Pyrene Biodegradation Capability of Two Different Microalgal Strains

Eman S. E. Aldaby[1] and Asmaa M.M Mawad[1*]

[1:]Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

[*]corresponding author: a.mawad@aun.edu.eg

[1]Coauther:- emanaldaby@aun.edu.eg

**Corresponding author* Fax: +20 882108109.

Abstract

Biological treatment is one of the most preferable strategies to complete and safe removal of pollutants. Pyrene (PY) is the first member of high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) that represents various concerns to biological life and human health. In this study, two different algal strains exhibited different capability to degrade PY along 30 days of incubation in the light. Dry weight, total chlorophyll, and carotenoids were growth parameters that were determined to detect robust of two algal strains to get used of PY as a source of carbon. *Oscillatoria* sp. could degrade 95% while *Chlorella* sp. could degrade 78.71 % of PY (50 mg/L) after 30 days of incubation. Both algal strains could completely remove 10 and 30 mg/L of PY. On the other hand, the degradation capability of *Oscillatoria* sp. was significantly exceeded than Chlorella sp. under the same incubation condition and at (50 and 100 mg/L) of PY.

Keyword: Carotenoids, *Chlorella* sp., Chlorophyll, kinetics, *Oscillatoria* sp., PAHs, Pyrene

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are considered the most serious environmental pollutants. According to their chemical structure, they consist of two or more fused benzene rings in linear, angular, or cluster arrangements. They characterized as a persistent compounds in the environment due to their low water solubility, relative stability or low volatility, and resistance to biological degradation (Sartoros *et al.*, 2005). They accumulate in the environment as a result of anthropogenic activities such as automobile exhausts, agricultural and waste incineration, forest fires, coal, and spillage of crude oil to water and soil; or due to nature activities such as combustion of fossil fuels, and petroleum products (Sartoros *et al.*, 2005, Haritash & Kaushik, 2009).

Removal of PAHs from the environmental contaminated sites is very important since most of them are known carcinogens and mutagens, as well as they, have many concerns to the public health (Grimmer *et al.*, 1982, Perera *et al.*, 1998, Luch, 2005). These pollutants gave several direct and indirect harmful effects on the human according to (Vijayaraghavan & Shanthakumar, 2015). Physical, chemical, and biological strategies have been used to remove these PAH compounds from polluted sites(Riser-Roberts, 1998, Gan *et al.*, 2009). However, these strategies are associated with some disadvantages such as high operation costs, low efficiency, and potential for secondary air or groundwater pollution due to the formation of toxic by-products. Biodegradation is an effective treatment strategy to remove PAH compounds from polluted sites because toxic microorganisms can decompose these organic substances into a simpler nontoxic product. By increasing in molecular weight of PAHs, the lipophilicity, environmental persistence, and genotoxicity increase. More specifically, pyrene is considered a genotoxic and bioaccumulative (Ahn *et al.*, 2010). It has many toxic effects on both human and animal health such as reproductive difficulties and causing cancer. Therefore, the maximum contaminant limit of pyrene in drinking water has been established at 0.2 µg/L (Paria, 2008). Additionally, The US Environmental Protection Agency (EPA) has listed 16 PAHs, including pyrene (PY), among the 129 priority pollutants (Jin *et al.*, 2007).During the past decades, several publications have reported that, pyrene can be degraded by different microbial species such as bacteria (Toyama *et al.*, 2011, Hesham *et al.*, 2014, Mawad *et al.*, 2016),

fungi (Hesham *et al.*, 2017), plants (Liste & Alexander, 2000), and microalgae(Chan *et al.*, 2006, Yan *et al.*, 2014). Among the microalgal strain some chlorophyta like *Scenedesmus platydiscus*, *Chlorella vulgaris*,, *Scenedesmus quadricauda, (Lei et al., 2007), chlorella* sp. MM3 *(*Subashchandrabose *et al*., 2017) *and Selenastrum capricornutum (Chan et al., 2006).*

Cyanophyta and eukaryotic microalgae have three different ways to remove Polyaromatic hydrocarbons from the environment; 1) adsorption of PAHs on the surface of algal cells depending upon the active groups present on that sufaces, 2) accumulation of PAHs within the algal cells and 3) transformation of PAHs which depending upon the enzymatic actions.the third method of removal is considered the effective one due to get rid of PAHs toxcicity (Semple et al., 1999, El-Sheekh et al., 2012).

