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Bioremediation of Wastewater using *Chlorella Vulgaris* Microalgae: Phosphorus and Organic Matter

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ABSTRACT: Wastewater contains many organic and inorganic contaminants that can cause serious damage to the environment and health of people, Therefore, they have to be eliminated before being downloaded into sensitive areas. Different methods of wastewater purification have been used for contaminants removal. However, they present some technical and economic limitations. Thus, new methods of nutrients removal by microalgae based on phytoremediation techniques, become promising methods due to its viability. The objective of wastewater treatment is to improve and purify the water. For that, the removal of all or some of the nutrients present in water is carried out, resulting in a reusable or suitable water to be returned to its natural environment. This study investigates the capacity of Chlorella Vulgaris microalga as a potential candidate for removing the phosphorous and organic matter from wastewater. The strain of microalga was cultivated in synthetic wastewater at room temperature with artificial illumination and aeration. Algal growth parameters such as pH, chemical oxygen demand, phosphorus, cell number, optical density and dry weight were measured during experimental period. Under these conditions, microalgae were able to remove the phosphorus concentration by more than 99%. By other hand, the chemical oxygen demand was reduced at 71%. After 9 days of cultivation, the biomass concentration increased from 0.05 to 0.57 g/L. The results of this study suggest that growing Chlorella Vulgaris microalgaein wastewater offers a new option of applying algae to manage the nutrient load. After then obtained biomas scan be used for biofuel production.

Key words: *Chlorella Vulgaris*, Bioremediation, Wastewater, Chemical oxygen demand, Phosphorus

INTRODUCTION

In the last years, it was estimated that a high volume of activities related to industrialization, urbanization or agriculture have been generated. All these activities increase the content of nutrients such as phosphorus and nitrogen in water reservoir. The eutrophication derived of these large nutrients levels produces an enrichment of water, especially in compounds of nitrogen and phosphorus, originating a fast growth of microalgae and higher forms of vegetable life, causing loss of species and loss of ecosystem function. Therefore, European Commission Directive (98/15/EEC) resolved that previously to water discharge, a reduction in nutrients level is necessary. However, certain substances can not be removed by conventional technologies used in wastewater treatment (Ternes, 1998;Türkerand Akmehmet, 2006).

The presence of phosphorus is due to the agricultural use of fertilizers, domestic and industrial wastewater, and atmospheric deposition. In general, the presence of this compound in wastewater comes from domestic wastewater. Municipal wastewaters may contain from 5 to 20 mg/L of total phosphorous, of which 1-5 mg/L is organic and the rest is inorganic (Ashton, 2013). Phosphorus removal from wastewater can take place by biological or chemical methods (Gillberg et al., 2003; Pastor, 2006). Chemical processes are efficient but they are expensive and present the huge amount of sludge that must be treated (Morse et al.,1998). For this, in the last decades many researchers have focused their studies on biological treatment (Tam and Wong, 1989; Stensel, 1991; Lau et al., 1995).

Nowadays, there are techniques of wastewater purification using microalgae that are able to remove nutrients from domestic wastewater more efficiently than traditional methods (Wang et al., 2009).Therefore, it is becoming in a very promising process (Harun et al., 2010). Moreover, this method can offer several advantages such as nutrients removal or low operating costs, because wastewater contains necessary nutrients for microalgae growth as well asa potentially valuable feedstock for the production of microalgal biodiesel(Olguín,

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2003; Patel et al., 2012).The biofuel production involves the following procedures: cultivation, harvesting, lipid extraction and transesterification. The microalgae cultivation in wastewater can reduce production costs of microalgal biodiesel because cultivation is the most expensive stage (Ryu et al., 2014).

Microalgae are eukaryotic and prokaryotic microorganisms that grow fast due to their unicellular or multicellular structure, low nutrient requirements and higher photosynthetic efficiency (Becker, 1994; Soha,2012). These microorganisms require phosphorous as essential nutrient for growing. Green microalga Chlorella Vulgaris is one of the most used in the world due to fast growth and high lipid content. By its characteristics, this microalga has multiples purposes, ranging from pharmaceutics products to health food (Rekha et al., 2012).Thus, the aim of the present work was to evaluate the uptake of phosphorus and growth of Chlorella Vulgaris in synthetic wastewater. Moreover, the elimination of organic matter was analysed by determining the chemical oxygen demand (COD).

