3.0 MATERIALS AND METHODS

3.1 Sample collection

Water samples were aseptically collected from different sites of the solar evaporation salt ponds at Kelambakkam, Near Chennai, in the state of Tamil Nadu, India. Then the samples were brought in laboratory and Dunaliella species were isolated within 24 hrs.

3.2 Isolation and identification of Dunaliella sp.

From the samples marine diatom contamination was eliminated by treating the samples with 5 mgL$^{-1}$ of GeO$_2$ (Germanium- di-oxide) for 3 days (Lewin, 1966) and then the blue green algal contaminant also was eliminated by treating them with 2000 ppm of streptomycin sulfate for 30 min under 30 mmol photons m$^{-2}$·s$^{-1}$ (Rengasamy et al., 1987). Then the samples were used for isolation.

Isolation was made by Serial dilution as follows

- Added about 1mL of sample in 9mL sterilized Dewarln's medium and mixed properly by vortex mixer. This is the first dilution and its gives $10^{-1}$ dilution.
- Then pipetted 1mL of the sample from the first dilution and transferred to another dilution. Like these up to $10^{-9}$ dilutions were made (1000μL for each sample of dilution).
- Dilutions $10^{-3}$ to $10^{-5}$ were chosen for the isolation of different Dunaliella sp. from the samples.
Finally 100µL of the diluted samples were spread on Petri plates containing Dewarln's agar medium and incubated at 20±2°C under continuous illumination (cool-white fluorescent, 1000 lux). The pH of the growth medium was adjusted to 7.5 with 1N NaOH or HCl.

Duplicates were done for each inoculation.

Cells from microcolonies on the Petri plates were isolated by micromanipulation. The algal cells were purified to aseptic conditions by streaking the cells repeatedly on the Dewarln's medium agar plate with 20% NaCl by picked up with sterilized inoculation loop from $10^{-3}$ to $10^{-5}$ of the diluted sample. The purified algal cells were transferred to liquid media. Then alga was axenized and eliminated the bacterial contamination by the triple antibiotic treatment (Droop, 1967). 1ml of the grown algae was transferred to the *Dunaliella* hypersaline medium with 0.86 M NaCl containing 0.02 g·L$^{-1}$ chloramphenicol, and 200 mg·L$^{-1}$ penicillin G for 25 days. The grown algae were streaked on Petri plates containing medium with the same antibiotics. The plates were incubated under 52.84 µmol photons m$^{-2}$s$^{-1}$ provided by the white fluorescent lamps.

Then the algal cells were transferred into liquid medium allowed for the growth of single strain. These liquid cultures were tested for bacterial contamination by plating on bacteriological media. Isolated and purified algal cultures were identified according to morphological description presented by Borowitzka and Siva (2007). After isolation, stock cultures were established for both the species under controlled laboratory conditions in Dewarln's medium under 30 mmol photons m$^{-2}$·s$^{-1}$ light irradiance in a 12:12 (light: dark) cycle and temperature 24±1 °C.
3.3 Medium used for isolation and maintenance of *Dunaliella* sp.

The axenic cultures were maintained in Dewarln's medium (Orset and Young, 1999). The medium was dispensed into 150-ml flasks and sterilized at 121°C at 15 Lb for 15 minutes. Both the *Dunaliella* sp. were inoculated under aseptic conditions in laminar airflow cabinet. Cultures were maintained in Erlenmeyer flasks as well as in agar slants.

**Chemical composition of the Dewarln's medium is as follows:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.14M</td>
</tr>
<tr>
<td>FeCl₃.6H₂O</td>
<td>4.81µM</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>0.54mM</td>
</tr>
<tr>
<td>*Na₂EDTA</td>
<td>0.12mM</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>0.13mM</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.18mM</td>
</tr>
<tr>
<td>ZnCl₂.4H₂O</td>
<td>0.10µM</td>
</tr>
<tr>
<td>CaCl₂.6H₂O</td>
<td>0.08µM</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄.4H₂O</td>
<td>7.28nm</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.08µM</td>
</tr>
<tr>
<td>Sea water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
<tr>
<td>*Agar</td>
<td>20g</td>
</tr>
</tbody>
</table>
*Preparation of Chelated Iron stock solution

Dissolved 10g of Na$_2$EDTA in 500mL of glass-distilled hot water and stirred well to make a solution. 0.81g of FeCl$_3$.6H$_2$O was dissolved in 500mL of 0.1N HCl and was slowly poured to the hot EDTA solution, with stirring. This solution was cooled before adding it to the media.

