CHAPTER - 3

MATERIALS AND METHODS

Materials

Glass wares

The glass wares involved in this study including Petri plates, conical flasks, measuring cylinders, beakers, test tubes etc. were bought from Borosil Pvt. Ltd., Mumbai, India.

Chemicals

The chemicals, salts and minerals used in this study were from Qualigens and Fisher Scientific. Especially, fine chemicals were bought from Himedia.

Instruments involved in this study

Autoclave, Cooling Centrifuge, Laminar Air Flow, pH Meter, Culture Rack, Electronic Weighing balance, Orbital Shaker, Microwave Oven, UV-Vis Spectrophotometer, Compound Microscope, Fluorescence Microscope, Ultra Sonic Bath, Cyclo-mixer, GC-MS, PCR, Electrophoresis Apparatus, SDS Page Apparatus, Gel Documentation Unit, Deep Freezer and DNA Sequencer etc.

Methods

General precautions

The white coloured lab coat, gloves and mask were utilized while working in the laboratory to avoid bacterial and fungal contamination. The working area was cleansed with 70% ethanol to avoid any mode of contamination.
Hazardous chemicals handling

Precautions were undertaken while working with hazardous and concentrated chemicals such as hydrochloric acid, sulphuric acid, acetic acid, and sodium hydroxide including other hazardous chemicals etc. Safety was ensured while handling some carcinogenic chemicals such as Ethidium bromide (EtBr).

Washing and sterilization of glass wares

All the glass wares including conical flasks, measuring cylinders, beakers and stock bottles etc. were washed and sterilized to prevent microbial contamination.

Washing and rinsing of glass wares

The glass wares were chemically sterilized overnight using 10% of 1M HCl to remove any depositions on the inner surface of the conical flasks and to kill germs. Then the glass wares were washed with 0.5% of Labolene a neutral liquid detergent and rinsed 3 to 5 times with tap water to remove the detergent and then once again rinsed with distilled water and kept in hot air oven at 80°C for 15 min.

Sterilization

The glass wares and culture media were steam sterilized using autoclave at 121°C and 15psi for 15 min.

Laminar air flow chamber

The working stage of the laminar air flow chamber was chemically sterilized with 70% alcohol. The autoclaved materials are transferred to laminar air flow chamber and illuminated with UV light for 15 minutes. The inoculation needle and loop were directly incinerated in a burning flame from Bunsen burner.
Waste disposal

The unwanted waste materials after the commencement of experiments were disposed safely and the other unwanted live biological materials were killed by decanting in an autoclave.

Methods

3.1 Field survey and waste water sample collection

The field surveys were carried out randomly in the three selected Thermal power stations including i) Vallur Thermal power station (VT), Tamil Nadu, ii) Kolaghat Thermal power station (KoT), West Bengal and iii) Kharergada Thermal power station (KhP), Maharashtra (Fig. 1 and 2). About 5 liters of waste water samples were collected from each site using 5 liters sterile plastic cans. The Physio-Chemical analyses were carried in all the waste water samples collected (Fig. 3.1 to 3.5).
Fig.3.1:  a – India map showing the three States chosen for survey,  
b – Topographical view showing the Three Thermal Power Station Sites.
Fig.3.2: Topographical view of three selected Thermal Power stations in India: a) Vallur, Tamil Nadu, b) Kolaghat, West Bengal and c) Khaperkheda, Maharashtra where waste water samples were collected.
Fig. 3.3: a–h, Waste water sample collection sites, Vallur Thermal Power Station, Chennai, Tamil Nadu, India.
Fig.3.4: a – h, Waste water sample collection sites, Kolaghat Thermal Power Station, West Bengal, India.
Fig.3.5: a – h, Waste water sample collection sites, Khaperkheda Thermal Power Station at Nagpur, Maharashtra, India.
3.2 Physio-Chemical analyses of waste water samples

The physio-chemical analyses including temperature, salinity, dissolved oxygen, oxidation reduction potential, specific conductivity, electrical conductivity and total dissolved solids were carried out in all the collected waste water samples by using YSI Multiparameter (Model: 600XL-B-O, 650MDS, YSI Incorporated, Yellow Springs, Ohio 45387, USA).