The kinetics of biodegradation is essential in the control of the bioremediation process (Lu *et al.*, 2012)**.** Biodegradation kinetics also provides a valuable explanation about the degradation pathways and reaction mechanisms. Therefore, application of kinetic data to the kinetic models provide an expectation of the rate at which a target toxic PAHs can be removed (Aryal & Liakopoulou-Kyriakides, 2013).

The major objective of this study is to evaluate the capability of two microalgal strains *Oscillatoria* sp*.* (Cyanophyta) and *Chlorella* sp. (chlorophyta) to degrade pyrene, determine the different concentrations of pyrene on the growth, dry weight and photosynthetic pigment contents of tested microalgae and to detect the most suitable model that describe PY biodegradation kinetics.

Materials and Methods

Biodegradation activity of algal strains on Pyrene

Two microalgal species *(Oscillatoria* sp. and *Chlorella* sp.) have been used in our study were isolated from Assiut region (Egypt) and cultivated on BG 11 medium for one week at room temperature for enrichment and activation the cells. Algal cells (OD750=0.3) were dispersed into a fifty milliliters of sterile BG11 supplemented with acetone-dissolved pyrene (PY) to a final concentration 50 mgL^{-1} followed by evaporation of acetone with gentle shaking. After 5 days intervals, aliquots of 5 ml were withdrown for determination the microalgal growth and degradation. Aliquots of BG11 medium containing algal cell without PY, served as the positive control. Cultures were incubated under the atmosphere condition at temperature 28±2 ° °C, at 100 µmol photons $m^{-2}s^{-1}$ of continuous PAR light, and 100 rpm agitation using a MiniOrbital Shaker (VWR, USA) for four weeks.

The growth of microalgae was monitored by OD 750 each experiment was carried out in duplicate and repeated twice, and the averages of four results were used for statistical analysis by T-test.

Pyrene Biodegradation Percentage by HPLC

Aliquots of algal growth sample (5ml) were withdrawn each 7 days extracted twice with an equal volume of ethyl acetate and passed through anhydrous sodium sulphate to get rid of any traces of water. The ethyl acetate extract then introduced to HPLC analysis (Hesham *et al.*, 2014).

To determine the remaining concentration of PY, calibration standard was prepared by dissolving different concentrations of PY (5-2000 μgml^{-1}) in ethyl acetate. Identification of the concentration of PY was performed using HPLC model Waters 600E equipped with autosampler waters 717 plus and dual wavelength UV detector model Waters 2487 (set at 254 nm). The condition of operation was as follows: Column: SUPELCOSIL ™ LC-PAH, 15 cm x 4.6 mm, 5µmInjection volume: 2.0 µl. Mobile phase: acetonitrile (A): water (W) isocratic program, HPLC grade Water%: 40% Acetonitrile % : 60 %.

Effect of the initial PY concentration on microalgal growth

Five different concentrations of PY (10, 30, 50, 100 and 200 mgL^{-1}) were separately supplemented to the microalgal (BG11) medium to determine the influence of the initial concentration on cell density (OD750), dry weight, and photosynthetic pigment contents.

Determination of the photosynthetic pigments content

Total Chlorophyll and carotenoids were extracted and monitored from 2 ml algal suspension by centrifugation and the growth media were decanted. Pigments were extracted in hot methanol for 10 minutes, according to the method described by (Marker, 1972). Cells debris was removed by centrifugation and the clear supernatant, which contains the pigments, was diluted to a definite volume. The absorbance was measured against methanol blank spectrophotometrically at the wavelengths of (663, 644 and 452 nm). The concentrations of each pigment fraction (total chlorophyll and cartenoids) was expressed as (µg/ml) culture

Determination of dry weight

Aliquots of algal suspension (20 ml) were filtered above a glass fiber filter. The filter paper with algal cells was dried overnight in an oven at 80 °C. After cooling reached to room temperature, they were reweighed and the dry weight was calculated. Data of dry matter contents were given as ($mg.ml^{-1}$) algal suspension.

Biodegradation kinetics of PY

The degradation kinetics of organic compounds pyreneby Microbial cells can be defined by following the kinetic equation (Jianlong *et al.*, 2002):

$$\gamma = (\gamma_m c)/(k+c) \quad (2)$$

Where γ is the biodegradation rate, $\gamma_m$is the maximum specific biodegradation rate, c is the substrate concentration, and k is the half-saturation constant. If c<<k, Eq. 2 can be reduced to:

$$\gamma = (\gamma_m c)/k \quad (3)$$

Eq. 3 is a typical first-order biodegradation kinetic model. Assuming $k_1=(\gamma m/k)$ and integrating it, Eq. 3 can be expressed as:

$$\ln c=a+k_1 t \quad (4)$$

Where k_1 is the first order kinetic constant and t is the time (per hour).The biodegradation half-life of first-order reaction can be also expressed as(Zeng *et al.*, 2004):

$$t_{1/2}=\ln 2/k_1 \quad (5)$$

If c>>k, another simplified equation can be derived from Eq. 2:

$$\gamma =\gamma_m \quad (6)$$

Eq. 6 describes a zero-order biodegradation kinetic model and the biodegradation rate constant $k_0=\gamma m$. Thus, the relation of PY concentration with time is given as (Jianlong *et al.*, 2002):

$$c=b+k_0 t \quad (7)$$

where k_0 is the rate constant for zero-order kinetics.

