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Collection site, collection and transportation

Rocky shores of Hong Kong (22° 13' N, 114° 12' E) experience a tropical monsoon climate with two distinct seasons, viz. winter (October-March, with mean air temperature 15-18 °C) and summer (May-September, with mean air temperature 25-32 °C, rock temperature up to 50 °C) (Morton and Morton, 1983; Williams, 1994). Mostly Dr. Sanjay Nagarkar, Post Doctoral Fellow, Department of Ecology and Biodiversity, The University of Hong Kong, Hong Kong collected cyanobacterial samples from intertidal rocks of Ap Lei Chau, Big Wave Way, Butterfly Beach, Cape d'Aguilar, Cheung Chau, Heng Fa Chuen and Silverstrand Beach.

Cyanobacterial biofilms were collected from the intertidal rocky shores by gentle scraping with sterile blades, or removed biofilm with rock chips using hammer and chisel and stored in plastic vials with few drops of sterile seawater. Samples were washed with sterile distilled water, blotted using blotting paper; air dried at room temperature (22-25 °C) and packed in such a way that samples were kept in dark for final transportation along with proper labeling to the National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, India.

Revitalization

Immediately on receipt of samples at NFMC, were transferred to 100 mL Erlenmeyer flasks containing modified MN medium (MMN) and ASN III medium (Rippka et al., 1979). Another set was transferred to MN and ASN III media devoid of combined nitrogen (NaNO₃) to support the nitrogen fixing forms. The flasks were then kept in dark at 25 ± 2 °C for overnight for rehydration and activation of growth. Samples were incubated at 25 ± 2 °C for seven days in diffuse light (<10 μE m⁻² sec⁻¹) and the light intensity was gradually increased up to 20 μE m⁻² sec⁻¹ after a week, keeping other incubation conditions same. Cyanobacterial samples started growing after a month, were sub-cultured in freshly prepared media and were incubated at 20-27 μE m⁻² sec⁻¹, 25 ± 2 °C and 12 h L/D cycle.

Isolation

Isolation of cyanobacteria was carried out following four different methods.

Streak Plate (STP): Cyanobacterial sample was homogenized using mortar and pestle with 2-5 mL of MN medium. The homogenate was serially diluted up to 10⁻⁵ in MN
medium. A loop full of sample was then streaked on to MN agar (1% for unicellular and 1.5% for filamentous forms) in parallel lines both back and forth and edge to edge. When the loop reached the center of the plate, turned it around 180° and continued streaking perpendicular to earlier direction. A set of plate containing no combined nitrogen was plated for isolation of heterocystous forms.

**Spread Plate (SP):** About 300 µL of diluted sample (obtained as mentioned above) was spread uniformly over the agar surface using a “L” shaped glass rod.

**Inoculation Plate (IP):** Cyanobacterial sample after observing under microscope, was dissected and a part of it was directly inoculated on the agar surface by using a sterile needle. Profuse growth away from the inoculation site due to their phototactic movement was exploited here to pick up few cells by sterile needle.

For above three methods, plates were incubated at 25 ± 2 °C, 14/10 h L/D cycle, with white fluorescent illumination of 20-25 µE m\(^{-2}\) sec\(^{-1}\) from the up, except for the Inoculation plate method, where unidirectional light was provided from one side. As soon as visible growth on agar surface was noticed, a small piece of agar block was observed under microscope. The isolated cells were picked up with sterile needle and inoculated in to fresh medium.

**Shaking Suspension Culture (SSC):** A small portion of cyanobacterial sample was inoculated directly to a 100 mL Erlenmeyer flask containing 40 mL of MN broth. The flasks were then incubated in an illuminated shaker (20 µE m\(^{-2}\) sec\(^{-1}\)) with a shaking speed of 50-60 rpm. Usually, unicellular forms appeared homogenously suspended in the medium at the center due to bouncy and unidirectional light. Few cells from the center was pipetted out with a sterile tip and inoculated into fresh medium.

**Microscopy**

Microscopic observation under bright field, dark field and photomicrography were made using Leitz diaplan microscope equipped with Photoautomat Wild MPS 46 (Leica, USA).