3.3 Isolation of microalgae

The collected waste water samples were brought to the laboratory and allowed to centrifuge at 8000 rpm for 15 min. to obtain the thick algal pellet. An inoculation loop full of algal pellet was used as inoculums and subjected to streak on Petri plates with solidified BBM medium using 2 % agar. The streaked Petri plates were then kept for incubation under light intensity of 120 μmol photons/m²/s⁻¹ on 12:12 h Light/Dark with 25 °C. All the plates were frequently monitored for every day and the arousing colonies were marked. Each and every pure microalgal colony was then allowed to streak on separate solidified BBM media to obtain pure microalgal cultures.

3.4 Pure microalgal culture maintenance

The isolated pure microalgal cultures were transferred to each of the 100 ml conical flasks containing 50 ml of liquid BBM medium for further studies. Then the microalgal cultures were further transferred to 400 ml of BBM medium in 1000 ml conical flasks for large scale optimization studies. At the same time all the isolated microalgal cultures were further sub-cultured for 20 days interval to maintain the pure microalgal colonies.
3.5 Identification of microalgae

All the isolated microalgae from the waste water samples of the three Thermal power stations of India were then subjected to identification. The microalgae were identified based on the morphological characteristic features viz., cell shape, size, flagella length, presence or absence of vacuoles, presence or absence of granules and presence or absence of pyrenoid etc. with the help of a compound binocular microscope OLYMPUS CH20i up to 100 X magnification and photographed using Sony digital camera. The microalgae were identified morphologically based on their distinctive characteristic features (Butcher, 1959, Carmelo and Grethe, 1997) and the instruction for the microscopical identification was suggested by Krishnamurthy Institute of Algology (KIA), Chennai.

3.6 Optimization of culture conditions

The optimization of culture conditions including different molar concentrations of sodium bicarbonate, growth kinetics, different photoperiod and different pH were studied for all the selected three microalgal species. For the optimization experiments 200 ml of BBM liquid medium was used in 500 ml of conical flasks were used to culture the microalgae and the absorbance values were measured at 680 nm using a UV-visible spectrophotometer (Hitachi U-2900) for 22 days with 24 hrs of interval.

3.6.1 Optimization of different molar concentrations of NaHCO₃

Different molar concentrations of Sodium bicarbonate 0.1 M, 0.5 M and 1.0 M were optimized towards the cultivation of the selected three microalgal
species and this experiment was done in triplicate, the average values were considered for the interpretation of results. About 200 ml of BBM liquid medium was prepared for each molar concentration and for each microalgal species involved in this study. The equation required for the measurement of different molar concentration of sodium bicarbonate is given below.

\[
1M \text{ Concentration of Sodium bicarbonate} = \frac{\text{Gram Molecular weight of Sodium bicarbonate in one litre of the given solution}}{84.01 \text{ grams of Sodium bicarbonate in 1 L of distilled water}} \times 1000
\]

Table 3.1: Different molar concentrations of Sodium bicarbonate studied

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Different molar Conc. of NaHCO\textsubscript{3}</th>
<th>Amount of BBM</th>
<th>Wt. of NaHCO\textsubscript{3} to be dissolved in 200 ml of BBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1</td>
<td>200 ml</td>
<td>1.680 mg</td>
</tr>
<tr>
<td>2.</td>
<td>0.5</td>
<td>200 ml</td>
<td>8.401 mg</td>
</tr>
<tr>
<td>3.</td>
<td>1.0</td>
<td>200 ml</td>
<td>16.802 mg</td>
</tr>
</tbody>
</table>

**3.6.2 Optimization of different photoperiod**

White inflorescence lamps (Crompton 40 W, Cool day light 6500 K) at an intensity of 2000 lux was employed and the culture room temperature was adjusted to 25°C. The three selected microalgae were further optimized for the selective duration of light for better growth and biomass yield. Different photoperiods with both light and dark including 24 hrs of dark, 8: 16 hrs of light and
dark, 12: 12 of light and dark, 16: 8 hrs of light and dark and at last 24 hrs of light were studied in triplicate. All the microalgal cultures were agitated twice a day throughout the experiment.

