Chapter 4

Material and Methods

Large amount of wastewater is generated, mainly due to anthropogenic activities, such as agricultural practices, urbanization, and industrialization. Water bodies receive pollution from many different sources, which vary both in quality and quantity depending on its source of generation such as; food industry, tannery, brewery industry, iron and steel industry, dye industry, nuclear industry, olive oil mills and textile industry etc. The relative severity of water quality varies according to a waste discharge from different sources, population growth and density, the extent of industrialization and economic situation. Various Physico-chemical treatment methods require a high amount of chemicals, energy, and manpower, but the biological treatment of wastewater is considered more eco-friendly and sustainable approach. However, these methods are costly and produce large amounts of sludge contaminated with chemical compounds, requiring further treatment. To overcome the drawbacks associated to the commonly used tertiary treatment methods, biological treatment using microalgae has been extensively studied in the last decades. Microalgae offer an alternative treatment approach in biological treatment as they are capable to remove nutrients and convert them into biomass. They comprise a large group of microorganisms accounting for >50,000 species living in diverse freshwater, brackish water, seawater, and wastewater environments. Different microalgae have been reported for successful removal of contaminants and bioremediation of effluents. There are different species of microalgae capable of growing effectively in polluted water, through their capability to utilize abundant organic carbon, inorganic N & P and toxic metals found present in polluted water and thus they can play an important role particularly during the final (tertiary) treatment phase of wastewater. Typically, a wastewater contains a rich supply of nitrogen and/or phosphorus, toxic organic pollutants, heavy metals etc. Direct discharge of wastewater into a water body can result in severe eutrophication and even vital disorder of the ecosystem. Thus the use of wastewater as a nutrient source seems to be an inevitable option when culturing microalgae for biofuel production. The significant advantage of algal processes in wastewater treatment over the conventional chemical-based treatment methods is the

potential cost saving and the lower level technology that is utilized, therefore making this approach more effective. Thus this research study is based on wastewater treatment using microalgae, which would help to reduce the cost of freshwater resource and simultaneously remediates the contaminated water and after remediation biomass can be used for biodiesel production.

4.1 Sampling sites and its location

Water is a most essential component of the environment, required for sustenance and survival of life on this planet Earth. Mismanagement of natural water resources coupled with population explosion, industrialization and urbanization has led most of the freshwater resources under stress. The assessment and monitoring of aquatic system thus requires appropriate restoration and conservation strategies for management of natural resources. Lake is a water body surrounded by land and receives water from precipitation and other local water bodies. It can be both man-made and natural. It receives pollutants from different sources which ultimately deteriorates the quality of water affecting aquatic system. The quality of lake water at a given time depends on interaction of many factors such as human influence, weather conditions, geology and characteristics of lake itself. In present research study 3 different lakes were selected for isolation of freshwater native microalgae.

a) Isolation of microalgae from lake water, Ahmedabad; Gujarat:

Ahmedabad, capital of Gujarat and 7th largest city of the India is located at 22.58°N Latitude; 72.35°E Longitude with an area of 464.14 km. In Ahmedabad there are many water reservoirs including rivers, lakes, ponds etc. Three different lakes were selected namely Chandola Lake (*22°59'14"N 72°35'12"E*); Vastral Lake (*22°59'37"N 72°39'26"E*) and Vinzol lake (*22°56'57"N 72°38'35"E*) situated in Northeastern part of Ahmedabad (Figure 4.1 and 4.2). The industrial belt comprising of 3,850 small and medium scale industries mainly situated in Eastern part of Ahmedabad city which discharge huge amount of wastewater, leading to water pollution and has adverse impact on environment and human beings. The lake water was collected randomly from different sites of the individual lakes and was characterized for various

physicochemical parameters: pH, EC (µs), TDS (ppm), TSS (mg/l), Alkalinity (mg/l), Inorganic phosphorous (mg/l), Organic phosphorous (mg/l), Nitrate (mg/l), Nitrite (mg/l), Ammonia (mg/l), Sulphate (mg/l), Total hardness (mg/l), COD (mg/l), DO (mg/l), BOD (mg/l) and Chloride (mg/l) (APHA 1988). The lake water was also analyzed for presence of heavy metal (lead, cadmium, chromium, arsenic and nickel) using ICP-AES (IIT Bombay). The characterization of lake water was done to assess the quality of lake water and presence of dominated strains of microalgae.

