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Growth Evaluation of Marine Microalgae *Chlorella* sp. BDU G91771 in Calcium Rich Ossein Effluent–A Bioremediation Perspective

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The aim of the present work is to study the growth and effluent parameters of the two ossein effluents. Growth parameters studied in this article are protein, chlorophyll, carbohydrate, moisture and the effluent parameters are alkalinity, nitrates, nitrites, ammonia, phosphates, sulphates, sulphites, calcium and magnesium. Calcium-rich effluent ossein was collected at three different clarifications stages from pioneer Jellice Industries Pvt, Ltd,, Cuddalore, which is gelatin manufacturing industry. The algae selected for the study was *Chlorella vulgaris* BDU G91771 which was obtained from the culture collection of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirapalli, India. The culture was maintained in F/2 medium under the continuous white light at an intensity of 20 µmol photon m^{-2} s^{-1} at 25 ± 2 ºC in a controlled culture room. The chosen organisms were grown in effluent diluted with seawater (1:1, 2:2 and 3:1) amended with fertilizer grade nutrient enrichment and phosphorus sources. The untreated effluent parameters were analyzed. *Chlorella vulgaris* was inoculated in F/2 medium and allowed to grow in ossein effluent for 7 days. The growth of the organism was measured by calculating its culture density, dry weight, carbohydrate, protein and chlorophyll. The growth parameters of microalgae revealed that the higher content of nitrate and ammonia in HTDS effluent served as the nitrogen source and supported microbial growth.

Keywords: Ossein effluent, *Chlorella vulgaris*, Growth parameters, Effluent parameters.

INTRODUCTION

Wastewaters from urban and industries must be treated before being excreted into the environment, in order to avoid unwanted effects such as pollution and hypertrophication [1]. Oswald and Gotaas [2] was first introduced the use of microalgae in the wastewater treatment process and has recognized much more interest in modern decades. Microalgae growth process such as eliminating impurities, preparing medium, purifying water and addition of nutrients are not necessary for the production process due to these main advantages the production cost is reduced [3]. Besides, during the process not only nutrients are removed from the waste but it also returned as an agricultural fertilizer to the terrestrial environment. Another benefit of utilization of microalgae in the wastewater treatment is photosynthetic CO_2 fixation *i.e.* it contributes to reducing the liberation of greenhouse gas carbon dioxide and it produces flue gas that possesses a lot of industrial uses which are reported in Van den *et al.* [4].

Bioremediation is a process that controlling pollution by using microorganisms as a catalyst to consume and break down (degradation) the hazardous toxic polluted chemicals into less or non-toxic chemicals [5,6]. Usage of cyanobacteria effluent treatments is a current trend and its initial experiments were proposed and practiced as effluent in agricultural and industrial fields. In worldwide, it helps to solve excessive richness of nutrients (eutrophication) in water bodies. The richness of nutrients enhances the dense growth of aquatic plants and it results in the reduction of nutrient level (toxic chemicals) in the water bodies [7]. Biomass production of cyanobacteria in large quantity can be used as a food for animals and important sources for amino acids production, light photo conversion, toxins and secondary production, production of enzymes, vitamin and hydrogen, nitrogen fixation and drug intermediates in pharmaceuticals [8-11]. Cyanobacteria are environmentally friendly and do not show any toxic effect to other biotic components. Due to the size, the separation of cyanobacteria biomass is much easier compared to other microalgae biomass

separations. The main organic component in the animal bone tissue is ossein (decalcified bone) which is obtained as a residue in the gelatin production during the clarification process. The major concerns in the disposal of the effluent ossein are a strong odour, high organic content, huge volume and high inorganic contents. Cattle bone decalcification produces a huge volume of calcium opulent effluent and they are dicalcium phosphate (DCP), low total dissolved solids (LTDS) and high total dissolved solids (HTDS). Dicalcium phosphate (DCP) is released during the acidification of the bones effluent formed after discharged, final washing followed by settlement of ossein is obtained and HTDS found at heat treatment of decalcified bones [2,12]. A marine microalgae *Chlorella* sp. BDU G91771 needs calcium, nitrogen, phosphorous as major nutrients for its growth. As the ossein effluent has major nutrients present work proposed to evaluate the growth of the organism in low total dissolved solids and high total dissolved solids ossein effluent to analyze the nutrient parameters in calcium rich ossein effluent.