**Purification and maintenance of culture**

The plating process was repeated until a uni-cyanobacterial form was obtained. In some cases, bacterial contamination was reduced by adding either 25 µg mL\(^{-1}\) ampicillin or 15 µg mL\(^{-1}\) tetracycline, or in combination of both in liquid culture media.
Irrespective of the methods adopted for isolation, all media contained 50 \( \mu \text{g mL}^{-1} \) cycloheximide (Loba Biochemie, India) to inhibit the growth of eukaryotic contaminants. All the purified cyanobacterial isolates are maintained in both MN liquid and MN slant in diffuse light at 25 ± 2 °C as stock cultures. Also another set is maintained in microcentrifuge tube containing MN agar in refrigerator at 4-7 °C.

**Identification**

Preliminary observation was made on the fresh field sample and identified species were targeted for isolation and purification. Identification of pure culture was carried out by using the taxonomic publications of Geitler (1932), Desikachary (1959), Starmach (1966) and Thajuddin (1991). Identification based on the above was also compared with Rippka et al. (1979) up to generic level. All identified, pure cultures of cyanobacteria were given a five digit isolate number followed by the name of culture collection i.e., BDHKU (BDHKU – Bharathidasan-Hong Kong University; first digit indicates the lot number of a collection containing samples, next two digits indicate sample number in that particular collection lot and the last two digits indicate the isolate number from a sample; for example, five digit isolate number 10101 represents: first lot of collection; first sample of the lot and first isolate from the sample) and deposited in marine cyanobacterial culture collection at the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India as well as in the Department of Ecology and Biodiversity, The University of Hong Kong, Hong Kong, SAR, China.

**Culture conditions**

The organisms were grown in 250 mL Erlenmeyer flasks containing 100 mL of ASN III medium. All the experimental cultures were incubated at 25 ± 2 °C, 14/10 h L/D cycle, with illumination of 27 \( \mu \text{E m}^{-2} \text{ sec}^{-1} \) by cool white fluorescent light (Philips). The cultures were agitated by hand on alternative days.

**Harvesting**

Cultures were harvested by centrifuging at 6,000 x g for 8-10 min at 4 °C. The pellet was thoroughly washed with distilled water and centrifuged as above. Fresh weight of the cyanobacterial pellet was noted after blotting it with filter paper. Dry weight was recorded after drying the pellet at 60 °C till a constant weight was obtained. A known amount of fresh weight biomass was taken for estimation of pigments,
proteins, lipids and fatty acids and for absorption spectra study. Results are the average of triplicates.

**Chlorophyll a estimation** *(Mac Kinney, 1941)*

Known amount of cyanobacterial biomass was suspended in 5 mL of cold 80% methanol and vortexed thoroughly. Covered the mouth of the test tubes with aluminium foil to prevent the solvent evaporation. Incubated the tubes in dark at 4-6 °C for 20-24 h of extraction. Tubes were then centrifuged at 5000 x g for about 5 min to obtain a clear yellowish-green supernatant. Re-extracting the pellet at room temperature ensured complete extraction. Pooled all the supernatants together and made up to a known final volume with 80% methanol. Absorbance at 663 nm was read in a spectrophotometer *(JASCO V-550, Japan)* against methanol blank. All the operations were done exclusively in dim laboratory light. Calculated the amount of chlorophyll using the following equation.

\[
\text{Chlorophyll a (µg)} = A_{663} \times 12.63 \times \text{volume of extractant.}
\]

**Carotenoids estimation** *(Jensen, 1978)*

Known amount of fresh weight biomass was extracted with 5 mL of 85% acetone in dark for 20-24 h at 4-6 °C. Centrifuged the contents at 5000 x g for 5 min and the supernatant collected was stored in the cold room. Repeated the extraction at room temperature by vortexing till acetone remains colourless. Pooled the supernatants and read the absorbance at 450 nm against acetone blank in a spectrophotometer *(Jasco V-550, Japan)*.

The amount of carotenoids was calculated using the following formula:

\[
\text{Carotenoids (mg)} = \frac{A_{450} \times V \times f \times 10}{2500}
\]

Where, \( V \) = volume of extractant, \( f \) = dilution factor and 2500 = extinction coefficient.