* Agar was used only for solid medium preparation.

3.4 Media optimization

The following physical and chemical parameters of media optimization was carried out in 250ml conical flask which contains 100ml of AS-100 medium (Vonshak, 1986).

Chemical composition of the AS-100 medium is as follows:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>8.0 mM</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.86 M</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>11.75 mM</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>8.25 mM</td>
</tr>
</tbody>
</table>
Trace metal solution (10.0 mL L⁻¹)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>MnCl₂.4H₂O</td>
<td>7.0 mM</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄H₂O</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Conc. H₂SO₄</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

*Chelated Iron solution (3.0 mL L⁻¹)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>0.09 mM</td>
</tr>
<tr>
<td>FeCl₃.6H₂O</td>
<td>0.06 µM</td>
</tr>
<tr>
<td>Sea water</td>
<td>1000mL</td>
</tr>
</tbody>
</table>

* Preparation of Chelated Iron stock solution:

Dissolved 10g of Na₂EDTA in 500mL of glass-distilled hot water and stirred well to make a solution. 0.81g of FeCl₃.6H₂O was dissolved in 500mL of 0.1N HCl and was slowly poured to the hot EDTA solution, with stirring. This solution was cooled before adding it to the media.

3.4.1 Effect of pH

The effect of different initial pH on the growth and β carotene production by selected Dunaliella species were studied using wide range of pH status. Initial pH of the each medium was adjusted to 7.5 to 9.0 with an increment of 0.5 units. Then
exponentially grown cells of *Dunaliella* species were inoculated into media with equal initial inoculum density (0.2 ± 0.1 × 10⁶ cells mL⁻¹). The cultures were incubated in a constant environment chamber under continuous illumination.

### 3.4.2 Effect of Temperature

Effect of different initial temperature on the production of biomass and β-carotene by selected *Dunaliella* species were studied using wide range 5°C to 25°C. The medium contained flasks were inoculated with equal initial inoculum density (0.2 ± 0.1 × 10⁶ cells mL⁻¹). The cultures were incubated in a thermo controlled growth chamber with continuous illumination. The remaining culture conditions were constant.

### 3.4.3 Effect of Light intensity

Influence of different light intensity on production of biomass and β-carotene by selected *Dunaliella* species were studied. Exponentially grown cells of *Dunaliella* Sp. were inoculated into medium and cultivated under varying light intensities range from 100 to 2000 μmol photon m⁻²s⁻². White light (tubular light, Philips Pvt., Ltd., India.) was used as light source for this study. After carotenogenesis the grown cells were harvested. Biomass and carotenoid contents were estimated. Light intensity was measured with a digital LUX meter (Lutron LX-101-Digital).

### 3.4.4 Effect of NaCl

Effect of different concentration of sodium chloride on production of biomass and β-carotene by selected *Dunaliella* species were studied. Exponentially grown *Dunaliella* species were inoculated into NaCl – free AS-100 medium amended with
different concentration of sodium chloride range from 1.0 to 5.0ML\(^{-1}\). The cultures were incubated in the growth chamber under continuous illumination. The samples were withdrawn for analysis. The division rates were calculated based on cell counts.

### 3.4.5 Effect of Nitrogen sources

Effect of various nitrogen sources such as ammonium chloride, sodium nitrate and urea on production of biomass and \(\beta\)-carotene by selected *Dunaliella* species was studied. In this study nitrogen source of AS-100 medium was replaced with different concentration of these nitrogen source range from 0.1 to 2.0 mM. Then inoculated exponentially grown and N- depleted culture of selected *Dunaliella* species and the cultures were incubated in a constant environment chamber under continuous illumination. The samples were withdrawn for analysis. The division rates were calculated based on cell counts.

### 3.4.6 Effect of Phosphate

To study the effect of phosphate on production of biomass and \(\beta\)-carotene by selected *Dunaliella* species, the media NaH\(_2\)PO\(_4\).2H\(_2\)O was replaced with various concentrations of sodium phosphate range from 0.1 to 1.0mM. Exponentially grown *Dunaliella* was inoculated and incubated in the growth chamber with continuous illumination.