EXPERIMENTAL

Selection of organism: Ossein effluents (HTDS and LTDS) obtained in the clarification process of gelatin production system was taken for the present study. The microalgae taken for the present work is *Chlorella vulgaris*. Unicellular, spherical green algae, cells are 5-10 micron in diameter, solitary or aggregated in irregular clumps, chloroplast cup or bowl-shaped. Its description and images are given in Table-1 and Fig. 1, respectively.

TABLE-1
DESCRIPTION OF *Chlorella vulgaris* ALGAE

Domain	*Eukaryota*	Order	*Chlorellales*
Kingdom	*Viridiplantae*	Family	*Chlorellaceae*
Division	*Chlorophyta*	Genus	*Chlorella*
Class	*Trebouxiophyceae*	Species	*Vulgaris*

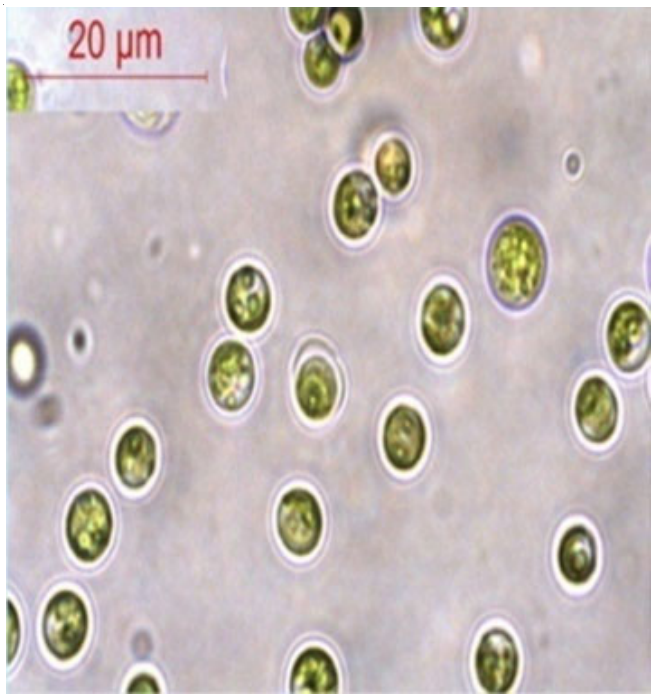

Fig. 1. Microscopic image of *Chlorella vulgaris*

Extraction and estimation of total protein: The main principle of the estimation of protein is the protein forms blue coloured complex with reagent Folin-Ciocalteu′s phenol. This is due to the reduction of phosphomolybdate (in reagent) by tryptophan and tyrosine which is present in the protein aromatic amino acids present in the protein is responsible for the deep blue colour of the complex and it varies from protein to protein.

To 0.5 g of washed pellet, 5 mL of 10 % trichloroacetic acid was added slowly and then left for 30 min in boiling water bath. The whole content was centrifuged at 500 rpm for 10 min and discards the remaining liquids. The pellets were treated with 1 mL of 1 N NaOH in boiling water bath for 30 min. Aliquot of 0.1 mL was taken from that and made upto 1 mL with double distilled water. To this, 4 mL of alkaline reagent was added then stand for 30 min and 0.5 mL of Folin-Ciocalteu′s phenol reagent was added further whole content was incubated for 30 min at room temperature. Finally, the absorbance of the solution was read at 750 nm and the amount of protein estimated by standard curve (bovine serum albumin) [13].

