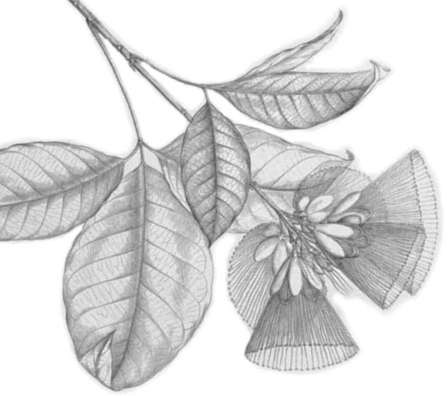


Toluene Degradation By Free Staphylococcus Gallinarum And Immobilized On Multi-Walled Carbon Nanotubes

Fatemeh Heydarnezhad [1]
Mehran Hoodaji [2]
Mahdi Shahriarinour [3]
Arezoo Tahmourespour [4]

ABSTRACT

Hydrocarbons pollution is a most important environmental and healthanxiety . Using free and immobilized bacteria could be a suitable attitude to find a proper bioaugmentation agent. A toluene degrading bacterium was isolated from oil-contaminated environs (located in Bandar-Anzali, Guilan, Iran). The strain was molecularly identified as *Staphylococcus gallinarum* ATHH41 (Accession number: KX344723) by partial sequencing of 16SrDNA gene. The response surface methodology (RSM) was expended for biodegradation of the toluene by ATHH41. The central composite design (CCD) was utilized to optimize pH, temperature, and toluene concentration by ATHH41. In accordance with the optimization purpose of the Design-Expert software, the optimum circumstances of toluene degradation were obtained when pH, temperature and toluene concentration were adjusted to 7.68, 31.73°C and 630.04 mg.l^{-1}, respectively. Multi-walled carbon nanotubes (MWCNTs) were used to immobilize the strain. Infrared spectroscopy and scanning electron microscopy showed that the cells adhered to the MWCNT surface and developed a biofilm. Results reveal that free cells were able to degrade 68.01% of the toluene as the sole carbon and energy source within 24 h under optimized conditions. The immobilized cells reached 95.68%.

Keywords: Carbon nanotube; Response surface methodology; Staphylococcus gallinarum ATHH41; Toluene.

[1] Soil Science Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran. fatemehheydarnezhad@yahoo.com
[2] Soil Science Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran. mehran.hoodaji1@gmail.com
[3] Basic Sciences Department, Rasht Branch, Islamic Azad University, Rasht, Iran. m.shahriarinour@gmail.com
[4] Basic Medical Sciences Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran. a.tahmoures.p@gmail.com

Widespread occurrence of accidental leakage and spillage of petroleum hydrocarbons from underground pipelines and storage tanks often contaminates groundwater and may pose health hazards to the nearby populace (Hilpert et al. 2015). Volatile monoaromatic hydrocarbons of crude oil and petroleum by-products, which are generally found together, are benzene, toluene, ethyl benzene, and xylene (BTEX). These contaminants are a source of health hazards and pollute surface and ground waters (Lee et al. 2018). The remediation of the contamination of BTEX compounds is difficult due to their relative water solubility (Yakout & Daifullah 2014). Hydrocarbon bioremediation depends on the biodegradation activity of soil bacteria. Bacterial biodegradation of these compounds is considered as the most active process in petroleum degradation, and bacteria are known as the primary degraders of spilled oil (Brzeszcz & Kaszycki 2018). The preparation of bacteria in a ready-to-use form that is appropriate to the contaminated site is one of the major issues in bioremediation. However, the use of free-living cells of oil degrading bacteria shows that they have limited efficiency and are not reusable in a continuous treatment system. Therefore, immobilization of bacterial cells on a solid support material is an approach to overcoming such problem (Nopcharoenkul et al. 2013). The technology of immobilized microorganisms can be applied in biological treatments to enhance the efficiency and effectiveness of biodegradation given the higher specific surface areas for microbial growth and better resistance against chemical toxicities and environmental stresses (e.g. pH, temperature, and toxic substances) compared to suspended cells (Wang et al. 2015; Yan et al. 2013). Bina et al. (2012) reported the efficiency of toluene adsorption to be 99.5% by multi-walled carbon nanotube in terms of 10 mg.l^{-1} of toluene, 1 g.l^{-1} of carbon nanotube, 10 min exposure time and neutral pH. Multi-walled carbon nanotubes (MWCNTs) are a capable candidate caused by the structure of their pores, the wide spectrum existence of surface functional groups, and their unique properties (Rahman et al. 2017). Anjum et al. (2019) showed that the surface modified MWCNTs presented a fast and efficient removal of BTX with the highest adsorption capacity. MWCNTs have got applications in various takes, such as the adsorption of pollutants due to their chemical, mechanical, electrical and thermal properties (Pourfayaz et al. 2013). MWCNTs have high surface area, hydrophobic property, and chemical and thermal stability. Thus, they can be suitable adsorbents for volatile organic compounds (Rahman et al. 2017).

