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Speeding up the successive clarification and bioremediation processes of anthracene-containing water using graphite/bacteria integrated columns

Tarek H. Taha[a,*], A. E. Mansy[b], Asmaa M. Youssif[c], Saad Alamri[d,e], Mahmoud Moustafa[d,f]

[a]*Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab City, 21934 Alexandria, Egypt, email: ttaha@srtacity.sci.eg*
[b]*Environment and Natural Materials Research Institute (ENMRI), City of Scientific Research and Technological Applications, "SRTA-City", New Borg El-Arab City, 21934 Alexandria, Egypt, email: amansy@srtacity.sci.eg*
[c]*Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt, email: asmaam.youssif@yahoo.com*
[d]*Department of Biology, College of Science, King Khalid University, P.O. Box: 9004, Abha, Saudi Arabia*
[e]*Research Center for Advanced Materials Science (RCAMS), King Khalid University, Abha 61413, P.O. Box: 9004, Saudi Arabia, email: amri555@yahoo.com*
[f]*Department of Botany and Microbiology, Faculty of Science, South Valley University, Qena, Egypt, email: mfmostfa@kku.edu.sa*

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ABSTRACT

Polycyclic aromatic hydrocarbons are a large group of chemicals that represent an important concern due to their widespread distribution in the environment and their harmful effects. The current study is aimed to accelerate the process of water clarification using successive stages of adsorption and biodegradation. For the optimized degradation of anthracene by isolate *Bacillus* sp., different parameters were examined such as pH, temperature, inoculum size and different concentration of anthracene. An integrated system of two separated columns was used to speed up the adsorption and biodegradation of anthracene. The HPLC results of anthracene adsorption showed that the free graphite powder succeeded to eliminate 43.9% of the water existing anthracene followed by 39.6% for the first and second columns, respectively. The sum of these percentages indicates the ability of both columns to remove 83.5% of the initial anthracene concentration (70 mg/L). The anthracene molecules that were adsorbed to both graphite forms were submitted to the biodegradation process using *Bacillus* sp. in separate tanks. After 24 h of incubation, *Bacillus* sp. was able to biodegrade 62.7% and 82.6% of anthracene adsorbed to free and immobilized graphite powders, respectively.

Keywords: Anthracene biodegradation; Graphite adsorption; HPLC; Alginate immobilization; Integrated clarification system

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that contain carbon and hydrogen atoms as main components. These atoms are almost arranged in the form of two or three aromatic rings. PAHs are environmental contaminants that resulted from the incomplete combustion of organic matter in automobile exhaust, petrochemical industries, or from the accidental spills of petroleum [1–3]. They are exhibiting multiple toxic properties and are classified as carcinogenic, mutagenic, and teratogenic compounds [4]. They have unique characteristics such as biomagnification, bioaccumulation, and persistent toxicity that categorize

* Corresponding author.

them as serious risks to human health and the environment. Consequently, they have raised a great environmental concern all over the world, which leads the USEPA to list 16 of them as priority pollutants [5,6].

According to the chemical nature of PAHs, their release to the environment may lead to their chemical oxidation, volatilization, photolysis, or adsorption on sediment of soil particles. However, the main pathway for their removal is almost via microbial degradation or transformation [5,7]. The biodegradation of PAHs is projected to be an environmental friendly and economic solution compared with other physical and chemical methods such as photolysis, combustion, ultrasonic decomposition, and landfilling [8,9].

Anthracene is one of the PAHs that is known for being a low molecular compound, which has three fused rings. It is almost one of the contaminants that existed in the sites contaminated with petroleum. Its origin is related to some activities such as incomplete combustion of organic matter or anthropogenic pyrolysis of petroleum products [7,10]. It shares structure with known carcinogenic compounds such as benzo(a)anthracene, and hence has been used as a useful molecule for modeling the metabolism of such counterpart PAH; however, it has not been known to exhibit any genotoxic or carcinogenic effects [11]. While it has been reported as skin sensitizers, which means that it can cause an allergic reaction in skins of humans and animals [7,12]. In addition, it has potential carcinogenic and mutagenic properties when combined with soil or water bodies. When released in the environment, it showed characteristics of being low volatile and low water soluble. It is accumulated in the soil and groundwater and increases year by year resulting in a non-negligible pollution [13,14].

It is a biodegradable compound that can be degraded in the environment by multiple species of bacteria and fungi. Many soil bacteria that are capable of degrading this PAH have been isolated a long time ago [15–17]. Multiple Gram-positive and Gram-negative bacteria are reported previously that are able to degrade it. These bacterial genera include *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Alcaligenes*, *Arthrobacter*, *Beijerinckia*, and *Micrococcus* [18–23]. In addition, recent reports detailed the ability of *Pseudomonas* species such as *P. putida*, *P. cepacia*, *P. paucimobilis* and *P. fluorescens* to degrade PAH [16,24–26].

However, in recent years, there has been an increase in the number of reports of novel PAH degraders, including *actinomycetes*, and even rhizosphere bacteria such as *Bacillus circulans* and *Kurthia* sp. SBA4 [27,28]. Others include *Escherichia coli* and *Thiobacter subterraneus* [29]. The present study focuses on degradation of anthracene by local bacterial isolate after its quick removal from contaminated water using a graphite-containing design. The study was extended to determine the optimum conditions for anthracene degradation, in addition to study the ability of alginate immobilized or free graphite powder to adsorb anthracene.

2. Materials and methods

2.1. Chemicals

All the chemicals used through the work were high-grade compounds with the following names and CAS No.: anthracene (CAS NO. 120-12-7), tris base (CAS NO. 77-86-1), boric acid (CAS NO. 10043-35-3), EDTA (CAS NO. 6381-92-6), agarose (CAS NO. 9012-36-6), graphite (CAS NO. 7782-42-5), acetonitrile (CAS NO. 75-05-8), sodium alginate (CAS NO. 9005-38-3), $CaCl_2$ (CAS NO. 10043-52-4), sodium citrate (CAS NO. 6132-04-3), NaCl (CAS NO. 7647-14-5).

Since the solubility of anthracene in water is 0.044 mg/L at 25°C, and since organic solvents such as acetone can greatly increase the amount of dissolved anthracene. In the current study, the acetone was used as the main solvent for the preparation of anthracene stock, which has been latterly used for the preparation of the tested concentrations 10–150 mg/L.

2.2. Sample collection

Contaminated soil sample was collected from a petroleum fueling station at Alexandria city. The soil was diluted in sterile normal saline solution into different dilutions (10^{-1}–10^{-7}), and was used for the isolation process.

2.3. Isolation of anthracene degrading bacteria

The isolation of anthracene degrading bacteria was performed according to the study by Tarafdar et al. [30] with some modifications. 50 μL of each soil dilution were spread over Bushnell Haas (BH) agar amended with 50 mg/L anthracene using sterile spreader. The plates were incubated at 30°C for 7 d till the appearance of bacterial colonies. The grown colonies were transferred to sterile LB agar plates for purification.

