International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Protocol to Establish Axenic Cultures for Diatoms of Fresh Water

Shristy Gautam[1], Aditi Arya[2], Vandana Vinayak[3]

[1]Diatom Research Lab, Department of Criminology & Forensic Science, School of Applied Sciences, Dr. H.S. Gour Central University, Sagar, M.P., 470003, India
Department of Biotechnology, Deenbandhu Chhotu Ram University of Science and Technology, Murthal, Sonipat, Haryana, 131039, India

[2]Department of Biotechnology, Deenbandhu Chhotu Ram University of Science and Technology, Murthal, Sonipat, Haryana, 131039, India

[3]Diatom Research Lab, Department of Criminology & Forensic Science, School of Applied Sciences, Dr. H.S. Gour Central University, Sagar, M.P., 470003, India

Abstract: ***Diatom axenic cultures have great potential importance in ecological, whole genome and other single genera based studies. The generation of axenic diatom cultures like other cell culture requires special treatment and requirement of macro, micronutrients vitamins and pH. However, since silica is the most important component of diatom cell wall it is most important component of the media too. The f/2 media with little modification served as the best media for fresh water diatoms collected from water bodies of Northern western India with culture conditions standardized at temperature 22°C, light intensity 18000 lumen per m^2, 16 hours/8 hours of light:dark conditions. The present study demonstrates the protocol to hinder the growth of fungi, bacteria and other green algae which contaminates the diatom culture. It was observed that antibiotic mixture of penicillin G and streptomycin in the ratio of 1:0.5 removed most of the bacterial growth in the culture media. The fungal spores were very rigid to remove from the culture plate and treatment with amphotericin B (2.5mg/L) did not reduce the appearance of fungal mycelium; however multiple centrifugation and washing with milliQ water gave relief from fungal spores. The axenicity of diatom culture is further confirmed using 16S rRNA gene based primers which showed that on blasting the amplicon the multiple sequence alignment had phylogeny resembling different diatom genera and species.***

Keywords: Diatom, Axenic culture, Antibiotics, 16S rRNA, f/2 media

1. Introduction

Diatoms are photosynthetic, unicellular algae and its axenic (bacteria and fungus free) culture is required for various types of studies at genomic and proteomic level [1-4], bacteria-diatom interaction, domoic acid production [5], extracellular polysaccharide matrix (EPA) characteristics [6, 7], morphological and physiological alteration [8], metabolic doping of elements like titania [9] and germanium [10] for construction of dye sensitized solar cell, analysis of climatic conditions, toxicity level of various inorganic and organic compounds [11-13] and lipid analysis [14-18]. Although diatoms are abundantly found in all open water bodies, culturing them is a tedious job due to various factors like sticky mucilage produced by diatoms which provides good medium for bacterial growth, low sensitivity to antibiotics in bacteria contaminating the culture and poor viability of diatoms after bacterial decontamination [19]. Various physical and chemical procedures (ultrasound treatment or differential centrifugation), antibiotic treatment were employed for obtaining axenic cultures and isolation of individual diatom cells for monoclonal culturing [20-25] but many a times they did not work.

Mainly diatoms are associated with extracellular bacteria, usually in close spatial contact. They show close association with heterotrophic bacteria, which includes: α-, β- and γ-proteobacteria, bacteriodetes and actinobacteria [26-32]. Often the diatoms use nitrogen provided by bacteria and bacteria are benefited from the carbon source produced by diatoms [33-35]. Diatoms are mainly associated with two heterotrophic (Proteobacteria and Bacteroidetes) bacteria phyla. Four diatoms were studied (*Coscinodiscus granii*, *Eucampia zodiacus*, *Rhizosolenia setigera* and *Skeletonema* sp.) which releases transparent exopolymer particles (TEP) which is an extracellular organic biomolecule in its active stage or as a product of lysis of cell [36, 37]. Diatoms in their active form use TEP to attract certain types of bacteria and bacteria start initiating attachment to TEP of diatoms and use as a nutrient source for bacteria. Diatoms and bacteria interaction have occurred in common habitat for more than 200 million years but do not always have beneficial effect. Some algicidal bacteria release acyl homoserine lactones (AHLs) a quorum sensing signal which enters the phycosphere of diatoms and kill them, The flavobacterim *Kordia algicida* releases a protease (>30KDa) against *Skeletonema, Thalassiosira* and *Pheodactylum*. The secretion of protease by bacteria occurred only when the bacterial cell density reaches a threshold which suggests that the quorum sensing signal regulates the algicidal activity [38]. Diatom also produces several antibacterial compounds in the form of fatty acids and esters, which plays an important role against algicidal bacteria. Diatom *Navicula delognei* secrete three antibacterial compounds such as fatty acids hexadecatetraenoic acid, octadecatetraenoic acid and ester (E)-phytol which display strong antibacterial activity against *Staphylococcus aurens, Staphylococcus epidermidis, Proteus vulgaris* and *Salmonelle enteric* Serovar *Typhimurium* [39]. Marine diatom *Pheodactylum tricornutum* inhibits the growth of gram positive bacteria by secreting palmitoleic acid and eicosapentaenoic acid (EPA) [40]. Some chytrids (primitive fungi) are host specific and they infect host cells,

extract nutrients and develop into mature sporangium which releases new zoospores [41, 42]. During epidemics, new fungal zoospores can be highly abundant in lake water and released approximately 70,000 spores per milliliter [43] [44]. In Netherland at lake Maarsseveen, *Asterionella formosa* diatom was heavily infected by chytrids *Zygorhizidium planktonicum* and *Rhizophydium planktonicum* and leads to succession of diatoms [43] [44]. There are number of defined fresh water media that are useful for growing wide variety of diatoms such as red-Schriber's [45] and Miquel's [46], Walne's or Coway [47]. The f/2 medium [48] is widely used for producing axenic cultures of diatoms collected from marine water as well as fresh water. Appropriate culture media having necessary macronutrients, micronutrients, vitamins, pH, chelator elements are used for the proper maturation of the diatom cells and also to see the physiological behavior, biological behavior which affects the growth under *in-vitro* condition [49].

