3. Materials and Methods

Chemicals and molecular kits

The chemicals used in the present study for microalgal cultivation and biochemical optimization were of laboratory grade. Molecular works were carried out with molecular biology grade chemicals and kits. The following kits were used in this study viz., Machery Nagel- PCR clean up gel extraction (Germany), QIA quick gel extraction kit from qiagen (USA).

Oligonucleotide NT

Oligo NT used for amplifying and subcloning of accD gene and for amplifying RNA ribosomal gene cluster region (molecular identification studies) of selected microalgal isolates were purchased from Sigma-Aldrich.

Buffer, media and solutions

All standard solutions, buffer and media were prepared according to Sambrook. (1989). Medium composition, Buffer, Solutions were included in Appendix.

Sterilization of glassware

All the glassware were soaked in chromic acid (10% Potassium dichromate in 25% Sulphuric acid) for 2 hours and washed in tap water. The glassware were cleaned with teepol solution and washed with tap water. Finally they were rinsed with distilled water and dried for overnight in hot air oven at 80°C.

Glassware, forceps and aluminium foil were wrapped with Kraft paper and sterilized using hot air oven at 160°C for 2 hours.
Sterilization of medium

Sterilization of medium was carried out using autoclave at 121°C, 15 psi for 20 min. Thermo labile chemicals were filter sterilized through 0.2 µm pore size Millipore filter before usage and added to the autoclaved media or buffer at a temperature of 50 to 60°C.

Microbial strain and vectors

Algae strains

Two reference strains namely *Chlorella vulgaris* designated as Ch.Ac Ref I and *Scenedesmus obliquus* designated as Sc.Ac Ref II were obtained from Centre for Advanced Science (CAS) in Botany, University of Madras for the present study.

Expression vector

For cloning procedure, pET-32a(+) expression vector was obtained from New England Bio lab (NEB).

3.1. Sample collection and microalgae isolation

3.1.1. Water sample collection

Fresh and marine water samples were collected from different locations of Tamilnadu including Chennai, Cuddalore, Trichy, Salem, Rameshwaram and Pudhucherry Union territory (Table 1). Figure 2 shows the location of water sampling sites. The sampling spots were chosen according to the visible algal population in the water system (water from beneath the rocks and water from stagnant pond water). The samples were filtered through 40 µ phytoplankton mesh. The fresh water filtrates were stored and transported in 1 mL of sterile Bold Basal Medium (BBM) and the marine filtrates in BBM incorporated with 2% NaCl. The sampling vials were stored at 4°C using cooler packs were transported to the lab. At
the time of sampling, the pH and temperature were recorded using pH testing meter model no PH009 and omron digital thermometer model no 246 respectively.

Table 1: Latitude and longitude of the sampling sites

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Districts</th>
<th>Latitude and Longitude</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattinapakkam beach area</td>
<td>Chennai</td>
<td>13.0336° N, 80.2687° E</td>
<td>3</td>
</tr>
<tr>
<td>Silver Beach</td>
<td>Cuddalore</td>
<td>11.7500° N, 79.7500° E</td>
<td>1</td>
</tr>
<tr>
<td>Kaveri river bead, Sri ramasamudram, Ayilooor</td>
<td>Trichy</td>
<td>11.2333° N, 78.1667° E</td>
<td>2</td>
</tr>
<tr>
<td>Serenity Beach</td>
<td>Pudhucherry union</td>
<td>11.9310° N, 79.7852° E</td>
<td>2</td>
</tr>
<tr>
<td>Rameshwaram Beach</td>
<td>Rameshwaram</td>
<td>9.2800° N, 79.3000° E</td>
<td>1</td>
</tr>
<tr>
<td>Mamangam pond</td>
<td>Salem</td>
<td>11.6500° N, 78.1600° E</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig. 2: Geographical location of water sampling sites in Tamil Nadu
3.1.2. Categorisation of water samples based on the presence of green algae members

Microalgae were identified based on their morphological features under light microscope at 400X magnification. According to the abundance of green algae strains water filtrates were divided into four types viz., very good sample with $10 \times 10^3$ µL abundance (class A), good sample with $5 \times 10^3$ µL abundance (class B), average sample with $1 \times 10^3$ µL abundance (class C) and unsuitable (class D) without any microalgae.

3.1.3. Isolation of unialgal strains

3 mL of the filtrate was inoculated in a separate flask with 100 mL of sterile BBM and incubated for a period of 21 days at 25°C under sunshades. After incubation, 12 mL of grown algal culture was taken and centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded to eliminate residual impurities and other microbial contaminants present in the filtrates. The remaining pellet was resuspended in sterile distilled water. This washing step was repeated for at least six times to remove all the contaminants. From the final pellet suspension, 1 mL was taken and inoculated on Bold Basal Medium (BBM) agar and incubated at the same condition mentioned above. After incubation the single colonies were picked and sub-cultured in 100 mL of BBM broth medium. A loopful of culture was placed on a microscopic slide and viewed at 400X magnification for the confirmation of unialgal strain (Parvin et al., 2007).
3.1.4. Axenization of isolates

Axenic state of microalgal culture is the state devoid of other type of algae, bacteria, microfungi and other microbes (Spolaore et al., 2006). To obtain axenic culture the unialgal strains were sub cultured in 50 mL BBM incorporated with antibiotics (Meropenum (2 µg/mL) and Nystatin (5 µg/mL). The conical flasks were incubated at 30°C in a rotary shaker at 200 rpm for 48-72 hours in dark condition. Then the cultures were streaked on BBM agar plate and their purity was confirmed after three days of incubation at 37°C. Axenated strains were maintained in sterile 200 mL BBM for further usage.

3.1.5 Primary cultivation of microalgae

5 mL of axenic culture was inoculated in their respective medium and incubated at 25°C under 15W illumination of yellow fluorescent light. The light and dark conditions were maintained (16h: 8h) for 21 days.

3.2. Screening of microalgae for lipid and biomass production

The algal isolates were cultivated on BBM, Bristol medium and Tris Acetate Phosphate (TAP) medium. The media compositions are mentioned in the following tables. (Table 2, 3 and 4)
Table 2: Chemical composition of Bold Basal Medium (pH-7.5)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in moles (M) (Except H₂SO₄)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitrate (NaNO₃)</td>
<td>0.0118</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂.2H₂O)</td>
<td>0.0170</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄.7H₂O)</td>
<td>0.00030</td>
</tr>
<tr>
<td>Dipotassium Hydrogen Phosphate (K₂HPO₄)</td>
<td>0.00043</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate (KH₂PO₄)</td>
<td>0.00129</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>0.000428</td>
</tr>
<tr>
<td><strong>Alkaline EDTA solution.</strong></td>
<td></td>
</tr>
<tr>
<td>Ethylene Diamine Tetra Acetic acid (EDTA)</td>
<td>0.000171</td>
</tr>
<tr>
<td>Potassium Hydroxide (KOH)</td>
<td>0.000553</td>
</tr>
<tr>
<td><strong>Acidified iron solution.</strong></td>
<td></td>
</tr>
<tr>
<td>Ferrous Sulphate (FeSO₄.7H₂O)</td>
<td>0.000018</td>
</tr>
<tr>
<td>Sulphuric Acid (H₂SO₄)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Boric Acid (H₃BO₃)</td>
<td>0.000185</td>
</tr>
<tr>
<td><strong>Trace metal solution</strong></td>
<td></td>
</tr>
<tr>
<td>Zinc Sulphate (ZnSO₄.7H₂O)</td>
<td>0.000031</td>
</tr>
<tr>
<td>Manganese Chloride (MnCl₂.4H₂O)</td>
<td>0.0000073</td>
</tr>
<tr>
<td>Molybdenum Trioxide (MoO₃)</td>
<td>0.0000049</td>
</tr>
<tr>
<td>Copper Sulphate (CuSO₄.5H₂O)</td>
<td>0.0000063</td>
</tr>
<tr>
<td>Cobalt Nitrate (Co(NO₃)₂.6H₂O)</td>
<td>0.0000017</td>
</tr>
</tbody>
</table>
Table 3: Chemical composition of Bold Basal Medium (pH-8.6)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in moles (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitrate (NaNO₃)</td>
<td>0.0294</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂.2H₂O)</td>
<td>0.017</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄.7H₂O)</td>
<td>0.003</td>
</tr>
<tr>
<td>Dipotassium Hydrogen Phosphate (K₂HPO₄)</td>
<td>0.0043</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate (KH₂PO₄)</td>
<td>0.00129</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>0.0043</td>
</tr>
</tbody>
</table>