2.4. Selection of potent anthracene degraders

Single colony of each isolated and purified bacterial isolates was aseptically cultivated in 10 mL LB broth followed by incubation at 30°C and 150 rpm for 24 h. A 1 mL of each culture was transferred to BH broth amended with 30 mg/L anthracene. All the tubes were incubated at 30°C and 150 rpm for 72 h. After incubation, the optical density (OD600 nm) of each bacterial isolate was measured and the bacterial isolate with highest optical density was considered as the potent anthracene degrading bacteria.

2.5. Molecular identification of the most promising isolate P1

2.5.1. DNA extraction

The genomic DNA of the isolate P1 was extracted using GeneJET Kit for DNA purification according to the manufacturer's instructions (Thermo Scientific, EU Lithuania).

2.5.2. PCR amplification and sequencing of 16S rRNA gene

The extracted DNA was submitted for PCR amplification of 16S rRNA gene using universal primers. The PCR composed of 12.5 μL of PCR master mix (Takara, Japan), 1 μL of the forward primer (5′-AACTGGAGGAAGGTGGGGAT-3′), 1 μL of the reverse primer (5′-AGGAGGTGATCCAACCGCA-3′), 1 μL of genomic DNA, and sterile injection water up to 25 μL. The PCR was carried out for 30 cycles of 94°C for 1 min, 55°C

for 1 min and 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using 1.5% agarose gel in TBE buffer (pH 8.5), where the electrophoresis was carried out at 150 V for 20 min. The gel was then examined using UV trans-illuminator and photographed using gel documentation. The obtained gene was then submitted for sequencing and the obtained sequence was examined and compared with the similar sequences in GenBank (www.ncbi.nlm.gov/blast). After that, the sequence was deposited in GenBank under a specific accession number. The phylogenetic tree of the obtained sequence and other similar sequences was performed using MEGA 5.1 program.

2.6. One variable at time optimization (OVAT)

Different parameters have been tested to determine the optimum conditions for obtaining the maximum degradation capacity.

2.6.1. pH

The ability of *Bacillus* sp. THTA to degrade anthracene at different pH values was determined. 10 mL of BH broth was prepared and 7 falcon tubes were adjusted with pH range from 3 to 11. All the tubes were first amended with 30 mg/L anthracene followed by inoculation with 100 μL of overnight culture of the bacterial isolate, in addition to an un-inoculated tube as a control. The tubes were incubated at 30°C and 150 rpm for 48 h. The optical densities at 600 nm were measured and the optimum pH value was determined.

2.6.2. Temperature

To determine the best temperature of *Bacillus* sp. THTA for maximum biodegradation capacity of anthracene, three different temperatures were tested. The prepared BH broth tubes (10 mL each) were inoculated with 100 μL of overnight culture of the bacterial isolate after the addition of 30 mg/L hydrocarbon. Each tube in addition to un-inoculated control was incubated at temperatures 25°C, 30°C, and 40°C for 48 h followed by the measurement of their optical densities for the determination of the highest growth.

2.6.3. Anthracene concentration

The maximum biodegradation capacity of *Bacillus* sp. THTA to degrade variable concentrations of anthracene was tested. Overnight culture of the bacterial isolate was inoculated into each tube after the amendments of the concentrations 10, 30, 50, 70, 100, and 150 mg/L of the hydrocarbon. The tubes were incubated at 30°C and shook at 150 rpm for 3 d. The ability of the isolate to degrade higher concentrations of the hydrocarbon was investigated through the measured optical densities at 600 nm.

2.6.4. Inoculum size

Variable amounts of the bacterial cells were tested for their ability to degrade a specific concentration of anthracene. LB cultivated cells were distributed as 2%, 4%, 6%, 8% and 10% into 10 mL of BH broth containing 70 mg/L anthracene. The tubes were incubated at 30°C for 48 h under shaking conditions at 150 rpm.

2.7. Antibacterial activity of graphite powder

The antibacterial activity of graphite powder against anthracene degrading isolate was examined using agar well diffusion method. Sterile LB agar plate was superficially inoculated with isolate P1 using sterile cotton swab. A central hole was made in the inoculated agar using sterile cork borer. A volume of 100 μL of 0.5% suspended graphite powder in sterile distilled water was added to the hole. The plate was kept at 4°C for 30 min to allow diffusion of the tested material. The plate was then incubated at 30°C for 24 h and the formation of a clear zone was investigated.

2.8. HPLC analysis

The concentration of anthracene was detected in all samples using HPLC instrument (Agilent 1100, Germany). Hypersil gold C8 column was used (150 × 4.6 mm). Before injection, all samples were subjected to centrifugation at 6,000 rpm for 10 min followed by drying at 60°C for 24 h. After dryness, the residual hydrocarbon pellets were dissolved in 75% acetonitrile and 25% H_2O as a mobile phase. Each sample was injected by 20 μL with flow rate 1 mL/min. A UV detector at 269 nm was used, and the concentration of each sample was calculated according to the concentration of the injected standard.

2.9. Immobilization of graphite and bacterial cells

In order to control and to collect easily each of the graphite powder and bacterial cells, each of them was separately mixed with sodium alginate solution for subsequent immobilization. The graphite–polymer mix and bacteria–polymer mix were prepared through the amendment of 50 mL of 1% sodium alginate/distilled water solution with 0.5 g of each of them, individually. Each mixture was stirred for 30 min using a magnetic stirrer till complete homogeneity and fair distribution of the bacterial cells or graphite powder inside the polymer. Each homogeneous mixture was then wisely dropped in $CaCl_2$ (3.5%) solution using syringe nozzle under stirring conditions. The formed beads were kept stirring for 15 min to allow complete solidification. The beads were filtered using filter paper followed by washing using distilled water to remove excess calcium ions and bacterial cells or graphite powder.

2.10. Designing of graphite/bacteria system for anthracene adsorption and biodegradation

2.10.1. Purification columns

An integrated system composed of two separated and combined columns was used in order to speed up the adsorption of anthracene followed by its biodegradation. The first column consists of a burette shaped glass column that contains either free graphite powder or alginate-immobilized graphite beads as a basic matrix for the adsorption

of organic pollutants such as anthracene. The anthracene polluted water was allowed to pass slowly through the column with a flow rate of 10 mL/h to support the effective adsorption of the pollutant on the matrix surface. The water was checked for the concentration of anthracene before and after its passage through the graphite column using HPLC. The obtained water was allowed to pass through a second graphite containing column in order to increase the amount of adsorbed anthracene as much as possible. The obtained water was again examined for its anthracene content using HPLC.