Efforts have been made to study the nutritional requirements of microalgae in laboratory conditions in an attempt to achieve a medium which gives constant feeding to the diatoms so that they do not perish before long and keep on splitting up and produce more lipid bodies [14]. Geologists considered fossil diatoms as the main source of the world's 30% crude oil [50]. Many diatoms have been screened through culturing to assess its relevance to produce biofuel feedstock (*Chaetoceros muelleri* Schutt, [16] *Thalassiosira pseudonana* Hasle & Hemidal, *Pheodactylum tricornutum* Bohlin, [51]; *Melosira varians* Agardh, *Stephanodiscus binderanus* (Kutz.) Krieger and *Cyclotella meneghiniana* Kutzing [18]. We can completely understand the ecological intricacies of diatoms by growing them on an artificial growth medium [52, 53]. Vinayak et al. [14] screened a diatom named *Diadesmis confevacea* that oozes oil spontaneously from their cells at the end of one month which could be utilized as biofuel using modified f/2 media. The present study focuses on generation of axenic cultures of different diatoms which are found in the fresh water bodies of north western region of India to study them for the various metabolic and molecular analyses.

2. Material and Methods

2.1 Sampling and culturing of water sample

Eleven freshwater water bodies were selected for the water collection and coded respectively as H01 to H11 as shown in fig 1 and 2. Two liters of water sample was collected in a clean plastic bottle from the littoral region of water body from 4-5 different areas of same water body.

SITE CODE	WATER BODY	DISTRICT OF HARYANA	LOCATION
H01	Karna Lake	Karnal	29°44'37.96" N 76°58'34.41" E
H02	Sannhit Sarover	Kurukshetra	29°57'54.67" N 76°50'08.47" E
H03	Saraswati River	Pehowa, Kurkshetra	29°59'36.19" N 76°36'43.77" E
H04	Tikkar Taal	Morni Hills, Panchkula	30°39'27.83" N 77°04'53.86" E
H05	Yamuna River	Karnal	30°11'05.38" N 77°45'24.80" E
H06	Sultanpur Lake	Sultanpur Bird Sanctuary, Gurgaon	28°27'42.53" N 76°53'24.46" E
H07	Yamuna River	Hathnikund, Yamuna Nagar	30°06'46.92" N 77°15'54.76" E
H08	Baghot Pond	Baghot, Mahendergarh	28°25'31.72" N 76°15'55.69" E
H09	Damdama Lake	Gurgaon	28°18'14.53" N 77°07'41.65" E
H10	Markanda River (Seasonal)	Sahabad, Ambala	30°10'44.61" N 76°51'38.06" E
H11	Ghaggar River	Cheeka, Kaithal	30°05'49.15" N 77°19'32.04" E

Figure 1: (a) Map of Haryana region, India showing 11 sampling sites (H01-H11), (b) Complete description of 11 sampling sites of Haryana region, India.

Figure 2: Pictorial representation of 11 sample collection sites (H01-H11) of Haryana region, India. (A) Karna Lake (H01), (B) Sannhit sarover (H02), (C) Saraswati River (H03), (D) Tikkartaal (H04), (E) Yamuna River (Karnal) (H05), (F) Sultanpur Lake (H06), (G) Yamuna River (Yamuna nagar) (H07), (H) Baghot Pond (H08), (I) Damdama Lake (H09), (J) Markanda River (seasonal) (H10), and (K) Ghaggar River (H11).

Physiological and chemical parameters were noted as described in Gautam et al. [8]. A part of water sample (1000 mL) was centrifuged thrice with clean MilliQ water at 3000 rpm for 5 minutes; with each time keeping the pellet and discarding the supernatant. About 50 µL of pellet was used for the preparation of slides following the protocol given by Biggs and Kilroy [54]. The diatoms were further identified using online diatom databases viz; Academy of Natural Sciences, Philadelphia (ANSP) (https://diatom.ansp.org/algae_image/), Diatoms of the United States (https://westerndiatoms.colorado.edu/) and Algal image database of India (AIDI) (http://indianalgae.co.in). The remaining pellet was inoculated on modified f/2 media plates (Table 1) [14, 48] and maintained in the culture room at 16:8 hrs of light and dark condition, temperature 25°C, light intensity of 18000 lumen per m^2.

Table 1: Composition of modified f/2 media

Sr. No.	*Media components*	*Stock concentration*	*Quantity to be used to make 1 liter of working media*
1.	$NaNO_3$	75.0 g/L dH_2O	1 mL
2.	$NaH_2PO_4.H_2O$	5.0 g/L dH_2O	1 mL
3.	$Na_2SiO_3.5H_2O$	30.0 g/L dH_2O	3 mL
4.	Trace Metal Solution (includes 7 compounds)	$Fecl_3.6H_2O$ (1.3 gm/L) $Na_2EDTA.2H_2O$ (8.7 gm/L) $CuSO_4.5H_2O$ (980 mg/100mL dH_2O) $Na_2MoO_4.2H_2O$ (630 mg/100mL dH_2O) $ZnSO_4.7H_2O$ (2.2 gm/ 100mL dH_2O) $CoCl_2.6H_2O$ (1.0 gm/ 100mL dH_2O) $MnCl_2.4H_2O$ (18.0 gm /100mL dH_2O)	1 mL
5.	Soil Extract	1 Kg/l	5 mL
6.	Vitamin B12	10 mg/10ml dH_2O	1 mL
7.	Vitamin H (Biotin)	10 mg/10 ml dH_2O	1 mL
8.	Thiamine HCl	200 mg	1 mL

2.2 Microscopy

After 7 days brown colonies of mixed diatoms population sprouted out along with bacteria, green algae and some possible fungal spores and mycelium as observed under the microscope at 10x magnification (fig 3).

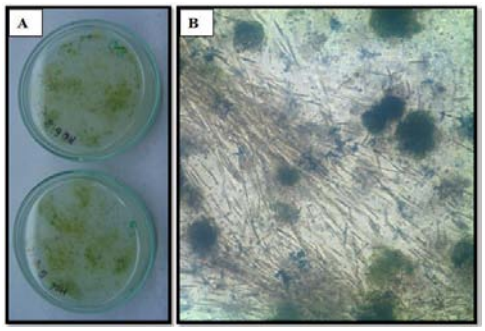

Figure 3: Presence of mixed colonies of different diatoms with green algae, bacteria and fungus, (A) Solid agar culture plate of f/2 media, (B) Compound microscopic image of the plates at 10 x magnifications.