Table 4: Chemical composition of Tris Acetate Phosphate (TAP) medium (pH-8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in moles (M) (Except acetic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>BEIJERINCK SALT SOLUTION</strong></td>
<td></td>
</tr>
<tr>
<td>Ammonium Chloride (NH₄Cl)</td>
<td>0.007</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄.7H₂O)</td>
<td>0.000830</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂.2H₂O)</td>
<td>0.00045</td>
</tr>
<tr>
<td><strong>PHOSPHATE SOLUTION</strong></td>
<td></td>
</tr>
<tr>
<td>Dipotassium Phosphate (K₂HPO₄)</td>
<td>0.00165</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate (KH₂PO₄)</td>
<td>0.00105</td>
</tr>
<tr>
<td><strong>HUNTNER TRACE ELEMENTS SOLUTION</strong></td>
<td></td>
</tr>
<tr>
<td>EDTA disodium salt (Na₂EDTA.2H₂O)</td>
<td>0.000134</td>
</tr>
<tr>
<td>Zinc Sulphate (ZnSO₄.7H₂O)</td>
<td>0.000136</td>
</tr>
<tr>
<td>Boric Acid (H₃BO₃)</td>
<td>0.000184</td>
</tr>
<tr>
<td>Manganese Chloride (MnCl₂.4H₂O)</td>
<td>0.00004</td>
</tr>
<tr>
<td>Ferrous Sulphate (FeSO₄.7H₂O)</td>
<td>0.000033</td>
</tr>
<tr>
<td>Cobalt Chloride (CoCl₂.6H₂O)</td>
<td>0.000012</td>
</tr>
<tr>
<td>Copper Sulphate (CuSO₄.5H₂O)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Ammonium Molybdate ((NH₄)MO₇O₂₄.4H₂O)</td>
<td>0.00000093</td>
</tr>
<tr>
<td>Acetic acid concentrated</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>
Algal culture (1 mL) was used as primary inoculum for 1000 mL of sterile synthetic medium. After inoculation, the flasks were incubated at 25°C. For 21 days light and dark condition were maintained (16h: 8h) using 15W illumination of yellow fluorescent light.

3.3. Biomass estimation studies

3.3.1. Determination of algal growth rate

About 1 mL of exponential phase (14 days old) culture was taken and the algal growth rate was calculated by the method described by Chandra et al. (2012). The Growth rate was recorded at 680 nm using spectrophotometer (Thermoscientific model no Evolution 201).

3.3.2. Biomass wet and dry weight

About 10 mL of 21 days old culture was taken and centrifuged at 4,000 rpm for 15 min. The pellet was washed twice with distilled water and wet weight was measured using electronic weighing balance. The pellet was dried in pre weighed aluminium dish at 60°C for 3 to 4 hours and the dry weight was measured (Rao et al., 2007).

3.3.3. Total carbohydrate estimation

About 10 mL of 21 days old culture was diluted with distilled water and kept for 3 hours in boiling water bath. To the sample, 5.0 mL of 2.5 N HCl was added and cooled to room temperature. Then it was neutralized with sodium carbonate until effervescence ceases. The above volume was made up to 100 mL with distilled water and centrifuged. The supernatant was used as working standard and it was taken in 0.2, 0.4, 0.6, 0.8 and 1.0 mL quantities in a series of test tubes. To the test
tubes 1.0 mL of phenol solution and 5.0 mL of 96% H2SO4 were added and shaken well. After 10 minutes the tubes were kept in water bath at 30°C for 20 minutes. The results were recorded at 490 nm using spectrophotometer (Thermoscientific model no Evolution 201). Glucose solution served as control and the total carbohydrate was calculated and recorded (Krishnaveni et al., 1984).

3.3.4. Total protein estimation

About 10 mL of grown algal samples were ground with potassium phosphate buffer (containing 20% polyvinyl pyrrolidone (PVP) and pH 7.4) using acid washed sand in a mortar and pestle and filtered through four layers of muslin cloth. An equal volume of 10% trichloro acetic acid (TCA) was added to precipitate the proteins in the filtrate. The precipitate was removed by centrifugation at 9500 rpm for 15 minutes and the pellets were resuspended in 1 N NaOH. The protein in the solution was estimated by Lowry’s method (Lowry et al., 1951). To 1mL of protein solution, 5.0 ml of alkaline copper sulphate solution was added and allowed to stand at room temperature for 10 minutes. Then 0.5 mL of diluted Folins-phenol reagent (Sigma- Aldrich) was added and mixed well. The solution was allowed to stand for 30 minutes and the absorbance was read at 595 nm using a spectrophotometer (Thermo Scientific Evolution 201). Bovine serum albumin (BSA) was used as standard.

3.3.5. Pigment characterisation

Estimation of Chlorophyll a and Chlorophyll b

Chlorophyll a and b were extracted from algal samples using 100% methanol. The samples were diluted 10 to 20 times using methanol depending on the cell concentration. The vials containing the samples were wrapped with aluminium foil
and stored at 4°C for 30 min. The samples were then centrifuged at 11,500 rpm for 10 min. The absorbance of the green supernatant was measured at 663 and 645 nm for Chlorophyll a and chlorophyll b, calculated using the equations described by (Hitkins et al., 1986).

Calculation,

\[
\text{Chl a (mg/g)} = (12.7 \times \text{OD } 663) - (2.69 \times \text{OD } 645) \times \frac{V}{1000 \times W}
\]

\[
\text{Chl b (mg/g)} = (22.9 \times \text{OD } 645) - (4.68 \times \text{OD } 663) \times \frac{V}{1000 \times W}
\]

**Estimation of β-carotene pigment**

β-carotene pigment is soluble in acetone. 10.0 mL of homogenous algal suspension was centrifuged at 5000 rpm for 10 minutes. The pellet was washed twice in distilled water and homogenized with 3.0 mL of acetone. The content was centrifuged at 5000 rpm for 5 minutes and the supernatant was taken and stored in the refrigerator. The extraction step was repeated until acetone become colourless. The supernatant was diluted with 10 mL of acetone. The absorbance was measured at 450 nm against acetone blank (Liaen-Jensen et al., 1978).