**Phycobilins estimation** *(Siegelman and Kycia, 1978)*

Known amount of cyanobacterial pellet was homogenized with 3 mL of phosphate buffer (0.05M, pH 6.8) by sonication in a Tarson tube and kept for freezing at -20 °C. Tubes were thawed quickly, centrifuged at 5000 x g for 5 min and
supernatant was stored at 4 °C. Repeated the process to ensure complete extraction. Pooled supernatant was again centrifuged at 5000 x g for 5 min to obtain a clear solution, read the absorbance at 652, 615 and 562 nm against phosphate buffer blank, in spectrophotometer (Jasco V-550, Japan). The amount of phycobilins was calculated using the following formula.

$$A_{615} - 0.474(A_{652})$$

C-phycocyanin (PC) = 5.34 mg

$$A_{652} - 0.208(A_{615})$$

Allophycocyanin (APC) = 5.09 mg

$$A_{562} - 2.41 (PC) - 0.0849 (APC)$$

C-Phycoerythrin (PE) = 9.62 mg

**Protein estimation** (Lowry et al., 1951)

To the chlorophyll extracted pellet, 5 mL of 10% TCA was added and boiled for 30 min in a water bath. Cooled the contents and centrifuged at 5000 x g for 5 min, dissolved the resulting pellet in 1 mL of 1N NaOH. An aliquot of 100 μL (10 μL in case of buffer extracted protein) was made up to 1 mL with distilled water. Blank (1 mL of distilled water) contained 100 μL of 1N NaOH (10 μL 0.0625 M Tris-Cl in case of buffer extracted protein). Added 5 mL of freshly prepared alkaline reagent, vortexed thoroughly and incubated for 3 min at room temperature. Then, Folin Ciocalteu's phenol reagent (500 μL) was added, mixed thoroughly and incubated for 30 min at room temperature and the absorbance was read at 750 nm in spectrophotometer (Jasco V-550). The amount of protein was calculated using bovine serum albumin as standard.

**Lipid estimation** (Folch et al., 1957)

About 500 mg fresh weight biomass was homogenized with glass powder adding 5-10 mL of chloroform / methanol (2:1). Filtered the homogenate through Whatman No.1 filter paper and collected in a tube. Left over's after filtration, was homogenized again in 5-10 mL of chloroform / methanol (2:1) and filtered. Repeated the process once again. All the filtrate was pooled in a single glass tube. Added 5-10
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mL of distilled water and vortexed thoroughly, to remove water-soluble impurities. Lower lipid layer was transferred to a new tube where about 2 g of sodium sulphate crystals was added to remove the moisture content. Filtered the same and evaporated to dryness in vacuum concentrator (Savant Speed Vac, USA). Amount of lipids recovered from a known biomass was determined gravimetrically.

Absorption Spectra

A known amount of cyanobacterial pellet was suspended in 5 mL of cold 95% methanol and vortexed thoroughly. Covered the mouth of the test tubes with aluminium foil. Incubated the tubes in dark at 4-6 °C for 20-24 h of extraction, centrifuged the content at 10,000 x g for about 5 min to obtain a clear supernatant. Absorption spectra (700-250 nm) of supernatant were recorded using a UV-visible spectrophotometer (JASCO V-550, Japan) against methanol blank. All the operations were carried out in dim laboratory light.

Fatty acids fingerprinting by GC (Miller and Berger, 1985)

Identification and quantification of fatty acids was done by the modified method of Miller and Berger (1985). A known amount of lipid was saponified by boiling with 1 mL of saponification reagent for 30 min. The sample was then boiled in a water bath at 80 °C for 20 min with 2 mL of methylation reagent. After cooling, 1 mL of extraction solvent was added and mixed thoroughly. Thereafter the lower aqueous phase was discarded and the remaining upper phase was washed with 3 mL of base wash solution. Finally, 2 μL of the organic phase was chromatographed in a gas chromatograph (5890A Hewlett Packard, USA) fitted with 10% DEGS column (6 ft x 3.12 mm) using a flame ionization detector. The conditions were: oven temp., 180 °C; detector temp., 230 °C; carrier gas, nitrogen at 30 ml min⁻¹. Fatty acids were identified and quantified by comparing the retention time and area of the authentic standards (Sigma, USA).