The individual brown colonies of diatoms were marked with the marker on the back side of petri plate and individually picked up with the help of sterilized platinum loop under microscope and streaked on fresh modified f/2 solid media plate.

2.3 Washing and Centrifugation

After few days the plates were observed to check the axenicity of diatom cultures microscopically. During the first batch of producing axenic culture from water sample contaminants like bacteria, green algae and fungi were always there and after numerous subculturing and treatments the pure or axenic cultures were produced. The best treatment to get rid of bulk bacteria, fungal spores and green algae was centrifugation; since diatoms being heavy due to their silica wall remained at the bottom and cells of green algae, bacterial and fungal spores floated at the top. The supernatant was discarded and pellet was washed with milliQ water and centrifuged at 3000 rpm for about 5-6 times. The washed pellet was again inoculated under laminar air flow work station on to the culture plate. The pure diatom colonies were generally obtained in 50% of the cases but to avoid any further contamination with bacteria and fungi they were treated with antibiotics and fungicides.

2.4 Antibiotic Treatment

The single cell from diatom colonies was picked carefully using sterile platinum loop under microscope kept in laminar air flow. The handpicked colonies were sub-cultured in a fresh media prior treated with a mixture of penicillin G (170 µg/mL) and streptomycin (85 µg/mL) [25] amphotericin B (2.5 mg/L) in the proportion of 1: 0.5 for approximately 18 hours before inoculation. The inoculated culture plates treated with antibiotics and fungicides were kept in a culture room and monitored after every second day under microscope at 40 x and 100 x magnification (Phase Contrast Microscope, Leica Microsystems Type DM LB2 with DC-200 camera, software- Leisz, Germany).

2.5 Monoculture preparation

Individual diatom colonies from the culture plates were picked after antibiotic and antifungal treatment. The brown colonies were carefully picked with sterilized platinum loop and sub-cultured on fresh culture plates in duplicates. The mixed colonies of diatom culture were then serial diluted to obtain axenic (pure) culture (fig 4).

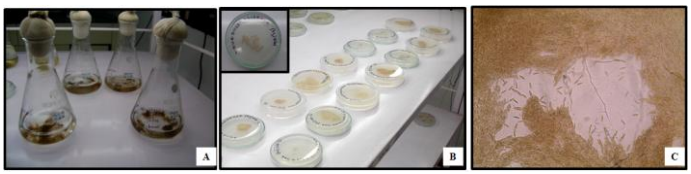

Figure 4: Axenic cultures (A) axenic culture of diatoms in liquid f/2 media, (B) axenic culture of diatoms in solid agar f/2 media, (C) diatom axenic culture observed at 10x magnification.

The individual diatom genera were later separated from mixed diatom population individually under microscope by single cell separation using sterile capillary tube 5mm in diameter by suction force. The individual axenic diatom cells were then inoculated on fresh media plate to observe the appearance of axenic monoclonal diatom colonies.

2.6 Testing the axenic diatom cultures

Confirmation of axenic culture of diatoms was done by inoculating the isolated diatoms in sucrose solution. Sucrose solution (2M) was prepared with sterile distilled water. 1 mL of freshly prepared sucrose solution (2M) was transferred in 1.5mL microcentrifuge tube and 50 µL of individual diatom culture was added into the solution along with phenol red as

an indicator. After 24 hours, 48 hours and 72 hours no turbidity and color change or reddish color or bubble was observed in the diatom containing culture tube [55] indicating sucrose not utilized by bacteria for fermentation. Slides of the diatom sample were prepared and observed under the microscope at 40 x magnification for the presence of bacteria cells if any in the culture media.

2.7 PCR method to generate axenic culture

The diatom monocultures obtained were processed further for DNA extraction. The pellet (~1mL) of diatom culture of particular genera/s were used for DNA extraction process following the standardized protocol of CTAB method [56]. The extracted DNA was quantified by UV-Vis spectrophotometer (UV-Vis 3000$^+$, Labindia) and qualitative study was done on 0.8% gel by gel electrophoresis (fig 5A). In the present study we have shown the DNA extraction of *Diadesmis confervaceae* which yielded 553.27ng/µl of DNA per 1mL of diatom culture. The protocol for DNA extraction for the rest obtained axenic monocultures was same but in present study that of *D. confervaceae* is discussed in detail.

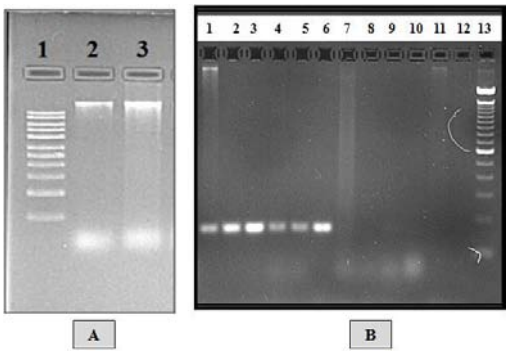

Figure 5: (A) Agarose (0.8%) gel run of DNA extraction from *Diadesmis confervacea* diatom extracted by CTAB method. Description of lane: Lane 1: 1Kb DNA ladder, Lane 2-3: Genomic DNA of *Diadesmis confervacea*, (B) Electrophoretic separation of PCR products of pure culture of diatom *Diadesmis confervacea* obtained with diatom primer specific to 16S rRNA at 135 bp. Descriptions of lane are as follows: Lane 1-6: *Diadesmis confervacea*, Lane 7-12: Blank and Lane 13: 100 bp DNA Ladder.

