Chapter 03

Materials and Methods

3.1 Dairy Waste Water Collection and Preservation:

The dairy effluent or dairy waste water was collected in disinfected bottles, from the milk processing plant named as Parag, situated at Mansurpur, Muzaffarnagar (UP), India, which used in this research work and stored at 4°C until utilized.

3.2 Physicochemical Analysis of Dairy Waste Water:

Firstly, filtration process is applied to remove all the suspended particles present in dairy effluent and afterwards, the physicochemical analysis was carried out by following water analysis standard protocols i.e., APHA, 2005.[151] While heavy metals were identified by ICP-MS. The dairy effluent sample was remediated with microalgae, to enhance the quality of effluent. The treated dairy waste water was again subjected to physiochemical analysis to draw comparative analysis of physicochemical properties of raw and treated water so as to assess the potential of microalgal species to treat dairy wastewater. For perceiving the depletion of nutrients, these following parameters were taken into consideration; pH, BOD, COD, total nitrate and total phosphate, before and after utilizing microalgae for remediation.

3.2.1 Colour and pH:

Dairy effluent sample was visually examined for colour. pH was measured by the use of digital pH meter. Standard buffer solution possessing pH 7 was utilized to calibrate the pH meter. To determine pH, dairy effluent was taken in a clean and dry beaker into which pH electrode was placed with the tip of electrode 6-8 cm dipped into the sample. pH depicted by the meter was recorded.

3.2.2 TSS (Total Suspended Solids):

To determine the TSS of the dairy wastewater, it was filtered with the help of filter paper and dried thoroughly. The filter paper was weighed before the sample was filtered. After filtration of the sample the filter paper was again oven dried. The TSS was estimated by the following formula:

TSS (mgL^{-1}) = Initial weight of paper-final weight of filter paper

3.2.3 TDS (Total dissolved solids):

Waste water was filtered to eliminate suspended particles. 250 ml of clear filtrate was dried at 100°C, in microwave oven, in the petri plate. TDS was estimated by the following formula-

$$TDS\ (mgL^{-1}) = \frac{T2-T1\times1000}{W}$$

Where,

T1= weight of empty Petri plate

T2= weight of oven dried Petri plate

W= volume of waste water taken (ml)

3.2.4. TS (Total Solids):

Total solids were calculated by addition of individual values obtained for TSS and TDS.

$$TS\ (mgL^{-1}) = TSS + TDS$$

3.2.5. DO (Dissolved Oxygen):

- Test sample were filled in a stopper BOD bottle of 100-300 ml. While pouring the sample and placing the stopper care should be taken to avoid any bubble formation.
- 1 ml of manganese sulphate and one ml of alkaline potassium iodide solution were added to the BOD bottles (carefully add the solution below the surface from the walls).
- In the next step the neck of the bottles was closed by the topper, bottles were shaken well. Bottles were left undisturbed so as to let any precipitate settle. If titration has to be conducted for a few days in that condition the sample can be stored at this stage of precipitation.
- For titration of sample a conical flask was taken to which 50-100 ml of sample was added again, taking care to avoid any air bubble.
- The sample should be titrated within 1 h of dilution of precipitates titration of sample was conducted against sodium-thio-sulphate solution, starch solution served as indicator. The initial dark blue colour turns to colourless, at the end point.

Calculation:

$$DO\ (mgL^{-1}) = [(ml \times N) \text{ no. of titrant} \times 8 \times 1000] \div V_1\text{-}V$$

Where, V_1= volume of waste water bottle after putting the stopper.

V = volume of manganese sulphate ($MnSO_4$) and potassium iodide (KI) added.

3.2.6. BOD (Biological Oxygen Demand):

Required Reagents:

$MgSO_4$ solution: 2.25 g MgSO4 (manganisium sulphate) dissolved in 100 ml of dH_2O.

$CaCl_2$ solution: 2.75 g $CaCl_2$ (calcium chloride) dissolved in 100 ml of dH_2O.

