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Exploring of the Green Algae Chlorella Vulgaris Potential for Phenanthrene Biodegradation

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Abstract

The distribution of Polycyclic Aromatic Hydrocarbons (PAHs), a group of toxic and persistent aromatic pollutants, in the environment is rapidly increasing. These compounds have adverse impacts on the health of living organisms. It is necessary to find an effective method for the elimination of PAHs from ecosystems. In the last few decades, phycoremediation as an effective, low-cost, and eco-friendly technology for the cleanup of different pollutants has gained great attention. Hence, the present study has focused on the potential of the green microalga Chlorella vulgaris for the degradation of phenanthrene as a toxic 3-ring PAH. The impact of phenanthrene on Chlorella vulgaris cells was evaluated by assay of algae growth, protein assay, and Gas Chromatography–Mass Spectrometry (GC–MS) analysis. Four different concentrations (2, 10, 25, and 50 mg L^{-1}) of phenanthrene were selected for the study. Intriguingly, optical density, as a growth factor of algae, was enhanced when treated with 2 mg L^{-1} of phenanthrene after 7 days of exposure in comparison to control. The cellular growth and total protein content were decreased as the concentration of phenanthrene increased from 10 to 50 mg L^{-1} at the same exposure. Furthermore, the GC/MS technique explained the biological degradation of phenanthrene in the present research, and accordingly, a number of intermediate byproducts were identified. The obtained results confirmed that phenanthrene is able to induce cytotoxicity in Chlorella cells in high concentrations, and subsequently, Chlorella vulgaris has noticeable potential in its biodegradation.

Keywords: Biodegradation, Chlorella vulgaris, Phenanthrene, Green microalgae, Phycoremediation.

1 | Introduction

During recent decades, Polycyclic Aromatic Hydrocarbons (PAHs) have become one of the largest groups of environmental pollutants [1-4]. PAHs are composed of at least two fused benzene rings [3]. PAHs are entered into the environment either by natural [4] or anthropogenic activities [3]. PAHs have harmful effects on the environment, living organisms, and humans owing to their mutagenic, toxic, and carcinogenic properties [7-10]. Therefore, PAH-contaminated sites remediation is a matter of challenge for ecologists [7], [8].

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Different physicochemical and biological techniques have been developed to preserve the environmental stability of the ecosystems versus PAHs [13-15]. Recently, phycoremediation that uses macro- or microalgae for environmental remediation has emerged as one of the promising, efficient, low-cost, eco-friendly biological approaches [14-18]. Microalgae have been introduced as an important biosystem for the treatment of wastewater, which currently gains a surge of interest because of their fast-growing pattern and cost-effectiveness [14-22]. In addition, microalgae culture for wastewater remediation provides a dual role of biotreatment in conjunction with the generation of valuable biomass for the production of biofuels such as bioalcohol and biodiesel [11], [14], [23-25]. Thus, wastewater cleanup coupled with biofuel production offers a powerful platform for environmental remediation.

A number of previous studies clearly demonstrated the considerable potential of microalgae in the bioremediation of wastewater [1], [2], [26-28]. Among several microalgal species, Chlorella vulgaris has been proposed as an appropriate biosystem for phycoremediation of pollutants in contaminated aquatic environments due to its specific vital features, including high growth rate, low food requirements, and ease of cultivation. Furthermore, Chlorella vulgaris has a very rigid cell wall with a significant capability for adapting to different environmental conditions [29-31].

Despite many advantages, the use of microalgae in the bioremediation of PAHs is still in its infancy stage. It is well known that some microalgal species are able to degrade many types of PAHs to less harmful components [14], [21], [20]. However, more studies are needed to investigate the role of different microalgal species to provide a better understanding of the microalgal impact on PAHs degradation.

In the present work, the potential of Chlorella vulgaris for the biodegradation of phenanthrene, as the representative of 3-ring and low-molecular-weight PAHs with high concentration [1], was examined. Phenanthrene is mentioned in the priority pollutants list of the United States Environmental Protection Agency (US-EPA) for monitoring in the environment [22], [23]. Its characteristics and chemical structure are presented in *Table 1*. Chlorella vulgaris species was treated with different concentrations of phenanthrene under experimental conditions, and subsequently its growth, the protein content, as well as remediation efficiency were examined. Furthermore, the Gas Chromatography–Mass Spectrometry (GC-MS) technique was used to identify intermediate products that were formed during the degradation process. The findings of this study deliver more data on PAHs phycoremediation as a raised area for further research.