3.6.3 Optimization of different pH

The different pH were chosen to optimize the proliferation of the three selected microalgae which are 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5 at 24 hrs of light condition. About three replicas were maintained for this experiment and the average values were taken for the exemplification of the results. All the culture flasks were shaken manually twice to obtain biomass yield.

3.6.4 Growth kinetics of microalgae

The growth kinetics of all the three selected microalgae was studied based on the optical density for every 22 days in order to choose the incubation period for the high yield of growth and biomass. The absorbance values were measured at 680 nm using a UV-visible spectrophotometer (Hitachi U-2900) for 22 days with 24 hrs of interval. This experiment was done as triplicate and the average values were interpreted for results.

The Growth kinetics (K) and generation times (G) of all the microalgae were calculated by the equations 1 and 2 (Qin, 2005).

Equation 1:

\[ K = \frac{\log OD_f - \log OD_i}{T \times 3.322} \]
Where,

\[
\text{OD}_f : \text{final optical density}, \quad \text{OD}_i : \text{initial optical density}, \quad T : \text{time in days}
\]

Equation 2: Following equation was used for Calculation of generation time (G) in days:

\[
G = \frac{0.301}{K}
\]

### 3.7 In vitro mass cultivation

All the three selected microalgae were further allowed to cultivate large scale in vitro in 5L conical flasks with 2L of BBM medium along with their respective optimized conditions at 25°C. All the conical flasks were agitated twice a day and kept for incubation under 24 hrs of illumination using white inflorescent lamps (Crompton 40 W, Cool day light 6500 K) at an intensity of 2000 lux.

### 3.8 Harvest and estimation of dry biomass

After 21 days of mass cultivation the 2L volume of three microalgal cultures were allowed to centrifuge at 8000 rpm for 10 min. under 20°C to obtain wet biomass. The wet biomasses were oven dried at 40°C for two to three days then after the complete removal of moisture, cooled down at room temperature and the resulted dry biomasses were conserved. The dry weight of the three microalgal biomasses was determined gravimetrically using an electronic weighing balance.

### 3.9 Determination of CO₂ fixation rate

The carbon content from the dried microalgal biomasses were determined by using a CHNS analyzer (CHNS analyzer model: Vario EL III from Elementar Analyser system GmbH, Germany). The analyzer was calibrated using a Perkin-
Elmer Sulphanilic acid as a standard to represent 100% carbon while beginning the experiment. The carbon content values of the three microalgae from their dry biomasses were determined and recorded. Then the bio-fixation rates of all the three studied microalgae were determined based on the formula derived by Tang et al. in 2009 which is given below.

\[ R_{CO_2} = C_c P \left( \frac{M_{CO_2}}{M_C} \right) \]

Where,

- \( C_c \) : Carbon content of the dry biomass in the microalgal samples
- \( M_C \) : Molecular weight of carbon
- \( M_{CO_2} \) : Molecular weight of \( CO_2 \)

### 3.10 Biochemical constituents of the three microalgae

#### 3.10.1 Extraction and estimation of Chlorophyll a and Chlorophyll b

About 100 ml of the microalgal cultures were centrifuged at 8000 rpm for 10 min. to yield concentrated biomass in the form of pellet. And the pellet was suspended in ice cold 90% acetone and left overnight undisturbed in dark condition. The whole content was then centrifuged at 1000 rpm for 10 min. to separate the extract from the cell debris. The pellet concentrated with cell debris was discarded and the supernatant was stored and used as a source for the estimation of pigments. Both the pigments chlorophyll a and b were estimated based on the method derived by Jefferey and Humphery (1975) and the equation given below.