Results and Discussion

Growth of microalgae and degradation of PY

The time-courses of microalgal growth and PY degradation with an initial concentration of 50 mgl−1 were determined at 100 rpm at 30 ˚C. As shown in Fig. 1a&b, the significant ($p\leq0.05$) increase in the algal biomass corresponding to the decrease of remaining concentration of PY was observed, after 30 days of incubation. However, the amount of PY removal by *Oscillatoria* sp. (95%) was significantly ($p\leq0.05$) higher than the PY removal by *Chlorella* sp. (78.7%).

Many algal species are capable of growing heterotrophically on different organic sources (Semple *et al.*, 1999). Many investigations discussed the role of algae on the degradation of crude oil components. Among these, the species of *Chlorella* sp. were widely used for biodegradation of pollutants (Subashchandrabose *et al.*, 2014, Subashchandrabose *et al.*, 2017). (Subashchandrabose *et al.*, 2017) mentioned that, Species of *Chlorella* sp. do not utilize organic compounds as carbon sources, but they could degrade them during the detoxification process. Photodegradation of PAHs by photosynthetic microorganisms as microalgae has been very well established, and the source of light plays a crucial role in yielding photodegradation products (Subashchandrabose *et al.*, 2014). In fact, the fluorescent light used in the present study might have little impact on photodegradation of PY as lately mentioned by (Subashchandrabose *et al.*, 2017)

In this study, the efficiency of two microalgal strains *Oscillatoria* sp. and *Chlorella* sp. to degrade 50 mg L^{-1} of PY was higher than the efficiency of *Chlorella* sp. MM3 (50 µM pyrene within 21 days in soil slurry system) that being combined with tween-80 used as a surfactant to make PY more available to the microalgae (Subashchandrabose *et al.*, 2017). In addition to the degradation efficiency in this study was higher than some bacterial strains such as and *Rhodococcus*sp (0.08 mg pyrene mL^{-1} day^{-1}) according to (Walter *et al.*, 1991).

Regarding to the microalgal growth, the curve of *Oscillatoria* sp exhibited rapid growth rate and significant ($p \leq 0.05$) higher algal biomass density (OD750=1.1) compared to *Chlorella* sp. (OD750=0.52), after 30 days of incubation. On the other hand, *Chlorella* sp. shifted the OD750 from 0.3 to 0.4 after 7 days of incubation which indicated short lag phase of 7 days, however this period extended to 14 days with *Oscillatoria* sp.

(Zhao *et al.*, 2005)used the optical density (OD.750nm) in order to following up growth of the different species of microalgae. The use of OD to follow microalgal growth has the advantage of being fast for sub-culturing in particular medium. The cell density of *Chlorella sp*. Strain MM3 increased from 1.1 × 105 cells mL−1 to 16.45 × 105 cells mL^{-1} within 7 days when it has been grown in presence of 50 µM pyrene. The degradation of pyrene was dependent on the concentration of algal biomass used, the more the biomass, the higher the degradation percentages (Lei *et al.*, 2006). In addition to biomass, cell density, cell wall composition and enzymes involved in PAH degradation might be important in determining the species- species variation in the degradation of pyrene (Lei *et al.*, 2007).

Effect of initial concentration on microalgal degradation efficiency

The capability of both algal strains to degrade PY was separately tested in BG 11 medium consist of different initial concentrations of PY (10, 30, 50, 100, 200 mg L^{-1}). Results in Fig 2 showed that, generally, the biodegradation ability decreased with the increase of PY concentration. The two algal strains could completely remove PY (100%) when the initial concentration of PY was 10 and 30 mg L^{-1}. On the other hand, when the initial concentration of PY was ranged from 50-200 mg L^{-1}, the biodegradation efficiency was significantly different. In addition to, the biodegradation efficiency of *Oscillatoria* sp. was significantly ($p \leq 0.05$) higher than *Chlorella* sp. at higher concentration of PY. The biodegradation efficiency of *Oscillatoria* sp. was 95, 83 and 68 % while for Chlorella sp. were 78, 66 and 49 % when the initial concentration was 50, 100 and 200 mg L^{-1}, respectively. Additionally, it was observed that, by increasing the initial concentration of Py, the algal growth decreased. The highest optical density was recorded at low concentration of 10-30 mg L^{-1} while the lowest one was recorded at initial concentration of 200 mg L^{-1}. However, the growth of *Oscillatoria* sp. was significantly higher than *Chlorella sp.* at tested initial concentration as shown in (Fig.2).