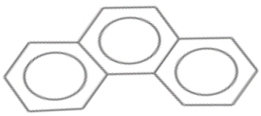
Chemical structure	
Chemical class	Polycyclic Aromatic Hydrocarbons
Molecular formula	$C_{14}H_{10}$
Mw ($gmol^{-1}$)	178.22

Fig. 1. Structure and characteristics of phenanthrene.

2|Material and Methods

2.1|Chlorella Cell Culture and Exposure Conditions

Chlorella Vulgaris was provided from the Culture Collection of Algae, Bushehr Shrimp Research Institute, Iran, and was cultured in BG11 medium under sterile conditions [6]. The stock of algal cultures was incubated

at a photon flux density of 80 μmol m^{-2} s^{-1} with a 12/12 h light/dark photoperiod at a room temperature of 25±2 °C with continuous aeration. The microalgal culture medium, including a nourishing mixture, was changed every week for the optimal growth of the algae.

The experiments were performed in 250-mL Erlenmeyer flasks, each including 100 mL BG11 medium and repeated 3 times for control (media without phenanthrene) and treated samples with 2, 10, 25, and 50 mg L^{-1} of phenanthrene. Various concentrations of phenanthrene were obtained from a stock solution of 1000 mg L^{-1} in acetone. The algal cells, in the exponential growth phase, were added to the culture media after complete evaporation of acetone and grown on a rotary shaker at 120 rpm for 48 h at 25 °C. After 7 days, the cells were harvested and used for different assays.

2.2 | Assay of Alga Growth

The growth of microalgae was evaluated by measuring the OD (optical density) of algal suspension at 600 nm as an indicator using a Ultraviolet (UV)/visible spectrophotometer [24]. Accordingly, treated chlorella vulgaris cells with phenanthrene at different concentrations (2, 10, 25, and 50 mg L^{-1}) were appraised along with a control every 24 h for 7 days.

2.3 | Protein Assay

The culture media were discarded by centrifugation and the algal pellets were frozen in liquid nitrogen and then homogenized in phosphate buffer (0.05 M, pH 7.0) at 4 °C. After centrifugation of the homogenates at 10,000 ×g for 10 min at 4 °C, the obtained supernatant was immediately used for the determination of protein content with Bovine Serum Albumin (BSA) as the standard reference using a Ultraviolet–Visible Spectroscopy (UV-Vis) spectrophotometer according to Bradford 1976 [25].

2.4 | Extraction of Sample for Degradation Study

No microalgae growth inhibition of phenanthrene as a toxic pollutant was observed at a concentration of 10 mg L^{-1}, and thus this concentration was selected for degradation studies. Accordingly, approximately 3.0×10^7 cells were inoculated into 100 mL culture media of Chlorella vulgaris containing 10 mg L^{-1} of phenanthrene. BG11 culture media with and without phenanthrene were maintained as control samples. After the 7th day of incubation, algal cells were discarded from the media using centrifugation at 3000 ×g for 5 minutes at 4°C. Then, the media were transferred to a decanter, and the organic compounds of the solution were extracted using 3 × 25 mL of diethyl ether. Subsequently, the collected organic fraction was evaporated at room temperature, and the remaining solid material was dissolved in 100 μL of absolute methanol [20]. Finally, the resulting products were analyzed by GC–MS.

2.5 | Gas Chromatography–Mass Spectrometry Analysis

GC-MS analysis was carried out to identify the possible byproducts resulting from the phenanthrene biodegradation by C. vulgaris. For the analysis of samples, an Agilent 6890 GC system and a GC-MS equipped with a Shimadzu GC-MS-QP 5050A gas chromatograph fitted with a DB-1 (polydimethylsiloxane, 60 m × 0.25 mm i.d.) capillary column was used with the following oven temperature program: 50°C for 5 min, 7 °C min^{-1} increase up to 290 °C, and 10 min hold time. The inlet and detector temperatures were 260 °C and 290 °C, respectively [20]. Helium was utilized as the carrier gas with a column flow rate of 0.9 mL min^{-1}. 1 μL of prepared extract was injected into the capillary column in the split mode. Mass spectra were obtained at a rate of 1.6 scans s^{-1}, electron energy of 70 eV, and mass/charge range of 30–650 m z^{-1}.

2.6 | Analytical Procedures

One-way Analysis of Variance (ANOVA) using multiple comparison tests based on Duncan, by applying SPSS 21 software, was conducted. The tests were performed in triplets. Statistical significance was considered at $p \leq 0.05$, and the obtained results were expressed as the mean ± standard deviation.