Chlorophyll: To study the growth and photosynthetic rates of algae chlorophyll estimation is an imperative biomass component. Chlorophyll-a absorbs 416 nm wavelength and chlorophyll-b absorbs 453 nm. A 0.5 g of cultured pellet was washed and suspended in vortex and 4 mL of methanol. To prevent solvent evaporation the mouth of the tubes are covered with aluminium foil. All the test tubes are incubated at 60 ºC in a water bath for 1 h in dark with occasional shaking to arrest the oxidation process in light. After 1 h, the whole content is centrifuged at 5000 rpm for 10 min and the supernant are added to another tube containing 80 % methanol [14]. The final sample was read at 663 nm and methanol used as a blank, the total chlorophyll was calculated by following equations:

$$\text{Amount of chlorophyll 'a'} = 6.4 \times (A_{416} - 0.79) \times A_{453}$$

$$\text{Amount of chlorophyll 'b'} = 5.87 \times (A_{453} - 0.24) \times A_{416}$$

Carbohydrate: The method can be used for estimation of total sugars in samples. The sugars in the presence of conc. H_2SO_4 get dehydrated and produce furfural (from hexoses) or 5-hydroxymethylfurfural (from pentoses), which when reacted with anthrone produces a coloured compound with λ_{max} of 625 nm. Pentoses, hexoses, heptoses and their derivatives yield a coloured product whereas trioses, tetroses and amino acids do not yield any coloured product.

To a 1 mL of cyanobacterial suspension added 4 mL of anthrone reagent. It was kept in a boiling water bath for 15 min. The tubes were cooled preferably in ice and the absorbance was read at 620 nm. The amount of carbohydrate was estimated from the standard curve prepared using a standard glucose solution [15].

Total moisture content: The culture was centrifuged at 5000 rpm for 10 min and the pellet was washed twice with distilled water. Pellet was taken in preweighed aluminium foil and it was dried at 50 ºC in a hot air oven until two or three concurrent weights remain the same [16-19].

Effluent parameters estimation

Alkalinity: In commonly natural water contains carbonates and bicarbonates in form of their salts which get hydrolyzed

in water and produces hydroxyl ions thus, raising the pH to make the water into more alkaline nature. Usually, alkalinity determined by titration the sample against strong acid (standard solution). The total amount of hydroxide and carbonate is determined by the first end-point (pH 8.3) using phenolphthalein indicator and the total amount of bicarbonate is determined by the second end-point (pH 4.5) using methyl orange indicator.

A 50 mL of sample solution was pipetted out in the conical flask and two-drops of phenolphthalein indicator added then, titrate against sulphuric acid and end-point is colourless. End-point reading is noted as 'p' then, added two drops of methyl orange to that same conical flask and continue to titrate till the colour changes from yellow to orange. Second end-point reading is noted as 't' and total alkalinity was calculated using the following equation, where the total alkalinity is expressed as mg L^{-1} of $CaCO_3$.

$$\text{Phenolphthalein alkalinity} = \frac{\text{mL of titrant 'P'} \times 1000 \text{ mg } CaCO_3}{\text{mL of the sample}}$$

$$\text{Total alkalinity (T) as mg/L } CaO_3 = \frac{\text{mL of titrant 't'} \times 1000}{\text{mL of sample}}$$

Chloride: Estimation of chloride ions in the sample solution is determined by titrating sample solution against silver nitrate solution using potassium chromate as an indicator. At the end point, the chloride concentration in the solution reaches to zero and silver ion converted into silver chromate which is in the reddish-brown product. Pipetted out 50 mL of water sample (if sea water is taken pipette 0.5 mL of sample) into a conical flask and added 0.5 mL of K_2CrO_4 indicator it gives yellow colour to the sample. Titrate the test solution by shaking it against standard silver nitrate solution till the development of reddish brown colour precipitate and repeated for concordant value. The blank titration was done by using 50 mL of deionized chloride free water sample and 0.5 mL of indicator. The total amount of chlorides present in the sample was expressed in mg L^{-1}.

