GPS and are isolated by streak plate method [13] maintained and preserved in the Fresh water cyanobacterial and microalgal repository at IBSD, Imphal, Manipur, India. The cyanobacterial cultures were grown in the Blue Green medium (BG-11) [14] media broth at temperature of 27±2ºC under the light intensity of 4000-5000 lux keeping the photoperiod time at 14 hours light and 10 hours dark period. The cultures were identified primarily upto generic or species level morphologically by using key books like Desikachary [15] and Komarek and Anagnostidis [16].

Isolation of cyanobacterial DNA

DNA extraction were carried out by taking mid to late logarithmic phase cultures.2 ml of the cultures were harvested by centrifugation at 10,000 rpm for 10 mins **in** 2ml eppendorf tubes. The pellets were washed with double distilled water atleast twice by adding 2ml of double distilled water with slight vortexing for 2 mins and centrifugation was done at 10,000 rpm (Eppendorf 5430R, Hamburg, Germany) for 5 mins. The pellets were washed finally by suspending with 2ml of TE wash buffer (10mM Tris-HCl [Sigma- Aldrich Co, USA], 1mM EDTA [SRL, Mumbai, India], pH 8.0) and centrifuge at 10,000rpm for 5 mins. The pellets were suspended in 1ml of Xanthogenate Sodium dodecyl Sulphate (XS) buffer (1% Potassium ethyl xanthogenate [Sigma- Aldrich Co, USA]; 100mM Tris-HCl, 20mM EDTA, pH 8.0; 1% sodium dodecylsulfate [Sigma- Aldrich Co, USA]; 800mM ammonium acetate [SRL, Mumbai, India]) and were homogenized by pestle and mortar. 1ml of the homogenized sample with buffer are transfered back in the 2ml eppendorf tube along with 100 µl of RNase A (1mg mL^{-1}) [Sigma- Aldrich Co, USA] by which is

mandatory and incubated in the waterbath at 70°C for $1^{1/2}$ hrs - 2hrs depending on the cultures having high polysaccharides sheaths content with slight vortexing (Spinix, Tarson, Kolkata, India) for 10 secs in between the incubation. After incubation, 1 ml of saturated phenol (Himedia, Mumbai, India) were added to the tube and vortex for 5mins.The tubes were then centrifuge at 12,000 rpm for 15 mins. The aqueous phase is removed gently and washed with equal amount of Chloroform: Isoamyl (24:1) [Himedia, Mumbai, India] in new eppendorf tube. Centrifuge at 12,000 rpm and remove the upper layer and added equal amount of chilled isopropanol (SRL, Mumbai, India) with gently mixing by inverting end to end. After incubation at -20°C for 10 mins, the tubes were centrifuged at 14,000 rpm for 10mins. The supernatant was removed and washed with 70% ethanol and centrifuge at 12,000 rpm for 5mins. The pellets were air dried and eluted in the 100 µl of sterile double distilled water or TE wash buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and stored at 4°C.The DNA was electrophorised (SEA 2000, Elchrom Scientific, Switzerland) on 0.8% agarose gel using TAE (Promega, Madison, USA) as buffer for 30 mins at 100V. The quantity and quality of DNA were observed using by taking 2 µl nanodrop by analyzing the ratio of absorbance at 260nm: absorbance at 280nm (eppendorf, USA). The experiments were repeated in triplicates independently.

16S rRNA PCR amplification

PCR amplication for different genera were undergone for partial 16S rRNA gene amplification [14]. Amplication was carried out by using 50ng of extracted DNA using PCR master mix made up of 1X Taq buffer with 1.5mM $MgCl_2$ (Genei, Mumbai, India), 200µM each of dNTPs (Himedia, Mumbai, India) solution, 1.25U Taq polymerase along with 0.3 µM each of cyanobacterial specific primers (IDT, New Delhi, India), cyanobacterial specific forward primer (1R-AGAGTTTGATCCTGGCTCAG) and reverse primer (740R CTACGCATTTCACCGCTAC) [IDT, India]. Thermal cycler PCR (Master cycler gradient, Eppendorf, California, USA) conditions were initial denaturation at 95°C for 5 mins, annealing at 45°C for 1min, extension at 72°C for 1min for 28 cycles for 25 µl reaction. The gel were ran at 1% agarose gel at 60V for 45 minutes. Universal primer for 16S rRNA like (536F-GTGCCAGCAGCCGCGGTRATA) and reverse (1488R-CGGTTACCTTGTTACGACTTCACC) [IDT, India] can also be use with 1X Taq buffer with 1.5mM $MgCl_2$ (Genei, Mumbai, India), 200µM each of dNTPs (Himedia, Mumbai, India) solution, 1.25U Taq polymerase along with 0.3 µM each of cyanobacterial specific primers (IDT, New Delhi, India) by PCR condition method initial denaturation of 95°C for 5 mins, annealing at 55°C for 1min and extension at 72°C for 1min for 35 cycles for 25 µl reaction.