Figure 4.1 – Sampling sites for isolation of microalgae

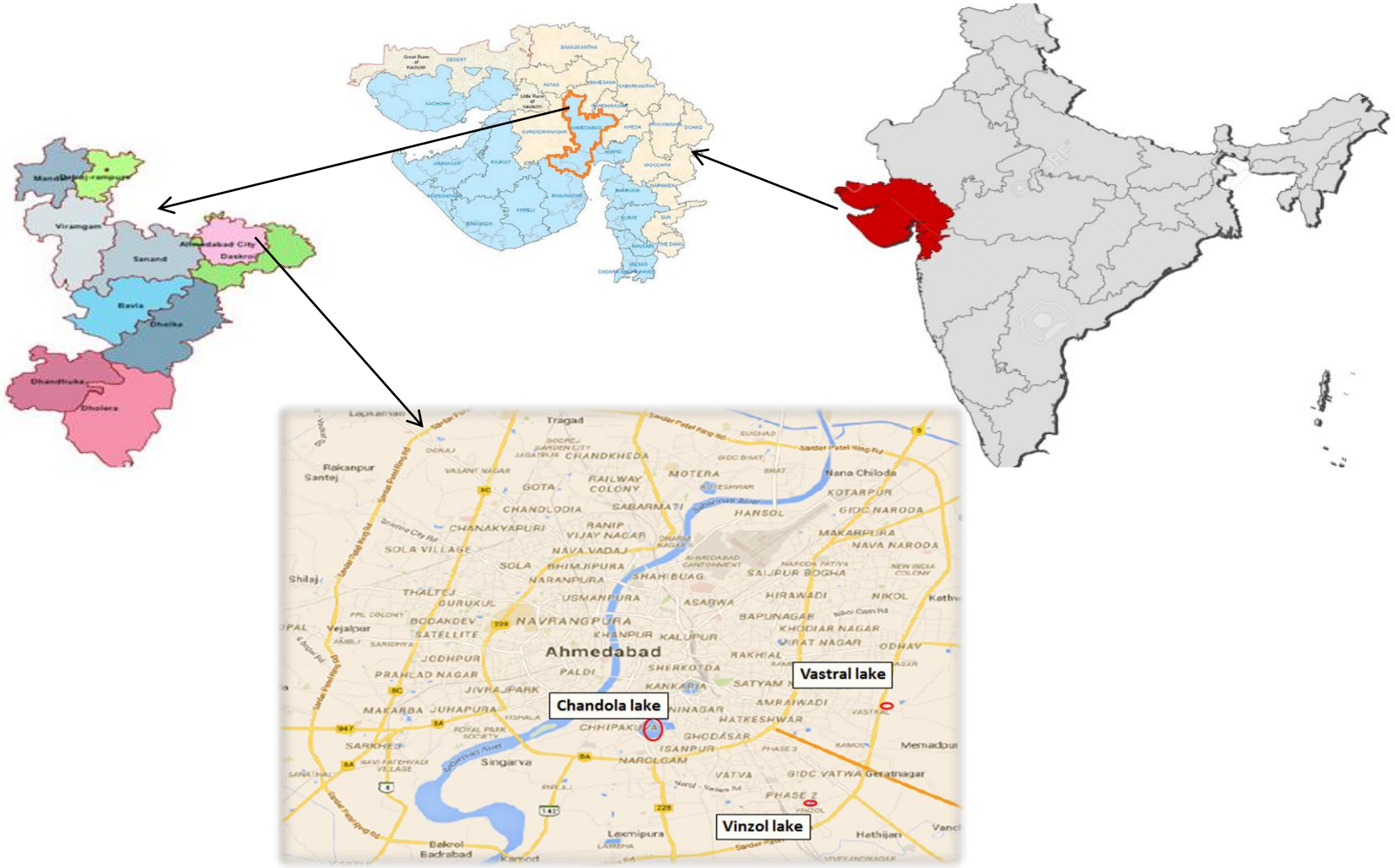

Figure 4.2 – Sampling site for isolation of Microalgae at Ahmedabad city

b) Collection of CETP effluent for phycoremediation study

The effluent samples were collected after tertiary treatment from common effluent treatment plant (CETP) Kalol, located in Gandhinagar Gujarat (Figure 4.3). The collected samples were characterized for different physicochemical parameters: pH, EC (µs), TDS (ppm), TSS (mg/l), Alkalinity (mg/l), Inorganic phosphorous (mg/l), Organic phosphorous (mg/l), Nitrate (mg/l), Nitrite (mg/l), Ammonia (mg/l), Sulphate (mg/l), Total hardness (mg/l), COD (mg/l), DO (mg/l), BOD (mg/l), Chloride (mg/l) (APHA 1988) and presence of heavy metal (manganese, cobalt, copper, zinc, lead, cadmium, chromium, arsenic and nickel) using ICP-AES (IIT Bombay). The microbial status has also been assessed in order to find out microbial diversity present in the effluent. The collected effluent was further used as a substrate for cultivation of microalgae to establish phycoremediation as a cost-effective and green technology in order to minimize pollution load in water reservoirs.

Figure 4.3 – Sampling site for collection of wastewater for Phycoremediation study

4.2 Experimental methodology

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4.2.1 Isolation of microalgae from different lakes in Ahmedabad

Collected water samples were taken to laboratory and isolation procedure of microalgae done within 24hrs as microalgal species are unable to grow in presence of some dominating species and disappear with time. All the three lake water was enriched using nutrient medium separately and isolation procedure was carried out.

a) Enrichment of water samples collected from different lakes: Water samples were collected aseptically randomly from five different sites of Chandola Lake (*22°59'14"N 72°35'12"E*); Vastral Lake (*22°59'37"N 72°39'26"E*) and Vinzol lake (*22°56'57"N 72°38'35"E*) situated in Northeastern part of Ahmedabad, Gujarat. The raw water samples (10 ml) from each lake was inoculated in three different flasks containing 100 ml BG-11 broth as a nutrient medium at 28±3°C under a photoperiod of 16:8 light-dark cycles in culture room for two weeks. The medium contains $NaNO_3$-1.5gm, $K_2HPO_4.3H_2O$ - 0.04gm, $KH_2PO_4.3H_2O$ - 0.2gm, Na_2EDTA - 0.001gm, Fe ammonium citrate 0.001gm, Citric acid - 0.006gm, Na_2CO_3- 0.02gm and 1 ml of trace metal solution per liter. The trace metal solution contains H_3BO_3 - 2.85 gm, $MnCl_2.4H_2O$- 1.8gm, $ZnSO_4.7H_2O$ - 0.02gm, $CuSO_4.5H_2O$ - 0.08gm, $CoCl_2.6H_2O$ - 0.08gm and $Na_2MoO_4.2H_2O$ - 0.05gm per liter.

b) Serial dilution and Plating of Microalgae culture: After initial cultivation of the stock cultures, unicellular microalgae were isolated using serial dilution, spread plate and streak plate methods. For serial dilutions samples were centrifuged at 1000rpm and washed repeatedly 2 -5 times with DDW and culture were diluted 10 times in a series in 10 different test tubes and incubated at 28±3°C with 16:8 light-dark cycles for growth. After that the test with growth was examined under light microscope coupled with camera for presence of different microalgae and spread plate was done. The serially diluted samples were spread plate for isolation of pure culture, where 100 µL of diluted sample was transferred to a Algae agar media plate ($NaNO_3$ – 1 gm/L, K_2HPO_4 - 0.25 gm/L, $MgSO_4$ - 0.513 gm/L, NH_4Cl - 0.050 gm/L, $CaCl_2$ -0.058 gm/L, Ferric chloride - 0.003 gm/L, Agar - 15 gm/L) and spread evenly under aseptic conditions and incubated in culture room at 28±3°C under photoperiod of 16:8 light-dark cycles for about 10 days. Grown microalgae cultures were streaked using sterile conditions onto additional sets of

Algae agar plates and incubated back in culture room under same conditions. Several times plating was done on sterile Algae agar plates until pure individual colonies were isolated.

c) Molecular identification of the isolated microalgae: The purity of the isolates was confirmed first by observing a drop of microalgae suspension under light microscope to ensure that isolated microalgae is not contaminated with the any other microalgae and latter the axenic culture were identified using 28S rDNA based molecular identification (Eurofins Genomics India Pvt. Ltd. Bangalore). The stock cultures were maintained with similar conditions as above on tissue culture racks in culture room. The isolated microalgae was further characterized for biochemical parameters initially i.e. protein content, carbohydrate content, lipid content, chlorophyll content, biomass productivity, lipid productivity etc.

4.2.2 Optimization of different parameters for growth of microalgae at laboratory scale

The growth rate and biomass yield of microalgae depends on its mode of cultivation. Therefore in order to find out suitable condition for growth of microalgae at laboratory scale the different isolated algae from lake water was optimized for different parameters i.e. pH, salinity, Nitrate, phosphate and different Nutrient medium (Table 4.1). The various parameters selected for optimization study has significant influence on algal growth, biochemical properties, biomass productivity and lipid synthesis. In present research study, the isolated pure cultures of microalgae were cultivated at laboratory scale under selected conditions to observe their influence on growth. The batch experiments were carried out for 25 days of cultivation period in 500 mL Erlenmeyer flask at 28±3°C under photoperiod of 16:8 light-dark cycles in culture room. During batch experiments the biochemical characterization of microalgae was done in order to find out effects of different conditions provided on the microalgae.