$$\text{Chloride (mg/L)} = \frac{\text{mL of titrant used} \times N \times 35.46 \times 1000}{\text{mL of sample}}$$

where N = normality of titrant

Salinity: The salinity of water sample is detected from the chlorinity of water sample.

$$\text{Chlorinity of sample} = \text{Chlorinity of Std. sea water sample} \times \frac{19.369 \times \text{Vol. of the titrant used for chloride}}{\text{Volume of } AgNO_3 \text{ used for standard water (19.3)}}$$

$$\text{Salinity ppt (\%)} = 0.03 + (1.805 \times \text{Chlorinity})$$

Nitrate: Nitrates in the sample solution were estimated by using 1,2,4-phenoldisulphonic acid and it forms 6-nitro, 2,4-acid (an alkaline salt) complex with the nitrates which gives yellow colour with the test solution. The alkaline salt absorbs 410 nm wavelength. For the estimation of nitrates, a series of 50 mL different concentration of nitrate solutions are prepared from standard solution (10 to 100 µg).

Pipette out 2 mL of each standard solution into different dry 100 mL beakers with the glass-rods. Added 1 mL of a brucine-sulphanilic acid reagent to each baker and followed by 10 mL of sulphuric acid solution is added. Then the solution was stirred gently for about 5 min and all the beakers were covered with the help of aluminium foil sheets along with the watch glasses. Kept all the beakers in the dark for 10 min at room temperature and yellow colours appeared in the beaker solutions. Again added 10 mL of double distilled water to each beaker and incubated in the dark for 30 min at room temperature and finally, the absorbance of each solution was measured at 410 nm. A graph was plotted between the absorbance values against the concentration of the standard nitrate solutions. Total nitrate content was determined by using the standard graph and expressed in mg L^{-1} [20-22].

Nitrite: Nitrite forms nitrous acid in acid medium and it is due to the diazotization of sulphanilamide. The diazonium salt when reacted with an aromatic amine, N-1-naphthylethylene diamine dihydrochloride, forms a red azo dye which is determined spectrophotometrically at 543 nm.

A 20 mL of the sample was taken in a 50 mL volumetric flask and added 1 mL of sulphanilamide reagent then mixed well. After 5 min interval, 1 mL Griess reagent is added and the whole content is made upto 50 mL by using double distilled water. Before measuring the absorbance of solution, the whole content is shaked well and read at 543 nm using distilled water as a blank. The amount of nitrite content was calculated from the standard graph and the amount of NO_2 nitrogen was expressed in mg L^{-1}.

Ammonia: Ammonia present in the sample solution can react with phenol and alkaline hypochlorite and forms indophenol blue. Nitroprusside or ferrocyanide used as a reaction catalyst. The absorbance of the reaction complex can be read by spectrophotometer at 640 nm.

To a test sample, 0. 4 mL of phenol reagent and 0.4 mL of nitroprusside reagent were added and mixed well. To this solution, 1 mL of oxidizing reagent was added and closes the tubes immediately then vortex and incubated for 1 h at room temperature in the dark room. Finally, measure the absorbance of solution at 640 nm in a spectrophotometer. The total amount of ammonia nitrogen was determined and expressed in mg L^{-1}.

Total phosphate: In the estimation of phosphate, total phosphorus was determined in the form of orthophosphates. The organically bound phosphorus is converted into inorganic phosphorus on oxidation with potassium persulphate because of the reduction of sulphuric acid. Phosphorous in the presence of ascorbic acid (reducing agent) is reduced to molybdenum blue with ammonium phosphomolybdate reagent.