RAPD analysis

The RAPD was carried out to assessed the DNA applicability to this method by using Illustra Ready-to-Go RAPD beads (GE Healthcare, UK) containing thermostable polymerases (AmpliTaq™ DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each dNTP in a 25 µl reaction volume), BSA (2.5 µg) and buffer [3 mM MgCl2, 30 mM KCl and 10 mM Tris, (pH 8.3)] with primer concentration of 5 pmol/µl in a 25 µl reaction volume along with 5-50ng of DNA. The RAPD for different genera were carried out by using six primers viz. primer 1 - (5'-d[GGTGCGGGAA]-3'); primer 2 - (5'-d[GTTTCGCTCC]-3'); primer 3 - (5'-d[GTAGACCCGT]-3'); primer 4 - (5'-d[AAGAGCCCGT]-3'); primer 5 - (5'-d[AACGCGCAAC]-3') and primer 6 (5'[CCCGTCAGCA]3').The PCR conditions were set for 45 cycles with initial denaturation at 95°C, 5 minutes then 95°C, 1 minute, annealing at 36°C, 1 minute and final extension at 72°C, 2 minutes using gradient Thermal cycler (Eppendorf, USA). All the primers show polymorphic bands. The RAPD were electrophorised at 2% agarose gel using TAE as buffer at 90V for 45 mins.

Results and Discussion

The cyanobacterial DNA of *Plectonema indica* Dixit, *Plectonema notatum* Schmidle*, Limnothrix redekei* (Van Goor) Meffert*, Anabaenopsis* sp., *Nostoc* sp., *Phormidium* sp., *Calothrix* sp., *Tolypothrix* sp., *Anabaena spiroides* Kleb, *Wollea* sp., *Cylindrospermum* sp., *Nostoc commune* Vaucher and *Phormidium tenue* (Menegh.) Gom were isolated by the modified Xanthogenate method belonging to families *Oscillatoriaceae, Nostocacea and Rivulariaceae* of different fresh water forms obtained from the Fresh water cyanobacterial Repository at IBSD, Imphal Manipur, India (Department of Biotechnology, Govt. of India funded) (Figure 1) which is considered high carbohydrate content..Apart from the genera given on

the (Table1), genera like *Oscillatoria*, *Lyngbya*, *Westiellopsis*, *Rivularia* and *Microchaete* can also be isolated using the above method. Quality and quantity of the DNA were also determined by using nanodrop The DNA quality range from the ratio 1.75-1.95 (Table 1) of A_{260}/A_{280} and the DNA yield obtained ranges from 85-450 ng μL^{-1} (Table 1). The DNA obtained from the extraction was found to have a size ranging from 15-20 Kb. It was observed that the polysaccharides and pigments associated with the DNA were able to separate out for those cultures with high polysaccharides content which were difficult to isolate using original XS method particularly for the fresh water forms found in North east region of India.
The PCR amplification for 16S rRNA partial gene were obtained from the genomic DNA obtained by the modified Xanthogenate method for the genera mentioned *Plectonema indica* Dixit, *Plectonema notatum* Schmidle*, Limnothrix redekei* (Van Goor) Meffert*, Anabaenopsis* sp., *Nostoc* sp., *Phormidium* sp., *Calothrix* sp., *Tolypothrix* sp., *Anabaena spiroides* Kleb, *Wollea* sp., *Cylindrospermum* sp., *Nostoc commune* Vaucher and *Phormidium tenue* (Menegh.) Gom getting the target DNA size of 600bp region (Figure 4).It shows that the PCR inhibitors are free and can be use effectively for the 16S rRNA.

