Indian Journal of Science and Technology, *Vol 11(29), DOI: 10.17485/ijst/2018/v11i29/127832, August 2018*
ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

Biodegradation Activity of Crude Oil by *Chlorella* sp. under Mixotrophic Conditions

Deimer Vitola Romero[1], Alexander Pérez Cordero[2] and Yorly Oviedo Garizado [3]

[1]Agricultural Bioprospection Research Group, Master's Students in Environmental Sciences Sue-Caribe, University of Sucre, Sincelejo, Colombia; deimervitolaromero@gmail.com
[2]Agricultural Bioprospection Research Group, University of Sucre, Sincelejo, Colombia; alexander.perez@unisucre.edu.co
[3]Master's Students in Environmental Sciences Sue-Caribe, University of Sucre, Sincelejo, Colombia; yoviedo@mail.uniatlantico.edu.co

Abstract

Objectives: The aim was to determine the biodegradation potential of crude oil by *Chlorella* sp. under mixotrophic conditions, as well as the capacity of production of biomass and photosynthetic pigments. **Methods / Statistical Analysis:** Concentrations of 5 g/L, 10 g/L, 15 g/L and 20 g/L crude oil were evaluated for 15 days with light intensity of 2500 lux, the oil mass removed, the production of biomass and photosynthetic pigments were measured. The treatments were carried out in triplicate and expressed as the mean ± the standard error. A ANOVA and Tukey test were carried out for the significant differences. **Findings:** The microalga Chlorella sp. under the conditions of mixotrophic growth showed the highest average mass removal of crude oil at concentrations of 10 g/L with a percentage of 96.64% on day 15, as well as high biomass production with 0.0786 g/mL and photosynthetic pigments on day 12 with 0.575 **µg**/mL for carotenoids and 1,740 **µg**/mL for chlorophyll-a, while chlorophyll b production was higher at the same concentration but on day 15 with 1,317 **µg**/mL. These results show that *Chlorella* sp. It has the capacity to grow under different concentrations of crude oil and this is positively influenced with high biomass production and high content of photosynthetic pigments with potential for biotechnological applications. **Improvements / Applications:** The mixotróficos growth conditions *in vitro* using crude oil as the sole source of carbon stimulate the productivity of algal biomass and photosynthetic pigments.

Keywords: Biodegradation, Chlorella, Microalga, Mixotrophic, GC-MS

1. Introduction

Environmental pollution has been positioned as the most important problem affecting the world[1], being one of the main environmental deteriorations of soil and water pollution by hydrocarbons of the oil[2], whose pollution is the responsibility of all sectors of the oil industry such as extraction, refinement, transport and consumption. Crude oil, also called black gold, is the most important natural resource in industrialized countries, but its production and transport can cause serious environmental pollution and interrupt the populations of organisms[3]. There is a lot of documented information about the serious damage caused by oil spills on ecosystems and on marine organisms, sediments, mammals, fish, coral reefs, birds, reptiles and surface water bodies[4-5].

In addition, the oil spilled on bodies of water creates a film on the surface that reduces the passage of sunlight responsible for photosynthesis[6] increasing the anaerobiosis of the system. Moreover, Total Petroleum Hydrocarbons (TPH) are an important group of persistent organic pollutants in the environment, with great toxic power for human health and many organisms[7]. Polycyclic Aromatic Hydrocarbons (PAHs) are the most toxic components of crude oil and are associated with carcinogenic, teratogenic and mutagenic effects in both

**Author for correspondence*

animals and humans[8,9] and given their nature hydrophobic can persist for long periods of time[10].

Bioremediation implies the use of living organisms and all their biochemical machinery to degrade and/or transform pollutants into less toxic forms of the environment[11], which has proved to be an effective, safe and less expensive technique[12].

However, a limitation of the bioremediation technique with microorganisms is the availability of nutrients such as nitrogen and phosphorus, affecting the speed of oil degradation[13]. Although the advances in molecular technologies on recombinant DNA have allowed the genetic improvement of many organisms and favor the speed of remediation[14] the use of this technique increases the costs in its application.

The main components of crude oil are naphthenes, asphaltenes, waxes, asphalts, aromatic hydrocarbons, resins and other volatile compounds such as benzene, toluene, ethylbenzene and xylene[15].Many compounds such as pyrene, benzo (a) pyrene and Criseno are carcinogenic, mutagenic and teratogenic[9] and therefore the U.S. Environmental Protection Agency (US-EPA) has considered 16 HAP as a priority contaminant. In addition to this, natural and accidental spills of crude are causing serious damage to life on the planet[16].