Phosphate Buffer (pH 7.2): 33.4 g $Na_2HPO_4.7H_2O$ (di sodium hydrogen phosphate), 21.75 g K_2HPO_4 (di potassium hydrogen phosphate), 1.7 g NH_4Cl (ammonium chloride), and 8.5 g KH_2PO_4 (potassium di hydrogen phosphate) were dissolved in 1L distilled water.

$FeCl_3$ solution: 0.2 g ferric chloride in 1L999 distilled water.

Sodium sulphite solution: 1.575 g $NaSO_3$ dissolved in 1L distilled water (0.025 N).

Procedure:

- DE sample was diluted by using aerated water.
- Total two sets were made ready, one set use as a blank while second for effluent.
- The second set for both blank and effluent were kept at 20^o C for 5 days' incubation.
- Each set was added with 2 mL $MnSO_4$ and 2 mL KI solution and blended thoroughly.
- Formed precipitate was dissolved by adding 2 mL concentrated H_2SO_4. A 50 mL of above effluent was poured into a flask and titrated against 0.0125 N Thio sulphate solution by applying starch indicator.
- BOD was estimated by using the formula.

$$\text{BOD mg/lit} = R \times 0.1 \times \text{Dilution Factor} \times 20$$

Where, $R = (SI - SF) - (BI - BF)$

SI – Sample Initial, SF- Sample Final

BI – Blank Initial, BF- Blank Final

3.2.7. COD (Chemical Oxygen Demand):

- A 20 ml of dairy effluent was taken into a flask of COD reflux unit.
  - 10 ml of 0.25 N $KMnO_4$ was added to the above effluent.
  - Shake well, after that add a pinch of mercuric sulphate.
  - Continue procedure with addition of 30 ml of H_2SO_4 in solution and place it into a water bath. Allowed it to stand for half an hour.
  - Contents of the flask were digested in a reflux condenser for 2 h.
  - The reflux condenses was disconnected and the mixture was diluted to 2 times the earliest volume by putting on distilled water in to this effluent. And the solution was allowed to cool at 28^0C and subsequently titrated reagent $K_2Cr_2O_7$ using 0.1m FAS; few drops of ferroin indicator.
  - Conversion of solution's colour from bluish green to red.
  - Reflux blank with the similar process using distilled water instead of sample.

$$\text{COD as mg } L^{-1} = (a - b) \times N \times 8000/ml\ sample$$

Where, a = volume in ml ferrous ammonium sulphate utilized for blank bottle

b = volume in ml ferrous ammonium sulphate used for effluent

N = normality of FAS

8000 = milli q water of $O_2 \times 1000$

3.2.8. Metals Analysis:

Metal analysis was done through ICP-MS method, Sample preparation is being followed in the Cytogene General Lab-ICPMS (Model; PerkinElmer SCIEX ELAN DRC-e), dairy effluent sample.

- Teflon crucible was washed with deionized water and 1N HCl.
- 0.1 g whole rock powdered sample was weighted and transferred into Teflon crucible and properly labeled.
- 5 ml HNO_3 and 10 ml of distilled water were added in ratio 1:2 to the effluent and put down on hot plate at 120°C (time).
- The sample further dries with 10 ml of HNO_3 repeatedly to ensure complete removal of fluoride.
- The process is repeated 3-4 times depending upon the matrix. Finally, clear sample aliquot ensures the complete dissolution.
- The sample aliquot is dissolved in 10 ml of 2N HNO_3 and the final volume is prepared up to 100 ml through addition of dH_2O.
- The prepared sample was stored in a cleaned PP container with proper labeling.
- The sample was subjected to geochemical analysis, to measure concentration of tracer elements.
- Apply data reduction and delivery to the user.

3.2.9. Nitrate Analysis:

Required reagents and apparatus:

- Nitrate free or de-ionised water was used for preparing solutions.
- **Nitrate Stock solution:** 0.7218 g $KNO_{3:}$ Dissolve in dH2O and dilute to one litter. conserve by adding two mL of chloroform per L; 1 mL= 100 µg NO_3^{-1}, shelf life up to 6 months.