PCR reaction of extracted DNA of diatoms was performed using Gene Amp® PCR System 9700 (Applied Biosystem, USA). The PCR reaction mixture was made to 25 µL with 1 µL (<50 ng/µL) of genomic DNA, 2.5 µL of 10x Buffer, 1 µL of 25mM $MgCl_2$, 1 µL of 40mM dNTPs, 1 µL of each primer (10 µM) and 0.4 µL (2 units) of Taq polymerase (Promega, Medison, USA). The PCR conditions were set to initial denaturation for 10 minutes at 95°C, amplification for 35 cycles at 95°C for 1 minute, elongation at 60°C for 40 seconds and annealing at 72°C for 1 minute and 30 seconds and finally 72°C for 10 minutes. Primers were designed based on 16S rRNA to test the axenicity of diatom using primer 3 software. The forward primer F1R1: 5'GGGAAGTATGCTCGCAAGAG3' and reverse primer 5' TCCAAACGGCACTTTCTTCT 3' amplified a 135 bp locus of 16S rRNA gene fragment in *Diadesmis confervacea* when observed under a run on 2% agarose gel. The PCR products were purified and sequenced by Sanger sequencing method (ABI 3730 XL DNA analyzer, Applied Biosystem, USA) using the ABI PRISM Big Dye Terminator cycle sequencing kit (Perkin-Elmer Corp., USA). All positions containing gaps and missing data were eliminated. The resulting sequences were analyzed for homology with those available from the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (fig 5B and 6). We have identified molecular relation between selected strain (*Diadesmis cnfervacea*) by aligning the multiple strains present in BLAST results by Clustal W method (multiple sequence alignment system for DNA and protein data) [57, 58] in Molecular Evolutionary Genetics Analysis (MEGA) 7 software [59] and designed phylogenetic tree using Kimura 2-parameter [60] for calculating the best overall probability of correct identification of an organism with 100 bootstrap [61] [62] [63] replications.

3. Result and Discussion

In the present study, we have generated and maintained 12 diatom axenic cultures from different water bodies of Haryana region, India (Table 2).

Table 2: Description of diatom (axenic) cultures obtained at different sites and in different seasons

Sr. No.	*Genera/species identified*	*pH*	*Season*	*Site Name*
1.	*Diadesmis confervacea* Kützing	7.8	Autumn Season	Sultanpur Lake (Gurgaon)
2	*Gomphonema parvulum* (Kützing) Kützing	7.8	Monsoon Season Autumn Season Pre-Winter Season	Baghot Pond (Mahendragarh) Ghaggar river (Chika, Kaithal)
3.	*Melosira varians* C. Agardh	7.8	Spring Season	Yamuna river (Yamuna Nagar) Damdama Lake (Gurgaon)
4.	*Gomphonema pseudoaugur* Lange-Bertalot	7.8	Monsoon Season	Saraswati River, Kurukshetra
5.	*Nitzschia palea* (Kützing) W. Smith	7.8	Summer Season Monsoon Season Autumn Season Pre-Winter Season Winter Season	Karna Lake (Karnal) Sannhit Sarover, Kurukshetra Yamuna river (Yamuna Nagar) Ghaggar river (Chika, Kaithal) Tikkar Taal (Mornihills) Sultanpur Lake (Gurgaon) Baghot Pond (Mahendragarh) Damdama Lake (Gurgaon) Saraswati River (Kurukshetra)
6.	*Nitzschia heufleriana* Grunow	7.8	Monsoon Season Autumn Season Pre-Winter Season Winter Season	Ghaggar river (Chika, Kaithal) Karna lake (Karnal) Sannhit Sarover (Kurukshetra) Tikkar Taal (Mornihills) Yamuna river (Yamuna Nagar) Saraswati River, Kurukshetra Baghot Pond (Mahendragarh)
7.	*Pinnularia saprophila* Lange-Bertalot, Kobayasi and Krammer	7.8	Spring Season Summer Season	Yamuna river (Yamuna Nagar) Damdama Lake (Gurgaon)
8.	*Nitzschia palea var. debilis* (Kützing) Grunow in Cleve and Grunow	7.8	Autumn Season	Yamuna river (Yamuna Nagar)
9.	*Cyclotella meneghiniana* Kützing	7.8	Spring Season	Yamuna river (Yamuna Nagar) Yamuna river (Karnal)
10.	*Placoneis elginensis* (Gregory) Cox	7.8	Spring Season Summer Season Pre-Winter Season	Tikkar Taal (Mornihills) Baghot Pond (Mahendragarh) Yamuna river (Yamuna Nagar)
11.	*Nitzschia inconspicua* Grunow	7.8	Autumn Season	Baghot Pond (Mahendragarh)
12.	*Seliaphora* Mereschkowsky	7.8	Spring Season	Damdama Lake (Gurgaon)

The present study showed that the antibiotics played a good role in removing bacteria from the culture medium and does not affect the growth of different diatom cultures. Diatoms were inoculated in the modified f/2 culture media when supplemented with antibiotics: penicillin G (170 µg/mL) and streptomycin (85 µg/mL) yielded pure colonies of diatom which was bacteria free and sterile. It was found that high concentration of antibiotics caused vacuolization of cytoplasm, destruction of chloroplast and even cell death before diatoms could be removed from the medium [19]. The fungal spores were controlled when treated with amphotericin B (2.5mg/L), however all the contaminations were checked by centrifuging the diatom cells in milliQ water. The diatoms due to their thick silica walls were heavy and sediment at the bottom whereas the green algae, bacterial and fungal spores/mycelium floated on the top which was discarded on each centrifugation. It was also observed that the temperature of 22°C, light intensity of 18000 lumen per m^2 and 16:8 hrs of light and dark conditions were best for the growth of diatoms. Out of 11 studied sites of Haryana region, India, 12 diatom genera/species *Diadesmis confervacea*, *Gomphonema parvulum*, *Melosira varians*, *Gomphonema pseudoaugur*, *Nitzschia palea*, *Nitzschia heufleriana*, *Pinnularia saprophila*, *Nitzschia palea var. debilis*, *Cyclotella meneghiniana*, *Placoneis elginensis*, *Nitzschia inconspicua*, *Sellaphora* sp. were obtained in the laboratory conditions (fig 7).