### 3.4.7 Effect of Trace element

Respective metal ion free AS-100 medium was supplemented individually with different concentration (ranging from 10 to 100 \(\mu\)gL\(^{-1}\)) of trace elements such as Iron as
FeSO$_4$. 7H$_2$O, Nickel as NiSO$_4$.7H$_2$O, Selenium as Na$_2$SeO$_3$, Copper as CuSO$_4$.5H$_2$O and Zinc as ZnSO$_4$.7H$_2$O, to study their effect on biomass production and β-Carotene by selected *Dunaliella* species. The trace element supplemented medium was inoculated with exponentially grown *Dunaliella* species and incubated in a constant environment chamber under continuous illumination. The samples were withdrawn for analysis. The division rates were calculated based on cell counts.

3.5 Growth rate estimation

Samples were withdrawn and growth was estimated microscopically using a counting chamber (haemocytometer). Since the algae are motile, one drop of HCl was added to arrest the motility. The specific growth rate (μ) (Division/day) was arrived at using the following formula.

$$\mu_{\text{max}} = \frac{\log N - \log N_0}{\log 2 \times t}$$

Where, log N- final, log N$_0$ - initial and t- time

3.6 Kinetics of substrate utilization

Substrate utilization kinetics of *Dunaliella* species grown in different concentration of different substrates (NO$_3^-$, NH$_4^+$, urea, phosphate, NaCl, Cu, Fe, Ni, Se and Zn) were determined by calculating $V_{\text{max}}$ and $K_s$ values by linear regression analysis using a Hyperbolic software (Hyper.exe, version1.1s, 1996) (Copyright by J.S. Easterby).
3.7 Determination of Dry Weight

The biomass was harvested from the culture broth by centrifugation. An aliquot 10ml was centrifuged at 4,000 rpm for 20 minutes and the microalgal biomass was placed in a preweighed watch glass. The biomass was dried in an oven at 60 °C for 12 hrs. The watch glass with the biomass was weighed and the net mass of the microalgal cells was determined by subtracting the final weight from the initial weight of the watch glass.

3.8 Extraction and Estimation of pigments

3.8.1 Extraction

Algal cells were pelleted by centrifugation at 2000 rpm for 10 minutes. The pellet was washed with AS-100 medium and again centrifuged. To the pellet 90% acetone was added and left in the dark for 1-2 hrs. with frequent shaking. After complete extraction it was again centrifuged at 2000 rpm for 15 min and the clear supernatant was used for spectrophotometric estimation of pigments.

3.8.2 Estimation of Chlorophyll content

The absorbance of supernatant was measured using 10 mm (Width) cuvette at 661.5 nm and 645nm in a double beam spectrophotometer (ELICO-Model SL 150 UV-VIS spectrophotometer) against acetone blank. Concentration of chlorophyll a, b and total content were calculated by the equation of Lichtenhaler (1987) and expressed as \( \mu g mL^{-1} \).
Chl a = 11.24 × OD 661.5 - 2.04 × OD 645.0

Chl b = 20.13 × OD 645.0 - 4.19 × OD 661.5

Total chlorophyll = Chl a + Chl b = 7.05 X OD 645.0 + 18.09 × OD 645.0

3.8.3 Estimation of Carotenoids content

Carotenoids were extracted in acetone as mentioned above and analyzed spectrophotometrically by measuring the absorbance at 480nm. Content of carotenoids was calculated according to method of Strickland and Parsons (1972). Content of carotenoids was expressed as µgmL⁻¹.

Carotenoids content (µgmL⁻¹) = E₄₈₀ x 10 (E- Denotes extinction at respective wavelengths)

3.9 Measurement of photosynthesis

Net photosynthetic rate of newly isolated Dunaleilla species cells which are grown at various culture conditions were measured using a DO (Dissolved oxygen) analyzer (Elico PE135). Temperature was controlled during photosynthetic measurements with an external recirculation water bath. The samples were pre-incubated in the dark for 3 min. Then the algal suspension was stirred magnetically and illumination was provided with a 100 watt incandescent bulb for another 55 min. The light intensity falling on the reaction vessel was equivalent to 75 to 85 watt. m². Light intensity was measured using a Lux meter (Lutron LX-101-Digital).
3.10 Flocculation technique for harvesting of Dunaliella Sp

Flocculation technique was used to separate the motile algal cells from the culture medium. Various flocculants, such as Al$_2$ (SO$_4$)$_3$, FeCl$_3$, and chitosan were employed. In all the experiments, 100mL of the culture was used in a glass-measuring cylinder. An experiment was carried out with the addition of varying concentrations of FeCl$_3$ (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0mM), Al$_2$ (SO$_4$)$_3$ (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2mM) and chitosan (0.01, 0.02, 0.04, 0.06, 0.08 and 0.10 mgL$^{-1}$). During the settling period, turbidity of the sample was measured at 750 nm. The percentage of recovery was calculated using the following formula,

$$\text{Recovery (}\%\text{)} = \frac{\text{OD}_{750}(t) - \text{OD}_{750}(t_0)}{\text{OD}_{750}(t_0)} \times 100$$

Where, OD$_{750}$ (t$_0$) is the turbidity of sample at time zero and OD$_{750}$ (t) is the turbidity at that time (t).