MATERIALS & METHODS

Strains of the eukaryotic green microalga Chlorella Vulgaris were supplied by the Algae Collection of the University of Vigo Marine Science Station (ECIMAT) at Toralla (Vigo, Spain).The microalgae strains were cultured on a synthetic wastewater medium. Chemical composition of synthetic medium was: 1000 mg/L of glucose, 95.5 mg/L NH_4Cl, 56.3 mg/L urea, 22.6 mg/L KH_2PO_4, 12.6 mg/L $FeSO_4.7H_2O$, 309 mg/L $NaHCO_3$ and 35 mg/L yeast extract. The pH value of the initial medium was approximately 8.90±0.09.

To carry out the experiments, the microalga Chlorella Vulgaris was inoculated in a 10% (volume inoculation/volume medium) in three Erlenmeyer flasks containing 1000 mL of synthetic wastewater. Tests were conducted under stationary condition at 26±2ºC and were supplied with an external light source (2x36W daylight normalized fluorescent lamps)with 14/10 of light/dark lightning cycle. Emission spectrum lamps was in the range of 400-700 nm. Agitation of medium was provided by continuously bubbling of air in the flasks. These conditions were maintained during 9 days.

Microalgae growth was determined daily by cell counting and optical density. The number of cells was determined by counting using a Neubauer hemocytometer and the optical density was measured at 680 nm using a spectrophotometer (Shimadzu UV-1800). This wavelength was selected due to the maximum absorbance was obtained at 680 nm when a culture sample was scanning between 550 and 800 nm. At the end of the experiments, algal biomass was determined gravimetrically. Agiven culture volume was harvested and then dewatered by centrifugation at 4000 rpm for 15 min. The microalgae were washed with distilled water and dried to constant weight in the laboratory oven at 60 ºC (Selecta Model Conterm) in order to determine dry weight biomass. The specific growth rates (μ) were determined in basis of the following equation in the exponential phase:

$$\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1) \quad (1)$$

Where X_1 and X_2 symbolizes the cell number (cell/mL) at the first day and end of cultivation, respectively and t_1 and t_2 (days) are the beginning and end time of cultivation, respectively. The equation (1) was previously used by Liu et al., (2011). Thus, daily growth of Chlorella Vulgaris microalgae was easily determined.

The culture was controlled daily by measuring pH, temperature, chemical oxygen demand (COD), phosphorus and optical density (OD) at 680nm. Samples for analysis were collected of culture medium and centrifuged by Selecta Model Mixtasel at 4000 rpm for 10 minutes before testing. Culture pH and water temperature were measured using a pH electrode Crison pH Meter Basic 20 and digital thermometer Quartz Electronic Thermometer, respectively. The COD was calculated according to Standard Methods (APHA 1998) and phosphorus (orthophosphate) were measured by the molibdovanate method according to Standard Methods 4500-P E (APHA, 1998). All the measurements were carried out by triplicate.

In order to analyse the removal of nutrients, liquid samples of culture medium were taken every 24 hours during 9 days. After that, the liquid samples were centrifuged and the supernatants were collected for analysis of nutrients.The percentage of nutrients removal was calculated by the following equation:

$$\%\ \text{Nutrient removal} = ((\text{Initial concentration} - \text{Final concentration}))/(\text{Initial concentration}) \times 100 \quad (2)$$

RESULTS & DISCUSSION

Initially, the synthetic sewage was inoculated with a strain of Chlorella Vulgaris microalgae and three phases in the growth of Chlorella Vulgaris microalga were observed during culture period. These individual stages do not always appear clearly differentiated. Different factors such as temperature, light or nutrient concentration in the medium can modify or even alter these phases (Becker, 1994). The growth of microalgae in terms of cell density is presented in Figure 1. This growth curve shows distinct phases:

1- Adaption (lag phase)
2- Exponential phase
3- Declining growth phase

The first days the lag phase happened and after that, the number of cells increased sharply (exponential phase). This can be checked based on the data shown in Fig. 1, where it can be observed that the first days the green microalga was adjusting and adapting to new environmental conditions. Thus, C. Vulgaris cells were able to adapt to the synthetic medium and low specific growth rate was achieved. This growth rate increased with cultivation time. The next days, once the strain was adapted to the culture medium, microalgae cells began to multiply quickly as a function of time, experiencing exponential growth with a specific growth rate of 0.4111/day (exponential phase). The first day of culturing a cell density of 3305.7±55.6 cell/mL (dry weight 0.06 g/L) was obtained while the maximum cell density was achieved on the sixth day with 25201.0±61.2 cell/mL (dry weight 0.57 g/L). Therefore, growth of Chlorella Vulgaris microalgae depends largely of nutrients present in the synthetic wastewater (Changfu et al., 2013). After sixth day, the next stage was reached (declining growth phase) in which the growth rate of the algal biomass was reduced due to physical or chemical constraints as the absence of nutrient content to sustain microalgal growth, oxygen deficiency or variation of pH (Becker, 1994).

Additionally, a cell counting was performed. This method is a relatively precise method however it needs a lot of time compared to the measure of optical density.Then, it is very useful establish a relationship between cell number and optical density.This relationship depends on culture conditions, such as culture media, air supply, size and weight of cells, etc. For that reason, a lineal correlation can only be found in the exponential phase of the growing (Rocha et al., 2003). The following linear equation between cell number (CN, cells/mL) and optical density at 680 nm (OD) was obtained:

$$CN = 14928 \cdot 10^4 OD - 3015.8 \text{ , } R^2 = 0.9768$$

On the other hand, microalgae growth determined using the optical density in the spectrophotometer is shown in Fig. 2. This method is the easiest and widely used to estimate algae growth. In the present study, growth curve based on cell density (Fig. 1) is more reliable than the growth curve obtained by optical density (Fig. 2). This is because the optical density was not appropriated for measuring cell growth due to possible interference opacity and turbidity of the medium, and the tendency of microalgae cells to form aggregates or agglomerates (Myers et al., 2013).

In addition, the pH value was measured throughout the nine days of culture. Fig. 3 illustrates the pH values obtained during the culture stage of Chlorella Vulgaris microalgae. These values varied in the range from 8.0±0.12 to 9.4±0.25, with a maximum pH value of the sixth day. Other authors have found that Chlorella vulgaris maintained the maximum growth rate in the wide range of pH between 6.0 and 9.0 (Makareviciene et al., 2011; Zhao et al., 2011).

Furthermore, it can be observed that pH was maintained within 8.4 and 9.2 during the lag phase with a low decrease at the end of this period. During the exponential phase, while algae were growing, the pH in-creased rapidly from 8.4 to 9.4. Some studies (Arbib et al., 2011; Zhao et al., 2016) revealed that pH increases during the growth of microalgae due to a shift in the chemical equilibrium system among carbon dioxide, carbonic, carbonate and hydroxide. In which, CO_2 firstly combines with H_2O to form H_2CO_3 which dissociates into HCO^-3 and H+. Then, the carbonic is dissociated in carbonate. During the growing, the amount of carbonate increases and carbonic decreases, resulting in the increase of hydroxide and the value of pH accord-