The variation between two microalgal strain in the degradation capability is indicating the higher initial concentrations of PY constrain the biodegradation process (Ziagova & Liakopoulou-Kyriakides, 2007). This decreased biodegradation efficiency of *Chlorella* sp. can be attributed to extending substrate toxicity on microalgal cell systems of the culture at high concentrations (Chang & Alvarez-Cohen, 1995)

The biodegradability of PAHs appeared to be also related to microalgal initial cell density. Many literatures reported that, the initial cell density controlled the ability of algae to attack the aromatic ring. (Chan *et al.*, 2006) reported that, the initial cell densities less than 10^4 cells mL^{-1} of *Selenastrum capricornutum* were insufficient for PAHs biodegradation. Also, (Lei *et al.*, 2006, Lei *et al.*, 2007) observed degradation of PAHs was proportional increase with increasing cell densities of different green microalgae such as *Scenedesmus platydiscus*, *Chlorella vulgaris*, *Selenastrum capricornutum*, and *Scenedesmus quadricauda.*

Effect of PY concentration on algal biomass and photosynthetic pigment contents

The algal biomass contents such as dry weight (mg mL^{-1}), total chlorophyll and carotenoid (µg mL^{-1}) were determined as the methods described above. Fig. 3 a&b illustrated that, the dry weight of *Oscillatoria* sp. was significant higher than of *Chlorella* sp. but the total chlorophyll and carotenoid contents of the later were higher than *Oscillatoria* sp. at various concentrations of PY. The low concentrations of PY (10-30 mgL^{-1}) enhanced the growth of *Oscillatoria* sp. and significantly increased its dry weight and total chlorophyll however, there was no significant variation in the carotenoid contents at the different concentrations of PY (fig.3 a). Interestingly, the results of *Chlorella* sp. exhibited the highest dry weight, total chlorophyll and carotenoid contents at 50 mgL^{-1} of PY without any significant difference in these biomass contents in either higher or lower PY concentrations (FIG.3b). The increased in chlorophyll accompanied with increased in the dry weight due to increase in photosynthesis. Chlorophyll content represented the internal indicator of photosynthetic capacity of organisms (Takáčová *et al.*, 2014).

The biochemical changes in microalgal system that induced by PAHs are widely used for accessing the toxicity of this contaminants (Subashchandrabose et al., 2017). Pyrene was revealed to be toxic and stimulate stress responsive antioxidant reactions in several microalgae(Lei et al., 2006). The present study showed that the carotenoids which represent antioxidant substances were highly induced by *Chlorella* sp. at high concentration of PY although it did not serve as a stress indicator with *Oscillatoria* sp.

The water solubility and lipophilicity as well as the molecular weight, of the PAHs compounds affect the degradation capablity of microorganisms. The 4-ring PAHs, pyrene, were easier to be degraded by *Selenastrum capricornutum* than phenanthrene, a 3-ring PAH(Chan *et al.*, 2006). Besides that, two flamentous fungi isolated from PAHs-contaminated soil, *Coniothyrium* sp. and *Fusarium* sp. preferentially decomposed high molecular weight PAHs (5–6 ring) than low molecular weight PAHs (Potin *et al.*, 2004) .

The biodegradation kinetics of pyrene

Biodegradation kinetic plays an important role in illustration the mechanisms of biodegradation as well as the reaction pathways. Application of experimental kinetic data to the kinetic models will be able to expect the rate of removal of toxic substance.

The kinetic results of the biodegradation of PY by two algal strains were shown in Table 1. As it can be revealed from this table, low correlation coefficient values (R^2= 0.89)was observed for first-order kinetics equation model at *Oscillatoria* sp. indicated that, this model was invalid with this strain however, it was valid with *Chlorella* sp. with high correlation coefficient value (R^2= 0.96). On the other hand, relatively high correlation coefficient values (R^2= 0.98 and 0.99) suggested that, the biodegradation kinetics of both *Oscillatoria* sp. And *Chlorella* sp. were well fitted with the zero-order kinetic equation model (Fig. 4).