3.11 Viability of cells

Cells viability was determined by the Evans blue method (Widholm 1972). 1 mL samples of each culture was centrifuged at 6000rpm, the supernatant was discarded, added 100 µL of 1% Evans blue solution, and incubated for 10 min at room temperature. The cells were then washed twice in deionized water. Finally, fresh preparations of the centrifuged samples were examined for the viability by light microscopy. Cells with broken cell walls appeared blue, as Evans blue solution diffused into the protoplasm and cell is stained blue.
3.12 Drying of *Dunaliella* biomass

In order to study the relative effectiveness of various drying processes, such as oven drying, sun drying, shade drying and freeze drying were studied, to find the most suitable process for drying and storage of biomass.

### 3.12.1 Sun drying

After harvested the wet biomass of *Dunaliella* species was spread on a thin layer of 0.5cm thickness on a aluminium tray and exposed to direct sunlight (32 ± 3°C) until completely dried. Then the sample was analysed for moisture content and also for the content of pigments. Time taken for complete drying and total loss of carotenoids were recorded and percentage loss of pigments were calculated.

### 3.12.2 Oven drying

This was done using hot air oven. Freshly harvested *Dunaliella* cells were spread as a thin layer in petriplate and placed in the oven at 45 ± 2°C. Then the sample was analysed once in 30 minutes for the content of moisture and content of pigments. Time taken for complete drying and total loss of carotenoids were recorded.

### 3.12.3 Shade drying

Freshly harvested biomass of *Dunaliella* species was spread on a thin layer of 0.5cm thickness on a aluminium tray and placed in the shade place at (20 ± 1°C) until completely dried. Then the sample was analysed for moisture content and also for the content of pigments. Time taken for complete drying and total loss of carotenoids were recorded and percentage loss of pigments were calculated.
3.12.4 Freeze-drying

This was carried out using a freeze drier for 7 hours by spreading the sample in a tray and the samples were analysed for moisture and pigments content after drying.

3.13 Extraction in organic solvents

Carotenoids were extracted from freeze-dried samples of both the *Dunaliella* species biomass using different polar and non-polar solvents such as acetone, methanol, petroleum ether, n- hexane, chloroform, and ethyl acetate. Five volumes of solvent was taken with the sample (5.0gm) in mortar along with glass powder and subjected for mechanical grinding for 2-3 minutes and the same was transferred into Borosil glass tubes and sonicated using MS-72 probe producing diameter 2.0 mm, with 70G horn of amplitude 200µ mss. This process was repeated 2-3 times or till the sample turned pale or white. Whole process was carried out under yellow light in order to minimize the loss due to photo degradation. This was centrifuged at 3000 rpm for 5 minutes and the supernatant was collected and absorbance was measured at 450 nm spectrophotometrically.

3.14 Moisture content

Moisture was estimated by drying a known amount of algal biomass in hot air oven at 70 ± 2⁰C to constant weight. This was cooled in desiccator and weighed. The loss in weight was expressed as percentage of moisture (AOAC, 1997).
3.15 Ash content

A known weight of the algal biomass was initially charred on a tared silica crucible and placed in a muffle furnace at 400- 450 °C for 6 hours till the charred material became white. The dish was allowed to cool to room temperature in a desiccator and reweighed. The difference in weight was taken as total ash content (AOAC, 1997).

3.16 Estimation of protein

Protein concentration of cell-free extract was estimated by the method of Lowry et al., (1951).

Reagents

Reagent A: Dissolved 2 g of sodium carbonate in 100 ml 0.1 N of NaOH solution.

Reagent B: Dissolved 500 mg of CuSO₄·5H₂O in 100 ml of 1% potassium sodium tartrate.

Reagent C: 2 ml of reagent B was mixed with 100 ml of reagent A

Folin- Ciocalteau reagent: Folin phenol was diluted with an equal volume of double distilled water.