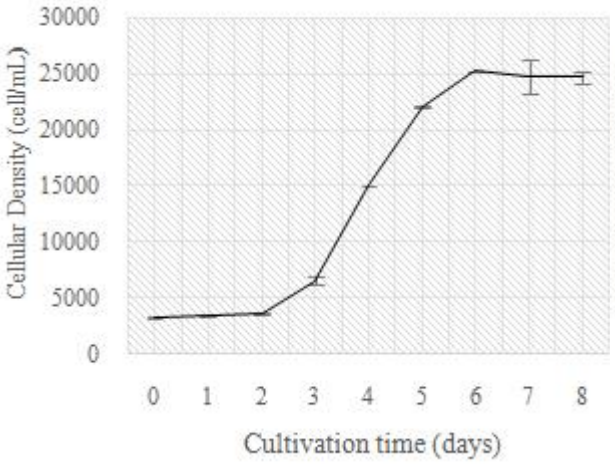

Fig. 1.Chlorella Vulgaris growth curve in terms of cell density

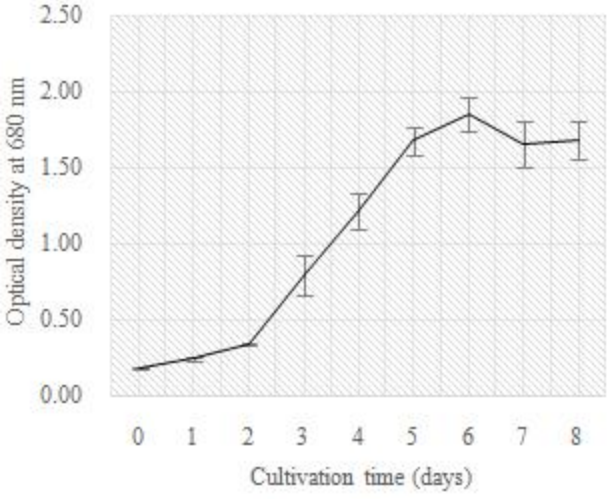

Fig. 2. Chlorella Vulgaris growth curve in terms of optical density at 680 nm

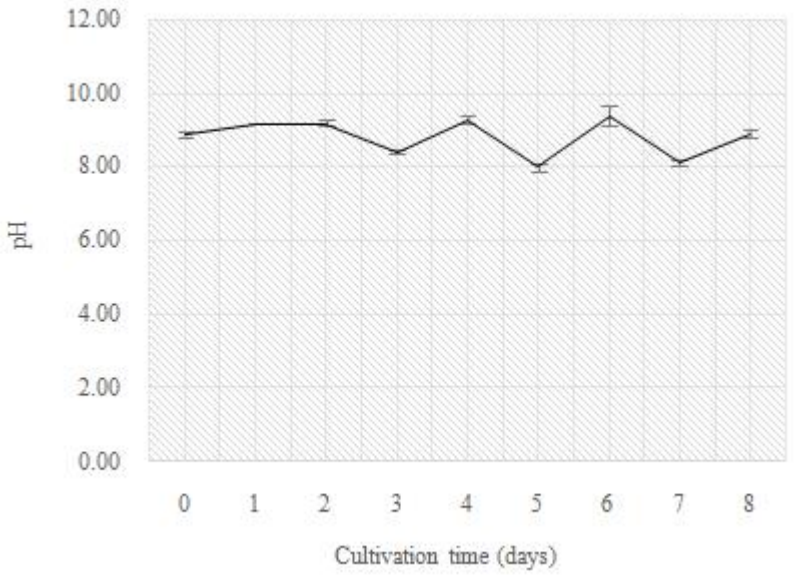

Fig. 3. pH values of Chlorella Vulgaris in synthetic medium

ing the following chemical equilibrium:

$$CO_3^{-2} + H_2O \leftrightarrow HCO_3^- + OH^-$$

One of the main nutrients in freshwater ecosystems and necessary for the growth microalgae is phosphorus. Phosphorus is used by microalgae for the synthesis of cellular constituents such as phospholipids, nucleic acids synthesis and associated reactions with cell division(Martinez et al., 1999; Richmond, 2004). In the present study, phosphorus values were measured daily and are shown in Fig. 4. According to data,an initial phosphorus concentration of 2.51 mg/L was obtained. Along days, this concentration was progressively decreased to a final concentration of 0.02±0.01 mg/L.So, after 9 days of culture, the removal efficiency of Chlorella Vulgaris microalgae in synthetic wastewater was 99.2%. Similar results were obtained by Wang et al., (2010) achieving up to 99% of phosphorus removal. On the other hand, Martinez et al.,(1999) reported that ScenedesmusObliquusmicroalgae were able to remove 97% of phosphorus.