- **Nitrate Standard Solution:** 100 mL of stock solution dilute to 1L with dH2O, store by adding two mL of chloroform per L; 1 mL = 10 µg $N\text{-}NO_3^{-1}$, shelf life up to 6 months.
- **Nitrate Standard Solution:** 100 mL of stock solution dilute to 1L with dH2O, store by adding two mL of chloroform per L; 1 mL = 10 µg $N\text{-}NO_3^{-1}$, shelf life up to 6 months.
- **HCl Solution, (1N):** Carefully added 83 mL conc. Hydrochloric acid to approximately 850 mL of dH2O and dilute to 1000 ml.
- Spectrophotometer

Procedure:

- **DE Treatment**: Add one mL hydrochloric acid to 50 mL DE and mix it properly.
- **Standard curve preparation:** calibration standards prepared between the stretch of 0-7 mg nitrate per 1000ml, dilute to 50 ml by adding volume of standard solution, and adding one ml of hydrochloric acid and mix it properly.
- Read absorbance against dH_2O set at 0. And 220 nm of wavelength used to obtain NO_3^{-1} to estimate intrusion because of dissolved organic substances.
- For calculation and standards, subtract the OD at 275 nm, from the OD taken at 220 nm to observed optical density because of NO_3^{-1}.

3.2.10. Phosphate Analysis: (Ascorbic acid method)

Required reagents and apparatus:

- 5N Sulphuric acid: Dilute 70 mL conc. Sulphuric acid to 500 mL dH_2O.
- Potassium antimony tartrate solution: Add 1.3715 g of potassium antimony tartrate in 400 ml dH_2O and dilute to half litre (500 ml), stored in glass bottle.

- $(NH_4)_26Mo_7O_{24}.4H_2O$ solution: Add 20 g of $(NH_4)_26Mo_7O_{24}.4H_2O$ in half litre (500 ml) of dH_2O, stored in a glass bottle.
- Combined reagent: Mix 5 mL $(K(SbO)C_4H_4O_6.1/2H_2O)$, 15 mL $(NH_4)_26Mo_7O_{24}.4H_2O$, 30 ml ascorbic acid and 50 mL 5N, H_2SO_4, kept this at room temperature for 4 hours, to make it stable.
- Phosphate stock solution: 219.5 mg of KH_2PO_4 (anhydrous), dissolve in dH_2O, 1 ml= 2.5 µg P.
- Spectrophotometer

Procedure

- Dairy effluent remediation: add 5 mL sample in to test tube, one drop of phenolphthalein indicator added in to it. Discard all red colour by adding 5 N sulphuric acid, then, add 0.5 ml of mixed reagent and shake well properly.
- After 20 min, measured absorbance of all samples of DE at 880 nm. While blank used as reference.
- Calibration curve preparation: calibration curve prepared from the range of standard between 0.15-1.30 mg L^{-1}. Use dH_2O blank with the combined reagent. After that plotting a graph by taking OD against the phosphate concentration.

Calculation:

$$\text{Phosphate } (mgL^{-1}) = \frac{\text{P from the calliberation curve} \times 1000}{\text{Effluent volume (ml)}}$$

3.3.1 Isolation of Microalgae from Dairy Wastewater:

The bold basal media is commonly utilized for cultivation of microalgae species. The media contain macronutrients such as Sodium nitrate, Di potassium hydrogen phosphate, Calcium chloride, Magnesium sulphate, Sodium chloride, Potassium di hydrogen phosphate, and micro-elements in trace amount; Boric acid, Manganese chloride, Sodium molybdate, Zink sulphate, Cobalt nitrate, and Copper sulphate etc. which are suitable for microalgae growth. [57,152]

3.3.2. Growth Media Preparation:

The composition of BBM is presented by the following Table no 3.1:

Table 3.1: Stock solutions of components utilized for preparation of Bold's Basal Medium