In addition to, the calculated zero-order rate coefficient (k_0) values of *Oscillatoria* sp. was higher than *Chlorella* sp. sp (Table 1), indicating the rate of PY biodegradation of *Oscillatoria* sp. exceed *Chlorella*. sp Also, from the first order kinetics model, the calculated half-life biodegradation period of *Oscillatoria* sp. and Chlorella sp. extended to about 137.3 and 346.5h (Table 1), respectively.

The HPLC metabolitlic products

The HPLC profile for PY and its metabolites were detected after 30 days of microalgal growth in BG11 medium that supplemented with PY. The HPLC chromatogram showed that there were five additional peaks that may indicate that there were five metabolic products. These peaks were detected due to PY degradation by *Chlorella* sp. while six metabolites were detected for *Oscillatoria* sp. as illustrated in (fig.5 a& b).That means the rate of PY degradation in *Oscillatoria* sp. more in *Chlorella* sp. which accompanied with increased in rate of growth in *Oscillatoria* sp. in compared to *Chlorella* sp. That may be due to the increase in the smallest compounds which can use as nutrient for the algal growth. For that, the rate of growth increased with the increased of degradation of PY. As a consequence of breaking down of aromatic cycle, that produced

organic acids. The biggest benefit of such systems can be described as the consumption of produced organic acids by microbial metabolism followed by subsequent synthesis of cell components and energy source (Takáčová *et al.*, 2014).

Conclusion

Pyrene was degraded by two different microalgal species *Oscillatoria* sp. and *Cholrella* sp. during 30 days of incubation. The first species exhibited higher biodegradation capability and higher growth parameters (OD750, total cholorphyll, carotenoids and dry weight) in the presence of different concentrations of PY. Biodegradation kinetics of PY of both microalgal species followed the zero-order kinetic equation model.

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List of figures caption

Fig.1 Time course of variation of microalgal growth (OD 750) and remaining Pyrene concentration (mgL^{1}) for *Oscillatoria* sp (a) and *Chlorella* sp (b) at 30 C for 30 days in light.

Fig. 2 Microalgal degradation capability and growth (OD 750) at different PY concentration. [a] Indicates statistically significant differences ($P < 0.05$) between the degradation percentage of *Oscillatoria* sp and *Chlorella* sp.

Fig 3 Effect of different initial PY concentrations on microalgal species *Oscillatoria* sp (a) and *Chlorella* sp (b) on microalga dry weight (mg ml-1) , total chlorophyll and carotenoid ($\mu g\ L^{-1}$) at 30 C for 30 days in light

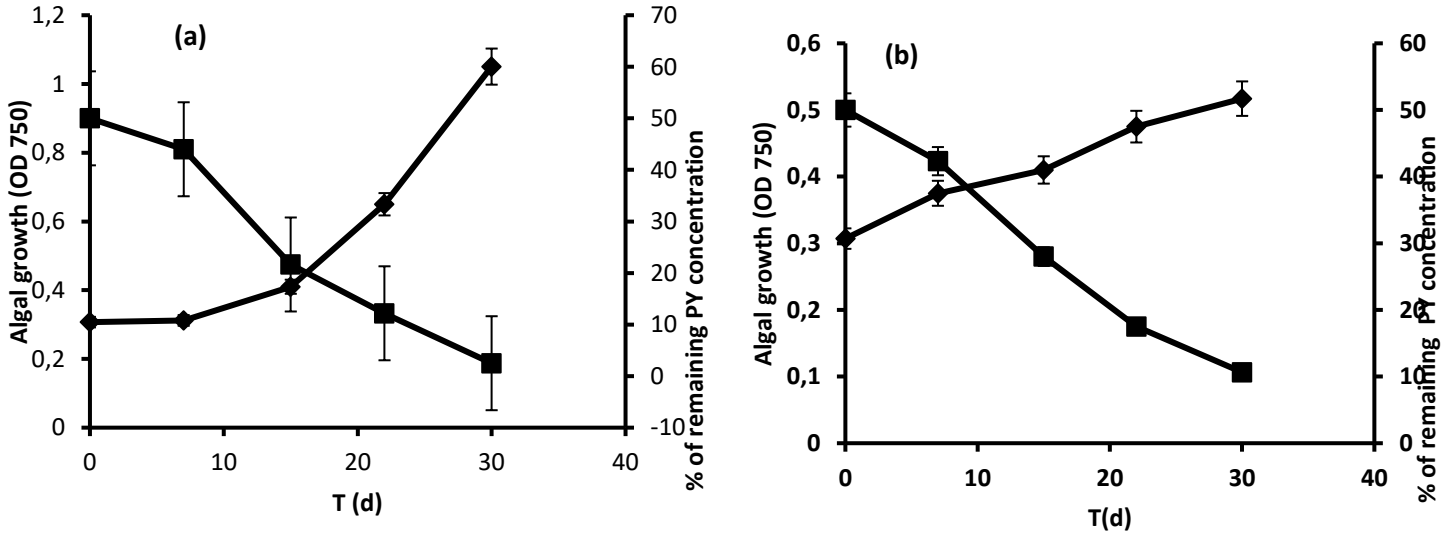
Fig. 4 zero and first-order kinetics equation (a&b).