The gradual reduction in phosphorus levels of culture medium isdue to the fact that this nutrient has been absorbed of wastewater byChlorella Vulgaris microalgae, nutrient necessary for its growth. Thus, phosphorus concentration in the medium is directly related to the growth of the microalga, as demonstrated earlier Xin et al.,(2010). Furthermore, it can be said that phosphorus concentration is often a limiting nutrient in microalgae growth (Elser et al.,1990) and the cells can assimilate and store this nutrient diminishing the amount of phosphate in the wastewater.

In this way, final phosphorus concentration of 0.02±0.01 mg/L would be within the requirements established by European Commission Directive 98/15/ ECC for discharge from urban WWTPs (Wastewater treatment plants) to sensitive zones.

COD is an indicator of the presence of organic matter in wastewater. In this research, this parameter was reduced significantly along the 9 days of culture (Fig. 5). So, in the experiments carried out, initial COD concentration in culture medium was 960±1.5 mgO_2/L whereas9 days later, this concentration was reduced at 277.3±48.9 mgO_2/L. Thus, removal percentage efficiency at the end of the culture was 71.1%. In this line, Wang et al., (2010) found that the COD removal efficiency varied widely with different types of wastewater from 50.9% to 83%. Other work was carried out by Lim et al., (2010) in which Chlorella Vulgaris microalgae were cultivated in textile wastewater getting COD removal efficiency from 38.3% to 62.3%. On the basis of the obtained results, it can be concluded that Chlorella Vulgaris microalga was be able to reduce more than half of the chemical oxygen demand in the synthetic wastewater studied.

The obtained results indicate that the levels of phosphorous and COD decreased rapidly due to the fast assimilation by Chlorella Vulgaris microalgae in nine days of culture in wastewater. Some studies have demonstrated that this microalga can grow faster in presence of organic acids or glucose that function directly as essential organic nutrients (Wang et al., 2010).This indicates that microalgae could assimilate some organic compounds, resulting in a rapid drop of chemical oxygen demand concentration during the first days of the culture.

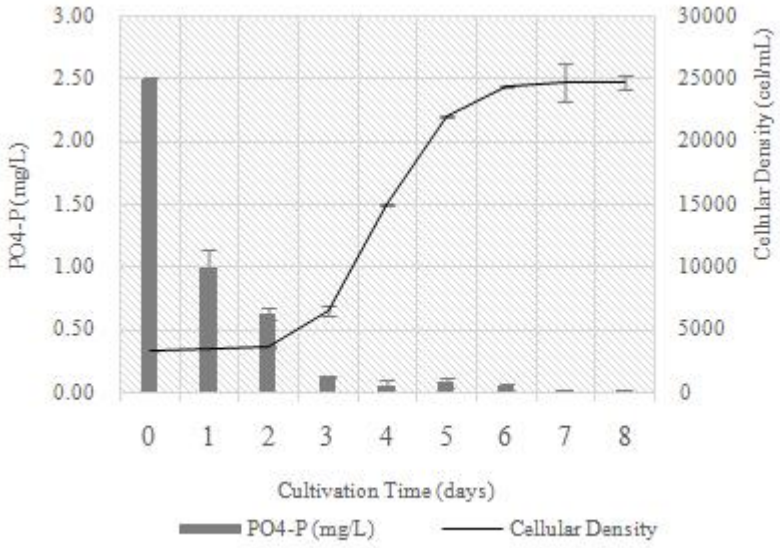

Fig.4. Variation of phosphorous concentration and growth curve of Chlorella Vulgarismicroalgae