3.3.6. **Total lipid extraction**

The Folch method developed in 1957 for total lipid extraction led to more than 95% lipid recovery from microalgae. 1g of dry weight of microalgal culture was taken and 20 mL of chloroform: methanol (2:1) ratio was added and centrifuged at 4000 rpm for 10 minutes. The lower layer was transferred into a fresh tube. The procedure was repeated for 3–5 times until the upper layer was colourless which indicated that the total lipid was extracted and the lipid extracts were evaporated using vaccum dessicator. The dried lipid content was measured using an electronic weighing balance.
3.4. Molecular identification of microalgal isolates
3.4.1. Isolation of genomic DNA from microalgae

Extraction buffer

About 24 g guanidinium thiocyanate (GuSCN) and 20 mL 0.1 M Tris-HCl (pH 6.4) was added to a 50 mL falcon tube. The content was kept in oven at 60°C to dissolve the GuSCN, which is followed by addition of 4.4 mL of 0.2 M EDTA (pH 8.0), then 0.5 mL Triton X-100. The solution was mixed well.

Binding buffer

About 24 g GuSCN and 20 mL 0.1 M Tris-HCl (pH 6.4) was added to a 50 mL Falcon tube and the content was kept in oven at 60°C to dissolve the GuSCN

Washing buffer

70% ethanol was mixed with 10 mM NaCl.

TE buffer

To 10 mM Tris-HCl buffer (pH 8.0) 1 mM EDTA (pH 8.0) was added and mixed well.

Procedure

To 50 mg of microalgal biomass and 0.6 mL of extraction buffer was added and macerated using a mortar and pestle placed on ice bath. After complete maceration the whole content was transferred into sterile 2.0 mL microfuge tubes and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was transferred to the spin column which was kept in a new sterile 2.0 mL microfuge tube. The centrifugation step was performed at 12,000 rpm for 30 sec. The flow through was discarded and to the spin column 500 µL of binding buffer was added. This is again centrifuged at 12,000 rpm for 30 sec and the flow through was
discarded. Subsequently 600 μL of washing buffer was added to the spin column and centrifuged at 12,000 rpm for 1 minute and the flow through was discarded. This washing step was performed twice. The spin column was placed in a new 1.5 mL microfuge tube and 200 μL of TE buffer was added and incubated at room temperature for 1 min. The tube was centrifuged at 12,000 rpm for 1 min. The collected buffer flow through containing DNA was stored at -20⁰ C until further use (Margam et al., 2012).

3.4.2. PCR amplification of ribosomal gene cluster gene region

Phylogenetic analysis based on single target region in ribosomal coding region would not give sufficient information to understand the evolutionary basis of new microalgal isolates. Maximum ribosomal cluster range was targeted which includes partial SSU, ITS and partial LSU region (Hall et al., 2010). Universal gene specific primers for chlorophyta were used (Table 5). Figure 3 illustrates the flanking pattern of ribosomal gene cluster region specific primers with approximate binding region. The amplified sequence was compared with existing NCBI sequence for further identification and phylogenetic tree construction

Table 5: Universal primer sequence for ribosomal gene cluster region

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>5' to 3' designation</th>
<th>Universal primer details</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL18F</td>
<td>TGTACACACCGCCGTC</td>
<td>SSU forward primer</td>
</tr>
<tr>
<td>UL28R</td>
<td>ATCGCCAGTTCTGCTTAC</td>
<td>LSU reverse primer</td>
</tr>
</tbody>
</table>
Fig. 3: Ribosomal gene cluster and universal primer binding region

**PCR reaction mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>dNTP mixture (2.5mM)</td>
<td>1.6 μL</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>DNA Template (50ng/μL)</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>13.2 μL</td>
</tr>
</tbody>
</table>

**PCR reaction condition**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Repeat</td>
<td>94</td>
</tr>
<tr>
<td>Annealing</td>
<td>29</td>
<td>54</td>
</tr>
<tr>
<td>Extension</td>
<td>cycles</td>
<td>72</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Cooling step</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
Agarose gel electrophoresis of PCR amplicons

**TBE Buffer (pH-8.0)**

| 90 mM Tris Base, 90 mM Boric acid and 0.2 M EDTA |

**DNA Loading dye**

| 1X TBE buffer, 5% (w/v) Sucrose, 0.04% (w/v) Bromophenol blue, 2% (w/v) SDS |

PCR amplicons were separated on 1% agarose gel prepared with TBE buffer and EtBr at a concentration of 1mg/mL. The amplified PCR products were mixed with DNA loading dye and loaded in appropriate wells on 1% agarose gel. The gel platform was placed in horizontal electrophoresis unit and electrophoresized at 100 V for 1 hour (Sambrook et al., 1989). The DNA bands were visualized using UV transilluminator.

**Gel elution procedure**

The PCR products separated on 1% agarose gel were eluted from the gel using Qiagen gel extraction kit. The amplified products were excised from the gel using a scalpel. 300 mg of gel slice was transferred to a microfuge tube and 500 µL of detergent free (DF) buffer (0.5 M ammonium acetate, 10 mM Magnesium acetate tetrahydrate and 1 mM EDTA, pH 8.0) was added and mixed by vortexing. The sample was incubated at 55°C for 10-15 minutes until the gel was completely dissolved. The tube was inverted very gently every 2 minutes during incubation. A DF column was placed in a collection tube and 800 µL of the dissolved gel sample was transferred to the DF column. The DF column was centrifuged at 10,000 rpm for 30 seconds. The fluid in the collection tube was discarded and 750 µL of wash solution was added to the DF column which was then centrifuged at 10,000 rpm for
30 seconds. The flow through was again discarded and the column was dried by centrifugation at 10,000 rpm for 2 minutes. To the centre of the DF column matrix 30 µL of DNA elution buffer was added. The column was allowed to stand for 2 min at room temperature and subsequently centrifuged at 10,000 rpm for 30 seconds for the elution of isolated DNA.

**Sequencing of the amplified DNA (using Sanger coulson dideoxy method)**

Sequencing was carried out with AB13730xl DNA analyzer (Applied biosystems). The exact base pair order in a particular DNA sequence could be determined by dye terminator sequencing. In this method the double stranded DNA fragment to be analysed was mixed with a single primer that bind to a strand complementary to the strand yet to be sequenced. DNA polymerase and nucleotide mixture containing ordinary dNTP’s and ddNTP’s that lack 3’ OH at polymerisation site and each nucleotide labelled with specific fluorescent dye was added. In a reaction based on the same principle as PCR, insertion of ddNTP terminated the extension of that particular strand. The insertion of these nucleotides was random but after a series of cycles the sequencing reaction contained mixture of copies of one strand ending at each base. During the separation of the strands by size in a capillary electrophoresis, the dyes of strands of equal length get excited when passing a laser and the specific light emissions were detected (Reece, 2004).

**3.4.3. BLAST of amplified sequences for species identification of isolates**

The ribosomal cluster region (partial SSU, ITS and LSU gene regions) of selected isolates were compared with similar sequence already deposited in NCBI database and the species was confirmed for both the isolates.
3.4.4. Construction of phylogenetic tree

Basic Local Alignment Sequencing Tool (BLAST) was used to search for similarity between sequences and phylogenetic tree was constructed by Neighbourhood joining method using MEGA 6.0 version software. The distance between closely related species was calculated using cluster analysis. It represented horizontal gene transfer between phylogenetically distinct groups.