However, there are different cleaning techniques in case of oil spills and can be classified into four main groups: physical methods, chemicals, natural processes, biological and a combination of these can be used to achieve effective cleaning[17], but when choosing the appropriate technique, it must have a good cost-benefit balance, and that is where the biological remediation processes gain prominence due to their low cost and high efficiency gaining wide scenarios in the recovery of contaminated sites[18].

There are many species of remedial microorganisms, including some species of microalgae such as *Monoraphidium braunii, Chlamydomonas reinhardtii, Chorella* sp.,etc., fungi such as *Tramates versicolor, Pleurotus eryngii, Phanerochaete chryososporium,* etc., and the bacteria *Pseudomonas aeruginosa, Rhodococcus erythropolis*, etc., which have catabolic pathways for the degradation of contaminants[11].

Algae are essential organisms in aquatic ecosystems and because they are primary producers they play an important role in the trophic chain, providing oxygen and organic substances to other life forms, being *Chlorella vulgaris* an important species due to its potential use to degrade or adsorb a variety of organic pollutants[19].

Therefore, the cultivation of microalgae in wastewater is spreading widely for the elimination of nutrients, control of the physical chemical parameters, as feedstock for the production of biofuel[20], elimination of antibiotics[21-22], removal of phenol and polycyclic aromatic compounds[23,24], thanks to its high adsorption capacity, bioaccumulation, biotransformation and biodegradation[25]. That is why in this investigation it was proposed to determine the potential of biodegradation of crude oil by the microalga *Chlorella* sp.

2.Methodology

2.1 Culture Medium and Growth of *Chlorella* sp

The biomass of *Chlorella sp.* was obtained under mixedotrophic conditions, supplementing the 2.5L of culture medium with 4mM of Nutrifoliar with a cell concentration of 1x106 UFC measured with 647nm wavelength[26], and maintained at 30±1°C for 18±1 days with light and constant agitation for 24 hours[27]. Daily measurements were taken on the same land with a UV-vis spectrophotometer (Spectroquant Pharo 300 from Merck).

Separation and washing of the biomass: Once the fiore actor enters the stationary phase (18±1 days), the microalgae will be separated by centrifugation at a speed of 4000 rpm for 5 min and washed with distilled water to dissolve any type of salt present in excess.

2.2 Biodegradation Activity of Crude Oil

Residual oil and algal biomass were extracted with petroleum ether, concentrated in a rotary evaporator under reduced pressure and temperature, then dissolved in n-hexane to be analyzed by gas chromatography coupled to spectrometry of mass (GC/MS)[28].

The cleaning percentages of the crude oil compounds were calculated using the following formula:

$$R(\%) = \left(\frac{C_0 - T}{C_0}\right) x100$$

Where R is the cleaning percentage of crude oil compounds by microalgae, C_0 is the initial mass of crude oil and T corresponds to the mass after each treatment.

2.3 Determination of Dry Weight

A volume of 5 mL of microalga suspension was taken, centrifuged and washed successively with sterile distilled water, then dried at 80°C to constant weight. The results were expressed in g/L of microbial suspension[28].

2.4 Estimation of Chlorophyll a, b and Carotenoids Pigment

A defined volume of 5 mL of the algal suspension was centrifuged at 8000 rpm for 10 min; after that, the algae granules were treated with 5 ml of ethyl alcohol and were kept in a bain-Marie for 30 min at 55°C, then centrifuged again. It was made sure that the color of the granules was white to guarantee the maximum extraction of pigments. The absorbance of the extract was measured in a Spectroquant Pharo 300 spectrophotometer from Merck at 650, 665 and 452nm. The calculations were made according to the formulas proposed[29] for chlorophyll-a, b and carotenoids.

3. Statistical Analysis

The results of each treatment will be compared by analysis of variance (ANOVA), the significant differences between the means will be determined using the Tukey range multiple test (P≤0.05).

The experiments were performed in triplicate expressing the results as the mean ± the standard error. The analyzes were performed in the InfoStat software.