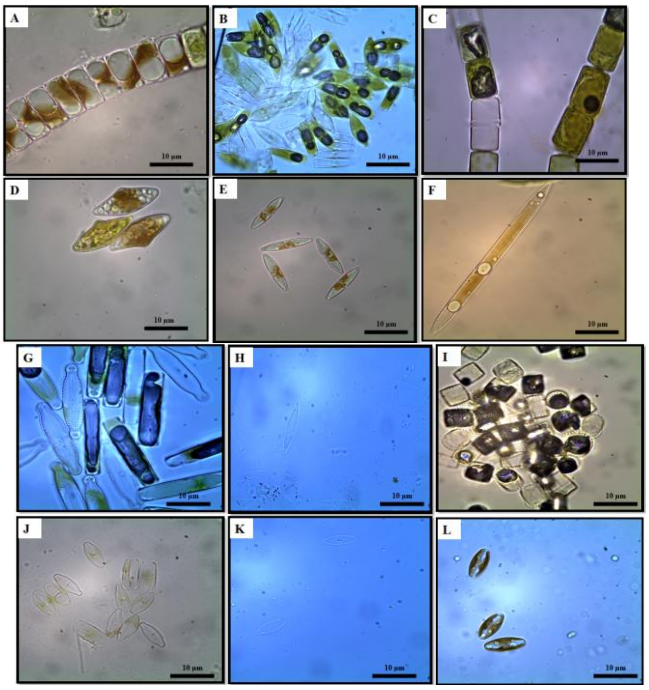

Figure 7: Diatom (axenic) cultures obtained and maintained in the laboratory conditions (A) *Diadesmis confervacea*, (B) *Gomphonema parvulum*, (C) *Melosira varians*, (D) *Gomphonema pseudoaugur*, (E) *Nitzschia palea*, (F) *Nitzschia heufleriana*, (G) *Pinnularia saprophila*, (H) *Nitzschia palea var. debilis*, (I) *Cyclotella meneghiniana*, (J) *Placoneis elginensis*, (K) *Nitzschia inconspicua*, (L) *Sellaphora* sp.

The extracted genomic DNA of pure culture of diatom (*Diadesmis confervacea*) was amplified using forward and reverse primers (F1R1) designed specific to 16S rRNA gene locus using primer 3 software. Results of sequencing were analyzed by blastn of NCBI for homologous sequences available in BLAST-NCBI database and were multiple aligned by the ClustalW method for DNA sequence data in MEGA 7.0.18 software. The molecular comparison of *Diadesmis confervacea* amplicon obtained from axenic was done by constructing phylogenetic tree by maximum likelihood method using MEGA 7.0.18 software. Maximum likelihood method is used to calculate the evolutionary distance between sequences from the observed differences between the sequences. This phylogenetic tree was designed for identification of common ancestors and for barcoding a particular diatom genera/species. For calculating the evolutionary distances and pairwise alignments, the overall probability of correct identification (PCI) was analyzed by using Kimura 2-parameter with 100 bootstrap methods for computing the standard error. Kimura 2 parameter model corrects for multiple hits with transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and those rates of substitution do not vary among sites. The MEGA 7.0.18 analysis had lead us to conclude that the nuclear encoded 16S rRNA sequence comprised 39 nucleotide sequences in the phylogenetic tree and all positions containing gaps and missing data were eliminated. There were a total of 59 positions in the final dataset. The above method analyzed all sequences of *Diadesmis confervacea* and resulted in a single Maximum Likelihood (ML) tree (fig 6).

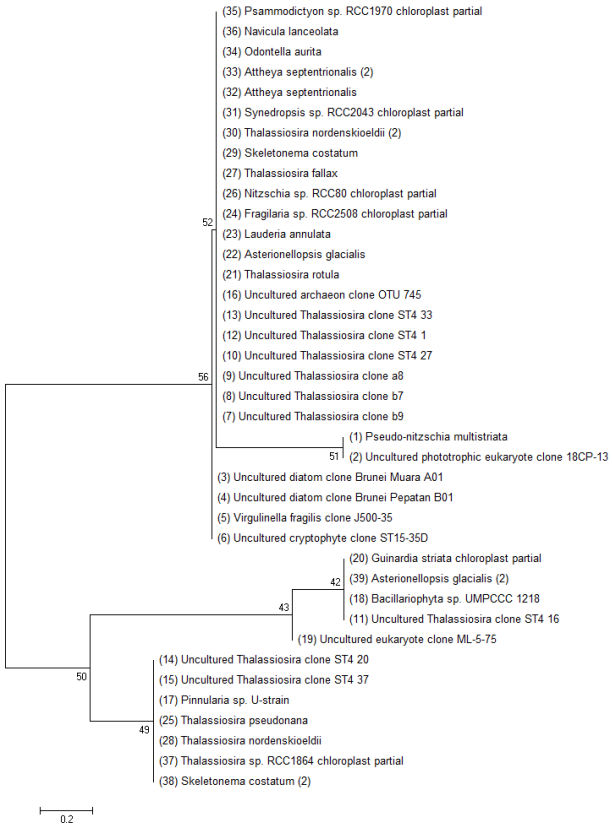

Figure 6: Molecular Phylogenetic analysis of 16S rRNA region of *Diadesmis confervacea* strain by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [60]. The tree with the highest log likelihood (-342.9187) is

shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 59 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [59].

The uncultured *Thalassiosira* clone (ST4 20 & ST4 37), *Pinnularia* sp. *Thalassiosira pseudonana*, *Thalassiosira nordenskioeldii*, *Thalassiosira* sp. (RCC 1864) chloroplast partial and *Skeletonema costatum* (boot strap value= 49%) species rooted the tree within clade I, with uncultured eukaryote clone ML5-75 (boot strap value= 43%) species forming a sister clade to *Guinardia striata* chloroplast partial, *Asterionellopsis glacialis*, *Bacillariophyta* sp. and uncultured *Thalassiosira* clone ST4 16 (boot strap value= 42%) within clade II. A clade III comprising *Psammodictyon* sp. (RCC1970), *Navicula lanceolata*, *Odontella aurita*, *Attheya septentrionalis*, *Synedropsis* sp. (RCC2043), *Thalassiosira nordenskioeldii*, *Skeletonema costatum*, *Thalassiosira fallax*, *Nitzschia* sp. (RCC80), *Fragilaria* sp. (RCC508), *Lauderia annulata*, *Asterionellopsis glacialis*, *Thalassiosira rotula*, uncultured archaeon clone OUT, uncultured *Thalassiosira* clone (ST4 33, ST4 1, ST4 27, a8, b7, b9) with boot strap value 52% and *Pseudonitzschia multiseriata* and uncultured phototrophic eukaryote clone (18 CP-13) with boot strap value 51%. A clade III also comprising with boot strap valve of 56% is uncultured diatom clone Brunei Muara A01, B01, *Virgulinella fragilis* clone (J500-35) and uncultured Cryptophyte clone (ST15-35D). The sequence analysis lead us to conclude that *Diadesmis confervacea* has molecular relationship with 13 numbers of diatoms genera/species, *Thalassiosira*, *Pinnularia*, *Skeletonema*, *Navicula*, *Nitzschia*, *Fragilaria*, *Guinardia*, *Asterionellopsis*, *Psammodictyon*, *Attheya*, *Synedropsis*, *Lauderia* and *Pseudonitzschia* along with *Virgulinella fragilis* (bacteria) and cryptophytes only. The molecular analysis of axenic culture further did not show any near lineage with fungi or bacteria or green algae thus proving that the axenic culture has only pure culture of *Diadesmis confervaceae* and is free from any contamination. The axenic cultures of diatoms would further be used for the study of toxicity in water bodies, for analysis of lipid content in diatoms in biofuel industries at small and large scales and have many applications in nanotechnology and biotechnology sectors.