Table 4.1: Conditions used for finding out suitable growth of microalgae

S.N.	Condition for optimization	Selected conditions range and type
1	**Nutrient medium**	**BG11:** $NaNO_3$ - *1500 mg/l*, K_2HPO_4 – *40 mg/l*, $MgSO_4.7H_2O$ – *75 mg/l*, $CaCl_2.2H_2O$ – *36mg/l* , Citric Acid – *6 mg/L*, Ammonium Ferric Citrate – *6 mg/l*, EDTA-Na_2 - *1 mg/l*, Na_2CO_3- *20mg/l*, H3BO3 – *2.86 mg/l*, $MnCl_2.4H_2O$ – *1.81 mg/l*, $ZnSO_4.7H_2O$ – *0.22 mg/l*, $Na_2MoO_4.2H_2O$ - *0.39 mg/L*, $CuSO_4.5H_2O$ – *0.08 mg/l* $Co(NO_3)_2.6H_2O$ – *0.05 mg/l* (Fazil et al., 2007).
		BBM (Bold basal medium): $NaNO_3$ - *0.25 g/l*, $MgSO_4.7H_2O$ – *0.075 g/l*, NaCl – *0.025 g/l*, K_2HPO_4 – *0.075 g/l*, KH_2PO_4 - *0.175 g/l* $CaCl_2.2H_2O$ - *0.025 g/l*, ZnSO4.7H_2O – *8.82 mg/l*, $MnCl_2.4H_2O$ – *0.44 mg/l*, MoO_3 – 0.71 mg/l, CuSO4.5H2O – *1.57 mg/l*, Co(NO3)$_2$.6H_2O – *0.49 mg/l*, H_3BO_3 – *11.42mg/l*, EDTA - *50 mg/l*, KOH - *31 mg/l*, $FeSo_4.7H_2O$ – *4.98 mg/l*, H_2SO_4 – *1 µL* , vitamin B1 – *10 µg*, vitamin B12 – *10 µg* (Ilavarasi et al., 2011).
		BM (Basal Medium): $Ca(NO_3)_2.4H_2O$ - *150 mg/l*, KNO_3 - *100 mg/l*, β-Na2glycerophosphate - *50 mg/l*, $MgSO_4.7H_2O$ - *40 mg/l*, EDTA-Na_2 - *2.71 mg/l*, Vitamin B12 - *0.0001 mg/l*, Thiamine HCL - *0.0001 mg/l*, Biotin - *0.01 mg/l*, $MnCl_2.4H_2O$ – *0.108 mg/l*, $ZnSO_4.7H_2O$ – *0.066 mg/l*, $Na_2MoO4.2H_2O$ – *0.0075 mg/l*, $FeCl_3.6H_2O$ – *5.888 mg/l*, $CoCl_2.6H_2O$ – *0.012 mg/l*, Trisaminomethane – *500mg/l* (Fazil et al., 2007).
		N8: KNO_3-*1000 mg/l*, KH_2PO_4 – *740 mg/l*, $Na_2HPO_4.2H_2O$ – *260 mg/l*, $CaCl_2.2H_2O$ – *13 mg/l*, Fe EDTA – *10 mg/l*, $MgSO_4.7H_2O$ – *50 mg/l*, Micronutrients – 1ml ($Al_2(SO_4)_3.18H_2O$ - *3.58 g/l*, $MnCl_2.4H_2O$ - *12.98 g/l*, $CuSO_4.5H_2O$ - *1.83g/l*, $ZnSO_4.7H_2O$ - *3.2g/l*) (Mandalam et al., 1998).
		M8: KNO_3 - *3000 mg/l*, KH_2PO_4 – *740 mg/l*, $Na_2HPO_4.2H_2O$ – *260 mg/l*, $CaCl_2.2H_2O$ – *13 mg/l*, Fe EDTA – *10 mg/l*, FeSO4.7H_2O – 130 mg/l, $MgSO_4.7H_2O$ – *50 mg/l*, Micronutrients – 1ml ($Al_2(SO_4)_3.18H_2O$ - *3.58 g/l*,

		$MnCl_2.4H_2O$ - *12.98 g/l*, $CuSO_4.5H_2O$ - *1.83g/l*, $ZnSO_4.7H_2O$ - *3.2g/l*) (Mandalam et al., 1998).
		RM (Rudic's medium): $NaNO_3$ - *300 mg/l*, K_2HPO_4 – *80 mg/l*, KH_2PO_4 – *20 mg/l*, $MgSO_4.7H_2O$ – *10 mg/l*, $CaCl_2.2H_2O$ – *58.5 mg/l* EDTA – *7.5 mg/l*, NaCl – *20mg/l*, H_3BO_3 – *0.3mg/l*, $MnSO_4.H_2O$ *1.5mg/l*, $ZnSO_4.7H_2O$ – *0.1 mg/l*, $(NH_4)6Mo_7O_{24}.4H_2O$ – *0.3 mg/l*, $CuSO_4.5H_2O$ - *0.08 mg/l*, $Co(NO_3)_2.6H_2O$ - *0.26 mg/l*, FeCl3.6H2O – *17 mg/l* (Fazil et al., 2007).
		CHU 13 medium: KNO_3 - *400 mg/l*, K_2HPO_4 – *80 mg/l*, $CaCl_2.2H_2O$ - *107 mg/l*, $MgSO_4.7H_2O$ –*200 mg/l*, Ferric Citrate – *20mg/l*, Citric acid – *100 mg/l*, $CoCl_2$ – *0.02 mg/l*, H_3BO_3 – *5.72 mg/l*, $MnCl_2.4H2O$ – *3.62 mg/l*, $ZnSO_4.7H_2O$ – *0.44 mg/l*, $CuSO_4.5H_2O$ – *0.16 mg/l*, Na_2MoO_4 – *0.084 mg/l*, 0.072 N H2SO4 – *1 drop*. (Chu 1942).
2	**pH**	Control, pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10
3	**Salinity (NaCl)**	Control, 0.5 NaCl gm/L, 1 NaCl gm/L, 2 NaCl gm/L, 3 NaCl gm/L, 4 NaCl gm/L and 5 NaCl gm/L
4	**Nitrogen content**	(-)100%, (-)50%, control, (+)50%, (+)100% (*from initial concentration present in nutrient medium*)
5	**Phosphorous content**	(-)100%, (-)50%, control, (+)50%, (+)100% (*from initial concentration present in nutrient medium*)

a) Optimization of different Nutrient medium: Growth rate and biomass production of microalgae depends on nutrient availability. Different nutrient medium proposed has different composition of micronutrients and macronutrients composition based on the natural habitat of algae. Therefore the study was carried out to evaluate the effect of different inorganic growth medium on microalgae isolate. The identified microalgal isolates were grown in selected nutrient media i.e. BG-11, BBM, RM, BM, N8, M8, RM and CHU-13 medium. Experiment was carried out in batch mode over a period of 25 days to evaluate their growth characteristics in selected medium. 500 ml Erlenmeyer flask supplemented with 250 ml of different culture medium was inoculated with 10 ml

identified microalgal culture separately and incubated at 28±3°C under photoperiod of 16:8 light-dark cycles in culture room. The growth of microalgae cultured in different medium was monitored regularly at an interval of 24hrs by measuring optical density at 680 nm using UV – visible spectrophotometer. Different biochemical properties were also assessed for each isolates.