4. Results and Discussion

4.1 Biodegradation Activity of Crude Oil

The analysis by GC/MS achieved the identification of 135 compounds present in the sample crude oil; of which 68 correspond to PAHs, achieving in all treatments removal percentages above 67% of the total compounds, while the highest percentage of removal was obtained on day 6 with 83% and an 82% removal of polycyclic aromatic hydrocarbons (Table 1). Also, from day 6 to day 15 more than 71% of the PAHs were removed, which shows that the phytoremediation is a viable biological strategy for the removal of compounds present in crude oil, such as PAHs, which have reported irreversible effects on human and animal health[9].

Table 1. Quantity of compounds identified by GC/MS in all treatments

	Crude oil	Removal of compounds (%)	HAPs	HAPs Removed (%)
Control	135		68	50
D1	45	67	25	63
D2	43	68	24	65
D3	35	74	24	65
D6	23	83	12	82
D9	37	73	19	72
D12	36	73	18	74
D15	36	73	16	76

With respect to the amount of crude oil mass removed, significant statistical differences were found (p-value<0.05) between the concentrations of crude oil used, as well as between the days of activity (Figure 1), showing the highest average removal rates of oil on day 15 (Figure 1 A) at the concentration of 10 g/L (Figure 1 B).

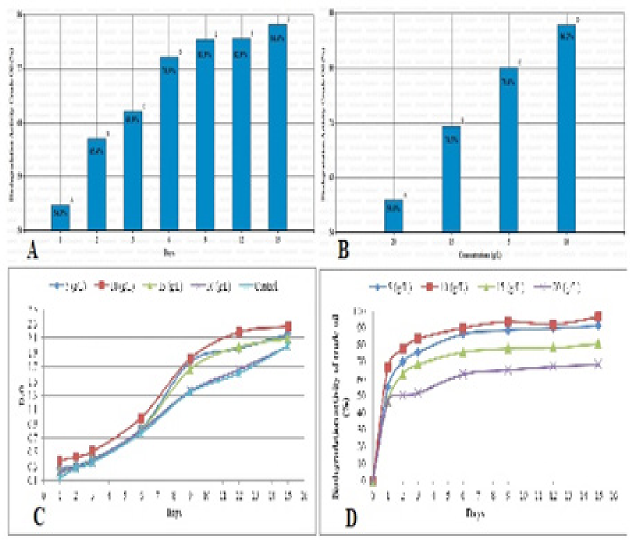

Figure 1. Biodegradation activity of crude oil by Chlorella sp. A: Tukey´s test for biodegradation activity for days. B: for concentrations. C: Effect of different concentration of crude oil on the growth of Chlorella sp. for days and D: by concentration.

However, there were no significant statistical differences (p-value>0.05) in the removal of oil between days 9 and 12. In addition, the concentration of 10 g/L was the one that showed the highest average growth of *Chlorella* sp. with an OD of 2.264 (Figure 1 C), as well as the highest removal percentage with 96.64% of crude oil (Figure 1 D), which demonstrates a high rate of oil removal at said concentration, this the most optimal, since if the concentration of oil is increased, the efficiency of the removal decreases. Nevertheless, it has been shown that *C. vulgaris* shows resistance when exposed to concentrations of 20g/L of oil, achieving a remediation of 94% of light hydrocarbons and 84% for heavy hydrocarbons after 14 days[3]. Therefore, it is demonstrated that algae can grow in the presence of organic contaminants and can be used as promising agents able to bio-transform and biodegrade aromatic contaminants present in the environment[30] and use them as nutrients for their growth[31].

It should be noted that the use of consortiums for the degradation of hydrocarbons are an important remediation strategy[28], since several studies have demonstrated the potential of microalgae-bacteria consortiums to biodegrade these pollutants under heterotrophic growth conditions[32]. However, the biological potential is determined by the presence of pollutants in the environment, which can affect the enzymatic activity of organisms, either increase or decrease that activity, mainly of the enzymes peroxidase and superoxide dismutase that is closely related to contaminant resistance and can be used as environmental biomarkers[33]. In addition, the peroxidase activity was determined over several species of marine microalgae, finding that low concentrations of parathion positively stimulates peroxidase activity, and this is reflected in an increase in cell division[34].

4.2 Determination of dry weight

Significant statistical differences were found between the dry weight of *Chlorella* sp. by concentration and by day, showing the highest average dry weight, the concentration of 10 g/L of oil with 0.0433 g/mL of algae (Figure 2 A), being the 15th highest average with 0.0488 g/mL (Figure 2 B), results superior to the control (Figure 2 C).