2.10.2. Dissolution of graphite beads

The graphite beads were dissociated before transferring to the treatment tank using a chemical treatment process. At first, each 1 g of graphite beads was transferred to 25 mL of the dissolution solution with pH 6.8 (0.55 M sodium citrate, 0.03 M EDTA, and 0.15 M NaCl). The mixture was incubated at 37°C for 10 min. The obtained graphite particles were then transferred to the treatment tank.

2.10.3. Treatment tank

The used graphite (both free and immobilized dissociated forms) was transferred to the treatment tanks that contain 50 mL BH broth inoculated with 10% of *Bacillus* sp. THTA (OD600 = 0.8) immobilized in alginate beads. Both containers were incubated at 30°C and 150 rpm for 24 h. The anthracene concentration (before and after biodegradation) was also determined using HPLC. It is worth mentioning that all performed steps included separate containers that only have 70 mg/L anthracene and were only submitted for a final biodegradation step using *Bacillus* sp. THTA as a control. Fig. 1 can represent the overall purification and bioremediation steps.

2.11. Adsorption isotherm models

The adsorption isotherm is very important for detection of how the adsorption molecules are distributed between liquid phase and solid phase when the adsorption process reaches equilibrium state. The isotherm also describes how solutes interact with adsorbents and optimize the use of adsorbents [31]. The resulted isotherm data were analyzed by fitting to different isotherm models to find the suitable model that can be used for design purpose. The most two well-known used isotherm models were the Langmuir isotherm model and the Freundlich isotherm model.

2.11.1. Langmuir isotherm model

Assumes monolayer adsorption onto a surface containing a finite number of adsorption sites of uniform strategies of adsorption with no transmigration of adsorbate in the plane of surface [31]. The linear form of Langmuir's isotherm model is given by the following equation [32]:

$$\frac{C_e}{q_e} = \left[\frac{1}{Q^\circ b}\right] + \left[\frac{1}{Q^\circ}\right] C_e \tag{1}$$

where C_e is the equilibrium concentration of the adsorbate (PAH) (mg/L), q_e is the amount of adsorbate adsorbed per unit mass of adsorbate (mg g^{-1}), and Q^o and b are Langmuir constants related to adsorption capacity and rate of adsorption, respectively. The essential characteristics of Langmuir's isotherm model can be expressed in terms of a dimensionless equilibrium parameter, R_L, which is defined by the following equation [33]:

$$R_L = \frac{1}{1 + bC_0} \tag{2}$$

where b is the Langmuir constant and C_0 is the highest hydrocarbon concentration (mg/L). The value of R_L indicates the type of the isotherm to be either unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$) or irreversible ($R_L = 0$).

2.11.2. Freundlich isotherm model

Assumes heterogeneous surface energies, in which the energy term in Langmuir equation varies as a function of the surface coverage [31]. The well-known logarithmic form of Freundlich isotherm model is given by the following equation [34]:

$$\ln q_e = \ln k_f + \frac{1}{n} \ln C_e \tag{3}$$

where q_e is the amount adsorbed at equilibrium (mg g^{-1}), C_e is the equilibrium concentration of the adsorbate (PAH), and k_f and n are Freundlich constants, n giving an indication of how favorable the adsorption process and k_f (g (mg/s)) is the adsorption capacity of the adsorbent. k_f can be defined as the adsorption or distribution coefficient and represents the quantity of PAH adsorbed onto graphite powder adsorbent for a unit equilibrium concentration. The slope $1/n$ ranging between 0 and 1 is a measure of adsorption intensity or surface heterogeneity, as its value gets closer to zero. A value for $1/n$ below 1 indicates a normal Langmuir isotherm while $1/n$ above 1 is indicative of cooperative adsorption [31].

2.12. Kinetic models

The investigations of the mechanism of adsorption take place by two kinetic models are pseudo-first-order model and pseudo-second-order model. The pseudo-first-order equation is represented as follows [35]:

$$\ln\left(q_e - q_t\right) = \ln q_e - k_1 t \tag{4}$$

where q_e, q_t are the amounts of PAH adsorbed (mg/g) at equilibrium and at time t (s), respectively, and k_1 is the rate constant adsorption (1/s). Values of k_1 were calculated from the plots of $\ln(q_e - q_t)$ vs. t.

The pseudo-second-order model can be expressed by the following equation [36]:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \tag{5}$$

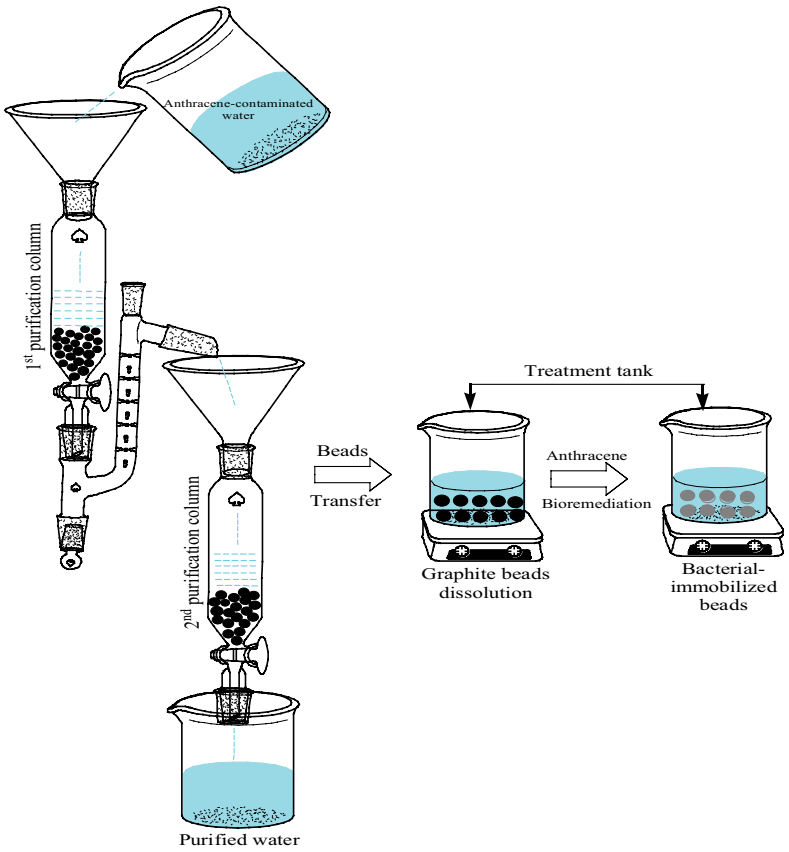

Fig. 1. Purification and bioremediation design of anthracene-contaminated water.

where k_2 (g/mg h) is the rate constant of second-order adsorption. If second-order kinetics is applicable, the plot of t/q vs. t should show a linear relationship. There is no need to know any parameters, q_e and k_2 can be determined from the slope and intercept of the plot. Also, this procedure is more likely to predict the behavior over the whole range of adsorption.