3.5. Screening of industrial effluents as a production medium for microalgae lipid

In the present study sago, sugar distillery, textile dyeing and silk processing industries were selected. The main reason for selecting various stages of effluents from sago, sugar distillery, textile dyeing and silk processing industries are mentioned as follows; (1) Sago industry effluent from tapioca wash unit is the major effluent discharged from this industry (Banu et al., 2006). This effluent can be a rich source of iron and other heavy metals which would support lipid production in algae. (2) Textile dyeing industry effluent from textile dyeing unit and final wash unit are of alkaline condition with various heavy metals such as manganese, cadmium, nickel and chromium. (3) Sugar Distillery effluent and distillery spent wash unit effluents were selected due to their heavy metal load comprised with zinc, iron, cadmium, manganese, nickel and chromium than any other industry (Samuel and Muthukkaruppan., 2011). (4) Silk processing industry effluent was previously applied for cyanobacterial growth. This is the first study to utilize silk degumming effluent and reeling unit effluent for microalgae lipid production.
3.5.1. Effluent collection

Four different industrial effluents viz., (1) Tapioca wash effluent (from sago industry located near thiruvakoundar bypass, Salem district), (2) Yarn dyeing unit effluent and final wash unit effluent (from textile dyeing industry located in Karur district), (3) Distillation unit effluent and distillery spent wash unit effluent (from sugar distillery industry located in Dharmapuri district) and (4) Silk degumming unit effluent and reeling unit effluent (from silk processing industry located in Seelanaikkanpatty, Salem district) were collected. The geographical map of the sampling sites was given in Fig. 4. The color, odour and temperature of effluents were recorded at the site of collection. The physico-chemical properties were analysed by following American Public Health Association (APHA), 2005 standard procedures.

Fig. 4: Geographical location of effluent sampling sites in Tamil Nadu
3.5.2. Physico chemical analysis of industrial effluent

3.5.2.1. pH

The pH of the effluent samples were analysed using pH meter (ELICO -116) and the results were recorded.

3.5.2.2. Total dissolved solids

About 100 mL of effluent sample was transferred to a porceline dish and evaporated on a steam bath. The dried residues were collected. This process was continued for 1 hour in a hot air oven at 180 ± 2°C and cooled in desiccators to balance the temperature and weighed. The process of drying, cooling and weighing were repeated to avoid the error in weight.

Calculation,

\[ \text{TDS (mg/L)} = \frac{(A-B) \times 1000}{\text{Vol.of water sample (mL)}} \]

Where

A-weight of dish + solids (mg)

B-weight of dish before use (mg)

3.5.2.3. Total suspended solids

The weight of the empty crucible was initially recorded. About 100 mL of the water sample was taken and shaken well. The water was poured into a separating funnel that was kept in the filter holder placed in the suction flask apparatus. After the filtration process was completed the crucible was removed slowly from the suction apparatus and placed in the drying oven. The crucible was dried for at least 1 hour at 103-105°C, cooled in desiccators and weighed. The process of drying, desiccating and weighing were repeated until the weight loss between two successive weighing was less than 0.5 mg. Finally the weight was recorded.
Calculation,
Total suspended solids (mg / L) = \( \frac{A - B}{C} \times 10^6 \)

Where
A-weight of filter + solids (g)
B-weight of filter (g)
C- volume of sample filtered (mL)

3.5.2.4. Chloride

About 100 mL of sample was taken into a porcelain dish. 1.0 mL of potassium chromate indicator solution was added and stirred well until reddish colour appeared. The solution was titrated with silver nitrate solution with constant stirring until the slightest perceptible reddish coloration persists.

Calculation,

\[
\text{Chloride as Cl}^- \text{ (mg/L)} = \frac{1000 \times (V_1 - V_2)}{\text{Vol.of water sample (mL)}} \text{ mg/L}
\]

Where
\(V_1\)-volume of silver nitrate required by the sample (mL)
\(V_2\)-volume of silver nitrate required by the blank (mL)

3.5.2.5. Total hardness

To 10 mL of the water sample about 6 mL of ammonia buffer and 3 drops of EBT indicator were added to the water sample. This solution was titrated against standard EDTA solution and the end point was calculated. This procedure has been repeated to obtain concordant value.

Calculation,

\[
\text{Total Hardness (mg/L)} = \frac{(T-S) \times C \times 1000 \text{ mg/L of CaCO}_3}{\text{Vol.of water sample}}
\]
Where

T- volume of EDTA for titration of the total hardness sample (mL)
S-volume of EDTA for titration of the blank (mL)
C- is calculated from the standardisation of the EDTA

3.5.2.6. Total alkalinity

About 40 mL of water sample was taken in a conical flask. Phenolphthalein indicator was added to the solution. The solution was titrated against HCl until pink colour disappears and to the same solution few drops of methyl orange indicator was added and titrated. The value was recorded when the colour change occurred from pale yellow to pale pink.

Calculation, Total alkalinity as CaCO₃

\[ T \text{ (mg/ L)} = \frac{100,000 \times A \times M}{V} \]

\[ T \text{ (mg/ L)} = \frac{100,000 \times B \times M}{V} \]

Where
A-volume of standard acid solution (mL) to reach the phenolphthalein end-point
B-volume of standard acid solution (mL) to reach the end-point of methyl orange
M-concentration of acid (mol /L)
V- volume of sample (mL)

3.5.2.7. Dissolved oxygen

To 100 mL of water sample, 2.0 mL of alkali azide and 2.0 mL of MnSO₄ were added. The precipitate formed was dissolved in concentrated sulphuric acid. To the solution 4 drops of starch indicator was added. This mixture was titrated with standard sodium thio sulphate solution and the titrated value was noted.
DO (mg/L) = \frac{(V_1 - V_2) \times 1000}{\text{Vol. of water sample}}

3.5.2.8. Biological oxygen demand (BOD)

The effluent samples were collected in the morning and incubated at 27°C for 5 days in a closed condition. Using DO meter the BOD of the effluents were measured and calculated.

Calculation, when using seeded dilution water:

\[
\text{BOD} = \frac{(D_1 - D_2) - (B_1 - B_2)}{P} \times f
\]

Where:
- \(D_0\) - DO of original dilution water
- \(D_1\) - DO of diluted sample immediately after preparation (mg/L)
- \(D_2\) - DO of diluted sample after 5 days’ incubation
- \(P\) - decimal fraction of sample used: \((P + p = 1.00)\)
- \(B_1\) - DO of dilution of seed control* before incubation;
- \(B_2\) - DO of dilution of seed control* after incubation;

\[
f = \frac{\% \text{ of seed in } D_1}{\% \text{ of seed in } B_1}
\]

Seed correction = \((B_1 - B_2) \times f\)

3.5.2.9. Chemical oxygen demand (COD)

About 10 mL of standard potassium dichromate solution (0.0417 mol/L) and 3 anti bumping granules were added to the water sample and mixed well. To the solution 30 mL of concentrated H\(_2\)SO\(_4\) containing silver sulphate was added slowly. The mixture was refluxed for 2 h and then cooled to room temperature. The solution was diluted with 150 mL of distilled water. Excess dichromate was titrated with
standard ammonium ferrous sulphate using 3 drops of ferroin indicator. The end-point was noted down with the colour change from blue-green to reddish-brown. The procedure was repeated for blank consisting of 20 mL of distilled water together with the reagents.