A 50 mL of test solution was taken in a beaker and heated till the volume was reduced to 15 mL and added 1 mL of perchloric acid and then heated until the volume becomes 5 mL. To this, 2 mL of phenolphthalein indicator was mixed and added NaOH solution dropwise until the solution turns into pink then it is made upto 50 mL using distilled water. Finally, 2 mL of mixed reagent was added followed by 2 mL of potassium persulphate was mixed then incubated for 10 min. The absorbance of test solution was measured at 882 nm and the total phosphorus content in effluent was determined by the standard curve.

Sulphate: Total sulphate was estimated by the formation of $BaSO_4$ complex with barium in acid solution and it forms

uniform size crystal when the glycerol-ethanol solution is used as a stabilizer. The quantitative estimation of $BaSO_4$ can easily be determined by spectrophotometer at a wavelength of 420 nm.

A 50 mL of test sample was taken then 10 mL of NaCl-HCl solution and 10 mL of glycerol-ethanol solutions were added one by one and kept this in the stirrer. At the same time, 0.15 g of barium chloride was added and kept this stirrer for 60 s at room temperature. The absorbance of the test solution was immediately read at 420 nm and the total amount of sulphate was measured.

Sulphide: The total amount of sulphide in the sample was determined by allowing sulphide to react with phenanthroline in the presence of an acidic medium and it gives an orange colour at the reaction time. This colouration is due to the reduction of phenanthroline.

To a 10 mL of test solution, 7 mL of acetate buffer (pH 3.5) and 3 mL of 0.1 % phenanthroline monohydrate were added and made up to 25 mL with double distilled water and incubated for 1 h at 25 ºC. The sample and standard solution absorbance were measured at 510 nm. From the standard curve, the amount of sulphide was calculated and expressed in mg L^{-1}

Calcium and magnesium: Calcium and magnesium ions forms a complex with Eriochrome black T which forms wine-red colour complexes with metal ions. Metal ions form the dye complex with disodium salt of EDTA giving the blue colour to dye complex and similarly, murexide indicator forms the pink coloured complex with metal ions. With the addition of disodium salt of EDTA, the Ca^{2+} forms a colourless chelate complex leaving behind a purple solution of the dye.

A 5 mL of test sample taken into a 250 mL conical flask and added 5 mL of ammonium buffer to this then diluted to 100 mL with double distilled water. A pinch of Eriochrome black T was added to this solution and warmed at 60 ºC then titrated against EDTA until the red turns into blue colour. The final end-point was noted as 'A'.

To another conical flask, pipette out 5 mL of water sample to this 5 mL of NaOH solution was added and made up to 100 mL using deionized water [23-26], then added a pinch of murexide indicator. The final solution was titrated against EDTA and the end point colour changes from pink to blue. The end-point was noted as 'B' and the total amount of Ca^{2+} and Mg^{2+} ions in the sample were calculated by the following standard equations:

$$\text{Amount of calcium in the sample (mg/L)} = \frac{F \times B \times 1000}{\text{Volume of sample}}$$

$$\text{Amount of magnesium in sample (mg/L)} = \frac{F \times A - B \times 1000}{\text{Volume of sample}}$$

where, A = volume of EDTA consumed by Ca and Mg (mL); B = volume of EDTA consumed by Ca alone (mL); A – B = volume of EDTA consumed by Mg alone (mL). Factor value for calcium (F) is 2 and Factor value for magnesium (F) is 1.2

RESULTS AND DISCUSSION

Estimation of protein, chlorophyll, carbohydrate and moisture content: From Table-2, total amount of protein, chlorophyll, carbohydrate and moisture content results were listed. The marine microalgae, *Chlorella* sp. was grown in two effluents namely high total dissolved solids (HTDS) and low total dissolved solids (LTDS). The undiluted ossein effluent didn't support the growth of the organism. In order to facilitate growth further, the effluents were amended with nitrogen (urea) and phosphorous (rock phosphate) sources. The organism was able to establish growth in the effluents HTDS and LTDS. When grown in LTDS 1:1 effluent, *Chlorella* sp. yields a maximum of 486.46 µg chlorophyll a and 483. 261 µg chlorophyll b, which was very low than the initial inoculum. However in HTDS effluent, the organism produced a maximum of 5825.37 µg and 5733.293 µg chlorophyll a and b, respectively. This was nearly double the amount of chlorophyll produced in the growth medium. From Fig. 2, it could be inferred that the organism is capable of growing in HTDS ossein effluent. This could be a good sign as growing microalgae could remediate the ossein effluent.