2.13. Determination of point of zero charge (pH_{pzc})

The pH_{pzc} of the graphite powder was determined by the method reported by Bhavsar et al. [37]. Briefly, 100 mg of sample were mixed in 25 mL water in glass beakers with continuous stirring. The pH of suspension was then adjusted to an initial pH value of 2, 4, 6, 8, 10 and 12 using 0.01 N HCl and 0.01 N NaOH. Each beaker was vigorously agitated in a shaker bath for 90 min. After settling for 10 min, the final pH of each solution was estimated carefully. The ΔpH (difference between final and initial pH) values were calculated and then were plotted against initial pH value. pH at the ΔpH was taken to be the pzc.

2.14. Scanning electron microscope (SEM)

The micrographs of graphite powder and bacterial cells in the free and immobilized forms were investigated using scanning electron microscopy (JSM 6360 LA, JEOL, Japan).

3. Results and discussion

3.1. Isolation of anthracene degrading bacteria

The diluted soil sample was spread over BH agar supplemented with anthracene. After the incubation period, five bacterial colonies were selected as presumptive anthracene degraders. The five isolates were purified and transferred to BH broth supplemented with anthracene. After 48 h incubation, the OD of the five isolates was measured at 600 nm. As shown in Fig. 2, all the isolates showed detectable optical densities that revealed the ability of all bacterial isolates to degrade anthracene as sole carbon and energy source. However, the optical densities showed varied measurements, which indicate that some isolates are more potent degraders of anthracene than the others. The potent bacterial isolate (P1) was selected and proceeded for the rest of the anthracene biodegradation experiments. Fig. 3 represents the simultaneous growth of bacterial isolate P1 and the percentage of PAH removal over 72 h of incubation using 30 mg/L initial concentration. Different bacterial species with anthracene degrading capacity have been previously isolated from contaminated soil, which is considered a rich source of microbes that have multiple beneficial activities [17,38].

3.2. Molecular identification of the most promising isolate (P1)

Molecular identification of the bacterial isolate was depending on the amplification and sequencing of 16S rRNA

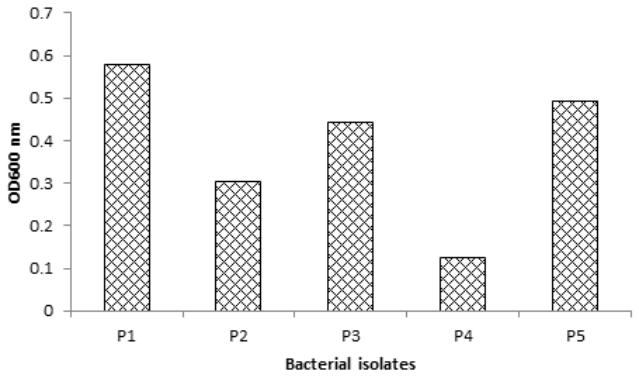

Fig. 2. Optical densities of the five bacterial isolates after incubation for 48 h in BH broth amended with 30 mg/L anthracene.

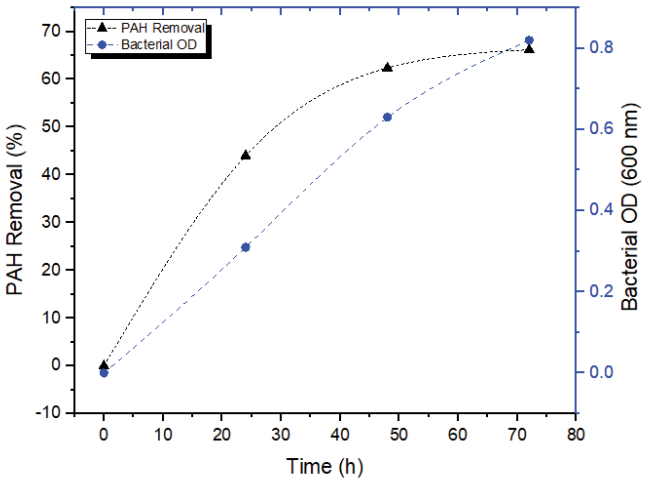

Fig. 3. Optical density of isolate P1 and the percentage of PAH removal over 72 h of incubation.

gene using universal primers. The obtained PCR product revealed the amplification of 350 bp of the ribosomal gene. The sequence of the amplified gene showed that P1 isolate is 100% similar to *Bacillus* sp. The obtained sequence was subsequently deposited in GenBank under the name *Bacillus* sp. THTA with the accession number MT192653. The phylogenetic analysis of the obtained sequence and related sequences in GenBank can be shown in Fig. 4.

Several literatures stated that *Bacillus* sp. capable of degrading anthracene at different concentrations and utilizing it as a sole carbon and energy source [17,39,40].

3.3. Optimization of anthracene biodegradation by OVAT method (one variable at time)

3.3.1. pH

Wide range of pH values was applied to investigate the optimum pH that can help *Bacillus* sp. THTA to degrade anthracene. Fig. 5a illustrates the wide range variation of anthracene biodegradation at different pH values. *Bacillus* sp. THTA was able to hardly resist the acidic and alkaline conditions with gradual growth increments at natural and slightly acidic or alkaline conditions. The bacterial growth was observed to be higher at pH values 6–8. However, the growth was decreased at pH 4–6 and 9–10 with minimum growth levels at pH 3 and 11.

All microorganisms (MO) can perform their activities at a certain pH range, that is, minimal, maximal and an optimal pH, where at optimal pH, the activity of the MO is significantly high. Any drastic changes in pH can interfere with cell wall and cell contents of the MO, and thus affecting its growth and metabolism [41]. Our results agreed with Bibi et al. [17] who mentioned that, at pH 7, the growth and activity of *Bacillus cereus* in anthracene degradation was high due to balanced ionic distribution inside and outside of the cells. Also, Jacques et al. [42] have shown that the anthracene degradation by *Pseudomonas aeruginosa* had the highest rate of degradation of 71% after 48 d at pH 7.0. On the other hand, Ahmed et al. [40] stated that *Bacillus badius* was isolated from the alkaline environment and the maximum degradation of anthracene was observed at alkaline pH 9.00. Also, Neelofur et al. [39] reported that maximum anthracene degradation by *Bacillus* sp. was observed at pH 5.

In general, the value of pH has a strong effect on both of the growth of microorganisms and on the solubility of the tested hydrocarbon. Some microorganisms prefer acidic or alkaline conditions, while some others prefer neutral conditions. For example, *Mycobacterium* sp., which is an acid-resistant Gram positive bacteria prefers the acidic condition, which makes them permeable for hydrophobic substrates [43]. Moreover, the secretion of naturally produced biosurfactants by some bacterial genera such as *Bacillus* and *Pseudomonas* increases the PAH solubility and hence bioavailability [44], which is another factor for PAH solubility rather than pH value.