The production of dry biomass of *Chlorella* sp. it is influenced by the exposure to crude oil, since it shows an increasing trend from the control to the concentration of 10 g / L, after which it drops suddenly in the concentration of 15 g/L to 20 g/L (Figure 2 C) possibly due to the intoxication of the microalgae with this contaminant, which is reflected in its low growth at these concentrations compared to 5 g/L and 10 g/L, but higher than the control (Figure 1 C).

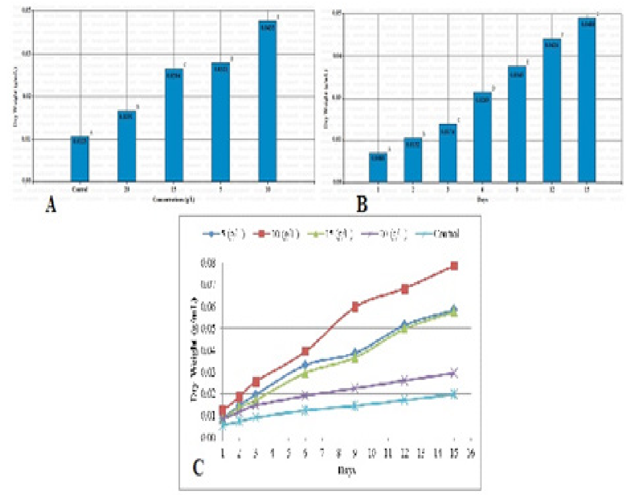

Figure 2. Determination of the dry weight of Chlorella sp. A: dry weight estimation by concentration. B: for day. C: dry weight ratio by concentration and day.

Chlorella sp. experience accelerated growth under mixedotrophic and heterotrophic conditions, compared to autotrophic growth[19,35], reaching dry biomass production yield between 0.050 to 0.10 g/mL under heterotrophic conditions, while in autotrophic cultures the yields were low, around 0.030 g/mL[2]. In addition, under heterotrophic conditions the dry weight production of *C. pyrenoidosa* was 0.0706 g/mL[36] and for *C. protothecoides* it was 0.048 g/mL, consistent results to those reported [37] in this investigation where high production of algal biomass is evidenced when it is subjected to different concentrations of hydrocarbons, because in these growing conditions it increases the yields of generation of ATP (mg of biomass generated per mg of ATP consumed) compared with autotrophic means[35]. Furthermore, the dry weight of *C. vulgaris* increases with the increase in the concentration of crude oil, which indicates that this pollutant stimulates its growth[3], therefore increases the production of biomass under mixotrophic conditions compared to heterotrophic[38].

Chlorella mixotrophic culture proved to be a good strategy to obtain high rates of cell density (Figure 1 C) and high yields of algal biomass (Figure 2 C) with the additional benefit of producing photosynthetic metabolites (Figure 3 D, E and F). Obtaining considerable quantities of algal biomass is very important, since it can be used for the production of biofuels such as biodiesel, bioethanol and biogas[39-41].

4.3 Estimation of pigments chlorophyll a, b and carotenoids

Significant statistical differences were found among all types of pigments, showing higher chlorophyll-a production with a mean of 0.96 μg/mL (Figure 3A) at a concentration of 10 g/L of oil (Figure 3 B), showing that the environments contaminated with crude oil favors the production of all the pigments with respect to the control (Figure 3 D, E, F). In addition, the highest average of these pigments was found on day 12; although this period did not show significant differences with day 9 (Figure 3 C).

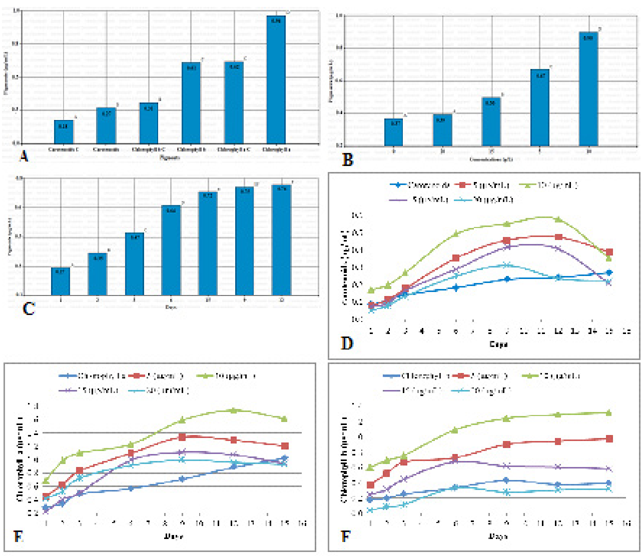

Figure 3. Estimation of pigment production. A: Tukey´s test by types of pigments. B: by concentration C: for day. D: Estimation of carotenoids. E: Chlorophyll-a. F: Chlorophyll-b.