This study aimed to isolate bacterial strain with toluene degradation ability, to molecularly identify toluene-degrading bacteria from oil-polluted soils, and to optimize the medium culture conditions to investigate the toluene biodegradation by free-living and MWCNTs-immobilized cells of isolate. In addition, the effects of MWCNTs concentration, environmental conditions such as pH, temperature, and toluene concentration were evaluated on the biodegradation efficiency of toluene.

Materials and Methods

Medium and Culture Conditions

Different polluted soil samples were gathered from the Caspian Sea (Bandar-Anzali, Guilan, Iran, (is located in the north of Guilan Province with the coordinates of 37 28' 16 North, 49 27' 44 East)). Samples were stored at 4ºC preceding to utilization. Toluene (purity of 99.5%) was filtration-sterilized and used as the sole carbon and energy sources to enrich culture media for the isolation of degrading bacteria. An amount of 5 g of soil sample was combined to 50 ml of mineral salt medium (MSM) complemented with 1% (V/V) toluene. The liquid mineral salt medium (MSM) comprised of ($g.l^{-1}$) 4 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.0011 g $FeSO_4.H_2O$, 0.01 g $CaCl_2$, and pH was regulated to 7 before autoclaving. The samples were incubated at 30ºC shaken at 150 rpm for 7 days. After an enrichment period, 1 ml of the culture was transferred into the fresh MSM medium and incubated at 30ºC shaken at 150 rpm (Zhang et al. 2013). After three subcultures, 0.1 ml of the culture was spread on MSM and nutrient agar plates and incubated at 37ºC for 24-48h.

Identification of Strain ATHH41 by 16S Rdna Sequence

The bacterial chromosomal DNA was extracted, using the method of CTAB, and identified by 1% agarose electrophoresis (Raieta et al. 2015). The forward primer was 27R-AGAGTTTGATCMTGGCTCAG and the reverse primer was 1502F-GGTTACCTTGTTACGACTT. For the PCR outcome system, states were as follows: 2.5µl DNA templates (70 ng/µl); 0.5 µL dNTP mixture (10 mM); 0.4 µL 27 F (10 ϱmol/L); 0.4 µL 1502 F (10 ϱmol/L); 1 µL 10X PCR Buffer (2.5) with $MgCl_2$ (50 mM); 0.3 µL Taq DNA polymerase (5 U/µl); 17.4 µL bringing up ddH_2O. The PCR amplification states were as follows: force-degeneration at 95ºC for 5 minutes, degeneration at 95ºC for 1 minute, annealing at 60ºC for 30 seconds and at 72ºC for 35 seconds, 30 cycles, with another extension at 72ºC for 5 minutes (Madueno et al. 2011). After purification, the PCR products were sent for sequencing by Iranian Biological Resource Center.

Design of Experiments and Modelling

Twenty runs and six replications of the central points were chosen to verify the initial pH, temperature and toluene concentration for the highest degradation of toluene. RSM with a three-factor, three-level CCD design was managed to optimize the response, Y (toluene degradation) of three variables:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_2 + b_{22}X_2 + b_{33}X_2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

where Y is the forecast response factor; X_1 is pH; X_2 is temperature (ºC); X_3 is toluene concentration (mg.l^{-1}), b_0, b_1, b_2, b_3, b_{11}, b_{22}, b_{33}, b_{12}, b_{13} and b_{23} are constant regression coefficients of the model, in which b_0 is the intercept term, b_1, b_2, and b_3 are linear coefficients, and b_{11}, b_{22}, and b_{33} are squared coefficients. On the other hand, X_1, X_2 and X_3 are independent factors. Combinations of factors (such as X_1X_2) represented the interaction between the individuals (Azaman et al. 2010). The genuine factor level relating to the coded factor levels are shown in Table 1. The ranges of factor levels for the experimental design were selected based on the original medium. The optimal culture conditions for maximum toluene degradation and the coefficients in the second-order polynomial (Eq. 1) were calculated by statistical analysis using the Design Expert Software (version 7.1).

Table 01. Levels and codes of variables for central composite design and related strains

	Level code				
Variables	-1.68	-1	0	1	+1.68
X_1	5.32	6	7	8	8.68
X_2	21.59	25	30	35	38.41
X_3	195.46	400	700	1000	1204.54

X_1: pH; X_2:Temperature (ºC); X_3:Toluene concentration (mg.l^{-1})
Source: The Author

Toluene Biodegradation Assay

The isolated Bacteria were grown at 30ºC, 150 rpm, in MSM medium containing 1% (v/v) of toluene for 24 h. The cells were harvested by centrifugation at 10,000×g for 10 min and washed twice in sterile MSM and re-suspended with one-tenth volume of medium. This cell suspension was operated as inoculum for consequent experiments. The toluene degradation was done by dissolving the residual toluene of the medium in 3 ml n-hexane and reading the optical density of the toluene against a blank at 200-400 nm in a UV-visible spectrophotometer (UV-vis-3600, Mapada) (Berlendis et al. 2010).