b) **Effect of pH on algae growth:** pH is one of the most important factor in algal cultivation as it can have significant impact on algal metabolism (Juneja et al., 2013). The initial pH of the selected medium depending on its growth was varied with pH ranging from pH 4 to pH 10. For pH optimization batch experiments were carried out in 500 ml conical flask having 250 ml nutrient medium. Each flask was inoculated with 10 ml microalgal culture under aseptic conditions. The pH of the medium was maintained by using 0.1N NaOH or 0.1 N HCl aseptically in laminar air flow before inoculating microalgae culture. Algal cultivation was carried out in similar conditions as mentioned above in section (a) and growth was monitored regularly by measuring optical density of microalgal culture at 680 nm using UV-visible spectrophotometer.

c) Effect of salt concentration on algae growth: Salinity is another important factor which influences physiological and biochemical mechanism of the microalgae growth and it also plays an important role in fatty acid metabolism. The batch experiments were carried out in order to study the effect of salinity on growth of the identified microalgae in 500 ml conical flask containing 250 ml selected nutrient medium supplemented with different doses of NaCl i.e. 0.05g/l, 1g/l, 2g/l, 3g/l, 4g/l and 5g/l. The nutrient medium without addition of salt was used as control. The flasks were inoculated with 10ml of each freshly prepared culture of different isolated microalgae culture separately and incubated at 28±3°C under photoperiod of 16:8 light-dark cycles in culture room over a period of 25 days. The growth rate and biochemical properties were monitored at regular interval as mentioned above.

d) Effect of Nitrogen concentration on algae growth: Nitrogen is one of the most important macronutrient required for growth and development of microalgal cells. It is an essential component required for the formation of proteins and nucleic acid in algal cells which directly affects the productivity of microalgae. The isolated microalgae were

evaluated for its biomass productivity and growth with different concentration of nitrate in selected nutrient medium. The nitrate concentration in selected medium was altered as control, 0%, -50%, -100%, +50% and +100% to its original concentration to observe its influence on algal growth. The batch experiments were performed in 500ml conical flask supplemented with the altered concentration of nitrate in nutrient medium separately and each flask was inoculated with 10 ml of each freshly prepared culture of different isolated microalgae. After inoculation, all flasks were incubated at 28±3°C under 16:8 light-dark cycles. The growth rate and biochemical properties were observed as mentioned above at a regular interval of time over a period of 25 days.

e) Effect of phosphorus concentration on algae growth: Phosphorous is another essential macronutrient required for growth and other metabolic activities of the algal cells. It is also a key component of phospholipids, nucleic acids, and ester phosphates. For studying the effect of different concentration of Phosphorous, batch experiments were performed in 500 ml Erlenmeyer flask containing 250 ml Nutrient medium. The phosphorous concentration was altered as control, 0%, -50%, -100%, +50% and +100% to its original concentration in selected growth medium. Each flask with different concentration of phosphorous content was inoculated with 10 ml of each freshly prepared culture of different isolated microalgae and all flasks were incubated at 28±3°C with 16:8 light-dark cycles in culture room over a period of 25 days. The growth rate and biochemical properties were monitored at regular interval as mentioned above.

4.2.3 Treatment of CETP effluent using microalgae

The wastewater samples used for Phycoremediation study was collected from common effluent treatment plant (CETP) Kalol, located in Gandhinagar (Gujarat). The CETP effluent contains various organic, inorganic pollutants which are highly toxic in nature and causes an adverse effect on the environment and living beings on exposure. Therefore proper treatment and management of wastewater generated is the prerequisite for every urban society in order to overcome water scarcity problem arising with increasing water pollution and a crucial factor for economic development worldwide. The samples collected after tertiary treatment in sampling canes sealed properly and transported to the laboratory for experimental studies. The CETP effluent samples were

characterized initially for different Physico-chemical, microbial and heavy metal content (as described earlier) and rest of the samples were refrigerated at 4°C for further research studies. In present research study, the collected CETP wastewater (3° treated) was used as a substrate for cultivation of microalgae; to find out nutrient removal efficiency of isolates from the effluent. The experiments were performed in 500 ml conical flask in batch mode containing 250ml medium as CETP effluent diluted with distilled water in different concentration 25%, 50%, 75% and 100%. All flasks were inoculated with algal culture and incubated at constant conditions of photoperiod 16:8 light-dark cycles, temperature 28±3°C in culture room for 25 days'. The algal growth was monitored on the basis of optical density at regular interval during the experiment. Different Physico-chemical parameters were determined (as mentioned in section 4.3) by standard methods as described in the APHA. All parameters were analyzed in triplicate. The removal of heavy metals present in the effluent i.e. Pb, Cd, Ni, As, Cr, Mn, Co, Cu, and Zn was also studied using ICP-AES.

The percent reduction in pollutants was estimated using following equation:

Pollution reduction (%) = (Initial concentration – Final concentration) × 100 / Initial concentration

Apart from wastewater characterization, a known volume of algal culture from experimental setup was also analyzed for various biochemical characteristic i.e. chlorophyll, lipid, biomass productivity, lipid productivity, dry weight (as mentioned in section 4.3.2) to observe the potential of CETP effluent to support growth of microalgae and appropriate concentration of CETP effluent with dilution for maximum algal growth.

4.2.4 Heavy metal remediation with a selected strain of microalgae

Presence of heavy metals in wastewater as a consequence of various industrial processes has become a key environmental problem worldwide. Heavy metals unlike other organic contaminants are a stable element and are not biodegradable in nature; hence its exposure can cause severe adverse effects on humans, plants, and animals. Various conventional treatment methodologies are being used for metal removal which requires high cost and

not eco-friendly. Thus the biological treatment using microalgae has gained much interest due to its significant efficiency, low-cost technology and integrated approach (i.e. bioremediation, biofuel production, carbon mitigation) making it more eco-friendly and green technology for metal removal.

The experiments were carried out for removal of selected heavy metal using potential microalgae. The potential microalgae and their mixed culture were selected for heavy metal removal study as these isolates have shown better results in phycoremediation of CETP effluent in comparison with other isolated microalgae. The experiments were set-up to investigate lead, cadmium, chromium removal by growing selected potential isolates of microalgae and their mixed culture in various concentration of selected heavy metal i.e. 5 ppm, 10ppm, 25 ppm and 50ppm. The batch experiments were carried out in 500 mL conical flask having 250 ml nutrient medium (BG-11 broth) amended with different concentrations of heavy metal separately. BG-11 medium without addition of heavy metal was used as a control. Each flask amended with heavy metal was inoculated with freshly prepared microalgae cultures under sterile conditions. Before inoculation algal culture was centrifuged at 3000 rpm for 5min and supernatant was discard and cultures retained were washed 3-4 times with DDW. All flasks inoculated with algae culture and incubated at constant conditions of photoperiod of 16:8 light-dark cycles, temperature 28±3°C in culture room and manually shaken to avoid sticking of cells to the walls of the conical flask for 15 days. The algal growth was monitored on the basis of optical density at regular interval. For metal removal analysis 10 ml culture was taken from each flask at different interval under a sterile condition in laminar air flow and then centrifuged at 5000 rpm for 15 min. The supernatants were used for estimation of different heavy metals after digestion with Nitric acid & Perchloric acid (APHA 1988) by ICP-AES (IIT Powai, Mumbai). All parameters were analyzed in triplicate. The percent reduction in Heavy metal was calculated as given below:

Percent reduction in Heavy metal = 100 (Initial Value - Final Value)/Initial Value

4.2.5 Enhance remediation of heavy metal using Nanocatalyst and algae-bacterium co-culture techniques

Further, for more efficient removal of selected heavy metals, three different combinations were made i.e. combination A, combination B, and combination C. Batch experiments were carried out for heavy metal removal for 15 days in 500 ml conical flask under controlled conditions.