Protein standard: 10 mg bovine serum albumin was dissolved in 10 ml of distilled water.
**Procedure**

Cell free protein was extracted by pretreatment of 20 mg freeze-dried biomass was suspended in 10 mL of lysis buffer for 20 min (Hurkman and Tanaka, 1986; Murphy and Prinsley, 1985). Then this well-mixed suspension was diluted with the lysis buffer such that the protein concentration in the diluted mixture was in the range of 0 and 1000 mg L\(^{-1}\). A 0.1 mL portion of this solution was placed in an eppendorf tube and 0.1 mL of SDS solution was added (Dulley and Grieve, 1975; Lees and Paxman, 1972). The resulting mixture was vortexed and added 1mL of Reagent C to the eppendorf tube. The tube was vortexed and after 10 min, 0.1 mL of Folin reagent was added. This was immediately followed by vortex mixing. After 30 min the absorbance of the sample was measured at a wavelength of 660nm by UV-Visible spectrophotometer. The same procedure was followed for the blank preparation.

The spectrophotometric absorbance was converted to protein concentration using a calibration curve established with BSA dissolved in distilled water. The protein content of the biomass was calculated using the following equation (Gonzalez Lopez et al., 2010):

\[
\text{Protein (\%, w/w)} = \frac{\text{CVD}}{m} \times 100
\]

Where, C is the protein concentration (mg L\(^{-1}\)) obtained from the calibration curve, V is the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg).
3.17 Estimation of lipids

Known amount of sample was taken and extracted with low boiling petroleum ether (40-60°C) in a soxhlet extraction apparatus for 8-10 hours. The solvent was evaporated and the lipids were estimated gravimetrically (AOAC, 1997).

3.18 Estimation of total carbohydrate

Total carbohydrate was estimated by Anthrone method (Hedge and Hofreiter, 1962)

Reagents

- 2.5 N HCl
- Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice cold 95% sulphuric acid. Prepare fresh before use.
- Standard Glucose: Stock- Dissolve 100 mg in 100ml water. Working standard 10 ml of stock diluted to 100 ml with distilled water. Glucose (5-25 mg) was used as standard.

Procedure

Hydrolyze the known amount of the sample by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cool to room temperature. Neutralize it with solid sodium carbonate until the effervescence ceases. Make up the volume to 10 ml and centrifuged at 10,000 rpm for 5 minutes. Collect the supernatant and take 0.5 ml and 1 ml aliquot for analysis. Added 4 ml of anthrone reagent and boiled for eight minutes in a boiling water bath. Then cooled rapidly at room temperature and measured
the green to dark green colour at 630 nm in UV-Visible spectrophotometer. The same procedure was followed for blank preparation. The values were expressed in terms of percentage dry weight. The amount of sugars was calculated from standard graph.

3.19 Estimation of ammonia

Residual ammonia was estimated by the phenol hypochlorite method (Solorzano, 1969).

Reagents

1. **Phenol alcohol solution**: 10g of reagent grade phenol dissolved in100ml of 95% ethyl alcohol.

2. **Sodium nitroprusside(0.5%)**: 1g of Sodium nitroprusside dissolved in 200ml glass distilled water and stored in amber bottle.

3. **Alkaline solution**: 100g of tri sodium citrate and 5g of sodium hydroxide dissolved in 500ml glass distilled water.

4. **Sodium hypochlorite solution**: commercial sodium hypochlorite solution (1.5N).

5. **Oxidizing solution**: 100ml of sodium citrate solution and 25ml of hypochlorite solution were mixed and used the same day.

Procedure

To 50ml, 2ml each of phenol and sodium nitroprusside solution followed by 5ml of oxidizing solution were added successively mixing thoroughly after each addition. The colour was allowed to develop at room temperature for 1hr. and the
absorbance was read at 640nm. The OD (optical density) value was interpolated in the standard graph and found the residual ammonia concentration.

3.20 Estimation of nitrate

Residual nitrate was estimated as nitrite after reduction by passing through a Cu-Cd column (Stickland and Parsons, 1972).

Reagents

1. Cadmium granules

2. Cu-Cd: Cd granules were cleaned with dilute HCl (6N) and copperised with a 2% solution of CuSO₄·5H₂O in the following manner.
   a. After washing Cd with 6 N HCl the granules were rinsed with distilled water till the colour of Cd granules was silver.
   b. 25g of Cd were swirled in 100ml portions of 2% CuSO₄·5H₂O solution until the blue colour partially faded, and then decanted. The procedure was repeated with fresh CuSO₄ until a brown colloidal precipitate was formed.
   c. This copperised Cd was washed with distilled water several times (10 to 12 times) to remove all the precipitated Cu. Colour of the treated Cd was black.