Stock Solution (BBM)	Volume (ml)	Components	Concentration (in stock solution)
1	10 ml	$MgSO_4.7H_2O$	0.75 g in 100 mL^{-1}
2	10 ml	$NaNO_3$	2.50 g in 100 mL^{-1}
3	10 ml	NaCl	0.25 g in 100 mL^{-1}
4	10 ml	KH_2PO_4	1.75 g in 100 mL^{-1}
5	10 ml	$CaCl_2.2H_2O$	0.25 g in 100 mL^{-1}
6	10 ml	K_2HPO_4	0.75 g in 100 mL^{-1}
7	10 ml	$ZnSO_4.7H_2O$	8.82 g in 100 mL^{-1}
Trace Elements	1 ml	$CuSO_4.5H_2O$	1.57 g in L^{-1}
		$Co(NO_3)_2.\ 6H_2O$	0.49 g in L^{-1}
		MoO_3	0.71 g in L^{-1}
		$MnCl_2.4H_2O$	1.44 g in L^{-1}
	1 ml	H_3BO_3	1.14 g in100 ml^{-1}
	1 ml	$EDTA.Na_2$	5.0 g in 100 ml^{-1}
		KOH	3.1 g in 100 ml^{-1}
	1 ml	$FeSO_4.7H_2O$	4.98 g in 1 L^{-1}
		$Conc.H_2SO_4$	1 ml (to acidify)

Stock solutions of different components were prepared and stored at 4°C. By addition of appropriate volume of each component the Basal Bold medium was prepared.

3.3.3. Culture Conditions:

The 10 ml of DWW was taken in to a 250 ml capacity conical flask, which contains 100 ml, BBM media (Bold's Basal Media). The pH of the broth was maintained 7.0 ± 3. Isolated microalgae strains, cultured in liquid BBM media at 26 ± 2 °C and illuminated with irradiance of 60 µmol m^{-2} s^{-1}, proper mixing at 100 rpm for fifteen days along with 12/12 h day-night regime. The culture was continuous supplied by CO_2 and air with a diffusion speed of 100 ml/min and 250 ml/min, individually. Some antibiotics, chloramphenicol (50 µg/ml) and ampicillin (100 µg/ml) added in to culture, to inhibit microbial growth.[9] The mixed microalgae strains, were appeared on broth, after 15 days. The successive streak plate procedure (with 100 µL culture) was followed for the isolation of pure or individual microalgae strains from miscellaneous culture. Repeated streaking was carried out on agar plate of Bold's Basal Medium, by using the above same cultivation conditions. The bacteria-free, individual microalgae colonies were obtained onto Bold's Basal Medium agar plates, after repeated streaking. Then microscopic analysis was done for morphological identification, by following Bellinger and Sigee.[153] After 15 days, when pure microalgae reach at the exponential phase, which gathered by centrifugation process at 3000 rpm for 10 min and utilized for dairy effluent or DWW treatment purposes.

3.3.4. Experimental Design:

The growth of isolated microalgae was optimized by using several dilutions of dairy effluent with Bold's Basal Media (BBM) [25% concentration (25 ml dairy effluent +75 ml Bold's Basal Media), 50% concentration (50 ml dairy effluent + 50 ml Bold's Basal Media), 75% concentration (75 ml dairy effluent + 25 ml Bold's Basal Media), 100% concentration (100 ml dairy effluent) and

Control (100 ml Bold's Basal Media)]. The growth was examined in terms of chlorophyll, at every third day up to the 15 days of batch cultivation. The OD was determined at 665 nanometre by applying Marker *et al* method.[154] Physicochemical properties of dairy effluent after treatment were measured and compared with untreated effluent.

3.4. Nutrient Removal Efficiency:

All isolated microalgae species were individually inoculated into flasks containing 100 mL autoclaved growth media. Which contain different dilutions of dairy effluent or dairy waste water (DWW) with BBM [25% concentration (25 ml DWW +75 ml Bold's Basal Media), 50% concentration (50 ml DWW+ 50 ml Bold's Basal Media), 75% concentration (75 ml DWW + 25 ml Bold's Basal Media), 100% concentration (100 ml dairy waste water) and Control (100 ml BBM)]. microalgae cultivated in growth media at 26 ± 2 °C and illuminated with irradiance of (60 µmol m^{-2} s^{-1}), continuous mixing (to avoid the biofilm formation of microalgae culture) at 100 rpm till to the end of batch culture i.e, 15 days, 12/12 h light-dark regime. Microalgal cultivated in BBM media was taken as control. Physicochemical properties of dairy effluent after treatment were measured and compared with untreated effluent. Microalgae was cultivated in different concentrations of growth media. After 15 days of microalgae cultivation in dairy effluent, removal percentage was determined. The 100 ml of culture (broth) was drawn off from all photo-bioreactor at every alternate day, to examined for chemical oxygen demand, nitrate, and phosphate reduction efficiency. The nutrient removal or eradication efficiency and reduction rate was determined by the using equations no. (iv) and (v). [9, 155, 156]