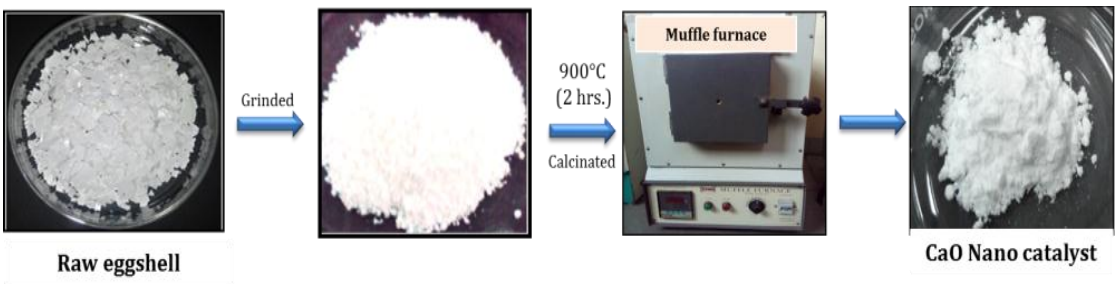
(I) Combination: A - Heavy metal removal using Nanocatalyst

a) Synthesis of Nanoparticle synthesis

There are several methodologies for nanoparticle synthesis i.e. chemical, biological and physical methods which includes various technologies depending on its applications. In present research study, two different nanoparticles were synthesized using physical methods (calcium oxide) and chemical method (magnesium oxide). These metal oxides play an important role in different areas of material sciences, chemistry and physics with their ability to adopt a vast number of structural geometries with an electronic structure having an insulator, metallic and semiconductor characteristics.

(i) Calcium oxide

Calcium oxide nanoparticles were synthesized using eggshells of chicken. Firstly the eggshells were collected and washed thoroughly using distilled water and dried in an oven at 80°C till dryness. The dried eggshells were crushed and calcinated at 900°C for 2 hrs. using muffle furnace. After that calcinated eggshell powder was placed in a desiccator for 30 min. to cool down and finally stored in the airtight vial for further characterization and application studies (L.M. Correia et al., 2014).



(ii) Magnesium oxide

A magnesium oxide (MgO) nanoparticle was synthesized by chemical method (Agarwal et al. 2015). In experiment 0.2M of Magnesium Nitrate Hexahydrate solution and 0.2M of NaOH solution was prepared in 200 mL of DDW separately in a conical flask. Then both the prepared solution was stirred continuously for 45 min. at room temperature on a magnetic stirrer until a clear solution is obtained. The 200ml of sodium hydroxide solution was further added to Magnesium Nitrate Hexahydrate solution dropwise and kept for continuous stirring on a magnetic stirrer for 2 hrs. After the completion of the reaction, the flask was kept under static condition for 6hrs in order to allow settling of precipitate formed. Latter the precipitate formed was separated from the solution by centrifugation at 1000 rpm for 15 min and washed with DDW repeatedly to remove impurities and then dried in an oven at 80°C (till dryness). The dried magnesium hydroxide sample was crushed in pestle-mortar and calcinated in a muffle furnace at 450°C for 3 hrs. After 3 hrs sample is cooled in a desiccator and placed in airtight vials for further characterization and application.

a) Characterization of synthesized Nanoparticle

Characterization of synthesized nanomaterial was carried out in order to obtain information of morphology and characteristics of nanoparticle before calcination and after calcination using SEM, TEM, XRD, FTIR and EDAX.

FTIR spectroscopy (Fourier transform infrared) of Calcium Oxide (CaO) and Magnesium Oxide (MgO) was obtained by mixing dried powder of synthesized material with potassium bromide (KBr) to form pellets and absorbance were recorded within the range of 4000 – 400 cm^{-1} (*Perkin Elmer Inc., USA*). Samples were analyzed for XRD pattern (X-ray diffraction) by continuous scanning mode with 2θ values ranging between 10 to 80° (*Bruker AXS D8 Advance*). The average crystallite size (D) was determined from the corresponding 2θ values using Debye-Scherrer equation:

$$D = K\frac{\lambda}{\beta \cos\theta}$$

Where K is crystallite shape factor (0.9), λ is the wavelength of the electron beam, β is the full-width at half-maximum or half-width ((FWHM) is in radian and θ (theta) is the position of the maximum of the diffraction peak. The detected peak positions were compared with those of the International Center for Diffraction Data Standard (JCPDS) patterns to identify the crystalline phases.

The SEM-EDAX (scanning electron microscope equipped with an energy dispersive spectrometer) was used to study morphology and quantification of elements (*model JSM 6490*) and size was analyzed using HR-TEM (high-resolution transmission electron microscope) with magnification range between 2000x - 1500000x at 200 kV (*Jeol /JEM 2100*).

(a) Application of nanomaterial in phycoremediation of selected heavy metals

Synthesized nanomaterial calcium oxide and magnesium oxide were used in different doses i.e. 1mg/L, 5mg/L and 10 mg/L for enhancing remediation of higher concentration of heavy (lead, cadmium, chromium) using mixed microalgal culture for heavy metal removal. The batch experiments were carried out in 500 mL conical flask having 250 ml BG-11 medium amended with different concentrations of heavy metal separately and control was taken without heavy metal. Each flask amended with heavy metal was inoculated with freshly prepared microalgae cultures under sterile conditions. Before inoculation algal culture was centrifuged at 3000 rpm for 5min and supernatant was discard and cultures retained were washed 3-4 times with DDW. All flasks inoculated with algae culture and incubated at constant conditions of photoperiod of 16:8 light-dark cycles, temperature 28±3°C in culture room for 15 days. The flasks were manually shaken to avoid sticking of cells to the walls of the conical flask at regular interval. The algal growth was monitored on the basis of optical density at regular interval. For metal removal analysis 10 ml culture was taken from each flask at different interval under a sterile condition in laminar air flow and then centrifuged at 5000 rpm for 15 min. The supernatants were used for estimation of different heavy metals after digestion with Nitric acid & Perchloric acid (APHA, 1998) by ICP-AES (IIT Powai, Mumbai). All parameters were analyzed in triplicate. The percent reduction in Heavy metal was calculated as given below.