\[
Chlorophyll \ a \ = \ 11.93E664 \ - \ 1.93E647
\]

\[
Chlorophyll \ b \ = \ 20.36E647 \ - \ 5.50E664
\]
Absorbance values were measured using 10 mm width cuvette at 664 and 647 nm by using a UV-Vis. Spectrophotometer.

3.10.2 Extraction and estimation of carotene

Dry microalgal powder was prepared by harvesting and concentrating microalgal cultures by using centrifugation at 8000 rpm for 10 min. from a fresh 100 ml of microalgal cultures and oven dried at 40°C for 2 days. About 100 mg of dry algal powder was allowed to extract carotene by using 3 ml of 2:1 ratio of ethanol and hexane mixture. After some time 2 ml of distilled water and 4 ml of hexane was added and vortexed for 5 min. The hexane was further aspirated and the absorbance values were measured at 450 nm using a UV-Vis spectrophotometer. Then the carotene content was calculated based on Prieto *et al.* (2011) method and the formula is given below.

\[ \text{Carotene (µg/ml)} = A_{450} \text{ nm} \times 25.2 \]

3.10.3 Extraction and estimation of carbohydrate

A 5 ml of fresh microalgal cultures were centrifuged at 8000 rpm for 10 min under 20°C and the pellet containing biomass was conserved. The resulted pellet was homogenized well with sodium phosphate buffer (pH – 6.8) and sonicated by using a sonicator (Equitron Ultrasonic cleaner) at 53 KHz for 10 min. under room temperature. Then the whole content was again centrifuged at 8000 rpm for 10 min. under 20°C and the supernatant was used as a source for the estimation of carbohydrate.
The total carbohydrate content was determined based on the method described by Dubois et al. in the year 1959. About 500 µl of the sample was allowed to react with 5% phenol and 2.5 ml of conc. sulphuric acid. The whole mixture was mixed and kept undisturbed under incubation for 15 to 20 min. at room temperature. The absorbance values were estimated optically at 490 nm by using a UV-Vis. spectrophotometer. The carbohydrate content of the unknown samples was estimated by comparing the values with known standard sample by construction of a standard graph. The D-glucose was used as a known carbohydrate to construct a standard graph.

3.10.4 Extraction and estimation of protein

About 5 ml of microalgal culture was centrifuged at 8000 rpm for 5 min. under 20°C. The concentrated biomass in the form of pellet was well homogenized using a mortar and pestle and subjected to sonication for 10 min. in a sonicator (Equitron Ultrasonic cleaner) at 53 KHz under room temperature. Then centrifuged at 1000 rpm for 10 min. under 15°C and resulted supernatant was conserved for the estimation of protein.

The estimation of protein in this present study was done based on the method described by Bradford (1976). The protein content of the unknown sample was determined in comparison with the standard graph of the known sample. The standard graph for protein was constructed using Bovine serum albumin as a standard known protein. About 100 µl of the extract was allowed to make up to 1 ml using 900 µl of distilled water. About 5 ml of Bradford reagent was added to make up the whole mixture 6 ml. After incubation for 15 to 20 min. under 20°C the mixture was allowed the measure the absorbance values at 595 nm using a UV-Vis. Spectrophotometer.
3.10.5 Extraction and estimation of lipid