Table 1: DNA quality and yield of cyanobacterial isolates extracted by modified XSP method

Name of isolates	Accession Nos.	DNA Quality (A_{260}/A_{280})	DNA Yield (ng μl^{-1})
Plectonema indica Dixit	65	1.85±0.01	453.0±0.04
Plectonema notatum Schmidle	75	1.84±0.34	293.0±0.01
Plectonema notatum Schmidle	95	1.81±0.12	84.2±0.28
Limnothrix redekei (Van Goor) Meffert	123	1.93±0.02	227.1±0.35
Anabaenopsis sp.	125	1.86±0.05	216.3±0.07
Nostoc sp.	131	1.96±0.23	255.9±0.36
Anabaena sp.	157	2.02±0.16	310.2±0.20
Anabaena circinalis Rabneh	163	1.96±0.32	377.5±0.31
Phormidium sp.	183	1.76±0.20	401.0±0.67
Calothrix sp.	206	1.90±0.67	215.3±0.78
Tolypothrix sp.	257	1.75±0.43	149.1±0.23
Phormidium tenue (Menegh.) Gom	258	1.96±0.12	167.0±0.06
Anabaena spiroides Kleb	284	2.05±0.19	110.0±0.21
Anabaena sp.	293	1.90±0.18	323.4±0.12
Plectonema sp.	503	1.94±0.01	592.2±0.40
Wollea sp.	650	1.92±0.02	239.0±0.31
Nostoc commune Vaucher	676	1.69±0.15	125.7±0.01
Limnothrix sp.	803	2.00±0.23	215.2±0.03
Cylindrospermum sp.	866	1.82±0.40	190.7±0.01

Values are mean with standard deviation

The quality of the DNA obtained by the modified xanthogenate method were assessed for RAPD PCR amplication for the genera viz. 65-*Plectonema indica* Dixit (ii) 75-*Plectonema notatum* Schmidle (iii) 95-*Plectonema notatum* Schmidle (iv) 123- *Limnothrix redekei* (Van Goor) Meffert (v) 125- *Anabaenopsis* sp.

(vi) 163- *Anabaena circinalis* Rabneh(vii) 183-*Phormidium* sp.(viii) 25-*Tolypothrix* sp. (ix) 258-*Phormidium tenue* (Menegh.) Gom (x) 287- *Plectonema* sp.(xi) 803- *Limnothrix* sp. (xii) 510- *Rivularia beccariana* (De Wilde) Geitler (xiii) 676- *Nostoc commune* Vaucher (xvi) 698-*Anabaena circinalis* Rabneh (xv) 828-*Phormidium* sp.(xvi) 883- *Calothrix* sp. The RAPD results were obtained for the 6 primers mentioned above. The RAPD polymorphic bands obtained from different genera were shown at (Figure 2) and (Figure 3). Many unique bands were also obtained for each primers. PCR amplicons were observed for all the cyanobacterial strains tested which indicate that the cyanobacterial DNA isolated were free from all PCR inhibitors and applicable for RAPD analysis.

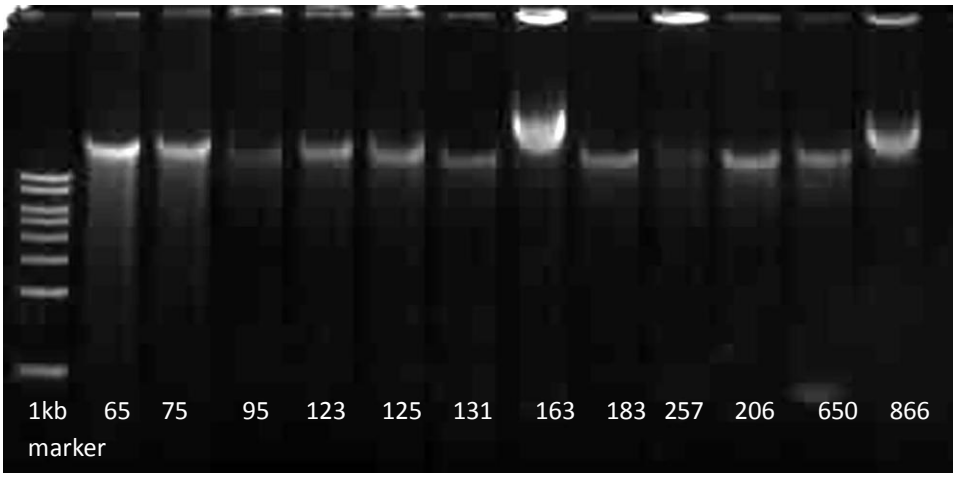

Figure 1: Cyanobacterial DNA isolated using modified XSP method: (i) 65- *Plectonema indica* (ii) 75- *Plectonema notatum* (iii) 95- *Plectonema notatum* (iv) 123- *Limnothrix redekei* (v) 125- *Anabaenopsis* sp. (vi) 131- *Nostoc* sp. (vii) 163- *Anabaena circinalis* (viii)183- *Phormidium* sp.(vii) 206- *Calothrix* sp. (viii) 257- *Tolypothrix* sp. (ix) 650- *Wollea* sp. (x) 866- *Cylindrospermum* sp.