On the other hand, the current-tested PAH has been initially dissolved in the organic solvent acetone, which allows high solubility concentration compared with water. Increasing the PAH solubility in water is almost matched with increasing temperature of the solvent. Andersson et al. [45] mentioned that an exponential relationship was found between the temperature of water and the degree of solubility of anthracene.

3.3.2. Temperature

The selected anthracene degrading bacterial isolate was tested for its ability to degrade 30 mg/L of anthracene at different temperatures. As shown in Fig. 5b, *Bacillus* sp. THTA was able to degrade anthracene at the three tested temperatures with varied activities. The optimum temperature was recorded at 25°C, where the highest optical density was measured. It can be detected from the graph that temperature increments can oppositely affect the bacterial biodegradation activity. The growth at 30°C was moderately suitable for the biodegradation of anthracene and resulted in an optical density lower than that recorded at 25°C and higher than that recorded at 40°C. At optimal conditions including temperature, the organisms have the ability to secrete a vast array of enzymes that are capable of degrading the surrounding toxic compounds. While

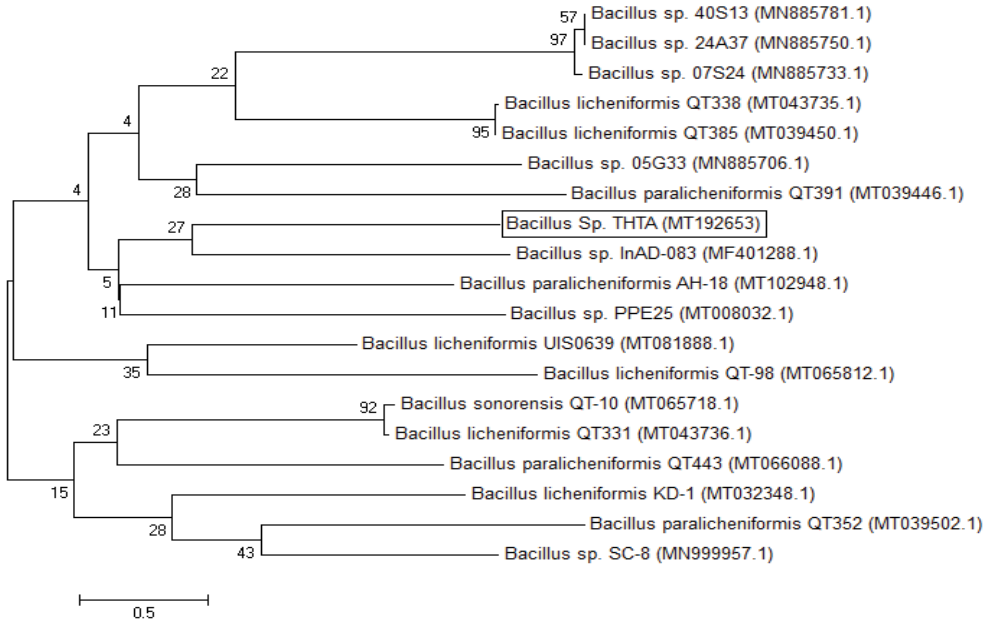

Fig. 4. Phylogenetic position of *Bacillus* sp. THTA among the related strains in GenBank. The branching pattern was generated by neighbor-joining tree method with 500 bootstrap and the Genbank accession numbers of the 16S rRNA nucleotide sequences are indicated in brackets.

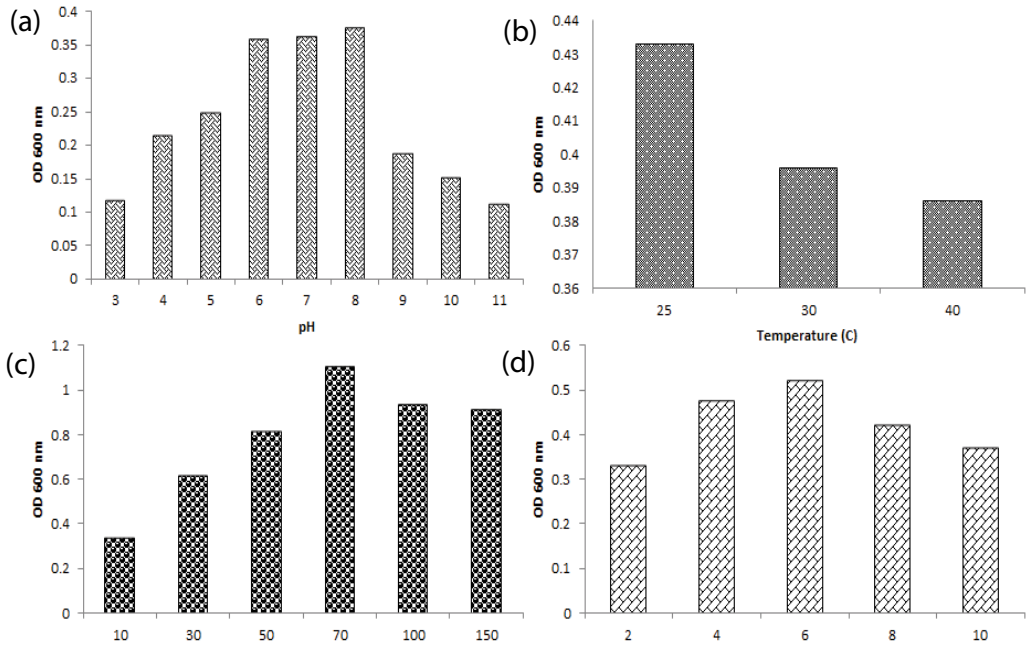

Fig. 5. Optimization parameters used for investigating the optimum conditions for the biodegradation of anthracene by *Bacillus* sp. THTA. (a) Effect of pH, (b) effect of temperature, (c) effect of anthracene concentration, and (d) effect of inoculum size.

the growing of microorganisms in unstable temperatures can block the access of the enzymes to their substrates, which may become insoluble at low temperatures or effect the confirmation of the enzymes at high temperatures [46].

In similar research to ours, Bibi et al. [17] mentioned that optimum growth and activity of *Bacillus cereus* was 30°C. While Ahmed et al. [40] stated that maximum degradation of anthracene by *Bacillus badius* was observed at 35°C. Neelofur et al. [39] have also reported that maximum anthracene degradation by *Bacillus* sp. was observed at 35°C.