Percent reduction in Heavy metal = 100 (initial Value - Final Value)/Initial Value

(II) Combination: B - Heavy metal removal using algae-bacterium co-culture

(a) Selection of potential bacteria isolates for metal remediation

A 10 ml of wastewater sample (CETP effluent) was taken and homogenized at 150 rpm on a shaker. A tenfold dilution series was prepared and 0.1ml of each dilution was inoculated in a test tube containing 3 ml of Nutrient broth supplement with 50mg/l of selected heavy metal (i.e. lead, cadmium, and chromium separately). The test tubes were incubated on a shaker for 2 weeks at 150 rpm. Then the tube showing positive growth for each metal was transferred into the fresh medium. Pure colonies were isolated on Nutrient agar medium and used for the further study (Fulekar et al., 2012).

b) Development of algae-bacterium co-culture

The microalgal-bacterial consortium was formed with Microalgae culture and heavy metal resistant bacterial consortium; where 5 ml of algae culture and 1ml mixed (1.0 × 107 cells/ml) bacteria consortium was inoculated into 30 ml Nutrient medium and all flask were incubated at 28±1ºC with 16:8 light-dark cycles in culture room for consortium development (7days). The algae bacterium co-culture developed was used for selected different heavy metal removal from the medium.

c) Heavy metal removal using algae-bacterium co-culture

The batch experiments were carried out in 500 mL conical flask amended with different concentrations of heavy metal separately and control was taken without heavy metal. Each flask amended with heavy metal was inoculated with freshly prepared microalgae cultures under sterile conditions. In heavy metal removal study, 20 ml of algae bacterium co-culture were inoculated into 500 ml flasks containing 230 ml Nutrient medium amended with selected concentration of heavy metals (lead, cadmium and chromium) separately and all flask were incubated at 28±1ºC with 16:8 light-dark cycles in culture room for 15 days in order to study heavy metal removal efficiency of developed algae

bacterium co-culture (Tang et al., 2012). After 15 days the biomass obtained was assessed for different biochemical properties. The supernatants were used for estimation of different heavy metals after digestion with Nitric acid & Perchloric acid (APHA, 1998) by ICP-AES (IIT Powai, Mumbai). All parameters were analyzed in triplicate. The percent reduction in Heavy metal was calculated as given below.

Percent reduction in Heavy metal = 100 (initial Value - Final Value)/Initial Value

(III) Combination: C Heavy metal removal using algae-bacterium co-culture in presence of nanomaterial developed.

Enhancement of selected heavy metal (Pb, Cd, and Cr) removal using microalgae mixed cultures were carried out in batch experiments. 230 ml of Nutrient medium was taken in 500 ml conical flask amended with different concentration of heavy metal i.e. 5 ppm, 10 ppm, 25 ppm and 50 ppm separately and inoculated with 20 ml algae-bacterium co-culture and 10 mg/L of CaO and MgO nanoparticles was added. After inoculation, all flask were incubated with 16:8 light-dark cycles at 28±1ºC for 15 days. The flasks were manually shaken to avoid sticking of cells to the walls of the conical flask at regular interval. The algal growth was monitored on the basis of optical density at regular interval. At the end of the experiment, biomass was harvested by centrifugation (6000rpm for 10 min) and used for biochemical characterization. The supernatants were used for estimation of different heavy metals after digestion with Nitric acid & Perchloric acid (APHA, 1998) by ICP-AES (IIT Powai, Mumbai). All parameters were analyzed in triplicate. The percent reduction in Heavy metal was calculated as given below.

Percent reduction in Heavy metal = 100 (initial Value - Final Value)/Initial Value

4.3 Analytical methods

4.3.1 Physico-chemical analysis of collected samples:

The important Physico-chemical properties of the collected polluted lake water sample and CETP effluent were analyzed for pH, electrical conductivity, alkalinity, Sulphate,

total phosphorus, hardness, chloride, nitrate, nitrite, ammonium, DO, BOD, COD, TDS using standard methods (APHA,1998). The details of these methods are as described in the following sections:

a) pH

Procedure: 10 ml of sample was transferred to 25 ml beaker and pH was measured with a pH meter using a glass electrode having a saturated potassium chloride calomel reference electrode.

b) Electrical Conductivity

Procedure: 10 ml of sample was transferred to 25 ml beaker and EC was measured with a conductivity meter.

c) Alkalinity

Procedure: Taken 25 or 50 mL, sample in a conical flask and add 2-3 drops of phenolphthalein indicator. If pink color develops titrate with 0.02 N H_2SO_4 till disappears or pH is 8.3. Add 2-3 drops of methyl orange to the same flask and continue titration till yellow color changes to orange. Note the volume of H_2SO_4 required.

Calculations: Alkalinity (mg/L as $CaCO_3$) = $\frac{(A-B) \times N \times 5000}{\text{Volume of sample, ml}}$

Where,

A = vol. of 0.02 N H_2SO_4 used for sample, mL

B = vol. of 0.02 N H_2SO_4 used for blank, mL

N = Normality of acid

d) Sulphate (Turbidimetric Method)

Procedure: The sample was filtered and 50 ml of it was taken in 250 conical flasks. 5 ml conditioning reagent was added accurately to it and mixed well. The flask was then kept constantly stirred with the help of magnetic stirring. Stirring was continued for 1

minute after addition of $BaCl_2$. Turbidity developed was measured at 420 nm using a UV-Visible spectrophotometer.

e) Total phosphorus

Procedure: Taken 2 mL of the water sample and mix with 30 ml of 60% $HClO_4$ in a 250 ml volumetric flask. The mixture was digested on a hot plate until the dark color disappears. The total digestion time was approximately 40 min. The mixture was cooled before bringing the volume up to 250 ml with distilled water. The contents of the flask were mixed properly and then allowed to settle. 10 ml of sample was transferred to 50 ml volumetric flask and 10 ml of the ammonium paramolybdate-vanadate reagent was added and the volume of the flask maintained up to 50 ml using distilled water. The absorbance of the sample was measured after 10 min at a wavelength of 420 nm.

f) Organic Phosphorus (Ascorbic method)

Procedure: Pipetted 50.0 mL sample into a clean, dry test tube or 125 mL Erlenmeyer flask. Add 1 drop phenolphthalein indicator (If a red color develops), then add 5N H2SO4 solution dropwise to just discharge the color. Add 8.0 mL combined reagent and mix thoroughly. After at least 10 min measure absorbance of each sample at 880 nm, using reagent blank as the reference solution.

g) Inorganic Phosphorus (Stannous Chloride Method)

Procedure: A well-mixed sample was filtered and 50 ml was taken into 250 ml conical flask and 2 ml of ammonium molybdate was added and mix well. Then 0.25 ml of stannous chloride was added and mixed well and color developed was measured at 690 nm.

h) Chlorine (Argentometric Method)

Procedure: 50 mL of sample was taken in a conical flask and titrated against $AgNO_3$ after adding 4 drops of 5% solution of potassium chromate as an indicator. Titration was performed until the chloride completely precipitated as AgCl and the first faint tinge of red Ag_2CrO_4 persists.