SODIUM DODECYL SULFATE- POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND STAINING (Laemmli, 1970)

Sample preparation

Samples for protein profile and assay of enzyme activities, were prepared from thoroughly washed cyanobacterial pellets, rewashed with extraction buffer (0.0625M Tris-Cl, pH 6.8). Cyanobacterial pellets were homogenized by using a pre chilled
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mortar and pestle in presence of glass powder (~0.5 mm) adding ice-cold extraction buffer. The samples were centrifuged at 15,000 x g for 15 min and repeated the process twice to obtain clear supernatant. The amounts of proteins in the above samples were estimated following Lowry et al., (1951) and used for both SDS-PAGE and activity staining of gels.

Casting of single or multigel

Sandwiched two (for single) or up to four (for multigel) clean glass plates (16 cm x 14 cm size) with 1.5 mm thick acrylic spacers. To cast three gels, a total of four plates were assembled, of which three notched plates were arranged in sequence and the fourth one was the regular plate (Saha et al., 2003a). The plates were clamped together with metal clips and poured the freshly prepared resolving solution (10%) into it. Layered 1 mL of water saturated iso-butanol over the gel solution and allowed the gel to polymerize at room temperature. Once it was polymerized, poured off the iso-butanol layer and washed the surface thoroughly with double distilled water. Freshly prepared stacking gel solution was added and inserted 1.5 mm thick comb with required number of wells. Allowed the stacking gel to polymerize at room temperature.

Electrophoresis

Electrophoresis was carried out at 20 ± 2 °C with 1.5 mm thick polyacrylamide gel in Tris-glycine buffer (pH 8.3) containing 0.1 % SDS as described by Laemmli et al. (1970). For SDS-PAGE, the samples were boiled for 3 min with sample buffer and centrifuged briefly before loading. A uniform amount (350 μg) of protein was loaded to each well. Samples were then electrophoresed at 50 V through the stacking gel (6%) and at 100 V through the separating gel (10%).

After completion of electrophoresis, indicated by marker dye at the bottom of the gel, disconnected the power supply and removed the glass plates from the apparatus. The gels were then fixed and stained with Coomassie brilliant blue R-250 (SRL, India) for three h and destained in a shaker (50 rpm) at room temperature.

NATIVE-POLYACRYLAMIDE GEL ELECTROPHORESIS (NATIVE-PAGE)

In case of native PAGE for esterase, superoxide dismutase, peroxidase and malate dehydrogenase, SDS was excluded from the running buffer as well as from the sample buffer. Also β-mercaptoethanol was omitted from the sample buffer. Loading of samples and electrophoresis conditions used were same as mentioned for SDS-
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PAGE. After electrophoresis, gels were recovered and stained for their respective activities as described below:

**Activity staining for Esterase** [E.C. 3.1.1.x] *(Wendel and Weeden, 1989)*

Reagents
- a) Phosphate buffer (100 mM, pH 6.2) : 100 mL
- b) α- naphthyl acetate : 50 mg
- c) β- naphthyl acetate : 50 mg
- d) Fast blue RR salt : 100 mg

Procedure: Both the substrates were dissolved quickly in 1 mL of acetone and mixed with phosphate buffer containing fast blue RR salt. The gel was immediately transferred to this solution and incubated at room temperature (22-25 °C) until black, red or magenta colour bands appeared. Rinsed with double distilled water to fix and stored in same water.

**Activity staining for Peroxidase** [E.C. 1.11.1.7] *(Fragerstedt et al., 1998)*

Reagents
- a) Na- acetate buffer (100 mM, pH 4.6) : 100 mL
- b) Hydrogen peroxide (30%) : 250 µL
- c) DAB : 30 mg

Procedure: Peroxidase activity was detected by incubating the gel in sodium acetate buffer containing DAB. The reaction was initiated by adding hydrogen peroxide and allowed up to the complete appearance of brown bands.