Removal rate (RR) (mg/L/d) = $(C_0 - Ct)/ (t_i - t_o)$ ……. (iv)

Removal percentage (RP) = $[(C_0 - C_t)/ C_0] \times 100$ …… (v)

Where, C_0 = initial concentration and C_t = final concentrations at time t_o (at 0 day) and t_i (at 15th day) respectively.

3.5. Estimation of Microalgal Biomass:

The microalgae suspension was centrifuged at 5000 rpm for 15 min. Further it was made to dry for 24 hours at 60°C. Thus, obtained dried biomass was measured gravimetrically. Microalgal cell growth was estimated by determining DCW and growth rate. Growth rate was monitored in dairy effluent and BBM media with different dilutions using as growth media. Absorbance was observed at 680 nm and values were converted to DCW (gL^{-1}), according to an earlier reported method.[9] A calibrated equation considering OD and DCW of microalgal cells for calculated biomass concentration by following the following Eq. no (i)

$$\text{Biomass concentration } (gL^{-1)} = \alpha \times Ab_{680} + \beta$$

Where, A_{680} is the density of microalgae at 680 nm and α and β are coefficients in equation.

At the 15th day, microalgal biomass was gathered, by ultra-centrifugation process at 6000 rpm for 15 min. Then, drying at 60 ^{0}C and after that dry cell weight was measured.

During the batch culturing, microalgae biomass productivity was calculated by following the Equation number (ii)

$$BP = (N_t - N_0)/ (t_t - t_0)$$

where, BP = Biomass productivity N_t = terminal yield of microalgae biomass (g L−1) at the end of batch cultivation, (t_t) and N_0 = initial amount of microalgae biomass (g L^{-1}) at t_0 (zero days)

The Specific growth rate (μ, d^{-1}), determined by using the Equation number (iii)

$$\mu = \ln (W_t/ W_0)/\Delta t$$

where, W_t = final dry cell weight and W_o, initial dry cell weight, Δ t = change in time (days)

microalgal cell doubling time (T_d), examined by following the equation no (iv)

$$(T_d) = \ln (2)/ \mu$$

3.6 Extraction and Identification of Microalgal Lipids:

Microalgae biomass was gathered by using ultra-centrifugation process at 6000 rpm for fifteen min, after 15 days of batch culture. Then, it was bathed in dH_2O to get rid of salt impurities and oven dried at 60 °C and mentioned as mg l^-. After that, microalgal lipid extraction process, accomplished by utilizing (1:2 v/v), a solution of $CHCl_3/CH_3OH$, by following the Bligh and Dyer protocol.[41, 157] The (1:2 v/v) of CH_3OH and $CHCl_3$, mixed into the dry microalgae biomass and kept at huge pitch soundwaves for disrupting intramolecular interactions, for about 10 minutes. The obtained slurry was centrifuged to eradicate all the dead cells and supernatants, transferred into the fresh test tube. $MgCl_2$ (0.034%) was added into the supernatant and after that centrifugation at 5000 rpm for 10 min. 2 N KCl was added to the lower phase of the microalgae culture and again centrifuged at 5000 rpm for 15 minutes. Again, organic solvent i.e., chloroform: methanol: water (3:47:48) was added to the lower phase followed by the process of centrifugation for 5 to 10 min. at 5000 rpm and the lower phase was collected. Lipid fraction was isolated by using separating funnel, from the chloroform layer. Anhydrous Na_2SO_4 (2 g), mixed into the lipid fraction, for removing wetness. Then, extracted microalgae lipid content, poured in to the other pre-weighted, round bottom flask. Microalgal lipid's weight was observed after evaporating $CHCl_3$. The lipid content expressed as the % percent composition of DCW of microalgae by using the Eq. no (v)

$$\text{Lipid Content \%} = \text{mass of lipid (g)} / \text{mass of microalgae (g)} \times 100$$