3.3.3. Hydrocarbon concentration

The tested concentrations of anthracene were started from a lower concentration at 10 mg/L to a higher concentration at 150 mg/L. Fig. 5c demonstrates the biodegradation

capacity of *Bacillus* sp. THTA at all tested anthracene concentrations. The measured optical density of the bacterial growth at 10, 30, and 50 mg/L indicates that these concentrations can be bear by the bacterial isolate and it is able to degrade higher concentrations. The highest bacterial growth was observed at 70 mg/L with lower growth at more anthracene concentrations indicating that 70 mg/L is the optimum concentration of the hydrocarbon biodegradation. However, the growth of the bacterial isolate at 100 and 150 mg/L was reasonable; indicating that the isolate can resist and degrade elevated concentrations of anthracene but with lower activity. We could attribute the lower OD600 nm of the *Bacillus* sp. THTA strain at lower hydrocarbon concentrations (10–30 mg/L) to the limited amounts of the carbon source. This limitation would decrease the microbial metabolism and division that are reflected by the measured optical density, especially after 3 days of incubation. Bibi et al. [17] colleagues mentioned that *Bacillus cereus* had achieved higher growth at an increased level of anthracene from 100 ± 1,000 ppm. Also, Swaathy et al. [47] reported that growth of *Bacillus licheniformis* MTCC 5514 was more pronounced with 300 ppm anthracene.

3.3.4. Inoculum size

It would be expected that increasing the number of bacterial cells to a certain limit would enhance the hydrocarbon's biodegradation process. For this reason, varied densities of the tested microbe were tested to determine the optimum density that would achieve the best biodegradation capability. Fig. 5d illustrates the activity of *Bacillus* sp. THTA to degrade 70 mg/L of anthracene using multiple cell densities. All the tested inoculum sizes showed potent anthracene biodegradation capacity especially 6%, which was recorded as the optimum. The biodegradation capacity order of the tested inoculum sizes could be summarized as: 2% < 4% < 6% > 8% > 10%. On the other hand, Bibi et al. [17] mentioned that maximum anthracene degradation was achieved by using 10% inoculum size of *Bacillus cereus*.

The variation in the inoculum size has a significant effect on the secretion of enzymes and hence on the biodegradation of the tested substrate. Increasing the inoculum size can increase the degradation activity to a certain limit [48]. However, lower inoculum size makes the microbial cells need more time to multiply and reach a sufficient number that allow them to produce enzymes and utilize the substrate. At a certain limit, the production of enzymes and degradation of the substrate can be decreased because the enhanced biomass would cause depletion of the nutrients and hence the metabolic activity would be decreased [49].

3.4. Antibacterial activity of graphite powder against *Bacillus sp. THTA*

The purpose of this experiment was to investigate if the graphite powder (as an anthracene adsorbent) will negatively affect the growth of *Bacillus* sp. THTA when coming in direct contact during the biodegradation process. As shown in Fig. 6, there was no detectable antibacterial effect of graphite on the growth of *Bacillus* sp. THTA and no clear zones were observed, indicating that graphite is completely safe and will not harm the microbial strain during the biodegradation test.

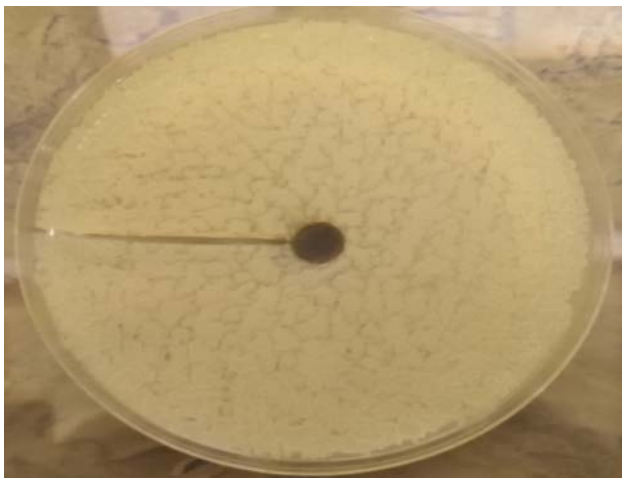

Fig. 6. Determination of antibacterial activity of graphite powder against *Bacillus* sp. THTA isolate using agar well diffusion method.

Most literature mentioned that graphite has not antimicrobial activity graphite has antimicrobial activity against *Bacillus* but has activity against *Escherichia coli*, *Streptococcus iniae*, *Proteus mirabilis*, *Staphylococcus aureus* and *Enterococcus faecalis* [50,51].

3.5. HPLC determination of anthracene concentration

Two separated and combined columns were used in order to speed up the adsorption of anthracene followed by its biodegradation. According to the complicated structure of anthracene; it almost needs a long time to be completely degraded by bacterial strains, which means that the polluted water will almost be contaminated and cannot be used for any water-dependent applications unless the source of contamination is effectively eliminated. The idea for the designed system was to find out a quick step for the purification of polluted water to be available for any required water-dependent application. However, the removed pollutant can be transferred for a specific treatment tank for its microbial biodegradation, and it does not matter how long time is required for completing its biodegradation process.

The obtained HPLC results showed that the free graphite powder succeeded to eliminate 43.9% of the water existing anthracene followed by 39.6% for the first and second columns, respectively. The sum of these percentages indicates the ability of both columns to remove 83.5% of the initial anthracene concentration (70 mg/L). However, the graphite beads were able to eliminate 29.6% of anthracene followed by 16.04% for the first and second columns, respectively. Both graphite beads columns were able to remove 45.64% of anthracene, which is significantly lower than the percentage recorded by free graphite powder' columns. These data revealed that the free graphite powder is more preferred for anthracene adsorption than immobilized powder. This could be attributed to the high surface area of free graphite powder that allows it to effectively adsorb anthracene better than its immobilized form.