**Activity staining for Superoxide dismutase** [E.C. 1.15.1.1] *(Beauchamp and Fridovich, 1971)*

Reagents
- a) Tris-Cl (50 mM, pH 8.0) : 100 mL
- b) Riboflavin : 4 mg
- c) EDTA : 2 mg
- d) NBT : 20 mg

Procedure: Combined all the ingredients and poured over the gel. Incubated in dark at room temperature (22-25 °C) for 30 min. It was then illuminated on a light box with
white fluorescent light (80 μE m⁻² sec⁻¹) for 30 min. Zones of SOD activity were revealed as achromatic regions on dark blue background.

**Activity staining for Malate dehydrogenase** [E.C. 1.1.1.37] (Wendel and Weeden, 1989)

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Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Tris-Cl (50 mM, pH 8.5)</td>
<td>100 mL</td>
</tr>
<tr>
<td>b) NAD</td>
<td>20 mg</td>
</tr>
<tr>
<td>c) Malic acid</td>
<td>300 mg</td>
</tr>
<tr>
<td>d) NBT</td>
<td>20 mg</td>
</tr>
<tr>
<td>e) PMS</td>
<td>4 mg</td>
</tr>
</tbody>
</table>

Procedure: First of all, malic acid was dissolved in Tris solution and the pH was set at 8.5. Then, remaining ingredients were combined and poured over gel. Incubated at room temperature (22-25 °C) till the blue or indigo bands appeared.

**MULTIPLEX RANDOMLY AMPLIFIED POLYMORPHIC DNA-POLYMERASE CHAIN REACTION (MULTIPLEX RAPD-PCR)**

**Template DNA preparation**

A mini scale isolation and purification of total DNA was followed by modified methods of Neilan (1995). About 2-3 mg fresh weight cyanobacterial biomass was washed thoroughly with sterile distilled water at least three times. The pelleted biomass was resuspended in 400 μL of resuspension solution containing RNase A. Added 100 μL of lysozyme (5 mg mL⁻¹ final concentration) and incubated at 37 °C for 60-80 min. Kept at room temperature for 5 min prior to adding 100 μL of SDS (10%) and 7 μL of proteinase K (20 mg mL⁻¹). Mixed well and incubated at 60 °C for 60-80 min. Transferred 200 μL of the content to another tube, which received an additional incubation at 95 °C for 10 min. The solution was brought to room temperature and was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, Sigma, USA). Centrifuged at 8,000 x g for 3 min and the supernatant was transferred to a new tube. Added 200 μL of TE buffer (50:10 mM) to the remaining lower phase and was re-extracted with 300 μL of phenol-chloroform-isoamylalcohol (25:24:1). Centrifuged at 8,000 x g for 3 min and pooled the supernatant with the interphase very carefully. Added 300 μL of phenol-chloroform-isoamylalcohol (25:24:1) and the extraction was repeated. Centrifuged at 8,000 x g for 5 min and transferred the supernatant along with clear white interphase to a new tube. Added 1 mL of distilled ethanol (-20 °C) and
incubated at -20 °C for 45-60 min. Centrifuged at 15,000 x g for 20 min at -10 °C for precipitating the total DNA. Decanted the supernatant and the pellet was washed overnight with 1 mL of 70% ethanol at 4 °C. Centrifuged at 15,000 x g for 20 min at 4 °C and the pellet obtained was vacuum dried (Savant Speed Vac, USA). Reconstituted with 50 μL of sterile double distilled water. 8 μL of the same was electrophoresed to check the presence of DNA in 1% agarose gel. Absorbance was read at 260 nm by diluting 10 μL of sample with 990 μL of water and estimation of DNA was carried out using following formula.

\[
\text{Concentration of original DNA solution (μg mL}^{-1}) = \text{Abs}_{260} \times 100 \times 50 \text{ μg mL}^{-1}
\]