Calculation,

$$\text{COD (mg/L)} = \frac{(a-b) \times c \times 8000}{V}$$

Where

- $a$- ferrous ammonium sulphate used for blank
- $b$- ferrous ammonium sulphate used for sample
- $c$- molarity of ferrous ammonium sulphate
- $v$- volume of sample

### 3.5.2.10. Estimation of Nitrate

About 5.0 mL of sample was taken in centrifuge tube and diluted with 20 mL of distilled water. The tubes were centrifuged at 3000 rpm for 10 min. This step was repeated until the solution become clear and 5.0 mL of clear solution was taken in glass evaporating dish and evaporated to dryness. Potassium nitrate was used as control. Then 1.0 mL of phenoldisulphonic acid was added to the dried residue and incubated at room temperature for 10 min. The whole content was transferred to 100 mL of volumetric flask and 0.2 mL of ammonium hydroxide was added and mixed well. The absorbance was recorded at 410 nm using UV Spectrophotometer (Thermoscientific Evolution model no 101) (Mussa et al., 2009).
3.5.2.11. Estimation of Phosphate

About 10 mL of sample was taken in a 50 mL measuring flask and diluted with 20 mL of distilled water. Potassium dihydrogen phosphate was used as control. About 2.0 mL of molybdate solution and one spatula of ascorbic acid crystals was added to the diluted sample and mixed well. Then slowly heat the mixture to boil and allowed it to cool for 15 min. The absorbance was recorded at 650 nm by using UV Spectrophotometer (Thermoscientific Evolution model no 101) (Mussa et al., 2009).

3.5.2.12. Estimation of Iron

About 10 mL of sample was diluted with 200 mL of distilled water in a volumetric flask. To 25 mL of aliquot, 4.0 mL of hydroxylamine hydrochloride solution and 4.0 mL of 0.3% o-phenanthroline solution was added and mixed well. The mixture was kept at room temperature for 10 min. Based on absorbance value at 508 nm using UV Spectrophotometer (Thermoscientific Evolution model no 101), iron concentration was estimated.

3.5.2.13. Analysis of heavy metals by atomic absorption spectroscopy (AAS)

Based on their absorbance against the blank at particular nm using specific hollow metal lamp (varies according to metal), the heavy metal concentration could be estimated. The following conditions were maintained to operate the instrument, depending upon the analytical procedure.

Acetylene and air were used as fuel and oxidant respectively in AAS for the determination of heavy metals quantitatively. The temperature of the flame was approximately 2300°C. Acetylene air oxidizing flame was used
along with the flow rate of about 1 to 3 L/min as per the recommended procedure. Peak energy was maintained over the range of 1 to 3V as per the recommended procedure.

**Determination of nickel**

Ni(II) concentration was analyzed by AAS method using a series of standard Ni(II) solution over the range 2-10 mg/L. The absorbance was measured at 232.0 nm against blank using nickel hollow-cathode lamp.

**Determination of chromium**

Cr(VI) concentration was measured by AAS method using a series of standard Cr(VI) solution over the range of 2-10 mg/L. The absorbance was recorded at 357.9 nm against blank using Chromium hollow-cathode lamp.

**Determination of lead**

Pb(II) concentration was estimated by AAS method using a series of standard Pb(II) solution over the range 2-10 mg/L. The absorbance was measured at 217.0 nm against blank using lead hollow cathode lamp.

**Determination of cadmium**

Cd(II) concentration was estimated by AAS method using a series of standard Cd(II) solution over the range 2-10 mg/L. The absorbance was measured at 228.0 nm against blank using cadmium hollow cathode lamp.

**3.5.3. Cultivation of algal isolates in industrial effluents**

The seven natural isolates and two reference strains were cultivated in 4 different industrial effluents for 21 days duration under yellow fluorescent light illumination at 25°C.
3.5.4. Biomass estimation studies

Determination of algal growth rate, biomass wet and dry weight estimation, pigment characterization, total carbohydrate, protein and lipid estimation were carried out as per the procedure (mentioned in section 3.3) for the algal cultures after 21 days of cultivation.

3.6. Large scale cultivation of selected isolates

During scale up process from lab condition there was a need for careful planning (Pulz, 2001). In the present study, three large scale cultivation methods viz., static, submerged and aerated cultivations were tested. Figure 5 shows the types of cultivation applied in the study. Further large scale cultivation was tested up to 16 L volume.

![Fig. 5: Large scale cultivation of microalgae isolates](image)

18a. Static type  18b. Submerged type  18c. Aerated type

3.7. Screening of isolates in combined effluent medium

3.7.1. Preparation of combined effluent medium

After cultivation in synthetic and effluent medium, the selected two isolates (MS3 and AY2) based on the biomass and lipid production were cultivated in combined effluent medium. An equal percentage of textile dyeing effluent and final wash effluent were mixed and designated as TDES medium. Similarly an equal
percentage of silk degumming unit effluent and reeling unit effluent from silk processing industry were mixed and designated as CRWS medium Fig. 6.

![Combined Effluent Medium](image)

**Fig. 6: Proportions of combined effluents**

Both the selected isolates were screened in combined effluent medium for 21 days under 15 W yellow fluorescent light illumination. After incubation chlorophyll a, lipid content, growth rate and total biomass were analysed by procedures mentioned in earlier section 3.3.

### 3.7.2. Physicochemical analysis of combined effluent medium before and after cultivation of microalgae

The physico-chemical changes in the combined effluent medium before and after cultivation of microalgae were estimated by following the procedures mentioned in section 3.5.2.

### 3.8. Screening of selected isolates in formulated medium

This step was necessary to find an efficient lipid production medium for the selected isolates. Both lipid production and biomass should be considered for better microalgae biodiesel feed stock production (Chisti *et al.*, 2007).

#### 3.8.1 Preparation of formulated medium

For the selected isolates *viz.*, *Asterarcys* sp. and *Neochloris* sp. and their respective synthetic medium Bristol and BBM were formulated with combined
effluent medium TDES and CRWS. In total, four sets of formulated medium were prepared SET I, II, III and IV and each of them in three proportions viz., 25:75, 50:50 and 75:25 ratio were provided to the isolates. SET I and II were prepared with Bristol medium (specific synthetic medium for Asterarcys sp.). SET III and IV was prepared with BBM medium (specific synthetic medium for Neochloris sp.). The cultivation conditions were maintained as mentioned in section 3.1.5. Figure 7 describes about the four sets of formulated medium and their proportions. After incubation chlorophyll a, lipid content, growth rate and total biomass were analysed by procedures mentioned in section 3.3.

![Fig. 7: Proportions of formulated medium](image)

### 3.9. Estimation of neutral lipid content in total biomass

#### 3.9.1. Quantification of neutral lipid by Nile red fluorescence method

Total neutral lipid was evaluated by Nile Red fluorescence based quantification (Chen et al., 2011). The samples were diluted twice with sterile distilled water and to the diluted cells; 2.0 mL of 20% (v/v) DMSO solution was added. The cells were stained with 4 μL of Nile Red (250 μg/mL in acetone) and vortexed for
30 seconds. The samples were analysed through fluorescence spectrophotometer and the neutral lipid content was estimated at 580 nm (Elsey et al., 2007).