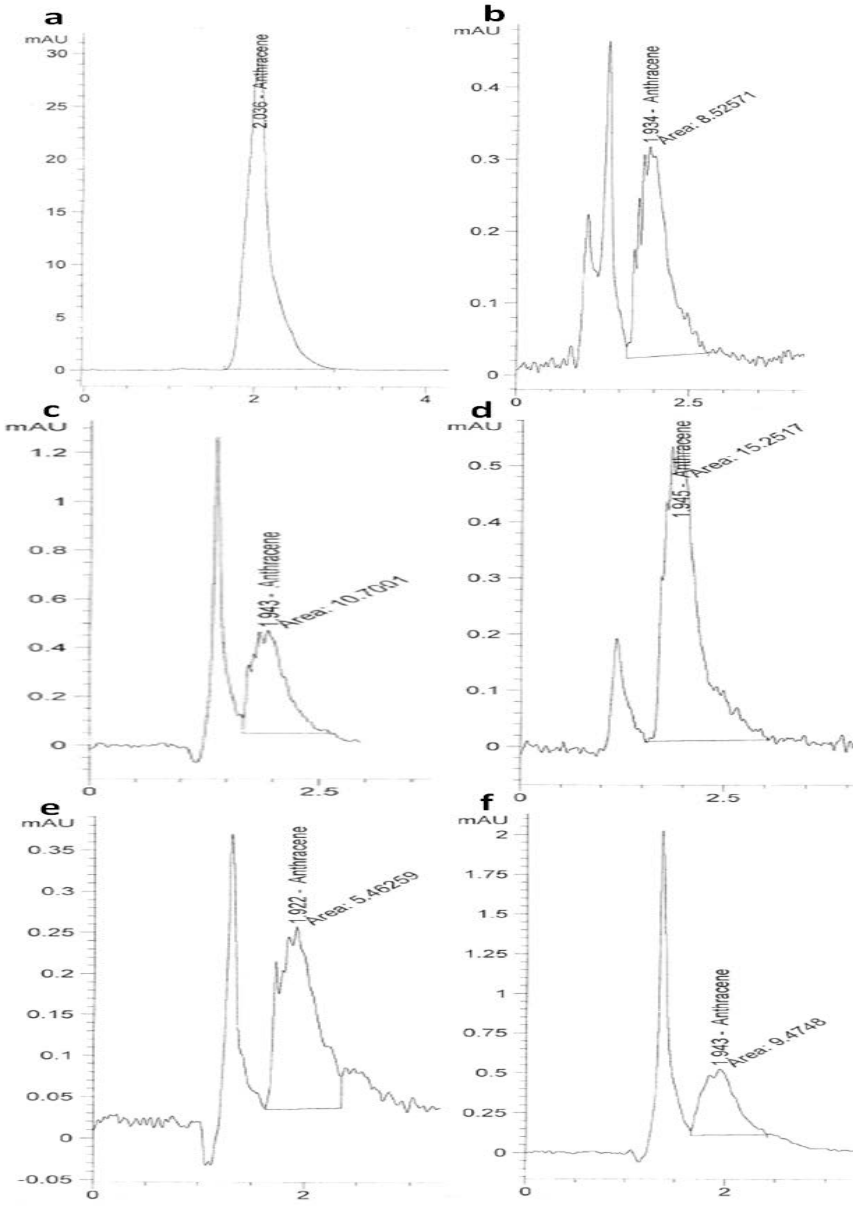

Fig. 7. HPLC chromatograms of anthracene concentration. (a) Standard anthracene, (b) after removal by free graphite powder in the first column, (c) after removal by immobilized graphite powder in the first column, (d) anthracene control, (e) after removal by free graphite powder in the second column, and (f) after removal by immobilized graphite powder in the second column.

On the other hand, the anthracene molecules that were adsorbed to both graphite forms were submitted to the biodegradation process using *Bacillus* sp. THTA in separate tanks. The obtained results revealed that, after 24 h of incubation, the isolate was able to biodegrade percentages of 62.7% and 82.6% of anthracene adsorbed to free and immobilized graphite powders, respectively (Fig. 7). These results are highly matched with anthracene concentrations obtained after the passage through the two columns, since the free graphite powder showed higher adsorption capacity than immobilized powder, which in turn assists the bacterial isolate to biodegrade the lower concentrations more effectively.

Bibi et al. [17] reported that maximum degradation of anthracene by *Bacillus cereus* was 82.29%. While, Jacques et al. [42] has shown that the anthracene degradation by *Pseudomonas aeruginosa* had the highest rate of degradation of 71% . Swaathy et al. [47] mentioned that *Bacillus licheniformis* MTCC 5514 capable of degrading 95% of anthracene.

3.6. Adsorption isotherm models

Figs. 8a and b presented both of Langmuir and Freundlich isotherm models. The characteristic parameters of both models are listed in Table 1.

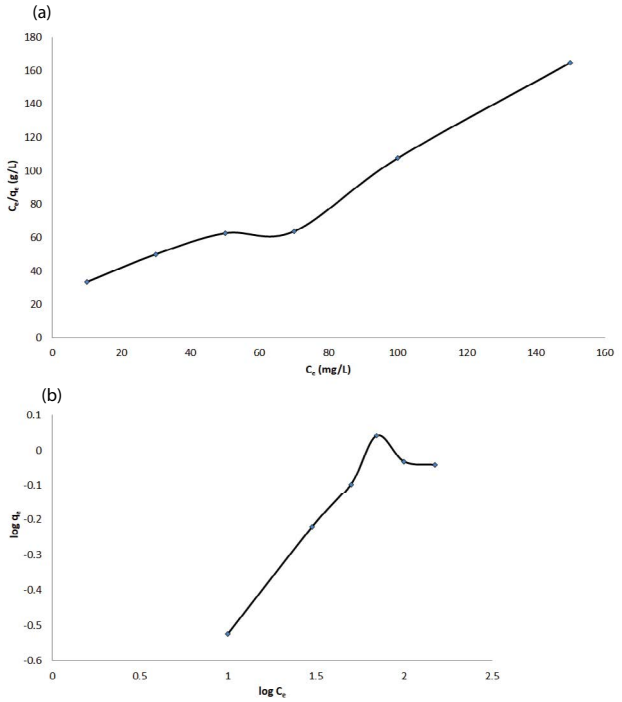

Fig. 8. Adsorption isotherm models, (a) Langmuir model and (b) Freundlich model.

Table 1
Adsorption isotherm models

Isotherm							
Model	Langmuir equation				Freundlich equation		
Parameters	Q^o	b	R^2	R_L	$1/n$	K_f	R^2
Value	1.077	0.055	0.9576	0.108	2.237	0.124	0.8371

The Langmuir model yielded a better fit (R^2 = 0.9576) than the Freundlich model (R^2 = 0.8371), which indicates that the current adsorption process follows the Langmuir isotherm model (Table 1).

The essential characteristics of Langmuir isotherm is R_L. Value of R_L was found to be 0.108. This result confirmed that the graphite powder was favorable for adsorption of PAH under condition used in this work.

The Langmuir isotherm models were chosen by assuming monolayer, homogeneous and finite adsorption sites on the surface of the catalyst, which is in good agreement with assumption of Saad et al. [52].

In the current study, the graphite powder follows the Langmuir isotherm models, and the calculated adsorbent pollutant (1.077) is close to its experimental value (1.76).

3.7. Kinetics

Figs. 9a and b present the pseudo-first and second order kinetics. The calculated kinetic parameters are listed in Table 2. As illustrated in the previous table, the correlation coefficient for the second-order model (R^2 = 0.966) was better than the first-order model (R^2 = 0.9347). This indicates the applicability of second-order model of the adsorption process of PAH on graphite powder, and this model could be used to determine kinetic parameters [53].

3.8. Point of zero charge

The point of zero charge (pH_{pzc}) of graphite powder was investigated at various pH ranges. As shown in Fig. 10, the pH_{pzc} of that powder was determined at 10.25. This result indicates that the pH above 10.25 becomes negatively charged, that favoring the adsorption of positively charged molecules or ions. While the pH before 10.25 is positively charged and hence favors the adsorption of negatively charged molecules or ions [54]. This explanation could suggest the favorable adsorption of anthracene on graphite powder at pH lower than 10.25. This may be attributed to the fact that PAHs play a role as negative charge carriers, since their surmised capability of soaking up free electrons [55].