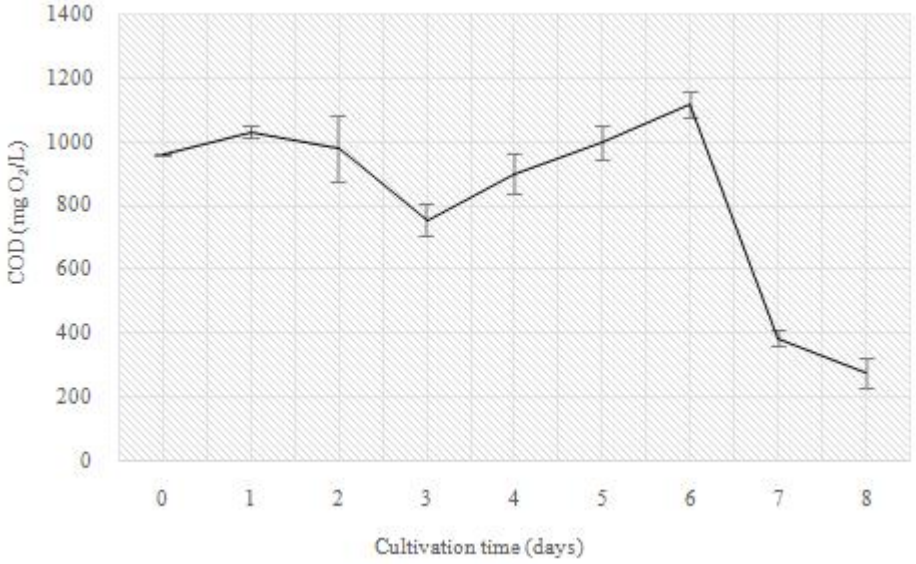

Fig. 5. Variation of COD concentration of Chlorella Vulgaris microalgae

CONCLUSIONS

In this study, the microalga Chlorella Vulgaris has shown great potential removing nutrients in wastewater,being able to remove phosphorus and COD within of environmental standard limits.The amount of phosphorus was nearly totally removed, indicating the ability of Chlorella Vulgaris to synthesize and accumulate it in their cells. COD removal efficiency (71%) was lower than phosphorus removal. Although the wastewater treatment by this microalga is efficient in nutrient reduction, the reduction of COD is not suitable to be used as a secondary wastewater treatment. However, if offers a new possibility for tertiary treatment in waters with a great amount of nutrients that they are not removed efficiently during the process. In this way, the use of Chlorella Vulgaris can reduce the nutrients level in wastewater and produce an appreciated biomass which it can be used for biodiesel production or obtaining valuable items. Therefore, it could be concluded that the bioremediation of wastewater using Chlorella Vulgaris is an effective and environmental option, since they improve the quality of the water and recycle valuable nutrients that can be used for other applications.

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REFERENCES

APHA. (1998). Standard methods for the Examination of Water and Wastewater. (American Public Health Association)

Arbib, Z., Ruiz, J., Álvarez-Díaz, P., Garrido-Pérez, C. and Perales, J. A. (2014).Capability of different microalgae species for phytoremediation processes: Wastewater tertiary treatment, CO2 bio-fixation and low cost biofuels production. Water research, **49,**465-474.

Ashton, Q. (2013). Phosphoric Acids - Advances in Research and Application: 2013 Edition(ScholarlyBrief. ScholarlyEditions)

Becker, E. W. (1994). Microalgae: Biotechnology and Microbiology. (New York)

Changfu, W., Xiaoqing, Y., Hong, L. and Jun, Y. (2013). Nitrogen and phosphorus removal from municipal wastewater by the green alga Chlorella sp. Journal of Environmental Biology, **34,** 421-425.

Elser, J. J., Marzolf, E. R. and Goldman, C. R. (1990). Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North America: a review and critique of experimental enrichments. Canadian Journal of Fisheries and Aquatic Sciences, **47,** 1468-1477.

Gillberg, L., Hansen, H. andKarlsson, I. (2003). About water treatment. KemiraKemwater. ISBN: 91-631-4344-5.

Harun, R., Singh, M., Forde, G. M. andDanquah, M. K. (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. Renewable and Sustainable Energy Reviews, **14(3),** 1037-1047.

Lau, P. S., Tam, N. F. Y. and Wong, Y. S. (1995). Effect of algal density on nutrient removal from primary settled wastewater. Environmental Pollution, **89(1),** 59-66.