Fig.5 HPLC chromatogram of microalgal species *Oscillatoria* sp (a) and *Chlorella* sp (b) after 30 days of PY degradtion

List of tables legends

Table1:-Biodegradation kinetics of pyrene of microalgal species (*Oscillatoria*sp.and *Chlorella* sp.).

List of Figures



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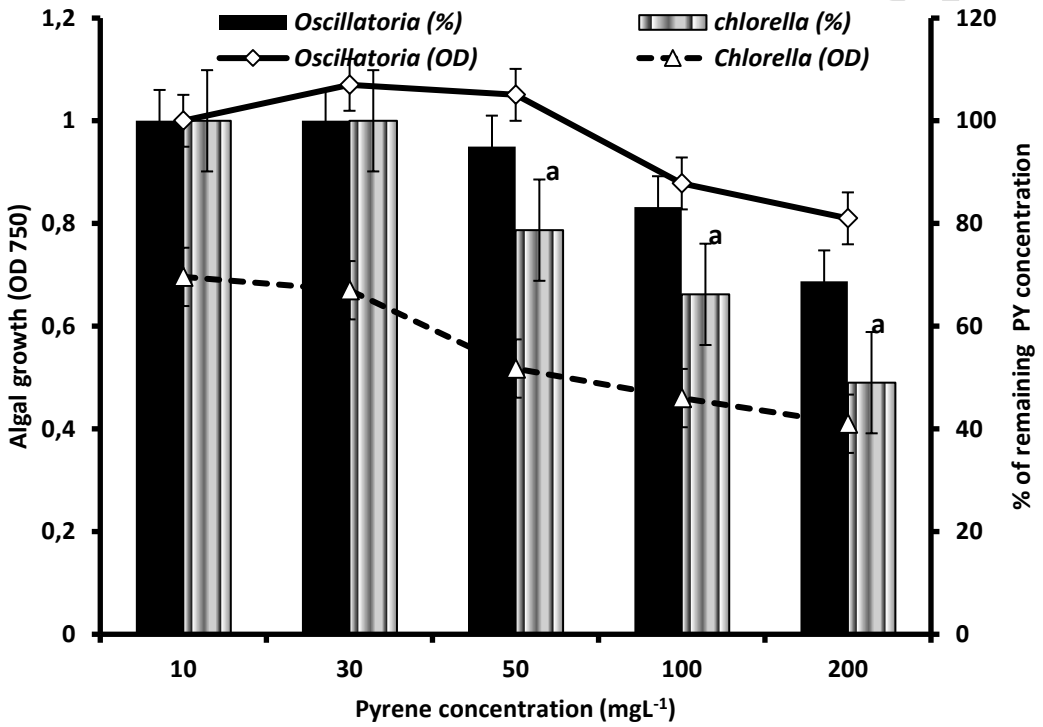