4. Conclusion

Though antibiotics and fungicides played an important role in producing pure cultures of diatoms but the cultures could be said axenic only when the PCR testing yielded sequences which show phylogeny tree near to class Diatomophyceae. In the present study diatom *Diadesmis confervacea* was used for testing axenicity using lab designed primers F1R1 at locus 16S rRNA. *Diadesmis confervacea* is a suitable and fast growing diatom in the lab known for their interesting property of continuously oozing oil [14]. The genomic DNA extracted, amplified for base pair position 135 bp and sequenced for their molecular phylogenetic analysis using maximum likelihood method at MEGA 7 software identified a molecular relationship between *Diadesmis confervacea* and other diatom genera found in the blast results in BLAST-NCBI database. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-342.9187) is shown (fig 6). The analysis involved 39 nucleotide sequences. The phylogenetic tree observed 13 numbers of diatoms, *Thalassiosira*, *Pinnularia*, *Skeletonema*, *Navicula*, *Nitzschia*, *Fragilaria*, *Guinardia*, *Asterionellopsis*, *Psammodictyon*, *Attheya*, *Synedropsis*, *Lauderia* and *Pseudonitzschia* with *Virgulinella fragilis* (bacteria, 01 number) and cryptophytes (01 number). The 12 numbers of axenic cultures generated in the laboratory conditions were used for further downstream applications like for analysis of heavy metal stress from diatom *Gomphonema pseudoaugur* [8], oozing of oil property from *Diadesmis confervacea* [14], study of formation of diatom nanotubes for the preparation of diatom solar panel from diatom *Nitzschia palea*. The f/2 diatom media with slight modification such as addition of northern region soil extract for generation of fresh water diatoms along with antibiotic and fungicide treatment helped in generating and establishing axenic culture of diatoms. The axenic cultures were further checked for their axenicity at molecular level using 16S rRNA primers which amplified diatoms belonging to class Diatomophyceae.

References

[1] Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M: The genome of the diatom Thalassiosira pseudonana: ecology, evolution, and metabolism. *Science* 2004, 306(5693):79-86.

[2] Kroth PG, Chiovitti A, Gruber A, Martin-Jezequel V, Mock T, Parker MS, Stanley MS, Kaplan A, Caron L, Weber T: A model for carbohydrate metabolism in the diatom Phaeodactylum tricornutum deduced from comparative whole genome analysis. *PloS one* 2008, 3(1):e1426.

[3] Sapriel G, Quinet M, Heijde M, Jourdren L, Tanty V, Luo G, Le Crom S, Lopez PJ: Genome-wide transcriptome analyses of silicon metabolism in Phaeodactylum tricornutum reveal the multilevel regulation of silicic acid transporters. *PLoS One* 2009, 4(10):e7458.

[4] Sheppard V, Poulsen N, Kröger N: Characterization of an endoplasmic reticulum-associated silaffin kinase from the diatom Thalassiosira pseudonana. *Journal of Biological Chemistry* 2010, 285(2):1166-1176.

[5] Douglas DJ, Bates SS: Production of domoic acid, a neurotoxic amino acid, by an axenic culture of the marine diatom Nitzschia pungens f. multiseries Hasle. *Canadian Journal of Fisheries and Aquatic Sciences* 1992, 49(1):85-90.

[6] Chiovitti A, Bacic A, Burke J, Wetherbee R: Heterogeneous xylose-rich glycans are associated with

extracellular glycoproteins from the biofouling diatom Craspedostauros australis (Bacillariophyceae). *European Journal of Phycology* 2003, 38(4):351-360.

[7] 7. Bahulikar RA, Kroth PG: THE COMPLEX EXTRACELLULAR POLYSACCHARIDES OF MAINLY CHAIN-FORMING FRESHWATER DIATOM SPECIES FROM EPILITHIC BIOFILMS1. *Journal of phycology* 2008, 44(6):1465-1475.

[8] 8. Gautam S, Pandey LK, Vinayak V, Arya A: Morphological and physiological alterations in the diatom Gomphonema pseudoaugur due to heavy metal stress. *Ecological Indicators* 2017, 72:67-76.

[9] 9. Gautam S, Kashyap M, Gupta S, Namdeo VK, Schoefs B, Gordon R, Joshi KB, Vinayak V, Jeffryes C: Metabolic engineering of TiO2 nanoparticles in Nitzschia palea to form diatom nanotubes: an ingredient for solar cells to produce electricity and biofuel†. *RSC Advances* 2016.

[10] 10. Jeffryes C, Campbell J, Li H, Jiao J, Rorrer G: The potential of diatom nanobiotechnology for applications in solar cells, batteries, and electroluminescent devices. *Energy & Environmental Science* 2011, 4(10):3930-3941.

[11] 11. Dickman M, Dixit S, Fortescue J, Barlow B, Terasmae J: Diatoms as indicators of the rate of lake acidification. *Water, Air, and Soil Pollution* 1984, 21(1-4):375-386.

[12] 12. Fritz S, Jugginsi S, Battarbeef R, Engstrom D: Reconstruction of past changes in salinity and climate using. *Nature* 1991, 352:22.

[13] 13. Potapova MG, Charles DF: Benthic diatoms in USA rivers: distributions along spatial and environmental gradients. *Journal of biogeography* 2002, 29(2):167-187.

[14] 14. Vinayak V, Gordon R, Gautam S, Rai A: Discovery of a diatom that oozes oil. *Advanced Science Letters* 2014, 20(7-8):1256-1267.

[15] 15. Vestal JR, White DC: Lipid analysis in microbial ecology quantitative approaches to the study of microbial communities. *Bioscience* 1989, 39(8):535-541.