Pigments are chemical species that absorb and emit light at a certain wavelength of the visible spectrum, being of absolute importance in the photosynthetic system of the microalgae, standing out three main groups (a) the chlorophylls: they are found in all the higher plants and photosynthetic algae; (b) carotenoids: present in most algae; and (c) phycobilins: exclusive of cyanobacteria and some red algae, all produced under autotrophic conditions[42].However, under heterotrophic conditions without luminosity significant amounts of chlorophyll have been produced[43], showing expression of the photosynthetic system[44].

Carotenoids are pigments that fulfill different functions during photosynthesis such as the capture of light, photoprotection thanks to their antioxidant capacity, the release of O_2, the dissipation of excess energy and the stabilization of the structure[45]. Therefore, its production is influenced by the pollutant, because concentrations of 5 g/L and 10 g/L of crude oil were the ones that showed the highest average production of these pigments compared to the control.

Similar results have reported a substantial increase in the production of chlorophylls and carotenoids by *C. kessleri* as the concentration of crude oil increased[28], whereas in heterotrophic media, greater production of these photosynthetic pigments has been shown in comparison with photoautotrophic means[38]. While the production of biomass by *Chlorella* sp. did not find statistical differences between algae exposed to natural growth conditions with direct sunlight and photoperiod of 12 hours of light and 12 of darkness, although the algae exposed to direct sunlight presented pale green color translated to low chlorophyll production possibly by photoinhibition[46].

This indicates that light intensity is an important factor when obtaining photosynthetic pigments of these microorganisms. The photosynthetic pigments chlorophyll and carotenoids are of high industrial value, because they can be used in cosmetology, pharmaceutical and food industry as additives[47-50].

5. Conclusions

Crude oil hydrocarbons stimulated the productivity of biomass and photosynthetic pigments during *Chlorella* sp. in mixotrophic conditions, since this pollutant acted as the only carbon source, which shows that the microalga metabolizes it, either by bio-degrading it, bio-transforming it and exploiting it for its growth and survival. So this research marks the course of the use of microalgae as an excellent bioremediation strategy applicable to oil spill tragedies, to alleviate negative environmental impacts.

6. Acknowledgment

The authors are grateful to the University of Sucre for providing its facilities for the development of this research, as well as the Agricultural Bioprospection research group for funding and support.

7. References

1. Reyes Y, Vergara I, Torres OE, Díaz M, González EE. Contaminación por metales pesados: implicaciones en

salud, ambiente y seguridad alimentaria. Revista Ingeniería, Investigación y Desarrollo. 2016 Jun; 16(2):66–77. https://doi.org/10.19053/1900771X.v16.n2.2016.5447