3 | Results

3.1 | Determination of Algal Growth

The effects of different concentrations of phenanthrene and incubation time on the microalgal growth in terms of optical density have been shown in *Fig. 1*. Intriguingly, after approximately 2 days of incubation, the growth of C. vulgaris was increased subsequent to the treatment with 2 mg L^{-1} phenanthrene, compared to the reference sample. According to Duncan's analysis, this increase in the biomass yield was significant after 4 days until the end of the treatment time. Indeed, the highest growth of C. vulgaris was observed when the cells were treated with 2 mg L^{-1} phenanthrene *Fig. 1*. The concentration of 10 mg L^{-1} of phenanthrene has remarkably lessened the optical density of C. vulgaris versus the control sample for the four days. However, 10 mg L^{-1} of phenanthrene had no significant effect on the optical density of C. vulgaris from the fifth day after treatment *Fig. 1*. While there was an inhibited growth, when 25 and 50 mg L^{-1} of phenanthrene was added to the media in comparison to the control at all treatment days; the cells in these media did not proliferate for 3 days. However, the algal density was gradually increased in the same media in the presence of 25 and 50 mg L^{-1} of phenanthrene from the fourth day *Fig.1*.

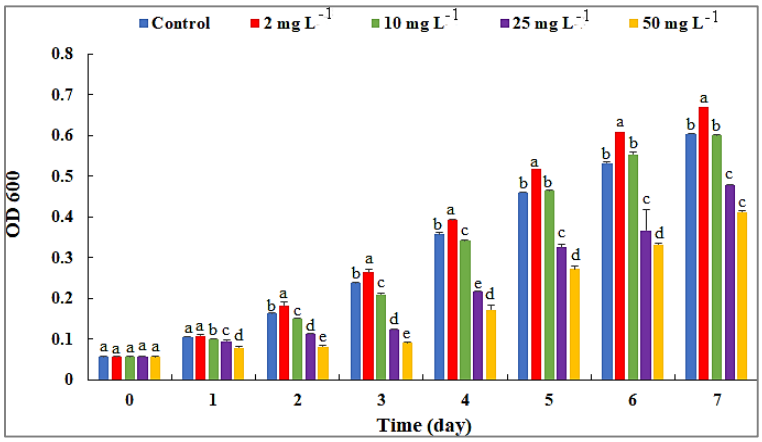


Fig. 2. The effect of different concentrations of phenanthrene on the growth of Chlorella vulgaris during 7 days.

3.2 | Estimation of Protein

Total protein content of Chlorella cells was assessed after 7 days of exposure to different concentrations of phenanthrene (0, 2, 10, 25 and 25 mg L^{-1}) *Fig. 2*. The protein content was not affected when treated with 2 mg L^{-1} of phenanthrene, but a statistically significant change in comparison with the control ($p< 0.05$) was observed from 10 mg L^{-1} and upper concentrations of phenanthrene.

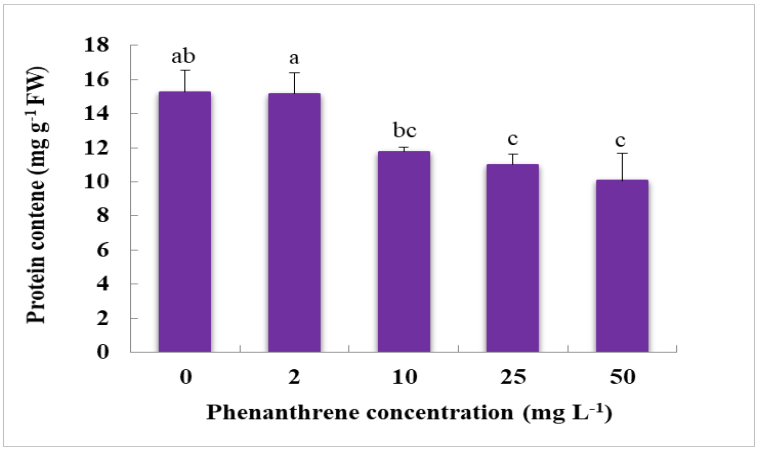


Fig. 3. Changes in the total protein of Chlorella cells treated with different concentrations of phenanthrene after 7 days of exposure. Different letters indicate significant differences ($P < 0.05$) according to Duncan Test (mean ± SE, n = 3).