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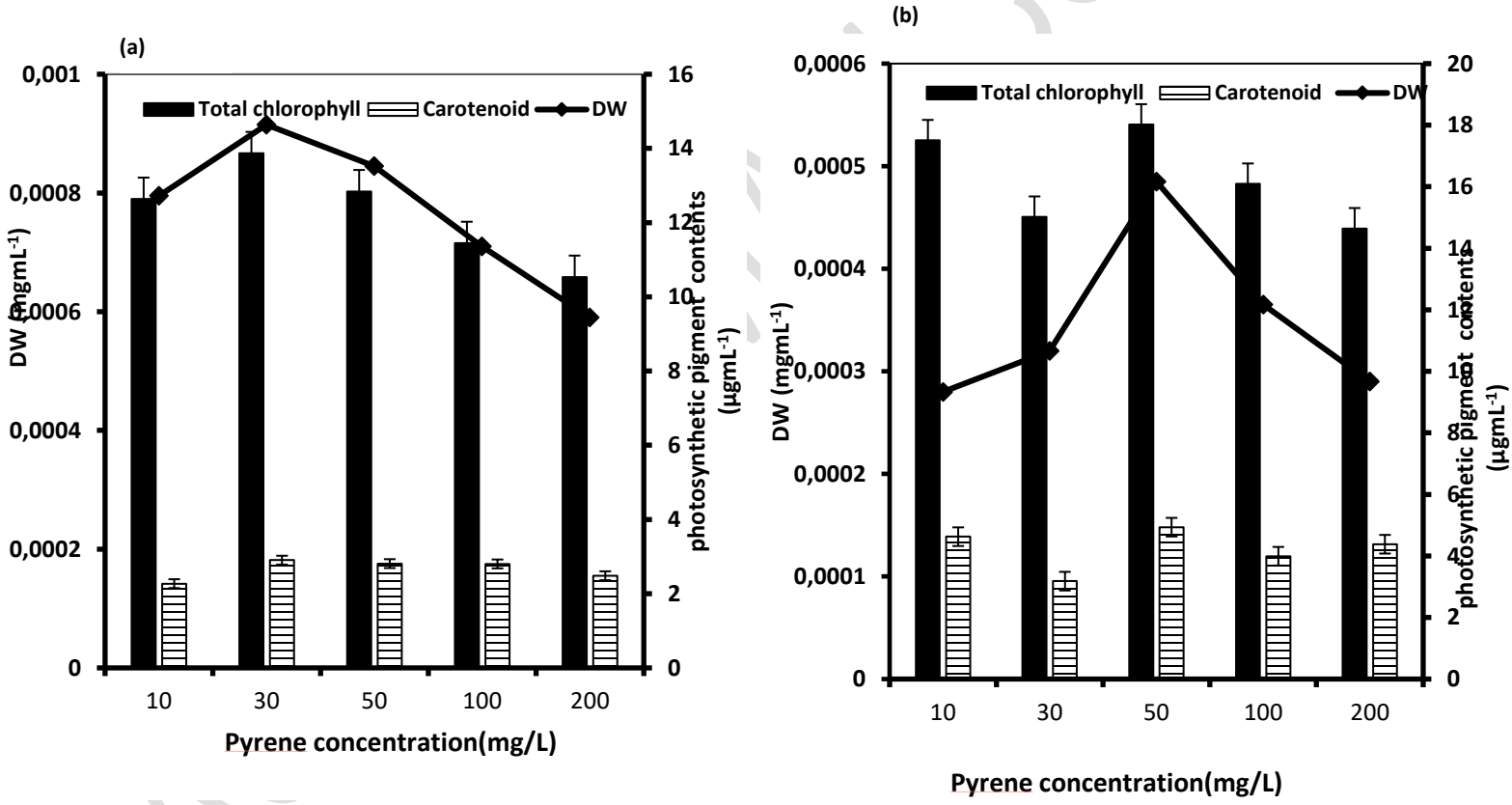
Fig 3