The microalgal biomass was harvested and concentrated from 50 ml of microalgal cultures by using centrifugation at 5000 rpm for 5 min. under 25°C. The obtained pellet was then homogenized and sonicated at 53 KHz for 10 min. under room temperature with the help of mortar and pestle and sonicator respectively (Equitron Ultrasonic cleaner). The total lipid content was then allowed to extract and estimate based on the method described by Folchs et al. (1957). About 6 ml of chloroform and methanol mixture (2: 1 ratio) was added to the homogenized biomass and vortexed with the use of a cyclomixer for 15 to 20 min. at room temperature (27 to 29°C). The extract was then centrifuged at 8000 rpm for 5 min. under 20°C and the obtained liquid phase was washed with 6 ml of 0.9% of NaCl and vortexed for 5 min. The whole mixture was centrifuged at 2000 rpm for 5 min. within 20°C resulted to form two phases. The lower phase was discarded; the upper solvent phase was conserved and stored in a separate container. The total lipid content was determined gravimetrically after complete evaporation of solvent from the stored extract.

3.11 Extraction and analysis of fatty acid methyl esters (FAME) by gas chromatography

About 20 ml of aqueous fresh algal samples were subjected to direct transesterification. A 1 ml of reagent 1 was poured to the fresh algal samples and vortexed for 5-10 sec. using a cyclomixer. The mixture was incubated in a water bath at 100°C for 5 min. and again vortexed for 5-10 sec. Then after incubated at 100°C for 25 min. reagent 2 was added and vortexed for 5-10 min. followed by
thermal incubation at 80°C and rapidly cooled down to 4°C. Reagent 3 was added the mixture and mixed gently up to 10 min. Two phases were seen blatantly from which, the lower phase was conserved. To the content 3 ml of reagent 4 was added followed by mixing for 5 min. and obtained upper phase was removed. The lower phase rich in fatty acid methyl esters were stored in a vial at 4°C in a refrigerator.

The tranesterified fatty methyl esters were analyzed with the help of a gas chromatography equipped with flame ionization detector (FID) (Perkin Elmer, USA). A SP-2560 column (100 m × 0.25 mm I. D., 0.20 µm) (Sigma, Germany) along with standard fatty acid Supelco 37 Component FAME mix from Supelco (Bellefonte, PA, USA) was employed. About 5 µl of the sample was injected and the GC conditions were injector temperature: 260°C; Column temperature: 140°C and detector temperature: 260°C. Helium was used as a carrier gas with the flow rate of 1 ml/min. The unknown FAMEs were determined in comparison with the retention times of the standard FAMEs (Supelco) using a mass spectra from NIST library.

3.12 Fourier transforms infra-red spectrometric (FT-IR) analysis of FAME

The FAME samples were analyzed under infra-red (Perkin Elmer model spectrum – I PC). The FT-IR spectra with the resolution of 4 cm⁻¹, Scan Number: 3 were performed after the evaporation of the lipid fraction on Thalium bromide tablets. The FT-IR spectrums of all the FAME samples were obtained as a percentage of transmission ranged from 450 cm⁻¹ to 4000 cm⁻¹.
3.13 Molecular identification of microalgae

3.13.1 Extraction of DNA

The genomic DNA was extracted by using InstaGene TM Matrix genomic DNA isolation kit as per the instructions described. The concentrated microalgal content was allowed to suspend in 1 ml of Milli-Q water and centrifuged at 10,000 rpm for 1 min. in a micro centrifuge tube. The supernatant was discarded and the pellet was incubated with 200 µl of InstaGene matrix at 56°C for 15 min. The content was vortexed for 10 sec. and incubated at 100°C in a boiling water bath for 8 min. The whole mixture was then centrifuged at 10,000 rpm for 2 min. the supernatant contains DNA. About 20 µl of the supernatant was used per 50 µl of PCR reaction.

3.13.2 PCR amplification

The 18S rRNA ITS regions (Universal primer) ITS-1 and ITS-4 were used to amplify using polymerase chain reaction MJ Research PTC-225 Peltier Thermal Cycler.