2. El-Sheekh MM, Hamouda RA, Nizam AA. Biodegradation of crude oil by Scenedesmus obliquus and Chlorella vulgaris growing under heterotrophic conditions. International Biodeterioration and Biodegradation. 2013 Aug; 82:67–72. https://doi.org/10.1016/j.ibiod.2012.12.015
3. Xaaldi A, Movafeghi A, Mohammadi-Nassab AD, Abedi E, Bahrami A. Potential of the green alga Chlorella vulgaris for biodegradation of crude oil hydrocarbons. Marine Pollution Bulletin. 2017 Oct; 123(1-2):286–90. https://doi.org/10.1016/j.marpolbul.2017.08.045 PMid:28844453
4. Afshar-Mohajer N, Li C, Rule AM, Katz J, Koehler K. A laboratory study of particulate and gaseous emissions from crude oil and crude oil-dispersant contaminated seawater due to breaking waves. Atmospheric Environment. 2018 Apr; 179:177–86. https://doi.org/10.1016/j.atmosenv.2018.02.017
5. Lee K (chair), Boufadel M, Chen B, Foght J, Hodson P, Swanson S, Venosa. A. Expert Panel Report on the Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments. Royal Society of Canada, Ottawa, ON. 2015.
6. Idris J, Ahmad Z, Eyu GD, Chukwuekezie CS. Oil spills hazard and sustainable mitigation approach: a review. Advanced Materials Research. 2013; 845:955–9. https://doi.org/10.4028/www.scientific.net/AMR.845.955
7. Moreira I, Oliveira O, Triguis JA, dos Santos A, Queiroz A, Martins C, Silva C, Jesus R. Phytoremediation using Rizophora mangle L. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's). Microchemical Journal. 2011 Nov; 99 (2):376–82. https://doi.org/10.1016/j.microc.2011.06.011
8. Paíga P, Mendes L, Albergaria JT, Delerue-Matos CM. Determination of total petroleum hydrocarbons in soil from different locations using infrared spectrophotometry and gas chromatography. Chemical Papers- Slovak Academy of Sciences. 2012 Aug; 66 (8):711–21.
9. Balachandran C, Duraipandiyan V, Balakrishna K, Ignacimuthu S. Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and naphthalene metabolism in Streptomyces sp. (ERI-CPDA-1) isolated from oil contaminated soil. Bioresource Technology. 2012 May; 112:83–90. https://doi.org/10.1016/j.biortech.2012.02.059 PMid:22425516
10. Duran R, Cravo-Laureau C. Role of environmental factors and microorganisms in determining the fate of polycyclic aromatic hydrocarbons in the marine environment. FEMS Microbiology Reviews. 2016 Aug; 40(6):814–30. https://doi.org/10.1093/femsre/fuw031 PMid:28201512 PMCid:PMC5091036
11. Sharma B, Kumar A, Shukla P. Contemporary enzyme based technologies for bioremediation: A review. Journal of Environmental Management. 2018 Mar; 210:10–22. https://doi.org/10.1016/j.jenvman.2017.12.075 PMid:29329004
12. Karigar CS, Rao SS. Role of microbial enzymes in the bioremediation of pollutants: a review. Enzyme Research. 2011 Jul; 7:1–10. https://doi.org/10.4061/2011/805187 PMid:21912739 PMCid:PMC3168789
13. Ron EZ, Rosenberg E. Enhanced bioremediation of oil spills in the sea. Current Opinion in Biotechnology. 2014 Jun; 27:191–4. https://doi.org/10.1016/j.copbio.2014.02.004 PMid:24657912
14. Kumar V, Baweja M, Singh PK, Shukla P. Recent developments in systems biology and metabolic engineering of plant–microbe interactions. Frontiers in Plant Science. 2016 Sep; 7: 1421. https://doi.org/10.3389/fpls.2016.01421 PMid:27725824 PMCid:PMC5035732
15. Sammarco PW, Kolian SR, Warby RAF, Bouldin JL, Subra WA, Porter SA. Distribution and concentrations of petroleum hydrocarbons associated with the BP/ Deepwater Horizon Oil Spill, Gulf of Mexico. Marine Pollution Bulletin. 2013 Aug; 73(1):129–43. https://doi.org/10.1016/j.marpolbul.2013.05.029 PMid:23831318
16. Tornero V, Hanke G. Chemical contaminants entering the marine environment fromsea-based sources: A review with a focus on European seas. Marine Pollution Bulletin. 2016 Nov; 112(1-2):17–38. https://doi.org/10.1016/j.marpolbul.2016.06.091 PMid:27444857
17. Motta FL, Stoyanov SR, Soares JBP. Application of solidifiers for oil spill containment: A review. Chemosphere. 2018 Mar; 194:837–46. https://doi.org/10.1016/j.chemosphere.2017.11.103 PMid:29223426
18. Kadri T, Magdouli S, Rouissi T, Kaur S. Ex-situ biodegradation of petroleum hydrocarbons using Alcanivorax borkumensis enzymes. Biochemical Engineering Journal. 2018 Apr; 132:279–87. https://doi.org/10.1016/j.bej.2018.01.014
19. Kong Q, Zhu L, Shen X. The toxicity of naphthalene to marine Chlorella vulgaris under different nutrient conditions. Journal of Hazardous Materials. 2010 Jun; 178(1-3):282–6. https://doi.org/10.1016/j.jhazmat.2010.01.074 PMid:20133058
20. Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan RR. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp. Bioresource Technology. 2010 Apr; 101(8):2623–8. https://doi.org/10.1016/j.biortech.2009.10.062 PMid:19932957
21. Santaeufemia S, Torres E, Mera R, Abalde J. Bioremediation of oxytetracycline in seawater by living and dead biomass of the microalga Phaeodactylum tricornutum. Journal of Hazardous Materials. 2016 Dec; 320:315–25. https://doi.org/10.1016/j.jhazmat.2016.08.042 PMid:27565856