Calculation:

$$\text{Chloride mg/L} = \frac{(A - B) \times N \times 35.45 \times 1000}{\text{mL sample}}$$

Where,

A = mL $AgNO_3$ required for sample

B = mL $AgNO_3$ required for blank, and

N = Normality of $AgNO_3$ used

(i) Hardness

Procedure: Take 50 ml of the water sample and 1ml of buffer solution was taken in a conical flask and mixed properly. Two drops of Eriochrome Black T indicator was added before titrating against EDTA solution. Titration was performed with continuous stirring. In the end, point color changed from wine red to blue.

Calculation:

$$\text{Hardness (EDTA) as mg } CaCO_3/l = \frac{\text{Volume of EDTA used (ml) X N X 50 X 1000}}{\text{The volume of sample ml}}$$

- **Calcium Hardness**

Procedure: Taken 50 mL of the sample in a conical flask and add 1 ml of NaOH to raise pH to 12 and add a pinch of Murexide indicator. Titrate immediately with EDTA till pink color changes to purple. Note the volume of EDTA required (A). Run a reagent blank. Note the mL of EDTA required (B) and keep it aside to compare end point of sample titrations. Calculate the volume of EDTA required by the sample. [C=A-B].

Calculation:

$$\text{Ca/ L hardness as } CaCO_3 = \frac{(V-B) \times N \times 50 \times 1000}{\text{Sample volume}}$$

Where;

V= volume of titrant used for sample mL

B = volume of titrant used for blank

N = determined Normality of EDTA

50 = equivalent weight of $CaCo_3$

- **Magnesium hardness**

Mg as CaCO3mg/L = total hardness – Ca hardness

j) Nitrite Nitrogen

Procedure: 1 ml sulfanilamide solution was added to 50 ml of sample solution taken in a beaker and kept the mixture for 2 to 8 minute to complete the reaction. 1 ml N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) solution was added and mixed immediately. After a period of 10 minutes, the absorbance was taken at 540 nm against reagent blank using distilled water instead of the sample.

k) Ammonium Nitrogen

Procedure: 50 ml of sample was taken in a 100 ml volumetric flask and 2 ml of sodium tartrate was added before maintaining the volume 90 ml by addition of distilled water. 5 ml Nessler's reagent was added and mixed thoroughly and final volume maintained was 100 ml. The content was mixed properly and kept for about half an hour. After that, absorbance reading was taken at 410 nm on a spectrometer and concentration was confirmed by the standard curve.

l) Nitrate Nitrogen

Procedure: 100 ml of the sample was taken in a conical flask and sufficient amount of silver sulfate solution was added to remove chloride (1mg/l Cl = 1 mg AgSO4 solution). Slightly heat the mixture and filtered the precipitate of AgCl. The filtrate was evaporated to dryness in a porcelain basin since the reaction must be effected in the virtual absence of water. After cooling, 2 ml of phenol disulphonic acid was added rapidly directly in the center. The basin was rotated to contact effectively with all the residual salt and the reagent was allowed to react for 10 minutes. 80 ml of distilled water was added and stirred the solution thoroughly. Yellow color was developed by adding 6 ml of liquid ammonia. Volume of the solution was maintained to 100 ml by adding distilled water.

The absorbance of the solution was measured by spectrophotometer at 410 nm wavelength against distilled water as a blank. The concentration of the nitrate of the sample was measured using standard curve.

m) Dissolve oxygen

Procedure: The samples were collected in a BOD bottle and 1mL of $MnSO_4$ followed by 1mL of alkali-iodide-azide reagent was added to a sample collected in the 300mL bottle up to the brim and stoppered immediately. The samples were mixed well by inverting the bottles 2-3 times and allow the precipitate to settle leaving 150mL clear supernatant. The precipitate is white if the sample is devoid of oxygen, and becomes increasingly brown with rising oxygen content. At this stage, add 1mL conc. H_2SO_4 and mix well till precipitate go into solution. Further 200 ml of this solution in a conical flask and titrate against standard $Na_2S_2O_3$ solution using starch (2mL) as an indicator.

Calculation:

$$\text{DO (mg/L)} = \frac{(0.2 \times 1000) \times (0.025\text{N}) \text{ ml of thiosulphate}}{\text{Volume of sample taken for titration}}$$

n) Biological oxygen demand

Procedure: Dilution water was prepared by adding 1 mL of each phosphate buffer, magnesium sulfate, calcium chloride and ferric chloride solutions for each liter of dilution water and mix thoroughly and pH was maintained around 7.0. After that BOD bottles were fill using sample diluted with prepared dilution water in duplicate; one set was kept in BOD incubator at 20°C for 5 days, and another set was analyzed immediately for DO. Similarly, blank was also prepared. After 5 days 1mL of $MnSO_4$ followed by 1mL of alkali-iodide-azide reagent was added to a sample collected in 300mL bottle up to the brim and stoppered immediately. The samples were mixed well by inverting the bottles 2-3 times and allow the precipitate to settle leaving 150mL clear supernatant. The precipitate is white if the sample is devoid of oxygen, and becomes increasingly brown with rising oxygen content. At this stage, add 1mL conc. H_2SO_4 and mix well till

precipitate go into solution. Further 200 ml of this solution in a conical flask and titrate against standard $Na_2S_2O_3$ solution using starch (2mL) as an indicator.

Calculation:

$$BOD\ (mg/l) = (D_0 - D_5) \times \text{dilution factor}$$

Where,

D_0 = initial DO in the sample and D_5 = DO after 5 days

O) Chemical oxygen demand

Procedure: Place 0.4gm $HgSO_4$ in 250 ml reflux and add 20 samples or sample diluted to 20 mL with distilled water, mix well the content and add clean pumice stones. Add 10 mL 0.25N $K_2Cr_2O_7$ solution and mix. After that add 30mL concentrated H_2SO_4 slowly through the condenser and reflux for minimum 2 hours. Cool reflux to room temperature and 2-3 drops ferroin indicator and titrate with 0.1M FAS. The sharp color change from blue-green to reddish green color reappears. Reflux blank was prepared in the same manner using DDW instead of a sample.

Calculation:

$$COD\ (mg/L) = (a - b) \times N \times 8000 / mL\ sample$$

Where, a = mL FAS used for blank

b = mL FAS used for sample

N = normality of FAS

8000 = Milieq. wt. of O_2 x 1000

p) Total Dissolved solid (TDS)

Procedure: 10 ml of sample was transferred to 25 ml beaker and TDS was measured with a conductivity meter equipped with TDS meter probe.

q) Heavy metal Digestion:

Different heavy metals after digestion carried out with Nitric acid & Perchloric acid (APHA,1998). Transfer a measured volume of well-mixed sample i.e. 100 ml in a beaker. In a hood add 5ml HNO_3 in a beaker and cover with a watch glass. Evaporate sample to 15 to 20 ml on a hot plate. Add 10 ml each of HNO_3 and $HClO_4$. Again evaporate gently on a hot plate until dense fumes of $HClO_4$ appear, and solution becomes clear. Cool, dilute to about 50 ml with DDW. Filter the solution and transfer the filtrate to clean volumetric flask. Cool, dilute, make up the volume and analyse for heavy metal determination using ICP-AES.