3.9.2. Total lipid (%) produced by the isolates

The relevance between chlorophyll a and lipid content should be studied for the quantification of actual lipid content of algal biomass. Chlorophyll a was measured using fluorescence excitation wavelength (Mu et al., 2001) of 663 nm and the emission wavelength of 675nm and calculated. The growth rate was measured at 680 nm and the neutral lipid content was quantified based on Nile red fluorescence estimation at 580 nm. The total lipid percentage was calculated using the formula described by Li et al., (2008) as follows,

Calculation,

\[
\text{Total Lipid percentage (\%) = \frac{\text{Growth rate} \times \text{neutral lipid content}}{\text{Chlorophyll a} \times \text{Total biomass}} \times 100}
\]

3.10. Analysis of total lipid from microalgae biomass

3.10.1. Analysis of total lipid using Fourier Transform Infra-Red (FT-IR) spectrometry

The total lipid was extracted as described by Folch. (1957) and was analysed through FT-IR spectroscopy (Instrument model Perkin Elmer model spectrum-I Pc). About 1 mg of lipid content was mixed with 5.0 mL of methanol and chloroform solvents in 2:1 ratio and placed into the sample holder. FT-IR spectra range was selected between the ranges 400 to 4000 cm\(^{-1}\). The obtained spectra of lipid samples were identified for structural changes in functional group. The functional group for lipid fraction was identified at 2926 cm\(^{-1}\). Based on the functional group and respective changes in lipid composition the specific formulated medium was selected for each isolate.
3.10.2. Morphological examination of algal isolates

3.10.2.1. Evans blue staining and light microscopic examination

Evans blue is the dye used to differentiate living and non-living cells. Adaptation of microalgae to adverse conditions was confirmed with 0.01% Evans blue stain. 10 µL of Evans blue stain was added to 100 µL of cell suspension and the microfuge tubes were incubated at room temperature for 10 minutes. After incubation the cells were examined under light microscope at 400X magnification (Crippen and Perrier.1974). The live and dead microalgae cultures were marked with symbols viz., + and – respectively.

3.10.2.2. Fluorescent microscopic analysis of neutral lipid

Nile red (9-(Diethylamino) -5H benzo [α] phenoxazin- 5- one) staining and fluorescent imaging were conducted to analyse intracellular lipid droplets. At first 0.5 mL of microalgae cells were collected by centrifugation at 1,500 rpm for 10 minutes and washed with 0.5 mL of physiological saline solution for six times (Chen et al., 2011). The washed cells were re-suspended in 0.5 mL of saline. To the cell suspension (1:100 v/v) nile red solution (0.1 mg/mL in acetone) was added and incubated for 10 minutes. After washing, the stained microalgal cells were observed using fluorescent microscopy (Model: Olympus IX-71)

3.11. Strain optimization for Acetyl CoA Carboxylase (ACCase) activity

3.11.1. Selection of light source for ACCase activity

The enzyme ACCase, follow a light dependent cascade. To start with, light parameter was standardized for each isolate by providing 15 W powerful yellow, green, blue, red and white light sources.
3.11.2. ACCase assay procedure

The ACCase activity was measured using a discontinuous spectrophotometric assay at 412 nm described by Willis et al. (2008).

Enzyme assay reaction mix

Each 250 µl ACCase assay reaction mixture contained

- 100 mM potassium phosphate (pH 8.0)
- 15 mM KHCO₃
- 5 mM MnCl₂
- 5 mM ATP
- Bovine serum albumin (BSA) (1µg/µL)
- 1 mM acetyl-CoA

Reaction mixture with 0.5 unit citrate synthase (Sigma) served as control and the standard curve was constructed by measuring the changes in acetyl-CoA level (µmol/ml) based on absorbance at 412 nm. The algae cells were permeabilised with 3.0 µL of 10% hexadecyltrimethyl-ammonium bromide (CTAB) and these permeabilized cells were tested for ACCase activity by determining the acetyl-CoA concentration.

Assay steps

1. In 1.0 mL microfuge tube 250 µL of reaction mixture was taken and 7.0 µL of permeabilized cells was added. This reaction mixture was incubated at 30°C for 30 min.
2. After incubation, 50 µL aliquots were transferred into a sterile microfuge tubes and 3.3 µL of 10% trifluoroacetic acid was added. The tubes were then placed on ice.
3. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatants were collected in sterile microfuge tubes and stored at 4°C.

4. 50 µL of supernatant was mixed with 950 µL of buffer [(100 mM KPO4 (pH 8.0), 2 mg/mL dithionitrobenzoic acid (DTNB), 20 mM oxaloacetate and 1 mg/mL bovine serum albumin)] and analysed for acetyl-CoA concentration by measuring absorbance at 412 nm using spectrophotometer (Thermoscientific model evolution model no101).

3.11.3. Biochemical optimization of isolates for ACCase activity

For biochemical optimization of ACCase, citrate and biotin metabolites were used where citrate is an allosteric activator and biotin is an analog for ACCase. These two metabolites were supplemented individually to the selected formulated medium and their effect on lipid composition was studied by FT-IR spectroscopy.

3.11.4. Analysis of microalgae cells by scanning electron microscopy (SEM) imaging

The effect of metabolites on microalgal cell morphology was analysed by observing SEM images. About 1.0 mL of 21 days old culture was fixed with 2.5% glutaraldehyde for 4 hours and, then centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in sterile distilled water. This washing step was repeated for four times and the samples were mounted on metal stubs and coated with gold for SEM analysis Model No Hitachi-3000 H) incorporated with INCA system software (Dwaish et al., 2011).
3.12. Analysis of FAMEs derived from lipids of biochemically optimised isolates

3.12.1. FAMEs preparation

From the biochemically optimized strain, lipids were extracted by Folch method, (1957) and in vitro lipid transesterification into fatty acid methyl esters (FAMEs) was performed for all the samples by following Lepage and Roy (1984) method. About 10 mg of lipid was dissolved in 2.0 mL of Chloroform and methanol solvent mixture (2:1) in a glass centrifuge tube. Then 1.0 mL of methanol and 0.3 mL of sulfuric acid as catalyst were added and vigorously shaken for 5 min. Finally the reaction mixture containing tubes were placed in water bath at 100°C for 10 minutes and the tubes were cooled to room temperature. To the mixture 1.0 mL of distilled water was added for phase separation where the upper layer was rich in water, methanol, glycerol, and sulfuric acid, and the lower layer was rich in chloroform and esters. This wash step was repeated twice. Chloroform was evaporated under laboratory hood at 25°C. The remaining esters were analysed through High performance liquid chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS).

3.12.2. Analysis of FAMEs using High performance liquid chromatography (HPLC)

The standard methyl ester which comprised equal volume of methyl stearate, methyl palmitate and methyl linoleate was used for the quantification of unknown microalgae FAME compounds. HPLC grade acetonitrile solvent was used as mobile phase. Peak area was calculated to know the exact concentration of FAME components. (Holcapek et al., 1999).
HPLC column and conditions

Desired sample amount (10 mg) was dissolved in 2 mL of methanol and chloroform in 2:1 proportion. The analysis was carried out in CTO-20A (Shimadzu, Japan) high performance liquid chromatography (HPLC) equipment with an UV detector range fixed at 205 nm. All chromatograms were generated by Lab Solutions software. A single Shim pack reversed phase column (250 mm X 4.6 mm, 5µm) was kept at 40° C (Carvalho et al., 2012). The injection volume of 10 µL and the flow rate of 1 mL per min with gradient elution of methanol and chloroform solvent mixture (2:1) were used. The yield of transesterification reaction was calculated by comparing sum of the peak areas of the chromatogram.

3.12.3. Analysis of FAMEs using Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

GC-MS analysis (Gopinath et al., 2009) was performed using Shimadzu QP2010 chromatography with silica column DB-5 ms (length 30m, 0.25mm intra diameter and 0.25 µm film layer thickness) and helium as carrier gas. Gas chromatography has a detection limit of 0.001 ppb. Split injection method with 1:200 ratio was used for gas chromatography separation.