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373 **Fig .4**

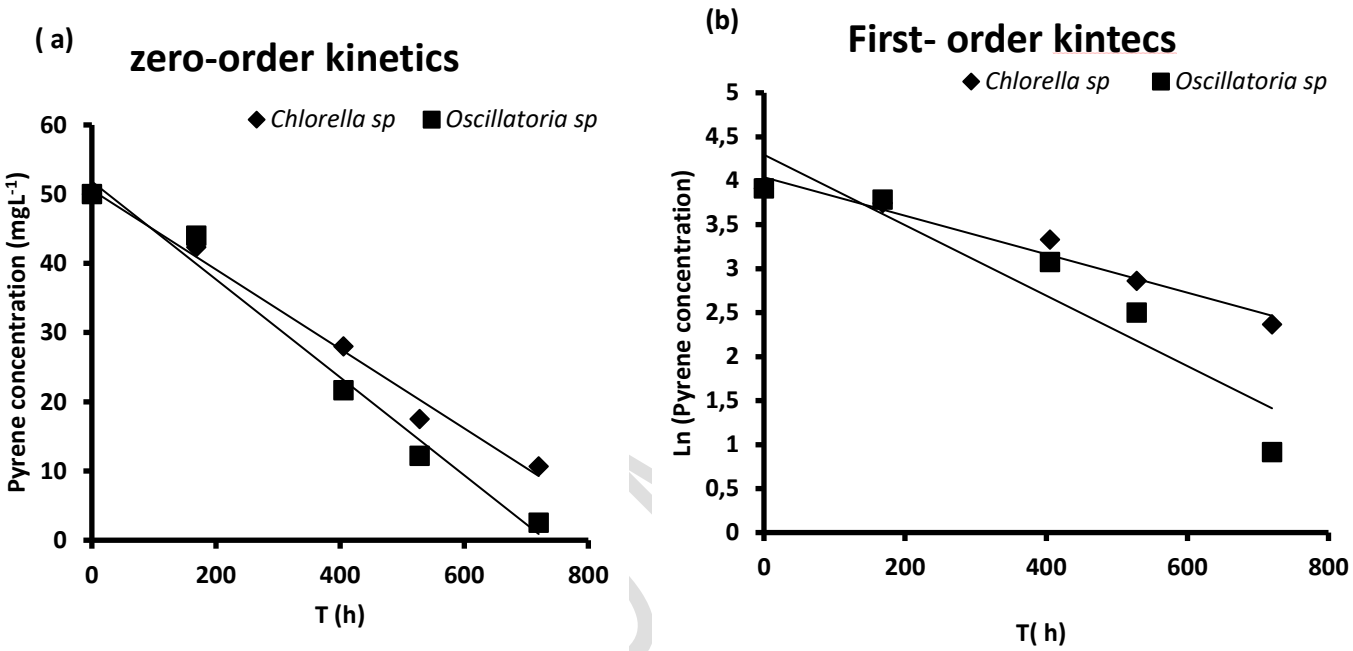

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Fig.5

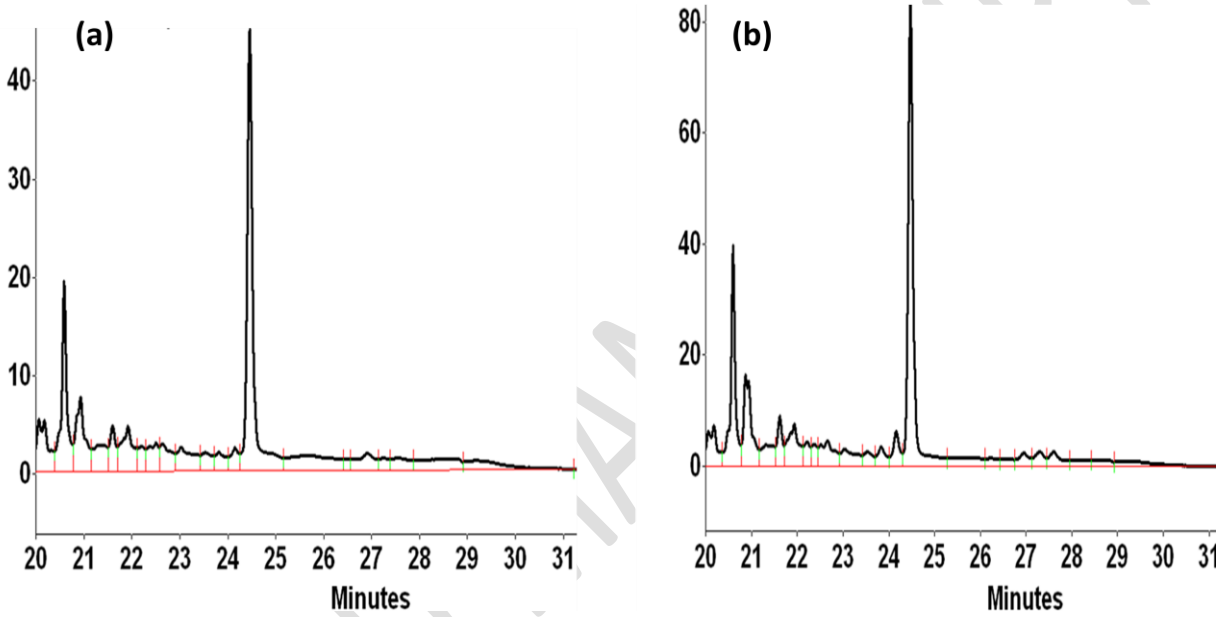

Fig. 5

Table:1

***Oscillatoria*sp**					***Chlorella* sp**				
Zero order kinetics		**First-order kinetics**			**Zero order kinetics**		**First-order kinetics**		
$k_0(h^{-1})$	**R^2**	**$k_1(h^{-1})$**	**$t_{1/2}(h)$**	**R^2**	**$k_0(h^{-1})$**	**R^2**	**$k_1(h^{-1})$**	**$t_{1/2}(h)$**	**R^2**
0.072	0.98	0.004	137.3	0.89	0.05	0.99	0.002	346.5	0.96