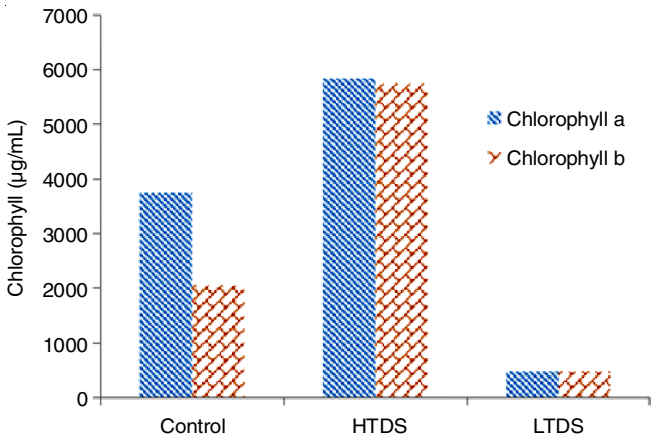

Fig. 2. Growth of *Chlorella* sp. in terms of chlorophyll a and chlorophyll b in ossein effluent HTDS and LTDS

The culture density is shown in Fig. 3, which also revealed the same result that HTDS effluent supporting more growth than LTDS. From Fig. 4, it was found that carbohydrate content of organism at the end of five days growth was higher in HTDS effluent with 80 mg/L as compared to 65 mg/L and 40 mg/L in LTDS and in control growth medium, respectively. In contrast, the protein content was lower in both HTDS and LTDS grown effluent compared to the control cultures (Table-2).

Effluent parameter estimation in HTDS and LTDS ossein effluent: The characteristics of two ossein effluents (LTDS and

TABLE-2
DETERMINATION OF PROTEIN, TOTAL CARBOHYDRATE, TOTAL MOISTURE CONTENT, CHLOROPHYLL 'a' AND CHLOROPHYLL 'b'

Medium	Protein (µg/mL)	Carbohydrate (µg/mL)	Dry weight (g)	A_{416nm}	A_{453nm}	Chlorophyll 'a' (µg/mL)	Chlorophyll 'b' (µg/mL)
Culture	0.610	40	0.101	1.5121	0.7950	3741.308	2062.864
HTDS	0.392	80	0.093	2.4904	2.2496	5825.376	5733.293
LTDS	0.198	65	0.106	0.2014	0.1893	486.46	483.261