Lipid productivity was estimated by following the Equation number (vi).

$$[LP = BP\times LC /100]$$

Where, LP = stands for microalgal lipid productivity ($mgL^{-1}d^{-1}$), BP = stands for biomass productivity ($mg\ L^{-1}d^{-1}$). While, LC = stands for lipid content percent (w/w).

The long chain free fatty acids or neutral lipids, identified and quantified by following GC-MS technique.[1] This process done by using the GC-MS QP2010 model (Shimadzu®), during this process, microalgae sample (lipids) was dissolved in Methyl alcohol and injected into the GC-MS column. The following operating conditions of GC-MS were used in analysis: first, 45 °C oven temperature for 2 min then enhanced up to 140 °C and finally reached to 280 °C by increasing 5°C per min and held for 10 min. The 2 µL sample was injected through helium gas with 1 mL/min flow rate. The sample components ionization was carried out 70 eV. The GC running time range was from 9.10 min – 52.0 min. NIST14.L library 2020 was searched for the comparison of unknown compounds with already existed compounds in NIST library. Hence, identification of unknown compounds on the basis of their retention times and mass spectra with already available compounds in the NIST database.

3.7. Molecular identification of isolated microalgae:

3.7.1. 18SrRNA sequencing:

- 18SrRNA sequences are used to study the phylogeny and taxonomy of isolated microalgae. It is widely used technique provide the information of genes and species name of the identical isolates. For identification of isolated microalgae species, first, gDNA was isolated from the pure culture by following the Smoker and Barnum method.[158].

DNA Extraction Procedure:

- Microalgae culture was micro-centrifuged at 14,000 rpm for 5 min. and homogenized by using 1 ml of extraction buffer and after that it was transferred in to the 2 ml-microfuge tube.
- The ratio 25:24:1 of (Phenol: Chloroform: Isoamly alcohol) was added into the microfuge tubes and mixed well.
- Microfuge tubes were centrifuged again for 15 min at 13,000 rpm.
- The upper phase was separated and shifted into the next tube and the ratio (25: 24:1) of phenol: Chloroform: Iso-amly alcohol was mixed again and shake well thoroughly.
- Again, the upper phase separated after centrifugation at 25^0C for 15 min at 14,000 rpm. Then it was transferred into a next tube.
- For the precipitation of DNA, add 0.1 ml of 3 M CH_3COONa with pH 7.0 and 0.7 ml of Isopropanol in to the solution.
- Incubation for 15 min, the tubes were centrifuged at 14,000 rpm, at 4ºC for 10 min.
- The DNA pellet washed two times, first with 70% ethyl alcohol and after that by using 100% ethyl alcohol and dried in air.
- The DNA pellet was dissolved in TE (Tris EDTA) buffer.
- 5 µl of RNAse A (10 mg/ml) mixed for the removal of RNA.
- An extracted DNA was isolated by means of 1.0 % Agarose gel electrophoresis. PCR was performed for the refined DNA using primers.

3.7.2 Amplification of DNA:

The amplification of 18S r DNA region in 10µl of reaction mixture contains,

Master mixture (taq polymerase, dNTPs, 2x buffer with Mg^+) - 7 µl

18s primers (forward and reverse) - 1 µl

Template DNA - 2 µ

Total reaction volume - 10 µl

Forward and reverse primer sequence which involved in the amplification of ITS rDNA sequence:

18s rDNA primers (Microalgae universal primers were used)

18s Forward primer : 5' GTAGTCATATGCTTGTCTC 3'

18s Reverse Primer : 5’ CTTCCGTCAATTCCTTTAAG 3’

The Sequencing Mixture Constitution and PCR Process Limitations are as follows:

PCR Condition:

Initial Denatueration (Temp: Time)	**Denaturation (Temp: Time)**	**Annealing(Temp Time)**	**Extension (Temp: Time)**	**Extension(Temp Time)**	**No. of Cycles**
95°C for 3mir	95°C for 30 sec	50°C for 30 se	72°C for 45 sec	72°C for 3 mir	35

Sequencing Reaction Composition:

10µl Sequencing Reaction

- Ready Reaction Mix (Big Dye Terminator): 4µl
- Milli Q Water: 3µl
- Primer (10pmol/λ): 2µl
- Template (100ng/ul): 1µl

For the molecular identification of the isolated microalgae sp., the 18S rRNA gene fragments were amplified with using the 18SrRNA-forward and 18SrRNA-reverse primers, (18S-

Forward primer: (5'GTAGTCATATGCTTGTCTC3'); 18S-Reverse primer: (5'CTTCCGTCAATTCCTTTAAG3') by following standard polymerase chain reaction procedure.[159] For polymerase chain reaction amplification needed, 10 µL reaction mixture containing; Big Dye Terminator Ready Reaction Mixture: 4µL, oligonucleotide primer: 2 µl; DNA template: 1 µl, Milli Q Water: 3 µl (all kits supplied by Himedia, India pvt. Ltd.). The thermal process was carried out at 95 °C temperature for 3 min, which continued through denaturation process at 95 °C for 1minute, annealing process at 50 °C, after that elongation process at 72 °C (35 cycles) and last continued by three minutes' extension at 72 ^{0}C. it was interpreted on 1.0% agarose gel as a single band after that polymerase chain reaction amplicon purified with the PCR purification kit. Then, sequencing reaction was carried out by using 18S rRNA-F and 18S rRNA-R primers (18S-Forward primer: (5'GTAGTCATATGCTTGTCTC3'); 18S-Reverse primer: (5'CTTCCGTCAATTCCTTTAAG3') utilizing Cycle sequencing kit, with the help of ABI 3730xl DNA Analyzer.

3.7 .3. BLAST (Basic Local Assignment Search Tool):

18SrRNA sequences were analyzed by BLAST tool. BLAST is the most common tool which is used to align and examine the protein as well as DNA sequences. BLAST program has many variants. BLAST p is utilized to compare the database of protein sequence with the query protein sequence. BLAST n is utilized to compare the database of nucleotide sequence with the query nucleotide sequence. BLAST X is used to compare the query nucleotide sequence against the database of protein sequence. BLAST mainly finds the regions of homology within the sequences and calculate the statistical significance of matches. It is used for the study of evolutionary relationships between members of family. Steps of analysis are shown as;