3.3 | Analysis of Phenanthrene Biodegradation and Identification of Byproducts

By employing GC–MS analysis, some intermediate compounds were identified during phenanthrene biodegradation by Chlorella vulgaris. In abiotic control (the media without algae containing phenanthrene) and biotic control (phenanthrene-free media containing algae), none of these compounds were detected by GC–MS. Accordingly, sixteen compounds have been successfully identified and listed in *Table 2*. The constituents were recognized by matching their spectra with those recorded in the Mass library (NIST21&107 and WILEY229).

4 | Discussion

The results of this study showed that 2 mg L^{-1} phenanthrene had a positive effect on growth and biomass production of C. vulgaris. Similar results have been reported with El-Sheekh et al. [21] on Scenedesmus obliquus and Chlorella vulgaris and Kalhor et al. [26] on C. vulgaris exposed to different concentrations of crude oil, who indicated the treatment of algal cells with crude oil led to high algal biomass production. In our previous study, increment in the dry weight, fresh weight, and cell density of C. vulgaris exposed to 2 mg L^{-1} of fluorene was reported [20]. It seems that the ability of C. vulgaris in the consumption of phenanthrene as a carbon source led to the optimum growth of algal cells at low concentrations.

The higher concentrations of phenanthrene led to the reduction of algal density. The negative effects of various contaminants on algal growth as a consequence of the induced cytotoxicity were formerly reported [1], [2], [36-38]. It mostly led to the disruption of vital and systematic functions of the algal cells and oxidative damage in the photosynthetic system [6], [12], [13], [29]. As a result, the restoration growth of cells at high concentrations of phenanthrene, 4 days after culture confirms that the microalgae Chlorella vulgaris is resistant to phenanthrene toxicity and able to recover, probably by a robust defense system.

The obtained results showed that total protein content was decreased by different concentrations of phenanthrene. Likewise, an investigation on the influence of acenaphthene and fluoranthene on the protein content of Chlorella vulgaris revealed a maximum reduction of 97% and 96%, respectively, on the 16th day [7]. Similar results were reported on Anabaena fertilissima using anthracene and pyrene, which showed a dose-dependent reduction in protein content [30]. It could be concluded that the inhibition of protein biosynthesis affected by phenanthrene toxicity could be due to the interruption of structural proteins and essential enzymes that participate in microorganism growth [31].

GC-MS analysis findings showed some intermediate metabolites during phenanthrene degradation *Table 2*. Based on the GC-MS analysis results, a possible pathway for phenanthrene degradation using Chlorella

vulgaris was suggested *Fig. 3*. In the proposed pathway, phenanthrene is subjected to aromatic rings cleavage to form the intermediates. As illustrated in *Fig. 3*, one of the initial steps in the phenanthrene biodegradation seemed to be the opening of the middle aromatic ring and subsequently the attachment of some groups, such as hydroxyl and amine groups, to the compound, which can lead to the formation of phenol, 4-(phenylamino) compound. The dioxygenase enzyme system in algal cells is reported to be mostly used for the biodegradation of PAHs like phenanthrene [30], [32]. Therefore, it can be assumed that early oxidation of polycyclic molecules using oxidizing enzymes led to the cleavage of aromatic rings. On the other hand, hydroxylation of xenobiotic molecules raises their hydrophilicity, which is required to augment their reactivity [33], [34]. Furthermore, the addition of an amine group into the aromatic structure can be under the influence of the media constitutes.

Afterward, more oxidation led to the formation of dibutyl phthalate and 1,2-benzenedicarboxylic acid, dioctyl ester, which are probably produced by the ring-cleavage of the dioxygenase system. The degradation pathway of PAHs by fungi and bacteria has been previously proposed, and phthalic acid and its derivations have been produced as the main byproducts [43-47]. Our previous results are also in agreement with the literature, indicating that dibutyl phthalate and 1,2-benzenedicarboxylic acid, dioctyl ester were formed during the biodegradation of fluorene by Chlorella vulgaris [20]. Eventually, complete cleavage of the benzene ring by oxidizing enzymes could yield the single-chain compounds and fatty acids such as tetracosanoic acid, methyl ester, hexadecanoic acid, ethyl ester, 9-octadecenoic acid, ethyl ester, and 2-hexadecen-1-ol, 3, 7, 11,15-tetramethyl. In accordance with our data, these compounds were reported in the biodegradation pathways of benzopyrene by Arthrobacter oxydans (B4), and pyrene by Mycobacterium sp. Strain KR2 [32] and acenaphthene and fluoranthene by Chlorella vulgaris [7]. In another study, tetradecanoic acid and benzene ring-containing metabolites were produced by cyanobacterium Anabaena fertilissima after exposure to 5.0 and 10.0 mg L^{-1} anthracene for 16 days. Additionally, erucic acid was detected in all of the treatments exposed to pyrene after 16-days [30].

