

Protocol to Establish Axenic Cultures for Diatoms of Fresh Water

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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 m², 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 titanium [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, bacteroidetes 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 flavobacterium *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 aureus*, *Staphylococcus epidermidis*, *Proteus vulgaris* and *Salmonelle enteric* Serovar *Typhimurium* [39]. Marine diatom *Pheodactylum tricorutum* 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,

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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 *Rhizophyidium 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 tricorntutum* Bohlin, [51]; *Melosira varians* Agardh, *Stephanodiscus binderanus* (Kutz.) Krieger and *Cyclotella meneghiniana* Kutzung [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.

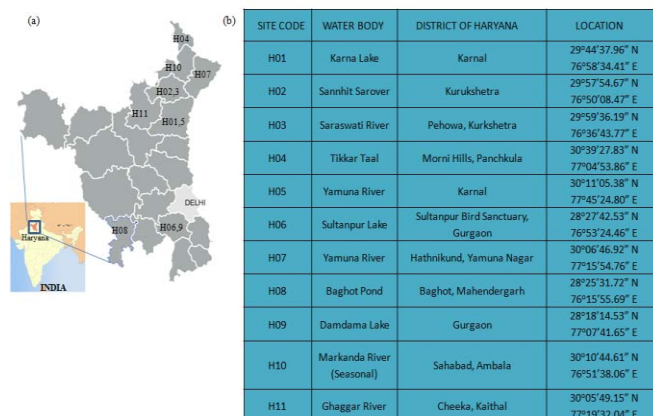


Figure 1: (a) Map of Haryana region, India showing 11 sampling sites (H01-H11), (b) Complete description of 11 sampling sites of Haryana region, India.



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 m².

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 ₂ O	1 mL
2.	NaH ₂ PO ₄ ·H ₂ O	5.0 g/L dH ₂ O	1 mL
3.	Na ₂ SiO ₃ ·5H ₂ O	30.0 g/L dH ₂ O	3 mL
4.	Trace Metal Solution (includes 7 compounds)	FeCl ₃ ·6H ₂ O (1.3 gm/L) Na ₂ EDTA·2H ₂ O (8.7 gm/L) CuSO ₄ ·5H ₂ O (980 mg/100mL dH ₂ O) Na ₂ MoO ₄ ·2H ₂ O (630 mg/100mL dH ₂ O) ZnSO ₄ ·7H ₂ O (2.2 gm/100mL dH ₂ O) CoCl ₂ ·6H ₂ O (1.0 gm/100mL dH ₂ O) MnCl ₂ ·4H ₂ O (18.0 gm/100mL dH ₂ O)	1 mL
5.	Soil Extract	1 Kg/l	5 mL
6.	Vitamin B12	10 mg/10ml dH ₂ O	1 mL
7.	Vitamin H (Biotin)	10 mg/10 ml dH ₂ O	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).

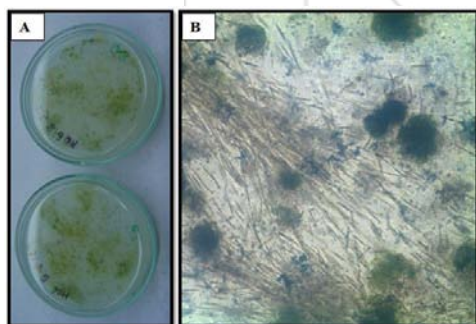


Figure 3: Presence of mixed colonies of different diatoms with green algae, bacteria and fungus, (A) Solid agar culture plate of f/2 media, (B) Compound microscopic image of the plates at 10 x magnifications.

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).

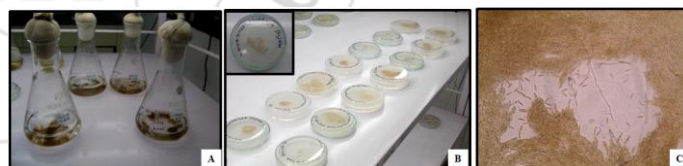


Figure 4: Axenic cultures (A) axenic culture of diatoms in liquid f/2 media, (B) axenic culture of diatoms in solid agar f/2 media, (C) diatom axenic culture observed at 10x magnification.

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 *Diadasmus confervaceae* which yielded 553.27ng/ μ l 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.