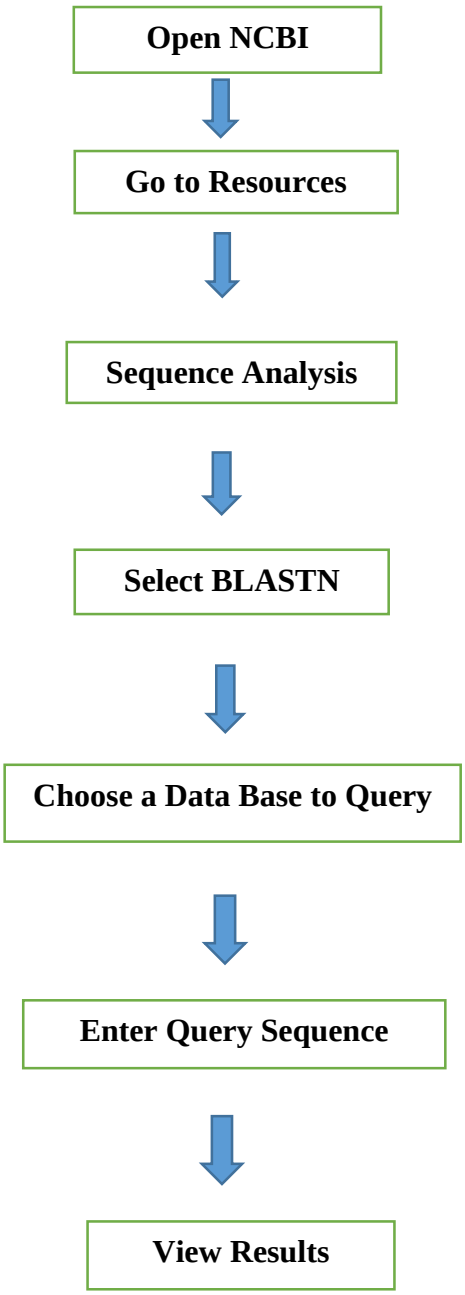

Figure 3.1: Steps of BLAST N Analysis

BLAST (Basic Local Alignment Search Tool) is a widely used bioinformatics tool for comparing nucleotide or protein sequences against a database to find similar sequences. The "BLAST N" analysis specifically refers to using BLAST for nucleotide sequence comparisons.

The following steps typically involved in a BLAST N analysis:

1. **Input Sequence**: The first step involves providing the nucleotide sequence which researcher need to compare against the database. This sequence could be DNA or RNA, and it represents the query sequence that you want to find matches for in the database.
2. **Database Selection**: Next, the selection of appropriate database against which one want to search for sequence similarities. The database contains a vast collection of nucleotide sequences from various organisms, including genes, genomes, and other nucleic acid sequences.
3. **Algorithm Selection**: BLAST offers different algorithms for sequence alignment, each with its own advantages and limitations. Researcher would choose the appropriate algorithm based on factors like the length of the query sequence, the size of the database, and the desired sensitivity of the search.
4. **Scoring Parameters**: BLAST allows the researcher to adjust various scoring parameters to customize the search sensitivity and specificity. These parameters include match scores, mismatch penalties, gap costs, and word size. Fine-tuning these parameters can optimize the accuracy and speed of the sequence alignment process.
5. **Sequence Comparison**: Once the input sequence, database, algorithm, and scoring parameters are specified, BLAST performs a sequence comparison using a heuristic approach. It identifies regions of similarity (alignments) between the query sequence and sequences in the database.
6. **Alignment Output**: BLAST generates an output that lists the alignments found between the query sequence and sequences in the database. Each alignment includes information about the degree of similarity, alignment length, sequence identity, and alignment score.
7. **Statistical Analysis**: BLAST provides statistical measures to assess the significance of the sequence similarities observed. These measures include E-values, which estimate the number of chance matches expected in a database of a given size, and bit scores, which quantify the strength of sequence similarity.

8. **Result Interpretation**: Finally, interpret the BLAST results to identify sequences in the database that are similar to researcher's query sequence. One could further analyze these matches to infer functional or evolutionary relationships, annotate genes, or explore sequence variations.

Overall, BLAST N analysis allows researchers to efficiently search large nucleotide sequence databases to identify sequences of interest and gain insights into the biological significance of sequence similarities.

The results obtained were evaluated for the following points:

1. Max score- highest alignment score
2. Tot score: sum of alignment score
3. Query cover: query length percentage
4. E value: No. of alignment expected by chance with calculated score.
5. Identity: The highest percentage identity for aligned sequence.

3.8. Phylogenetic Analysis:

To find out the phylogenetic connection of isolated microalgae species, developed standard sequences of 18S rRNA was lined up with multiple sequence alignment by utilizing Clustal W software program. Evolutionary relationships of texa were evaluated by using the Neighbour-joining method (system software aligner) which issued evolutionary and attribute-based homology.[160]

Jukes-Cantor method used to examine the genetic stretch between two (02) sequences.[161] Weighbor, distance-based tree was utilized for understanding the difference between nucleotides. Jukes and Cantor formula was used in determining the distance. While creating the distance matrix, only alignment positions are utilized, whereas the alignment inserts are neglect and 200 was the lowers comparable position. The Weighbor tree was generated with 4 alphabet size and 1000 length size. Bootstrap technique, which utilized for calculating the sampling distribution by resampling replacement with the original one. when creating evolutionary relationships, based on thousand replicates, followed for the determination of heartiness of the evolutionary tree.[162]