GC-MS program

The gas chromatography programming parameters are as follows; Initial temperature was maintained at 80°C for 2 min, increased to 100°C for 1 minute and maintained at 210°C for 1 minute. Followed by this temperature was increased to 280°C for 5 minutes.
Mass spectrometer analysis was performed with following parameters:

- Potential ionization/electron energy was 70eV
- Ion source temperature was 250°C
- Interface temperature was set to 280°C
- Full mass data was recorded between 0-400 daltons per second.

**GC-MS fatty acid identification**

Fatty acids were identified using Gas Chromatography and Mass Spectrometry. Methyl esters were identified by comparing the mass spectra with the literature data. Carbon number of the methyl esters was determined by calculating molecular ion peak value that appeared in mass spectra. Characteristics of fatty acid methyl esters were most powerful at m/z in the range from 70 to 80. The base peak value of 74 was taken as a constant range for the entire methyl ester samples. The widest peak in each group represented the fragment C\textsubscript{n} H\textsubscript{2n-1}O\textsubscript{2} and in a simpler way can be stated as

\[
C_x = \lfloor (m-74)/14 \rfloor + 2
\]

Where \(x\)=carbon number (FAME), \(m\)=molecule mass that appeared in the peak of mass spectra, Molecule mass of CH\textsubscript{2} is 14 (Knothe et al., 2003).

**3.13. Isolation and cloning of accD gene in selected isolates**

The accD partial gene was amplified using accD forward and reverse primers. The details of all the primers used for accD gene amplification and cloning were listed in Table 6. The PCR reaction mixture and conditions are as follows.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>accD Forward</td>
<td>5’ CTT GGA TYG AAR ATC AAC GAA 3’</td>
<td>accD partial sequence forward primer</td>
</tr>
<tr>
<td>accD Reverse</td>
<td>5' TCH CCT  AAC ATA SCA AAR CT 3’</td>
<td>accD partial sequence reverse primer</td>
</tr>
<tr>
<td>RAGE adapter 1</td>
<td>5’ CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CGG CCG CCC GGG CAG GT 3’</td>
<td>RAGE adapter 5’ end</td>
</tr>
<tr>
<td>(AP-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAGE adapter 2</td>
<td>3’ H2N CCC GTC CA 5’</td>
<td>RAGE adapter 3’ end</td>
</tr>
<tr>
<td>(AP-2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSP3</td>
<td>5’ GTGGTGTTCACAGCAAGTTTTTGCTATGT 3’</td>
<td>3’ end gene specific forward primer</td>
</tr>
<tr>
<td>GSP 3’</td>
<td>5’ GTGTTACAGCAAGTTTTTGCTATGT 3’</td>
<td>3’ end gene specific reverse primer</td>
</tr>
<tr>
<td>GSP 5</td>
<td>5’ CAGCATCTTGTAATCCTGGTCTTCTTT 3’</td>
<td>5’ end gene specific forward primer</td>
</tr>
<tr>
<td>GSP 5’</td>
<td>5’ CATCTTGAATCCTGGTCCTTCTT 3’</td>
<td>5’ end gene specific reverse primer</td>
</tr>
<tr>
<td>accD cloneF</td>
<td>5’ CGGGATCCATGTCATTTCTTTCTT 3’</td>
<td>Subcloning 3’end forward primer</td>
</tr>
<tr>
<td>accD cloner</td>
<td>5’ GGAATTCTATTTAAAAACAGGTCGAAT 3’</td>
<td>Subcloning 5’end reverse primer</td>
</tr>
</tbody>
</table>
**PCR reaction mix**

- 10X buffer : 10.0 µL
- dNTP mixture (2.5mM) : 8.0 µL
- Forward primer : 5.0 µL
- Reverse primer : 5.0 µL
- Taq Polymerase : 1.0 µL
- DNA Template (50ng/µL) : 1.0 µL
- MilliQ water : 50.0 µL

**PCR reaction condition**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Repeat</td>
<td>94</td>
</tr>
<tr>
<td>Annealing</td>
<td>29</td>
<td>65</td>
</tr>
<tr>
<td>Extension cycles</td>
<td>68</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>68</td>
<td>5 min</td>
</tr>
<tr>
<td>Cooling step</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

The amplified accD partial sequence product was eluted with Qiagen gel elution kit procedure and sequenced using Sanger coulson dideoxy method as mentioned previously in the section 3.4.2. The obtained sequence was used for designing Rapid Amplification of Genomic DNA (RAGE) primers to synthesize full length accD gene.
3.13.1. Isolation of full length accD gene of microalgae isolates and vector

**pET-accD construction**

5 µg of DNA isolated from *Neochloris* sp. was digested with blunt end restriction endonucleases such as DraI, EcoRV, DpnI and SspI. The digestion was carried out overnight at 37°C. The digested products were analysed by running through 1% agarose gel. Further DNA fragments were purified by Phenol: Chloroform: isoamyl alcohol extraction followed by chloroform extraction. Then the DNA was precipitated with 80% ethanol and air dried and dissolved in 10 µL of TE buffer. The RAGE adapters were ligated to the 10 µL of digested microalgal genomic DNA,(2 µL of 10X ligation buffer, 5 µM of RAGE adapter, 10 units of T4 DNA ligase (NEB) and 20 µL of sterile milliQ water). Ligation reaction was performed overnight at 16°C.

**RAGE adapters**

The adapter had one long arm and one short arm. The amino group at the 3’ end of the short arm of the adapter prevented any extension from the short arm. The adapter specific primer carried the same sequence as the long arm and would bind only if the priming of the gene specific primer formed a product which extended to the 5’ end of the long arm of the adapter.

**Random amplification of genomic DNA end (RAGE) PCR reaction**

The ligation mixture was diluted to 1:10 ratio and 1 µL of the diluted mixture was used as template for the PCR reaction. The primary PCR reaction was done with the adapter specific primer (AP-1) and accD gene specific GSP 5 primer for 5' end of accD gene. The 3’ end of accD gene was amplified with
GSP 3 primer. The PCR reaction mixture for 3' and 5' ends consisted of following components.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation mixture</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>0.4 μM Primer AP-1</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>0.4 μM gene specific primer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>200 μM dNTP mixture</td>
<td>8.0 μL</td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>5.0 μL</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>50.0 μL</td>
</tr>
</tbody>
</table>

**PCR conditions**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Repeat</td>
<td>94</td>
</tr>
<tr>
<td>Annealing</td>
<td>29</td>
<td>65</td>
</tr>
<tr>
<td>Extension</td>
<td>cycles</td>
<td>68</td>
</tr>
<tr>
<td>Final extension</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Cooling step</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

The PCR products were amplified with nested adapter specific primers AP-2, accD gene specific primer GSP 5' and GSP 3'. Thus RAGE PCR products were confirmed. The PCR reaction mixture for the amplification of 5' end and 3' end consisted of
Primary PCR product : 1.0 µL  
0.4 µM Primer AP-2 : 2.5 µL  
0.4 µM gene specific primer : 2.5 µL  
200 µM dNTP mixture : 4.0 µL  
10X reaction buffer : 2.5 µL  
Taq DNA Polymerase : 1.0 µL  
Sterile MilliQ water : 25.0 µL

**PCR Conditions**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Repeat</td>
<td>94</td>
</tr>
<tr>
<td>Annealing</td>
<td>29</td>
<td>65</td>
</tr>
<tr>
<td>Extension</td>
<td>cycles</td>
<td>68</td>
</tr>
<tr>
<td>Final extension</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>Cooling step</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

The amplified product obtained from the reaction using accD gene specific primer GSP 5' and GSP 3' with adapter primer (AP-2) was purified using QIA quick gel elution kit (Qiagen, USA).