3. Ammonium chloride solution: 12.5g of ammonium chloride was dissolved in 50ml glass distilled water. 5ml of this was diluted to 200ml and used as dilute ammonium chloride solution.
Preparation of reduction column

A glass wool plug was inserted into the bottom of a column (20cm X 0.35cm glass tube attached to a funnel) and filled with glass-distilled water. Copperised Cd granules were added to produce a column 18.5cm in length. The column was washed with 20ml of dilute ammonium chloride solution and activated by passing 100ml of solution containing 1mgL⁻¹ NO₃⁻ N standard solution and 2ml of concentration ammonium chloride solution. A flow rate of 7 to 10ml per min was maintained.

Procedure

To 50ml of sample 1ml of ammonium chloride solution was added and passed through the column and collected at a rate of 7 to 10ml.min. The first 25ml was discarded and the rest of the sample was collected. To 3ml of the reduced sample were added 1ml each of NEDD and sulfanilamide solution and left at room temperature. After 20min the colour was read at 540nm. Passing KNO₃⁻ solution through the column at different concentrations drew the standard curve. Column efficiency was tested periodically by running 2, 5 and 10 µM NO₃⁻. The OD value was interpolated in the standard graph and found the residual nitrate concentration was found.

3.21 Estimation of Urea

Residual urea was estimated by modified manual method using diacetylmonoxime reagent (Mulvenna and Savidge, 1992).
Reagents

**Reagent A:** Dissolved 8.5g diacetylmonoxime in 250ml distilled water together with 10ml of thiosemicarbanide solution, 0.95g in 100ml distilled water.

**Reagent B:** 300ml concentrated H$_2$SO$_4$ diluted to 535ml with distilled water together with 0.5 ml Ferric chloride solution (0.15g in 10 ml distilled water).

Procedure

For the analysis, 35ml of sample was placed into a 100ml conical flask specifically reserved for urea analysis and cleaned prior to use by rinsing with 10% HCl followed by distilled water. To each aliquot, 2.5ml of Reagent A was added. The solution was mixed by swirling each flask five times. Immediately following the mixing, 8.0ml of Reagent B was added. Each solution was again swirled five times and the flasks were covered tightly with aluminum foil. The flasks were then kept in dark for 24h in room temperature, adopting room temperature. The absorbance of the sample was measured at a wavelength of 520nm by UV-Visible spectrophotometer. The same procedure was followed for the blank preparation. The OD value of sample was interpolated in the standard graph and found the residual urea concentration was found.

3.22 Estimation of phosphate

Residual phosphate was estimated by colorimetric method

Reagents

- Molybdate reagent: Dissolved 12.5gm of ammonium molybdate in 250mL of 10N sulphuric acid. Then made up 500mL with distilled water. (10N H$_2$SO$_4$ was
prepared by mixing 139mL of concentrated sulphuric acid with 361mL of distilled water).

- Aminonaphthyl sulphonic acid (ANSA) solution: Dissolved 0.5g of ANSA in 198mL of 15% sodium metabisulphite and then added 5mL of 20% sodium sulphite. The solution warmed, stirred well, filtered and stored in a brown bottle before used.

- Standard solution: 87.75mg of NaH$_2$PO$_4$·2H$_2$O was dissolved in 250mL of distilled water. 4mL of this stock solution was diluted to 100mL with distilled water to prepare standard graph.

**Procedure**

1mL of sample was taken and made it up to 9mL with distilled water. Then added 1mL of molybdate reagent followed by 0.5mL of ANSA solution was added. After 10 min the absorbance of the sample was measured at a wavelength of 620nm by UV-Visible spectrophotometer. The same procedure was followed for the blank preparation. The OD value of sample was interpolated in the standard graph and found the residual phosphate concentration was found.

3.23 Estimation of heavy metals

Residual heavy metal (Cu, Fe, Ni, Se and Zn) concentration was analyzed by direct aspiration of the sample solution into a Perkin-Elmer model 2380 flame atomic absorption spectrophotometer (AAS). All metals were analysed using lean-blue air acetylene flame.
3.24 Statistical Analysis

The mean of three replicates was taken for all the experimental conditions for statistical analysis by Software - MINITAM Release 12.2. The Pearson product moment correlation coefficient calculated to measure the degree of linear relationship between the variables. The correlation coefficient assumes a value between -1 and +1. If one variable tends to increase as the other decreases, the correlation coefficient is negative. Conversely, if the two variables tend to increase together the correlation coefficient is positive.