Table 1. Formed intermediate metabolites during phenanthrene degradation by Chlorella vulgaris.

Number	Compound	Retention Time (min)	Molecular Formula	KI
1	Pentadecane, 2,6,10-trimethyl	30.408	C18H38	1652
2	Tetradecane, 2-methyl	30.608	C15H32	1665
3	Benzenesulfonamid, N-butyl	31.975	C10H15NO2S	1794
4	Heptadecane, 3-methyl	32.442	C18H38	1774
5	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	33.508	C20H40O	2116
6	Dibenzothiophene, 3-methyl	33.833	C13H10S	1853
7	Octadecane, 2-methyl	33.917	C19H40	1864
8	Octadecane, 3-methyl	34.042	C19H40	1874
9	Phenol, 4-(phenylamino)	34.358	C12H11NO	-
10	Tetracosanoic acid, methyl ester	34.650	C25H50O2	2712
11	Dibutyl phthalate	34.908	C16H22O4	1914
12	Nonadecane, 3-methyl	35.583	C20H42	1974
13	Hexadecanoic acid, ethyl ester	35.642	C18H36O2	1975
14	9-Octadecenoic acid, ethyl ester	38.125	C20H38O2	2175
15	Benzenamine, 4-nitro-N-phenyl	38.958	C12H10N2O2	1886
16	1,2-Benzenedicarboxylic acid, dioctyl ester	42.600	C24H38O4	2860

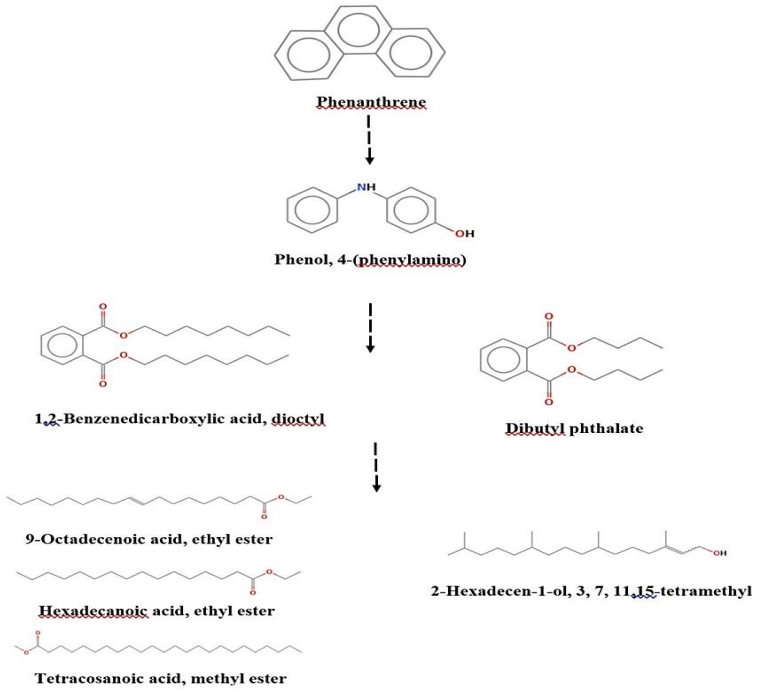


Fig. 4. Proposed pathway for the degradation of phenanthrene by Chlorella vulgaris.

5 | Conclusion

The obtained results conclusively revealed considerable potential of Chlorella vulgaris in the biodegradation of phenanthrene as a persistent and toxic environmental pollutant. Phenanthrene induced cytotoxicity in the algal species in a dose-dependent manner, which was determined through the decline of optical density and total protein content. Furthermore, some intermediate metabolites were detected during the phenanthrene degradation process, which proposed a possible metabolic pathway for phenanthrene degradation in Chlorella vulgaris microalgae. Since Chlorella vulgaris shows considerable resistance against the pollutants, it can surely be an attractive target for future phycoremediation studies. Accordingly, complementary studies are required to find out the complete degradation pathway and a biological remediation scheme for PAHs by Chlorella vulgaris.

Authors' Contributions

All aspects of the research and manuscript preparation were carried out by the author. The author has read and approved the final version of the manuscript.

Data Availability

All data supporting the reported findings in this research paper are provided within the manuscript.

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Conflict of Interest

The author declares that they do not have any conflict of interest.

Consent for Publication

The author confirms consent for the publication of this work

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants performed by the author.

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