Preparation and Characterization of MWCNT

1 g of MWCNTs (5-10 nm inner diameter, 20-30 nm outer diameter, surface area >110 m^2.g^{-1}, purity above 98%, US Research Nanomaterials, Houston, TX, USA) was soaked in 60 ml of HNO_3 and H_2SO_4 (3:1) and dispersed using a probe sonicator for 3 h (Zhang et al. 2011). The suspension was filtered through a 0.45 μm membrane filter, and the MWCNTs were washed with deionized water until neutral pH was reached; then, it was dried for 12 h at 60ºC and stored for further use (Pan et al. 2007). The MWCNTs were characterized by the scanning electron microscopy (SEM) (AIS-2100, Seron Technologies, Gyeonggi-do, Korea) and the Fourier transform infrared spectroscopy (FT-IR) (Perkin Elmer, Waltham, MA, USA).

Bacterial Immobilization by MWCNTs for Toluene Removal

MWCNTs were dispersed in sterile distilled water to yield concentrations of 0.005, 0.025, 0.05, 0.25 g.l^{-1} under ultrasonication for 30 min. Then, 10 μL of bacterial suspension with a density of 0.5 McFarland were re-suspended in MSM and 100 μL of MWCNTs suspensions were added. After incubation for 24 h with shaking at 150 rpm, toluene degradation was determined by dissolving the residual toluene of the medium in 3 ml n-hexane and reading the optical density of the toluene against a blank at 200-400 nm in a UV-visible spectrophotometer.

SEM Observations and FT-IR Analysis

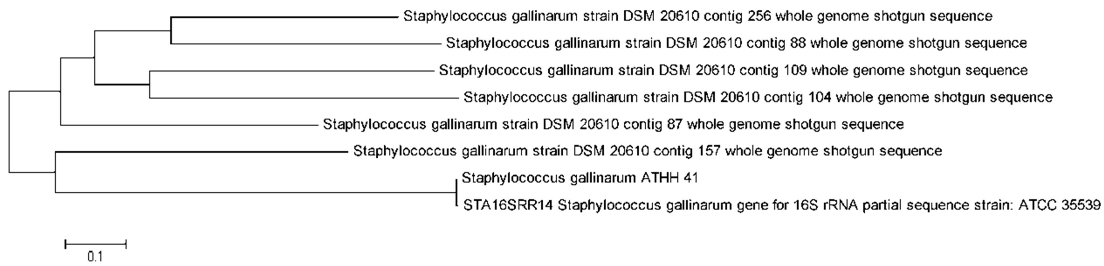
The carbon nanotubes with adhered cells were analyzed by SEM after being rinsed three times with sterile distilled water to remove unattached cells. The surface organic structures were studied by FT-IR. The spectra were recorded at 4 cm^{-1} and 0.01 cm^{-1} of resolution between 4000 and 500 cm^{-1} using a Perkin Elmer Spectrum one series model instrumental analysis with the KBr disc method.

Results

Toluene Degrading Isolate Characterization

After sampling from oil-contaminated soils and enrichment procedures in MSM toluene-containing medium, toluene-degrading bacterial strain was isolated. The bacterium in the strain surviving presence of toluene isolated in this study was designated as *Staphylococcus gallinarum* ATHH41. *Staphylococcus gallinarum* ATHH41 cells were cocci-shaped, gram-positive, catalase, nitrate positive, and oxidase negative. Almost complete sequences of the 16S¬rDNA of the strain *Staphylococcus gallinarum* ATHH41 (1380 bases) were determined. The BLAST algorithm downloaded from the Genebank database (<http: //www.ncbi.nlm.nih/BLAST>) exhibited 99% identified with the closest match for *Staphylococcus gallinarum* ATCC35539. The strain reported in this paper has been deposited in the GeneBank database under the accession number of KX344723. Fig. 1 shows a phylogenetic tree of *Staphylococcus gallinarum* ATHH41 that was constructed using the MEGA (version 5.2) (Tamura et al. 2011).

Figure 01. Phylogenetic tree of the 16S rDNA sequence of Staphylococcus gallinarum ATHH41 and related strains



Source: The Author

RSM MODEL DEVELOPMENT

Instead of optimizing medium composition by one factor at a time approach, the statistical RSM design provides the opportunity to determine the optimal conditions in any given parameter by establishing the relationship between factors and predicted responses (Myers et al. 2016). The RSM design was applied to obtain the precise factor values, which results in the higher toluene degradation. The results are summarized in Table 2.