22. Xiong JQ, Kurade MB, Jeon BH. Biodegradation of levofloxacin by an acclimated freshwater microalga, Chlorella vulgaris. Chemical Engineering Journal. 2017 Apr; 313(1):1251–7. https://doi.org/10.1016/j.cej.2016.11.017
23. Gao Q, Wong YS, Tam N. Removal and biodegradation of nonylphenol by different Chlorella species. Marine Pollution Bulletin. 2011; 63(5-12):445–51.
24. El-Sheekh M, Ghareib M, El-Souod G. Biodegradation of phenolic and polycyclic aromatic compounds by some algae and cyanobacteria. Journal of Bioremediation and Biodegradation. 2012 Dec; 3:1–9. https://doi.org/10.4172/2155-6199.1000133
25. He N, Sun X, Zhong Y, Sun K, Liu W, Duan S. Removal and biodegradation of nonylphenol by four freshwater microalgae. International Journal of Environmental Research and Public Health. 2016 Dec; 13(12):1239. https://doi.org/10.3390/ijerph13121239 PMid:27983663 PMCid:PMC5201380
26. Infante C, Angulo E, Zárate A, Florez JZ, Barrios F, Zapata C. Propagación de la microalga Chlorella sp. en cultivo por lote: cinética del crecimiento celular. Avances en Ciencias e Ingeniería. 2012; 3 (2):159–64.
27. Sánchez E, Garza M, Almaguer V, Sáenz I, Li-án A. Estudio cinético e isotermas de adsorción de Ni (II) y Zn (II) utilizando biomasa del alga Chlorella sp. inmovilizada. Ciencia UNAL. 2008; 11 (2):168–76.
28. Hamouda RA, Sorour NM, Yeheia DS. Biodegradation of crude oil by Anabaena oryzae, Chlorella kessleri and its consortium under mixotrophic conditions. International Biodeterioration and Biodegradation. 2016 May; 112:128–34. https://doi.org/10.1016/j.ibiod.2016.05.001
29. Sengar H. Charakterlsiarung einer synckronkultur yon Scenedesmus obliquus (Characterization of the synchronous culture of Scenedesmus obliquus). Planta. 1970 Sep; 90 (3):243–66.
30. El-Sheekh MM, Hamouda RA, Biodegradation of crude oil by some cyanobacteria under heterotrophic conditions. Desalination and Water Treatment. 2014 May; 52 (7-9):1448–54. https://doi.org/10.1080/19443994.2013.794008
31. Sforza E, Cipriani R, Morosinotto T, Bertucco A, Giacometti GM. Excess CO2 supply inhibits mixotrophic growth of Chlorella protothecoides and Nannochloropsis salina. Bioresource Technology. 2012 Jan; 104:523–9. https://doi.org/10.1016/j.biortech.2011.10.025 PMid:22088657
32. Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R. Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. Environment International. 2013 Jan; 51:59-72. https://doi.org/10.1016/j.envint.2012.10.007 PMid:23201778
33. Chen S, Chen M, Wang Z, Qiu W, Wang J, Shen Y, Wang Y, Ge S. Toxicological effects of chlorpyrifos on growth, enzyme activity and chlorophyll a synthesis of freshwater microalgae. Environmental Toxicology and Pharmacology. 2016 Jul; 45:179–86. https://doi.org/10.1016/j.etap.2016.05.032 PMid:27314761
34. Xu JY, Fu SW, Tang XX, Li YQ. Toxic effects of parathion on four kinds of marine microalgae. Marine Environmental Science. 1998; 4:10–2.
35. Pérez-García O, Bashan LE, Hernández JP, Bashan Y. Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic and mixotrophic cultivation of 1 C. vulgaris immobilized with Azospirillum brasilense. Phycologia. 2010 Aug; 46 (4):800–12. https://doi.org/10.1111/j.1529-8817.2010.00862.x
36. Wu ZY, Shi CL, Shi XM. Modeling of lutein production by heterotrophic Chlorella in batch and fed-batch cultures. World Journal of Microbiology and Biotechnology. 2007 Sep; 2 (9):1233–8. https://doi.org/10.1007/s11274-007-9354-2
37. Sun N, Wang Y, Li YT, Huang JC, Chen F. Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic Chlorella zofingiensis (Chlorophyta). Process Biochemistry. 2008 Nov; 43 (11): 1288–92. https://doi.org/10.1016/j.procbio.2008.07.014
38. Miazek K, Remacle C, Richel A, Goffin D. Beech wood Fagus sylvatica dilute-acid hydrolysate as a feedstock to support Chlorella sorokiniana biomass, fatty acid and pigment production. Bioresource Technology. 2017 Apr; 230:122–31. https://doi.org/10.1016/j.biortech.2017.01.034 PMid:28187341
39. Yusoff MF, Xu X, Guo Z. Comparison of fatty acid methyl and ethyl esters as biodiesel base stock: a review on processing and production requirements. Journal of the American Oil Chemists' Society. 2014 Apr; 91(4):525–31. https://doi.org/10.1007/s11746-014-2443-0
40. Prajapati SK, Choudhary P, Malik A, Vijay VK. Algae mediated treatment and bioenergy generation process for handling liquid and solid waste from dairy cattle farm. Bioresource Technology. 2014 Sep; 167:260–8. https://doi.org/10.1016/j.biortech.2014.06.038 PMid:24994683
41. Piemonte V, Di Paola L, Iaquaniello G, Prisciandaro M. Biodiesel production from microalgae: ionic liquid process simulation. Journal of Cleaner Production. 2015 Jan; 111:62–8. https://doi.org/10.1016/j.jclepro.2015.07.089
42. Morales-Sánchez D, Martinez-Rodríguez OA, Martinez A. Heterotrophic cultivation of microalgae: production of metabolites of commercial interest. Journal of Chemical Technology & Biotechnology. 2016 May; 92(5):925–36. https://doi.org/10.1002/jctb.5115