[16] 16. McGinnis K, Dempster T, Sommerfeld M: Characterization of the growth and lipid content of the diatom Chaetoceros muelleri. *Journal of Applied Phycology* 1997, 9(1):19-24.

[17] 17. Vieler A, Wilhelm C, Goss R, Süß R, Schiller J: The lipid composition of the unicellular green alga Chlamydomonas reinhardtii and the diatom Cyclotella meneghiniana investigated by MALDI-TOF MS and TLC. *Chemistry and physics of lipids* 2007, 150(2):143-155.

[18] 18. Sicko-Goad L, Andresen NA: Effect of growth and light/dark cycles on diatom lipid content and composition1. *Journal of Phycology* 1991, 27(6):710-718.

[19] 19. Shishlyannikov SM, Zakharova YR, Volokitina NA, Mikhailov IS, Petrova DP, Likhoshway YV: A procedure for establishing an axenic culture of the diatom Synedra acus subsp. radians (Kütz.) Skabibitsch. from Lake Baikal. *Limnol Oceanogr: Methods* 2011, 9:478-484.

[20] Spencer C: On the use of antibiotics for isolating bacteria-free cultures of marine phytoplankton organisms. *Journal of the marine biological association of the United Kingdom* 1952, 31(01):97-106.

[21] Brown R, Bischoff H: A new and useful method for obtaining axenic cultures of algae. *Phycol Soc Am News Bull* 1962, 15:43-44.

[22] Jones A, Rhodes ME, Evans SC: The use of antibiotics to obtain axenic cultures of algae. *British Phycological Journal* 1973, 8(2):185-196.

[23] Daste P, Neuville D, Victor-Baptiste B: A simple procedure for obtaining clonal isolation of diatoms. *British Phycological Journal* 1983, 18(1):1-3.

[24] Kobayashi K, Kobiyama A, Kotaki Y, Kodama M: Possible occurrence of intracellular bacteria in Pseudo-nitzschia multiseries, a causative diatom of amnesic shellfish poisoning. *Fisheries science* 2003, 69(5):974-978.

[25] Bruckner CG, Kroth PG: Protocols for the Removal of Bacteria from Freshwater Benthic Diatom Cultures1. *Journal of phycology* 2009, 45(4):981-986.

[26] Brachvogel T, Schweitzer B, Simon M: Dynamics and bacterial colonization of microaggregates in a large mesotrophic lake. *Aquatic Microbial Ecology* 2001, 26(1):23-35.

[27] Knoll S, Zwisler W, Simon M: Bacterial colonization of early stages of limnetic diatom microaggregates. *Aquatic Microbial Ecology* 2001, 25(2):141-150.

[28] Schäfer H, Abbas B, Witte H, Muyzer G: Genetic diversity of 'satellite'bacteria present in cultures of marine diatoms. *FEMS Microbiology Ecology* 2002, 42(1):25-35.

[29] Makk J, Ács É, Márialigeti K, Kovács G: Investigations on the Danube gravel-biofilm diatom-associated bacterial communities. *Biologia (Bratislava)* 2003, 58(4):729-742.

[30] Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T: Marine diatom species harbour distinct bacterial communities. *Environmental Microbiology* 2005, 7(6):860-873.

[31] Bruckner CG, Bahulikar R, Rahalkar M, Schink B, Kroth PG: Bacteria associated with benthic diatoms from Lake Constance: phylogeny and influences on diatom growth and secretion of extracellular polymeric substances. *Applied and environmental microbiology* 2008, 74(24):7740-7749.

[32] Zakharova YR, Adel'shin R, Parfenova V, Bedoshvili YD, Likhoshway YV: Taxonomic characterization of the microorganisms associated with the cultivable diatom Synedra acus from Lake Baikal. *Microbiology* 2010, 79(5):679-687.

[33] Carpenter EJ, Janson S: Intracellular cyanobacterial symbionts in the marine diatom Climacodium frauenfeldianum (Bacillariophyceae). *Journal of Phycology* 2000, 36(3):540-544.

[34] Villareal TA: Nitrogen-fixation by the cyanobacterial symbiont of the diatom genus Hemiaulus. *Marine ecology progress series Oldendorf* 1991, 76(2):201-204.

[35] Martínez L, Silver MW, King JM, Alldredge AL: Nitrogen fixation by floating diatom mats: A source of new nitrogen to oligotrophic ocean waters. *Science* 1983, 221(4606):152-154.