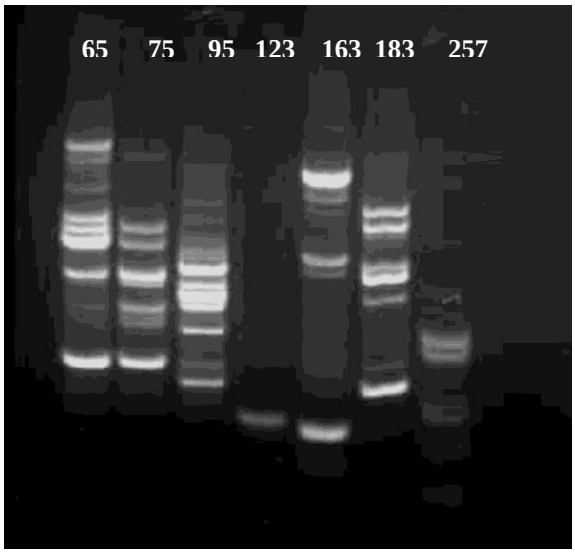

Figure 2: RAPD markers obtained from cyanobacterial DNA using modified XSP method (i) 65-*Plectonema indica* (ii) 75-*Plectonema notatum* (iii) 95- *Plectonema notatum* (iv) 123- *Limnothrix redekei* (v) 125- *Anabaenopsis* sp. (vi) 163- *Anabaena circinalis* (viii) 183-*Phormidium* sp.(vii) 25-*Tolypothrix* sp.

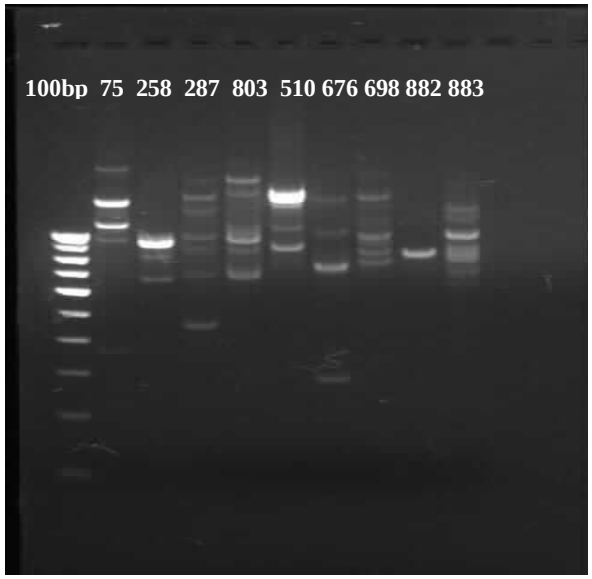

Figure 3: RAPD markers obtained from cyanobacterial DNA using modified XSP method (i) 75- *Plectonema notatum* (ii) 258- *Phormidium tenue* (iii) 287- *Plectonema* sp. (iv) 803- *Limnothrix* sp. (v) 510- *Rivularia beccariana* (vi) 676- *Nostoc commune* (vii) 698- *Nostoc calcicola* (viii) 828- *Calothrix marchica* (vii) 883- *Anabaena* sp.