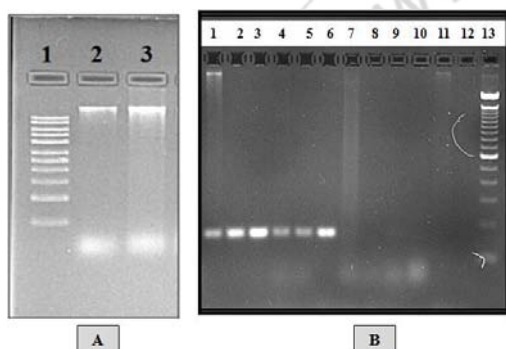


Figure 5: (A) Agarose (0.8%) gel run of DNA extraction from *Diadasmus confervaceae* diatom extracted by CTAB method. Description of lane: Lane 1: 1Kb DNA ladder, Lane 2-3: Genomic DNA of *Diadasmus confervaceae*, (B) Electrophoretic separation of PCR products of pure culture of diatom *Diadasmus confervaceae* obtained with diatom primer specific to 16S rRNA at 135 bp. Descriptions of lane are as follows: Lane 1-6: *Diadasmus confervaceae*, 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 μ L with 1 μ L (<50 ng/ μ L) of genomic DNA, 2.5 μ L of 10x Buffer, 1 μ L of 25mM MgCl₂, 1 μ L of 40mM dNTPs, 1 μ L of each primer (10 μ M) and 0.4 μ L (2 units) of Taq polymerase (Promega, Madison, 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 *Diadasmus confervaceae* 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 (*Diadasmus confervaceae*) 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.	Genus/species identified	pH	Season	Site Name
1.	<i>Diadasmus confervaceae</i> Kützing	7.8	Autumn Season	Sultanpur Lake (Gurgaon)
2.	<i>Gomphonema parvulum</i> (Kützing) Kützing	7.8	Monsoon Season Autumn Season Pre-Winter Season	Baghot Pond (Mahendragarh) Ghaggar river (Chika, Kaithal)
3.	<i>Melosira varians</i> C. Agardh	7.8	Spring Season	Yamuna river (Yamuna Nagar) Damdama Lake (Gurgaon)
4.	<i>Gomphonema pseudoangur</i> Lange-Bertalot	7.8	Monsoon Season	Saraswati River, Kurukshetra
5.	<i>Nitzschia palea</i> (Kützing) W. Smith	7.8	Summer Season Monsoon Season Autumn Season Pre-Winter Season Winter Season	Karna Lake (Karnal) Sanhit Sarover, Kurukshetra Yamuna river (Yamuna Nagar) Ghaggar river (Chika, Kaithal) Tikkar Taal (Morni hills) Sultanpur Lake (Gurgaon) Baghot Pond (Mahendragarh) Damdama Lake (Gurgaon) Saraswati River (Kurukshetra)
6.	<i>Nitzschia kuetzingiana</i> Grunow	7.8	Monsoon Season Autumn Season Pre-Winter Season Winter Season	Ghaggar river (Chika, Kaithal) Karna lake (Karnal) Sanhit Sarover (Kurukshetra) Tikkar Taal (Morni hills) Yamuna river (Yamuna Nagar) Saraswati River, Kurukshetra Baghot Pond (Mahendragarh)
7.	<i>Pinnularia saprophila</i> Lange-Bertalot, Kobayasi and Kramer	7.8	Spring Season Summer Season	Yamuna river (Yamuna Nagar) Damdama Lake (Gurgaon)
8.	<i>Nitzschia palea</i> var. <i>debilis</i> (Kützing) Grunow in Cleve and Grunow	7.8	Autumn Season	Yamuna river (Yamuna Nagar)
9.	<i>Cyclotella meneghiniana</i> Kützing	7.8	Spring Season	Yamuna river (Yamuna Nagar) Yamuna river (Karnal)
10.	<i>Pinnularia eigenensis</i> (Gregory) Cox	7.8	Spring Season Summer Season Pre-Winter Season	Tikkar Taal (Morni hills) Baghot Pond (Mahendragarh) Yamuna river (Yamuna Nagar)
11.	<i>Nitzschia inconspicua</i> Grunow	7.8	Autumn Season	Baghot Pond (Mahendragarh)
12.	<i>Selaphora</i> Merschikowsky	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 m² 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 *Diademsis 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).