4.3.2 Biochemical analysis

All the chemicals used were of analytical grade and reagents were prepared using milli-Q water. The precision of analysis was ascertained by triplicate analyses and the results were reported as mean values.

a) Proteins

Procedure: Total protein contents of algae were determined colorimetrically by the method of (Lowry et al., 1951).To 10 mg of dried algal powder taken in a test tube, 5ml of 1N NaOH was added and allowed to stand for 24 hrs. at room temperature. 0.5 mL of the extract was pipetted into a separate test tube and 5 ml of freshly prepared alkaline copper tartrate reagent was added followed by 0.5ml of 1N Folin – Ciocalteu phenol reagent. The contents were mixed thoroughly and allowed to stand for 20 min for color development. The absorbance was then read at 750 nm against a reagent blank using Dynamic HB 20 UV-Visible spectrophotometer. Bovine serum albumin was used as standard and the results were expressed as a percentage of dry weight of algae.

b) Total Carbohydrates

Procedure: Total carbohydrate contents of algae were determined colorimetrically by phenol - H_2SO_4 method (Dubois et al., 1956). To 5 mg of dried algal powder taken in a test tube, 10 ml of 5% trichloroacetic acid was added and heated in a water bath at 80 - 90°C for 3 hrs. After cooling to room temperature, the volume was made up to 10 ml

with milli-Q water. 0.2 ml of the extract was pipetted into a separate test tube and 1 ml of 5% phenol was added followed by rapid addition of 5 ml of cone. H_2So_4. After cooling the contents of the tube, absorbance was measured at 490 nm against a reagent blank using Dynamic HB 20 UV-Visible spectrophotometer. Glucose was used as standard and the results are expressed as a percentage of dry weight of algae.

c) Chlorophyll content

Procedure: Chlorophyll synthesis is influenced by the interaction of various environmental factors such as light, temperature, oxygen, moisture, metallic ions and nutrients. Stress in any factors results in a reduction in chlorophyll synthesis, which varies from species to species. The chlorophyll pigments in microalgae were estimated following the method of Arnon (1949). 10 ml of algal culture was centrifuged at 5000 rpm for 10 min. and the supernatant was discarded after that 10mL of 5000–10000rpm for 5mins. The supernatant was transferred and the procedure was repeated till the residue becomes colorless. The absorbance of the solution was read at 645nm and 663nm against the solvent (acetone) blank. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation:

Total Chlorophyll: 20.2(A645) + 8.02(A663)
Chlorophyll a: 12.7(A663) – 2.69(A645)
Chlorophyll b: 22.9(A645) – 4.68(A663)

d) Estimation of dry cell weight

The known volume of microalgae culture was centrifuged at 5000 rpm for 10 min and washes three times with distilled water. The biomass pellets were collected and dried in hot air oven at 80°C until dried completely. Dried biomass calculated by the gravimetric method in g/L dry cell weight.

Calculation:

$$\text{Dry cell weight (gm/L)} = \frac{W1 - W2}{\text{Volume of sample}} \times 100$$

Where,

W1 = pre-weight of the vial with the sample

W2 = post weight of the vial with sample after drying

e) Optical density

Procedure: OD (optical density) was used to represent the algal growth in the culture medium by recording increase of cell number at a regular interval (i.e. lag phase, exponential growth phase, stationary phase and death phase) during the incubation period. The optical density was measured at 680nm wavelength by HB 20 Dynamica UV-Visible Spectrophotometer at regular interval during experiments.

f) Lipid content

Procedure: The oil content in the biomass was determined according to Modified Bligh and Dyer (1959). The cells were harvested by centrifugation at 10,000 rpm for 10 min. Re-centrifugation of cells with distilled water was done to remove salts and then dried in an oven at 80°C. A mixture of 0.5 ml of PBS solution composed of 8 mM Na_2HPO_4, 140 mM NaCl, 2mM NaH_2PO_4, pH 7.4) and glass beads (0.5mm) was added to the algal cells and cells were disintegrated by high speed centrifugation for 4 minute after that 3 mL of Methanol and chloroform were added to the dried algal powder in 1:2 ratio centrifuged for 10 min. at 3000 rpm and left it at room temperature for 18 hrs. After 18 hrs. distilled water was added for phase separation and the mixture was again centrifuged for 10 min. at 3000 rpm. The lower supernatant layer with lipid was collected in pre-weight vial and solvent was evaporation in hot air oven at 50°C. Lipid content was calculated in % dry cell weight.

Calculation:

$$\text{Lipid content (\%)} = \frac{\text{Dry cell weight}}{\text{Lipid weight}} \times 100$$

g) Biomass and lipid productivity

The biomass productivity, BP (mg/l/d) and LP (mg/l/d) was calculated (Nayak et al. 2013) by using following equation:

- **Biomass productivity (mg/l/d/) = Ca × 1000/t**

  Where: Ca = concentration of biomass (g/L) at the end of the experiment

  t = duration of cultivation (days)

- **Lipid productivity (mg/l/d/) = C_L × DCW/t**

  Where: **C_L =** concentration of lipid at the end of the experiment

  DCW = Dry cell weight (g/L)

  t = duration of cultivation (days)

4.3.3. Microbial analysis

a) Isolation of Bacteria

Procedure: The different bacteria were isolated by using serial dilution and spread plate technique from wastewater sample collected from tertiary treated common effluent treatment plant, Gandhinagar. Nutrient agar was used for bacterial isolation composed of peptone-10gm; meat extract-3gm; sodium chloride - 5gm; distilled water-1L; agar-30gm; pH-7.4. 1 mL of wastewater sample was suspended in 9 mL of distilled water and vortex properly. Different dilutions prepared i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} of the suspension was applied on the nutrient agar plates using spreader and plates were incubated at 37°C for 24 hrs. The developed colonies were counted as CFU/ml of the sample.

CFU/ml of original sample = No. of Colonies / Inoculum size (ml) x Dilution factor

For isolation of pure and discrete colonies of bacteria from mixed culture; the selected colonies were streaked using sterile loop dipped into a suitably diluted suspension and

streaked on the sterile nutrient agar plate to obtain pure colonies of the bacteria-free from other microbial contaminations.

b) Morphological characterization and identification of the isolated bacteria

Procedure: The microbial world is very diverse in nature, which make necessary to classify them into different groups based on their similarities. Therefore in order to classify isolated bacteria primarily, it is compared with the description of previously recognized groups of bacteria. However, it is necessary that a culture should be pure culture and then isolates were identified based on the morphological characteristics which include: cell shape, size, structure, arrangement, staining reactions, colonial appearance, pigmentation, surface, optical characteristics, margin/edge of the isolates etc. (Patel et al., 2006). Further, the pure cultures were identified using 16S rDNA gene sequence from BioGene, GSBTM, Gandhinagar, Gujarat.