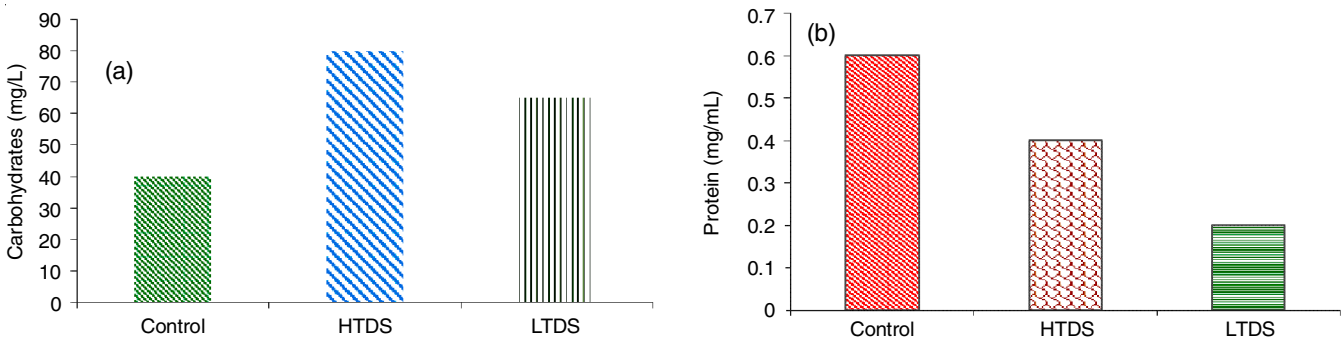

Fig. 4. Carbohydrate (a) and protein content (b) of *Chlorella* sp. BDUG91771 in different ossein effluent

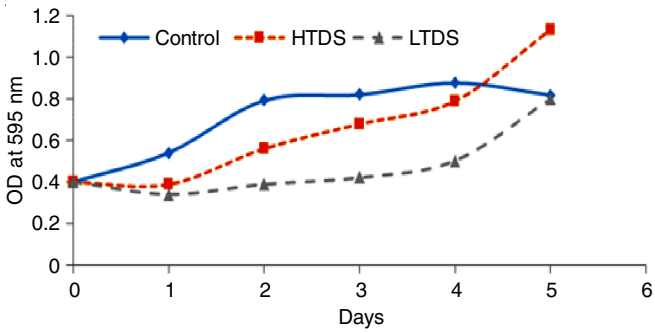

Fig. 3. Growth of *Chlorella* sp. BDU G91771 in terms of culture density measured at 595 nm

HTDS) were highly variable because they were collected at different stages of treatment. In general, HTDS was characterized by yellowish colour, high calcium, magnesium and dissolved solids. The nutrients in HTDS and LTDS ossein effluents are given in Table-3. The HTDS effluent contained higher nitrate while the nitrite was higher in LTDS effluent. The high nitrate and ammonia in HTDS effluent might serve as the nitrogen source and support microalgal growth. No heavy metals were found in the effluent.

TABLE-3
EFFLUENT PARAMETERS IN HTDS AND LTDS OSSEIN EFFLUENT

Parameters	LTDS (mg L^{-1})	HTDS (mg L^{-1})
Alkalinity	3.8	19.7
Nitrate	0.1779	2.037
Nitrite	1.6075	0.0671
Ammonia	0.2193	2.253
Total phosphate	0.9172	1.872
Sulphate	0.5077	0.0756
Sulphite	0.4567	0.0935
Calcium	120	6400
Magnesium	40	840

From chlorophyll a, chlorophyll b and culture intensity of the organism measured during the culture period in different effluents, it was observed that among the two different ossein effluents tested, HTDS effluent supported better growth than the LTDS effluent. This may be due to the presence of higher nitrate (2.037 mg L^{-1}) and ammonia (2.253 mg L^{-1}) in the HTDS effluent compared to its level in LTDS effluent. Uma *et al.* [22] have utilized two marine cyanobacterium, *Oscillatoria willei* BDU 130791 and *Phormidium valderianum* BDU20041 in treating seawater diluted ossein effluent. In this study, the effluents were not diluted with sea water and used as such.

The growth exhibited in the HTDS effluent is very promising that the organism could be used to treat the effluent in further experiments. The abundance of calcium in the effluent is another unique way of remediating the effluent, the excess calcium along with bicarbonate can be sequestered as calcium carbonate and it could be precipitated and removed, later thus making the wastewater more usable for irrigation.

Conclusion

In the present study, *Chlorella* sp. is used to analyze the growth and nutrient parameters in calcium-rich ossein effluent. The present study not only aims at bioremediation of ossein effluent by *Chlorella* sp. but also gives the idea about the microbial growth in the same. It is concluded that the higher content of nitrate and ammonia in HTDS effluent serve as the nitrogen source and support microbial growth. These microalgae wastewater treatment is eco-friendly and offers the advantage of a cost-effective way of nutrient removal and biomass production.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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