Liu, J., Huang, J., Sun, Z., Zhong, Y., Jiang, Y. and Chen, F. (2011). Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic Chlorella zofingiensis: Assessment of algal oils for biodiesel production. Bioresource technology, **102(1),** 106-110.

Makarevi?ien?, V.,Andrulevi?i?t?, V., Skorupskait?, V. andKasperovi?ien?, J. (2011). Cultivation of Microalgae Chlorella sp. and Scenedesmus sp. as a Potentional Biofuel Feedstock. Environmental Research, Engineering and Management, **3(57),** 21-27.

Mart??nez, M., Jiménez, J. M. and El Yousfi, F. (1999).Photoautotrophic consumption of phosphorus by Scenedesmusobliquus in a continuous culture. Influence of light intensity. Process Biochemistry, **34(8),** 811-818.

Morse, G. K., Brett, S.W., Guy, J.A. and Lester, J.N. (1998). Review: Phosphorus removal and recovery technologies. Science of the Total Environment, **212,** 69-81.

Myers, J. A., Curtis, B. S., Curtis, W. R. (2013). Improving accuracy of cell and chromophore concentration measurements using optical density. BMC Biophysics, **6,** 4.

Olguín, E. J. (2003).Phycoremediation: Key issues for cost-effective nutrient removal processes. Biotechnology Advances, **22(1-2),** 81-91.

Pastor, L. (2006). Investigations on the recovery of phosphorus from wastewater by crystallization. (Dissertation.com, Florida)

Patel, A., Barrington, S. andLefsrud, M. (2012). Microalgae for phosphorus removal and biomass production: A six species screen for dual purpose organisms. Global Change Biology Bioenergy, **4,** 485-495.

Sharma, R., Singh, G. P. and Sharma, V. K. (2012). Effects of Culture Conditions on Growth and Biochemical Profile of Chlorella Vulgaris. Journal of plant Pathology & Microbiology, 3(5).

Richmond, A. (2004). Environmental effects on cell composition. In Anonymous Handbook of Microalgal culture-Biotechnology & Applied phycology, 83-93. (Oxford, UK)

Rocha, J. M. S., Garcia, J. E. C. and Henriques M. H. F. (2003). Growth aspects of the marine microalga Nannochloropsisgaditana. Biomolecular engineering, **20,** 237-242.

Ryu, B., Kim, E. J, Kim, H., Kim, J., Choi, Y. and Yang, J. (2014). Simultaneous treatment of municipal wastewater and biodiesel production by cultivation of Chlorella vulgaris with indigenous wastewater bacteria. Biotechnology and Bioprocess Engineering, **19(2),** 201-210.

Soha, S. M. M. (2012).Microalgal Biotechnology: Prospects and Applications. In Plant Science, 275-341.

Stensel, H. D. (1991). Phosphorus and nitrogen removal from municipal wastewater: principles and practice. (Richard Sedlak, Editor)

Tam, N. F. Y. and Wong, Y. S. (1989). Wastewater nutrient removal by Chlorella pyrenoidosa and Scenedesmus sp. Environmental Pollution, **58(1),** 19-34.

Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. Water research,**32(11),**3245-3260.

Türker, S. M. and Akmehmet, B. I. (2006). A case study on algal response to raw and treated effluents from an aluminum plating plant and a pharmaceutical plant. Ecotoxicology and environmental safety, **64(2),** 234-243.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y. andRuan, R. (2010). Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. Applied Biochemistry and Biotechnology, **162(4),** 1174-1186.

Xin, L., Hong-ying, H., Ke, G. and Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. Bioresource Technology, **101(14),** 5494-5500.

Zhao, B., Zhang, Y., Xiong, K., Zhang, Z., Hao, X. and Liu, T. (2011). Effect of cultivation mode on microalgal growth and CO2 fixation. Chemical Engineering Research and Design, **89(9),** 1758-1762.

Zhao, Y., Ge, Z., Lui, H. and Sun, S. (2016). Ability of different microalgae species in synthetic high-strength wastewater treatment and potential lipid production. Journal of Chemical Technology & Biotechnology.