[36] Fukao T, Kimoto K, Kotani Y: Production of transparent exopolymer particles by four diatom species. *Fisheries science* 2010, 76(5):755-760.
[37] Passow U: Production of transparent exopolymer particles (TEP) by phyto-and bacterioplankton. *Marine Ecology Progress Series* 2002, 236:1-12.
[38] Paul C, Pohnert G: Interactions of the algicidal bacterium Kordia algicida with diatoms: regulated protease excretion for specific algal lysis. *PloS one* 2011, 6(6):e21032.
[39] Findlay JA, Patil AD: Antibacterial constituents of the diatom Navicula delognei. *Journal of natural products* 1984, 47(5):815-818.
[40] Desbois AP, Lebl T, Yan L, Smith VJ: Isolation and structural characterisation of two antibacterial free fatty acids from the marine diatom, Phaeodactylum tricornutum. *Applied microbiology and biotechnology* 2008, 81(4):755-764.
[41] James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J: Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 2006, 443(7113):818-822.
[42] CANTER HM: Studies on British chytrids: XXVI. A critical examination of Zygorhizidium melosirae Canter and Z. planktonicum Canter. *Journal of the Linnean Society of London, Botany* 1967, 60(381):85-97.
[43] DONK EV, Ringelberg J: The effect of fungal parasitism on the succession of diatoms in Lake Maarsseveen I (The Netherlands). *Freshwater biology* 1983, 13(3):241-251.
[44] Bruning K: Infection of the diatom Asterionella by a chytrid. I. Effects of light on reproduction and infectivity of the parasite. *Journal of plankton research* 1991, 13(1):103-117.
[45] Gopinathan C: Differential growth rates of micro-algae in various culture media. *Indian Journal of Fisheries* 1986, 33(4):450-456.
[46] Miquel P: De la culture artificielle des Diatomées. *Le Diatomiste* 1892, 1(9):93-99.
[47] Walne PR: Culture of bivalve molluscs: 50 years' experience at Conwy: Fishing News Books Ltd.; 1979.
[48] Guillard RR, Ryther JH: STUDIES OF MARINE PLANKTONIC DIATOMS: I. CYCLOTELLA NANA HUSTEDT, AND DETONULA CONFERVACEA (CLEVE) GRAN. *Canadian journal of microbiology* 1962, 8(2):229-239.
[49] Pelczar M, Chan E, Microbiology NKN: Concepts and Applications. In.: McGraw-Hill Inc., New York; 1993.
[50] Ramachandra T, Mahapatra DM, Gordon R: Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels. *Industrial & Engineering Chemistry Research* 2009, 48(19):8769-8788.
[51] Eizadora TY, Zendejas FJ, Lane PD, Gaucher S, Simmons BA, Lane TW: Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum (Baccilariophyceae) during starvation. *Journal of Applied Phycology* 2009, 21(6):669-681.
[52] de Jong L, Admiraal W: Competition between three estuarine benthic diatom species in mixed cultures. *Marine ecology progress series Oldendorf* 1984, 18(3):269-275.
[53] Debenest T, Silvestre J, Coste M, Pinelli E: Effects of pesticides on freshwater diatoms. In: *Reviews of environmental contamination and toxicology*. Springer; 2010: 87-103.
[54] Biggs B, Kilroy C: Stream periphyton monitoring manual. National Institute of Water and Atmospheric Research, Christchurch, New Zealand. In.; 2000.
[55] Boczar BA, Beitler MK, Liston J, Sullivan JJ, Cattolico RA: Paralytic shellfish toxins in Protogonyaulax tamarensis and Protogonyaulax catenella in axenic culture. *Plant Physiology* 1988, 88(4):1285-1290.
[56] Doyle J, Doyle J: Genomic plant DNA preparation from fresh tissue-CTAB method. *Phytochem Bull* 1987, 19(11):11-15.
[57] Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ: Multiple sequence alignment with Clustal X. *Trends in biochemical sciences* 1998, 23(10):403-405.
[58] Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research* 1994, 22(22):4673-4680.
[59] Kumar S, Stecher G, Tamura K: MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution* 2016:msw054.
[60] Kimura M: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution* 1980, 16(2):111-120.
[61] Nei M, Kumar S: Molecular evolution and phylogenetics: Oxford university press; 2000.
[62] Felsenstein J: Inferring Phylogenies. 2003.
[63] Efron B, Efron B: The jackknife, the bootstrap and other resampling plans, vol. 38: SIAM; 1982.

Author Profile

Shristy Gautam received the B.Sc (Zoology, Hons.) from University of Delhi in 2008 and M.Sc. degrees in Biotechnology from Dr. B. R. Ambedkar University, Agra in 2010. She is currently pursuing Ph.D in Biotechnology from Deenbandhu ChhotuRam University of Science and Technology, Murthal, Sonipat, Haryana, India. She has also cleared CSIR-UGC NET in Life science in December 2009. She has received "Best Scientific Paper Award" in Biological science, oral presentation at XXII All India Forensic Science Conference June 26-28, 2012, cash prize of 2500 Rs on topic titled- "Forensic Palynology: a review" authored by Shristy Gautam, Dr. Vandana Vinayak and Dr. Aditi Arya. The Second Prize" (cash prize of Rs.800) and certificate on poster presented on the topic "Genetic Engineering of diatoms to increase the lipid percentage: an alternative source for biofuel" authored by- Shristy Gautam, Vandana Vinayak, Anshuman Rai on the occasion of National Science day Celebration-2014 on the theme "Fostering Scientific Temper",28th February, 2014".

Dr. Aditi Arya received the B.Sc (Medical) from Dayanand Post Graduate College, Hisar (Kurukshetra University, Kurukshetra, Haryana) in 1999. She has done her M.Sc (Biotechnology & Molecular Biology) in 2002 and Ph.D (Biotechnology & Molecular

Biology) in 2005 from CCS Haryana Agriculture University, Hisar, Haryana. She was awarded Merit Scholarship 2002-2005 (Ph.D.) and Merit Scholarship 1999-2002 (M.Sc.), Award for second position in B.Sc. Medical in Kurukshetra University, Kurukshetra, 1999 and State Merit Scholarship, 1995 (10+2) and Merit Scholarship, 1993 (Matriculation). She also conferred Best Paper award during National Seminar on "Physiological and Biotechnological approaches to improve plant productivity "organized at Centre for Plant Biotechnology, CCSHAU, Hisar from March 15- 17, 2008. She was J. Technical Officer, Biotechnology Division- Centre for Plant Biotechnology, HSCST, CCS HAU New Campus, Hisar from October, 2007 to February, 2010. Since February, 2010 she is working as Assistant Professor in Department of Biotechnology at Deenbandhu Chhotu Ram University of Sc. & Tech., Murthal.

Dr. Vandana Vinayak received the B.Sc (Medical) from Hans Raj Mahila Mahavidyala, Jalandhar; (Guru Nanak Dev University, Amritsar) in 1996 and M.Sc. (Biochemistry) from Panjab Agricultural University, Ludhiana, India in 1999. She has done her Ph.D (Biochemistry) from Kurukshetra University, Kurukshetra, India in 2008. She has 4 research projects of DST-GOI (completed), DBT-GOI (completed), UGC-GOI (going on) and DST Nanomission, GOI (going on) and she has awarded meritorious student award FCMS-2011 at City Montessori School, Lucknow, the only school with its name in Guinness book of world record. She has awarded "NOEL DEERR GOLD MEDAL AWARD" for best paper presentation by His Excellency former President of India Dr. A.P.J Abdul Kalam on occasion of 70th Annual Convention of STAI on 26th August' 2009 held at Udaipur India and awarded Gold medal Award as "YOUNG SCIENTIST AWARD" in Molecular Biology at ~~a~~ National seminar on Physiological and Biotechnological approaches to improve plant productivity on March 17' 2008. She was state merit scholarship awardees during college and university. She was Senior Scientific Officer, Biology Division- Diatom Research Unit at Forensic Science Laboratory (H), Madhuban, Karnal, Haryana, India and now she is working as Assistant Professor at Department of Crim. & Forensic Science, Dr. Hari Singh Gour central University, Sagar, M.P.