**Standardization and PCR condition**

Multiplex RAPD-PCR conditions were optimized for 50 μL reaction in terms of template DNA concentration (10, 50 and 100 ng), primer concentration (2.5 and 10 pmole), annealing temperature (35, 40, 42 and 45 °C), enzyme concentration (1 and 2 U), Mg²⁺ concentration (1.5 and 3 mM). After standardization, a single multiplex RAPD-PCR was performed in a total volume of 50 μL containing 1.5 mM MgCl₂, 200 μM dNTPs mixture, 10 pmol of each primer (details given below), 2 U of enzyme, and 10 ng of DNA. Thermal cycling was performed in Mastercycler gradient (eppendorf, Germany) according to the following program: initial denaturation at 94 °C for 4 min; 28 cycles of 94 °C for 20 sec, 42 °C for 30 sec, 72 °C for 60 sec; and a final extension at 72 °C for 6 min. All the above PCR reagents used is components of DyNAzyme™ II DNA Polymerase Kit (Finnzymes, Finland) and the primers were obtained from commercial source (Integrated DNA Technologies, Inc., USA).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>% G+C</th>
<th>Mol. Wt.</th>
<th>Tm (°C)</th>
<th>Source</th>
</tr>
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<td>2982.0</td>
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<td>2942.0</td>
<td>34.9</td>
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</table>

**Agarose gel electrophoresis**

1 g of agarose was dissolved in 50 mL of 0.5 X TBE buffer by microwaving and cooled to 45-50 °C. Added 5 μL of ethidium bromide solution (0.05 %, w/v), mixed
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thoroughly without developing any air bubbles and poured on horizontal gel electrophoresis apparatus or on casting boat (Genei, India; Broviga, India). Quickly inserted comb and allowed to solidify at room temperature. Submerged the gel by filling the tank with 0.5 X TBE buffer. 10 µL of sample containing PCR products coupled with 4 µL of loading buffer was loaded into the well and allowed to run at 50 V for about 4 h. Simultaneously, 8 µL of “ready to use DNA size standard” (λ DNA Hind III digest and φX174 DNA Hae III digest) was run after heating at 65 °C for 5 min.

GEL DOCUMENTATION AND ANALYSIS

Gel image of native-PAGE and SDS-PAGE was captured with CCD camera as well as with hp scanjet 5470c (HP Precisionscan Pro 3.1) immediately after staining. The banding pattern in terms of their relative mobilities (R_m) were obtained by the software provided with the gel documentation system (Alpha Imager™ 2200, USA).

Gel image of multiplex RAPD-PCR product was visualized in UV illuminator and captured by CCD camera of gel documentation system. The size of each RAPD-PCR product was determined comparing marker lane using above software.

CONSTRUCTION OF DENDROGRAMS

Morphological characters were scored by presence or absence of a particular character as 1 and 0 respectively. The morphological characters used were according to conventional system of taxonomy such as unicellular, unicellular colonial, trichomatous or filamentous; if unicellular is it spherical; sheath lamellated or un-lamellated, thick sheath or thin sheath; if filamentous branched or unbranched; heterocyst present or absent; plane of cell division in first/second direction, intercalary transverse or others; presence of endospores, nannocytes, hormogone and calyptra/special apical cell. These qualitative data were used for construction of dendrogram using squared euclidean distance and within groups linkage of hierarchical cluster analysis method.

Quantitative data on the amounts of various pigments, total protein and lipids as variables were used to generate dendrogram based on squared euclidean distance and within groups linkage of hierarchical cluster analysis method. In case of fatty acid and absorption spectra, both qualitative and quantitative data were used following the above method of cluster analysis.
The electrophoretic banding pattern for each enzyme and SDS-PAGE protein profiles were independently scored as presence or absence of bands in each genotype. In case of enzyme based cluster analysis, either data of single enzyme or a combination of two, three or four enzymes were used. With RAPD-PCR, presence or absence of a particular size of DNA product was considered as a variable. Bands were detected based on visual appearance on the monitor by changing light filters and magnification. Relative mobility ($R_m$) or molecular mass of the bands were determined by the software provided with the gel documentation system (Alpha Imager™ 2200, USA) and the data were fed as 0 or 1 (absence or presence) in Microsoft Excel sheet based on spreadsheet principle. These data were used in a statistical software package SPSS version 6.0 for dendrogram analysis. The dendrograms were constructed using squared euclidean distance and within groups linkage of hierarchical cluster analysis method.