43. Pérez-García O, Escalante F, De-Bashan L, Bashan Y. Heterotrophic cultures of microalgae: metabolism and potential products. Water Research. 2011 Jan; 45 (1):11–36. https://doi.org/10.1016/j.watres.2010.08.037 PMid:20970155
44. Morales-Sánchez D, Tinoco R, Kyndt J, Martínez A. Heterotrophic growth of Neochloris oleoabundans using glucose as a carbon source. Biotechnology for Biofuels. 2013 Jul; 6 (100):1–13. https://doi.org/10.1186/1754-6834-6-100
45. Markou G, Nerantzis E. Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. Biotechnology Advances. 2013 Dec; 31(8):1532–42. https://doi.org/10.1016/j.biotechadv.2013.07.011 PMid:23928208
46. Nurachman Z, Hartini H, Ridhani RW, Kurnia D, Hidayat R, Prijamboedi B, Suendo V, Ratnaningsih E, Goretty PLM, Nurbaiti S. Tropical marine Chlorella sp. PP1 as a source of photosynthetic pigments for dye-sensitized solar cells. Algal Research. 2015 Jul; 10:25–32. https://doi.org/10.1016/j.algal.2015.04.009
47. Chen P, Min M, Chen Y, Wang L, Li Y, Chen Q, Wang C, Wan Y, Wang X, Cheng Y, Deng S, Hennessy K, Lin X, Liu Y, Wang Y, Martinez B, Ruan R. Review of the biological and engineering aspects of algae to fuels approach. International Journal of Agricultural and Biological Engineering. 2009 Dec; 2 (4):1–30.
48. Hosikian A, Lim S, Halim R, Danquah MK. Chlorophyll extraction from microalgae: a review on the process engineering aspects. International Journal of Chemical Engineering. 2010 Mar; p.32–43. https://doi.org/10.1155/2010/391632
49. Draaisma RB, Wijffels RH, Slegers PME, Brentner LB, Roy A, Barbosa MJ. Food commodities from microalgae. Current Opinion in Biotechnology. 2013 April; 24(2):169–77. https://doi.org/10.1016/j.copbio.2012.09.012 PMid:23084075
50. Maki K, Yurko-Mauro K, Dicklin M, Schild A, Geohas J. A new, microalgal DHA- and EPA-containing oil lowers triacylglycerols in adults with mild-to-moderate hypertriglyceridemia. Prostaglandins Leukot Essent Fatty Acids. 2014 Oct; 91 (4):141–8. https://doi.org/10.1016/j.plefa.2014.07.012 PMid:25123060