Table 3.2: The 18S rRNA primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence Details</th>
<th>Number of Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1</td>
<td>TCCGTAGGTGAACCTGCGG</td>
<td>19</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCTCCGCTTTATTGATATGC</td>
<td>20</td>
</tr>
</tbody>
</table>
About 1 µl of the template DNA was incubated with 20 µl of PCR reaction solution. The PCR was amplified using the above mentioned primers with the following PCR conditions. The initial denaturation was at 94°C for 45 to 55°C min. primers were added followed by annealing at 72°C for 2 min. And final extension was at 72°C for 10. About 35 amplification cycles were used to amplify the fragments. The amplified DNA fragments (10 µl) were separated in 1.5% agarose gel using submarine gel electrophoresis at 71 V for 2 hrs and analyzed in and compared with the DNA ladder (2µl).

3.13.3 Gene sequencing and submission to GenBank

The removed and unincorporated PCR primers and dNTPs from PCR products were removed by Montage PCR Clean up kit (Millipore). The PCR product was sequenced using ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA Polymerase (FS enzyme) (Applied Biosystems, USA). The single-pass sequencing was performed on each of the template for 18S primers. The fluorescent labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). Sequence date was aligned and analyzed for identifying the samples.

The sequenced 18S rRNA genes were submitted to NCBI GenBank and the accession numbers were retrieved. The phylogenetic analysis was performed using the multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments (Edgar, 2004) the resulted aligned sequences were cured using the program Gblocks 0.91b. The poorly aligned positions and divergent regions
(removes alignment noise) were removed (Talavera and Castresana, 2007). The PhyML 3.0 aLRT program was used to construct the phylogenetic tree and HKY85 as substitution model. The bioinformatic program Tree Dyn 198.3 was used to render the tree (Dereeper et al., 2008). The PhyML program was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster.

3.14 Determination of the Carbonic anhydrase enzyme based on the molecular weight

3.14.1 Extraction of protein

About 15 ml of microalgal samples were centrifuged at 8000 rpm for 10 min. under 5°C the pellet was resuspended in PBS with 2 % triton – X (10 ml) and again centrifuged at 8000 rpm for 10 min. The greenish yellow supernatant was discarded and the pellet was again resuspended in PBS and centrifuged at 8000 rpm for 10 min. The pellet was then resuspended with extraction buffer (3.75 ml) with 1 aliquot of protease inhibitor cocktail and 10 ml of buffer. About 2 ml of acid washed glass beads was added and vortexed for 30 sec. and placed on ice for 30 sec. and procedure was repeated after 30 min. The whole suspension was the sonicated on ice bath for 5 min. and again vortexed for 5 min. the temperature was maintained below 5°C. Then revolved at 10,000 rpm for 5 min. the pellet was discarded and the obtained supernatant was conserved.

Two volumes of 10 % of TCA and two volumes of ice cold acetone containing 0.07 % DTT was mixed and incubated for overnight at -20°C. The whole content was then allowed to centrifuge at 10,000 rpm for 20 min. below 4°C the
obtained pellet was conserved and the supernatant was discarded. About two volumes of 80% of acetone and two volumes of ice cold acetone containing 0.07% of DTT was added and kept undisturbed at -20°C for 30 min. Centrifuged at 10,000 rpm for 10 min. below 5°C and the whole procedure was repeated for three times until a blue green tinch of chlorophyll and other pigment was removed. At last, the obtained pellet was stored at -80°C and the extracted protein samples were allowed to separate using SDS-PAGE (Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis).

3.14.2 SDS-PAGE separation and determination of carbonic anhydrase enzyme

The two glass plates were spaced using 1 mm thickness spacers at all the margins except the top and sealed with the help of molten agar. Two iron clips were used to hold the plates without disturbing the seal. Then the seal was ensured with GD water to check for any leakage. About 30 ml of 12% resolving gel was prepared which constitutes 30% acrylamide and bis-acrylamide mix, GD water, 1.5 M Tris HCl (pH – 8.8), 10% SDS, 10% APS and TEMED. The whole preparation was carefully allowed to pour in between the sealed glass plates. Intense care was taken to avoid the formation of air bubbles and kept undisturbed for 3 to 5 min. to polymerize the gel. The GD water was added on top of the surface to avoid oxidation with the contact to air.