The full length accD gene was generated from RAGE PCR products. Sub cloning primers accD clone F and accD clone R primers that were flanked with BamHI and EcoRV restriction enzyme sequence in terminal region were used. The full length accD gene sequence was obtained after PCR reaction.
Restriction digestion of accD gene and vector

For cloning the accD gene full length accD gene obtained from RAGE PCR and the expression vector pET-32a(+) (Fig. 8) were double digested with BamHI and EcoRV enzymes in separate reactions.

![Fig. 8: Plasmid pET-32a(+) map with BamHI and EcoRV restriction enzyme sites](image)

The reaction mixture consists of

- **Template accD gene (or) pET 32a(+)**: 5 µL
- **BamHI restriction enzyme**: 1 µL (20 units)
- **EcoRV restriction enzyme**: 1 µL (20 units)
- **10X reaction buffer**: 2 µL
- **Sterile MilliQ water**: 20 µL

The reaction mixture was incubated at 37°C for 4 hours. The digested product was dephosphorylated by addition of 0.5 µL of calf intestinal alkaline phosphatase.
phosphatase (obtained from New England Biolabs (NEB) to the digestion reaction mixture after 10 min. The digested products were purified by phenol: chloroform (1:1; v/v) extraction and precipitated using 95% ethanol. The precipitate was washed with 70% ethanol, air dried and dissolved in 20 µL of 0.1X TE buffer.

**Ligation of accD and pET-32a(+) digested product**

The accD gene and pET-32a(+) were double digested with BamHI and EcoRV. Then digested products were ligated to form pET-32a-accD construct.

The ligation mixture consisted of the following components

- **Vector: insert (1:3 ratio)** : 8.0 µL
- **10X ligase buffer with 1mM ATP** : 1.0 µL
- **T4 DNA ligase (NEB)** : 1.0 µL (1 unit)

This reaction mixture was incubated at 16°C for over night.

**3.13.2 Transformation in microalgae cells using glass bead**

Glass bead with 5 micron particle size were used for transformation purpose (Kindle, 1990). 1mL of *Neochloris* sp. cells were added with 2 µL of ligated mixture, 1mg of glass bead (5 micron size) and 0.4mg of PEG and vortexed for 5 minutes and viewed under the microscope for morphological changes. The vortexing procedure was continued for another 5 minutes if no cell damage was observed. After this procedure the *Neochloris* sp. was streaked on BBM medium incorporated with ampicillin antibiotic (100 µg/mL) for selection purpose.

The plasmid pET-32a-accD was extracted using the method described by Birnboim and Doly, 1979 and the method is as follows. 10 mL of exponential phase (16 days old) culture was pelleted out and was resuspended in 250 µL of GTE buffer
(50mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA). After an incubation period of 5 minutes at room temperature, the cells were lysed by adding 200 μL of lysis buffer (200 mM NaOH, 1% sodium dodecyl sulphate [SDS]). Samples were mixed and placed on ice for 5 minutes. The solution was neutralized by adding 150 μL of potassium acetate solution (5 M potassium acetate, pH 4.8). Cell debris and chromosomal DNA were subsequently removed from the solution by centrifugation at 11,000 rpm for 5 minutes. The plasmid DNA was further purified by phenol/chloroform extraction and ethanol precipitation as mentioned earlier. The purified plasmid construct was analysed on 1% agarose gel.

3.13.3. Relative quantification of cDNA using real time PCR

The real time data based on cDNA quantification was conducted for studying the expression of accD gene level in microalgae.

3.13.3.1. Total RNA isolation from microalgal transformants

Total RNA isolation was performed with Trizol reagent (Sigma). 100mg of cloned microalgae sample was homogenized in a prechilled mortar and pestle using liquid nitrogen. The suspension was transferred to 2.0 ml sterile microfuge tube and 1.0 mL of Trizol reagent was added. The tube was kept at room temperature for 5 min. To the mixture 2.0 mL of chloroform was added and vortexed for 15 seconds. Then the tube was kept at room temperature for 15 minutes and centrifuged at 12000 rpm for 15 min at 4ºC. The aqueous phase was transferred into new sterile 1.5 mL microfuge tube and 0.5 mL of isopropanol was added and mixed well. Then the tube was allowed to stand for 10 min at room temperature and centrifuged at 12000 rpm for 10 minutes at 4ºC. The supernatant was removed and the pellet was
washed with 1.0 mL of 75% ethanol. Finally the pellet was dissolved with DEPC treated water. The RNA was confirmed by running formaldehyde agarose gel electrophoresis (Rio et al., 2010).

**Formaldehyde gel for RNA**

1. **DEPC treated water**

   About 2.0 mL of DEPC (Diethyl pyrocarbonate) was added to 1 L of miliQ water and mixed well and incubated at 37°C overnight. The treated water was autoclaved and stored at room temperature.

2. **10x MOPS**

   About 41.2 g of MOPS was dissolved in 800 mL of DEPC treated water and sodium acetate (50mM) was added. The pH was adjusted to 7.0±0.1 with NaOH. About 20 mL of 0.5M EDTA (pH 8.0) was added and the volume was adjusted to 1000 mL with DEPC treated water. The solution was filter sterilized and stored at room temperature.

3. **RNA loading dye**

   This consisted of 62.5% deionized Formamide, 1.14 M Formaldehyde, 1.25 X MOPS buffer, 200 µg/mL bromophenol blue, 200 µg/mL Xylene cyanol and 50 µg/mL Ethidium bromide.

4. **Procedure**

   The gel platform and comb were first wiped with distilled water and then with 70% ethanol. For preparation of 50 mL of 1.4% gel, 0.7 g of agarose was mixed in 43.75 mL water and melted. To this 0.89 mL of formaldehyde and 5 mL of 10 X MOPS buffer were added. Agarose solution was mixed well and poured carefully in gel
platform. The gel was allowed to set at room temperature for 45 minutes. The comb was removed without disturbing the well and placed in the electrophoresis tank with adequate buffer. 10 µL of sample was added with 20 µL of RNA sample loading buffer and mixed well. Just before loading, the sample was heated to 65°C for 10 minutes and then cooled on ice. RNA samples were loaded carefully into the wells. In the electrophoresis tank constant voltage and power supply was maintained. The isolated RNA was later visualized using UV-transilluminator (Rio et al., 2010).

3.13.3.2. Relative cDNA quantification method

PCR reaction mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>2.0</td>
<td>µL</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>6.0</td>
<td>µL</td>
</tr>
<tr>
<td>Real time PCR master mix</td>
<td>10</td>
<td>µL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.0</td>
<td>µL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.0</td>
<td>µL</td>
</tr>
<tr>
<td>Total</td>
<td>22.0</td>
<td>µL</td>
</tr>
</tbody>
</table>

RT PCR program

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature(°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
</tr>
</tbody>
</table>

69
RT–PCR quantification method was an efficient tool in studying the expression level of a particular gene in the cells. The quantification of cDNA was performed in two-step procedure. In the first step, cDNA was prepared from RNA by reverse transcription with random hexamers serving as primers (Morse et al., 2005); This RT-PCR was sufficient for amplification and detection of accD gene (target) and GAPDH gene (reference) using Light Cycler PCR real time software. The crossing point (Cp) value denoted the cycle number of detection threshold for the gene of interest (GOI) from the RT-PCR. Based on these values the expression level of accD gene was identified.