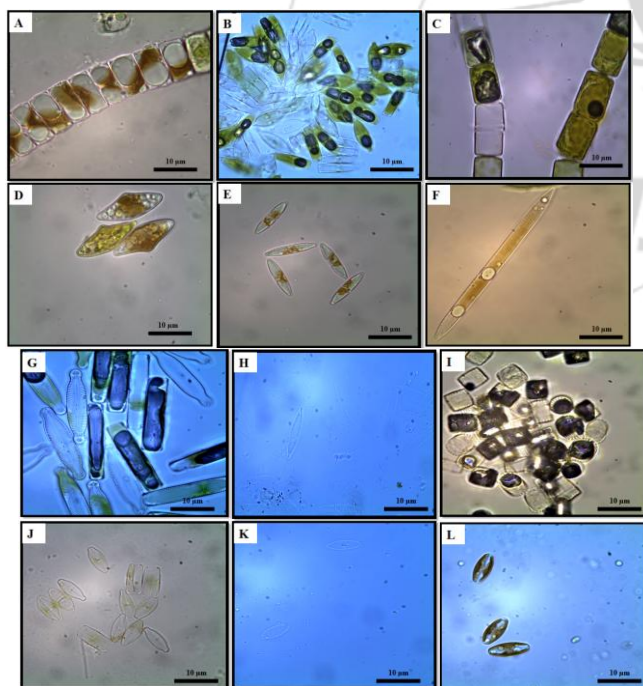


Figure 7: Diatom (axenic) cultures obtained and maintained in the laboratory conditions (A) *Diademsis confervacea*, (B) *Gomphonema parvulum*, (C) *Melosira varians*, (D) *Gomphonema pseudoaugur*, (E) *Nitzschia palea*, (F) *Nitzschia heufleriana*, (G) *Pinnularia saprophila*, (H) *Nitzschia palea var.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

The extracted genomic DNA of pure culture of diatom (*Diademsis 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 *Diademsis 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 *Diademsis confervacea* and resulted in a single Maximum Likelihood (ML) tree (fig 6).

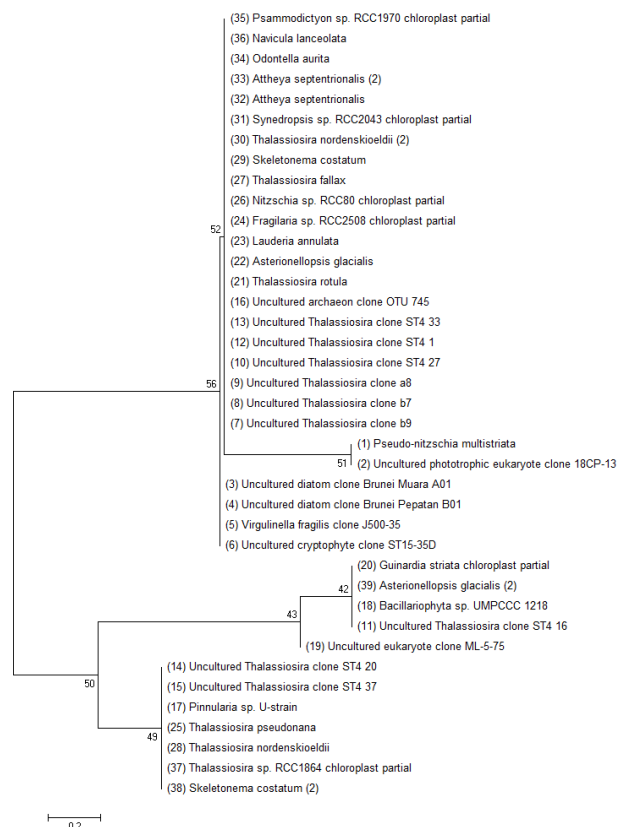


Figure 6: Molecular Phylogenetic analysis of 16S rRNA region of *Diademsis confervacea* strain by Maximum Likelihood method

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 nordenskiöldii*, *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 *Psammodyctyon* sp. (RCC1970), *Navicula lanceolata*, *Odontella aurita*, *Attheya septentrionalis*, *Synedropsis* sp. (RCC2043), *Thalassiosira nordenskiöldii*, *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 value of 56% is uncultured diatom clone Brunei Muara A01, B01, *Virgulinema fragilis* clone (J500-35) and uncultured Cryptophyte clone (ST15-35D). The sequence analysis lead us to conclude that *Diademes confervacea* has molecular relationship with 13 numbers of diatoms genera/species, *Thalassiosira*, *Pinnularia*, *Skeletonema*, *Navicula*, *Nitzschia*, *Fragilaria*, *Guinardia*, *Asterionellopsis*, *Psammodyctyon*, *Attheya*, *Synedropsis*, *Lauderia* and *Pseudonitzschia* along with *Virgulinema 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 *Diademes 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 *Diademes confervacea* was used for testing axenicity using lab designed primers FIR1 at locus 16S rRNA. *Diademes 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 *Diademes 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*, *Psammodyctyon*, *Attheya*, *Synedropsis*, *Lauderia* and *Pseudonitzschia* with *Virgulinema 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 *Diademes 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.

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