Then the 5% of stacking gel was prepared and the composition includes 30% acrylamide and bis-acrylamidemix, GD water, 10 M Tris HCl (pH – 6.8), 10% SDS, 10% APS and TEMED. The remaining water present on the surface of the
resolving gel was removed with the help of the Whatman No.1 filter paper. Then the 5% stacking gel was poured over the top of the resolving gel and the 1 mm thick comb was used to form wells on the gel and the whole set up was undisturbed for 5 min. The spacer placed at the bottom of the gel was removed before placing the gel plate to the unit. The comb was removed cautiously without disturbing the well’s after casting and GD water was poured to all the wells to avoid oxidation. After removing water from all the wells, protein ladder (5 µl) and samples (20 µl) were slowly loaded to the wells using 10 µl micro-pipette. Tris-Glycine (tank buffer) buffer was then subjected to pour to each of the tank and the cathode and anode electrodes were fixed to their respective slots of their respective tanks. Initially 100 V of electric current was fixed with the help of the power pack and gradually increased to 120 V for the separation of protein. After the incubation period of 5 hrs for the separation of the protein, the gel was carefully taken out from the glass plates and washed twice with Milli-Q water.

3.14.2.1 Silver staining of gel

The obtained gel was treated with the Hypo solution for 1 min. and again washed twice with Milli-Q water. Silver nitrate solution was added to the gel in a tray and kept in a gel shaker for 20 min. in dark to prevent oxidation. After incubation with silver nitrate the gel was washed with developers until the protein bands were obviously visible. The developers were drained followed by the addition of stop solution and stored. The gel image with blatant protein bands were clearly photographed and recorded.
3.15 Determination of the carbonic anhydrase gene from selected microalgae

About 200µl of the microalgal samples were allowed to ground to fine powder with liquid nitrogen using a mortar and pestle. Exactly 2 ml of the solution-I was then added and grounded by complete homogenization to complete denaturation of protein. The whole content was subjected to thaw completely along with continuous grinding. Nuclease enzyme free water (800 µl) was added while grounding the mixture and transferred to 2 ml micro centrifuge tubes and kept undisturbed for 5 min. at room temperature. To the solution, 200 µl of chloroform was mixed and vortexed for some 5 to 10 sec. and kept at room temperature without disturbing. The solution was then allowed to centrifuge at 13,000 rpm for 10 min. at 4°C, the obtained upper phase was transferred into fresh 2 ml micro centrifuge tubes. About 0.6 volumes of isopropanol was served to it and vortexed briefly to 5 to 10 sec. Then the content was centrifuged after incubation for 10 min. at 13,000 rpm for 10 min. under 4°C, the obtained supernatant was discarded. The deposited pellet containing DNA was washed with 70% of ethanol; air dried and diluted with 50 µl of DEPC- treated water. The extracted and isolated DNA was then separated and analyzed using 0.8% Agarose gel using submarine agarose gel electrophoresis.

The isolated DNA was then amplified along with designed forward and reverse primer for carbonic anhydrase gene. About 20 µl of the total volume of the PCR reaction mixture constitutes 10 µl of master mixture, 1 µl of 1µM each of carbonic anhydrase forward and reverse primers CA-FP (5’ GAATGTGGTGCTGCTGCTA 3’) and CA-RP (5’ GGCCTGTGGGAAGTGGAACT 3’) and 100 ng of template DNA. The PCR
conditions were as follows: denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, primer-specific annealing temperature at 49.7°C for 45 sec and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were then separated and resolved by using 1.2% agarose gel with 1X TAE buffer. The resulting gel was then pre-stained with 10 mg/ml of ethidium bromide.