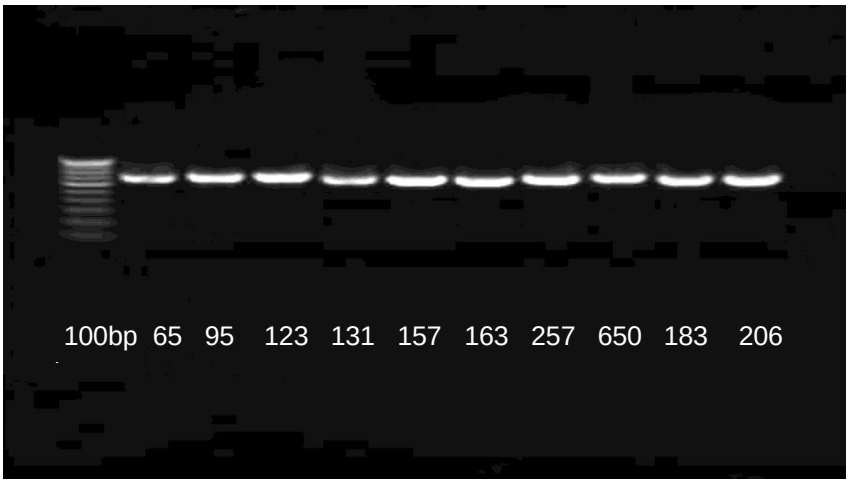

Figure 4: Partial amplicon of 16S rRNA obtained using modified XSP method: (i) 65- *Plectonema indica* (ii) 95-*Plectonema notatum* (iii) 123- *Limnothrix redekei* (iv) 131- *Nostoc* sp. (v) 157- *Anabaena* sp. (vi) 163- *Anabaena circinalis* (vii) 257- *Tolypothrix* sp. (viii) 650- *Wollea* sp. (ix) 183- *Phormidium* sp.(x) 206- *Calothrix* sp.

The Xanthogenate-SDS with Phenol (XSP) method was first described by Tillett and Neilan (2000) mainly for RNA isolation. But in this study it was found that it can be suited for DNA isolation also with much greater ease than the XS method of Tillett and Neilan (2000) by applying modifications like mechanical grinding using pestle and mortar with the lysis buffer with certain amount of homogenized sample showing more repeatability of obtaining DNA against using without mechanical treatment. The steps like addition of saturated phenol (1:1) after incubation for short duration and vortexing helps in the complete removal of protein and sometimes separation of DNA with polysaccharides, pigments and proteins. Steps

like keeping at ice for precipitation of proteins after incubation were omitted as saturated phenol were added. Here the presence of RNA along with DNA is prominent but can be removed effectively by adding 100 µl RNAse of 1mg/ml during incubation or 20-60 µl after elution. Precipitation using isopropanol at -20°C for just 10 minutes is very effective. The hour and temperature of incubation i.e.$1^{1/2}$ or 2hrs and 70°C can be use as per the genera and amount of sample needed should be implemented and the volume of the homogenized sample should not be greater than 1ml.

A number of methods exist for DNA isolation from cultured cyanobacteria, but applications of these techniques to natural water or sediment samples have yielded mixed results. [17] [9]. Tiellet and Nielan (2000) had developed DNA isolation and RNA isolation from which little modifications the protocol is made in which they developed high quality DNA isolation from cultured and environmental samples from the genera like *Lyngbya*, *Pseudanabaena*, *Aphanizomenon*, *Nodularia*, *Anabaena* and *Nostoc* but it failed to describe properly for the isolation of genomic DNA for the genus *Phormidium* of fresh water forms which contains high carbohydrates [10].DNA extraction and modifying the XS method of Tillett and Neilan (2000) by Mete Yilmaz *et al*. [18] reported improved methods for removing inhibitory substances from DNA of environmental samples. XS-PEG—the XS method was employed, except that the DNA was precipitated with 7% final concentration of PEG 8000 (2) XS-3% PVPP—the XS method was employed, except that 3% PVPP was included in the XS buffer. (3) XS-3% PVPP-PEG—the XS-3% PVPP approach was used, except that the DNA was precipitated with 7% PEG 8000 and 10 mM $MgCl_2$. (4) PVPP-Prewashing but the use of PEG or PVP usually consumes more time and subsequently add to the cost of DNA isolation [19].

The cultures studied by this method were lake water and sediment samples contained cyanobacteria species but were found not to be effective on the cyanobacterial cultures obtained from fresh water forms and acidic soils of North East India. The range of DNA isolation were limited to cultures like *Anabaena* sp., *Planktothrix* sp., *Cylindrospermopsis raciborskii* and *Microcystis* sp. comparing to modified xanthogenate method. Dhananjaya et al., 2012 [19] described isolation of genomic DNA from various cyanobacteria including from North East India like *Aulosira fertillissima, Anabaena doliolum, Anabaena oryzae, phormidium fragile, tolypothrix tenuis, Plectonema boryanum, Plectonema tomasinianum, Cylindrospermum muscicola, Hapalosiphon intricatus, Oscillatoria acuta, Calothrix geitonos and Microchaete ubberima* but found to use hazardous 2-mercaptoethanol and procedures were lengthy compare to the present modified xanthogenate method.

Conclusion

In this study, it showed that the modified xanthogenate (XSP) method can be used for fresh water cyanobacterial genomic DNA extraction with high quality and quantity for successive processing for PCR analysis and other related studies of DNA of cyanobacteria as per the other methods available. This method also provides applicability to isolation of genomic DNA to broader range of genera of cyanobacteria as no specific isolation kit for cyanobacterial DNA is available which can cover the entire genera of cyanobacteria.

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