3.9. Scanning electron microscope

The micrographs of free and immobilized graphite powder and bacterial cells were investigated using scanning electron microscope. As shown in Fig. 11a, the plain alginate capsule showed a smooth solid surface, indicating proper interaction between the polymer chains and the used cross linker.

However, the immobilized graphite capsule showed a rough and irregular surface structure, which reflects wide distribution of graphite particles inside the alginate polymeric material (Fig. 11b). While, the free graphite powder showed multiple foliar-like structures, these structures almost have the same size, and look flat-shaped from their both sides (Fig. 11c). These foliar structures usually provide large surface area that would assist for the efficiency of the

Table 2
Pseudo-first and pseudo-second order kinetics

Kinetics								
Parameter	Initial concentration (mg/L)	Q_e (exp)	First-order			Second-order		
			k_1	q_e	R^2	k_2	q_e	R^2
Value	150	1.76	0.015	7.418	0.9347	0.1	1.8179	0.966

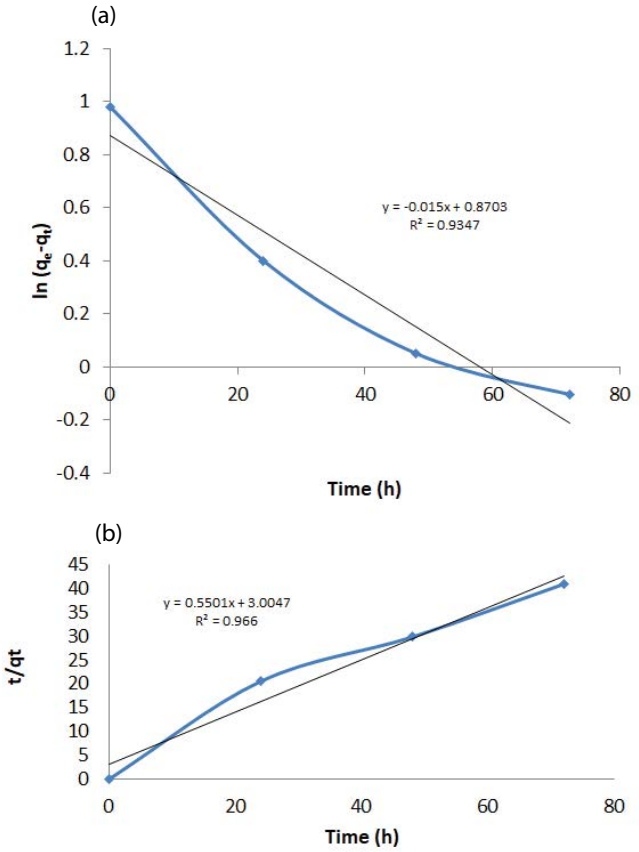

Fig. 9. Kinetics study, (a) pseudo-first order and (b) pseudo-second order.

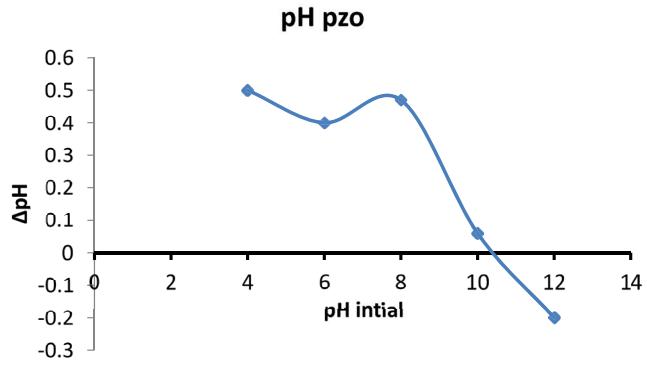

Fig. 10. Point of zero charge (pH_{pzc}) of graphite powder.

used application. These shapes with increased surface areas allow the graphite to adsorb as much organic substances as possible. This indicated that graphite powder in its free form would be able to remove anthracene compounds more efficiently than alginate-immobilized graphite that is approximately embedded inside the alginate capsule and has a reduced surface area. These observations are almost matched with the data obtained by HPLC analysis that proved the ability of free graphite powder to adsorb anthracene efficiently (Fig. 7).

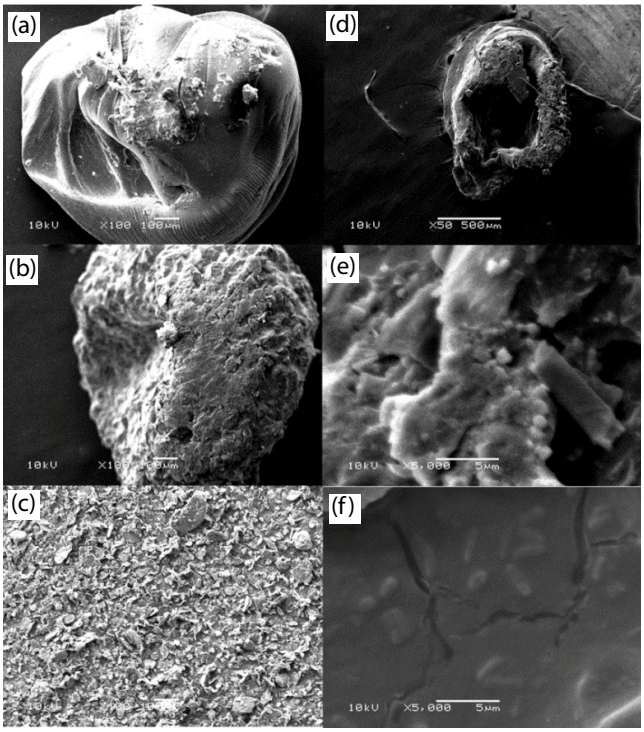

Fig. 11. SEM of the free and immobilized graphite and bacterial cells inside alginate capsules. (a) Plain alginate capsule, (b) graphite-immobilized capsule, (c) free graphite powder, (d) bacteria-immobilized capsule (external shape), (e) bacteria-immobilized capsule (internal shape), and (f) free bacterial cells.

On the other hand, the bacterial cells appeared in the form of bacilli or coco-bacilli shapes (Figs. 11e and 11f). The cells are homogeneously distributed inside the cavities of the polymeric capsules indicating proper immobilization process (Fig. 11e).

4. Conclusion

Successful removal of anthracene from contaminated water was achieved using a designed multiple columns-depending system. The system was effectively able to adsorb anthracene on free graphite powder using two successive columns. The adsorbed anthracene was transferred to a treatment tank to be degraded using local bacterial isolate that has been identified as *Bacillus* sp. THTA. The SEM micrographs revealed the existence of foliar-like shapes of free graphite powder that resulted in large surface area allowing the free powder to remove high percentages of anthracene better than graphite-immobilized beads. The designed system showed a potent accelerating process for quick removal of anthracene from contaminated water, with subsequent biodegradation in a separate treatment tank.

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