Table 02. RSM design for the three factors and their experimental results

	Factors			Toluene biodegradation (%)	
Run order	X_1[a]	X_2	X_3	Experimental[b]	Predicted
1	7	30	195.46	60.703	60.40
2	6	25	400	48.016	47.90
3	8	25	400	57.155	57.14
4	6	35	400	59.168	60.36
5	8	35	400	67.958	67.52
6	7	21.59	700	54.941	55.38
7	5.32	30	700	51.229	50.22
8	7	30	700	69.636	69.22
9	7	30	700	69.722	69.22
10	7	30	700	69.729	69.22
11	7	30	700	69.501	69.22
12	7	30	700	68.301	69.22
13	7	30	700	68.394	69.22
14	8.68	30	700	65.393	66.21
15	7	38.41	700	64.537	63.91
16	6	25	1000	55.313	55.89
17	8	25	1000	68.8	67.74
18	6	35	1000	55.505	55.66
19	8	35	1000	65.179	65.43
20	7	30	1204.54	65.251	65.36

[a] X_1: pH; X_2: Temperature (ºC); X_3: Toluene concentration ($mg.l^{-1}$).
[b] The results are presented as the means of duplicates.
Source: The Author

TOLUENE BIODEGRADATION

By applying multiple regression analysis to the experimentally determined data in Eq. (1), the regression coefficients were estimated and the following second-order polynomial equation was obtained for toluene biodegradation:

$$Y = 69.22 + 4.75X_1 + 2.45\,X_2 + 1.48X_3 - 3.89X_1^2 - 3.39X_2^2 - 2.24X_3^2 - 3.17X_2X_3 \qquad (2)$$

The predicted optimum levels of X_1, X_2, X_3were obtained by applying regression analysis (Eq. 2), and they were 7.68 of pH, 31.73ºC of temperature, and 636.04 mg.l^{-1} of toluene concentration, respectively. The prediction of toluene biodegradation was 70.73%. The coefficient of determination (R^2) of the regression for the response related to significant effects on the model was 0.96, which means that the sample variation of 96% for toluene degradation was attributable to the factors. The adequacy of the full quadratic model of toluene degradation was also evaluated with ANOVA. Model summary statistics in Table 3 indicated the adequacy of the models including linear, two-factor interactions, and quadratic terms. Linear and interaction models for toluene degradation were significant.

Table 03. Analysis of variance for response surface quadratic model obtained from experimental design

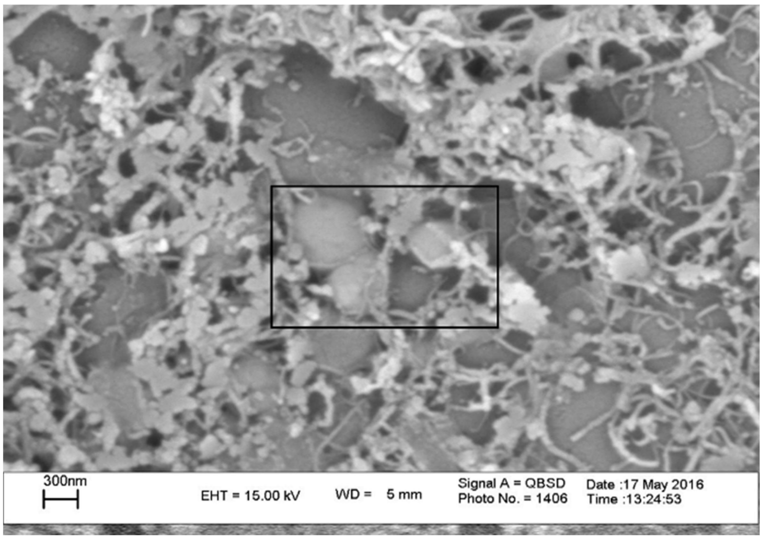
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	0.023	9	$2.595*10^{-3}$	26.88	<0.0001***
X_1	$3.162*10^{-3}$	1	$3.162*10^{-3}$	32.76	0.0002***
X_2	$1.042*10^{-3}$	1	$1.042*10^{-3}$	10.79	0.0082**
X_3	$6.851*10^{-4}$	1	$6.851*10^{-4}$	7.10	0.0237*
X_1^2	0.013	1	0.013	134.65	<0.0001***
X_2^2	$1.213*10^{-3}$	1	$1.213*10^{-3}$	12.56	0.0053**
X_3^2	$3.322*10^{-3}$	1	$3.322*10^{-3}$	34.42	0.0002***
X_1X_2	$1.431*10^{-3}$	1	$1.431*10^{-3}$	14.83	0.0032**
X_1X_3	$4.061*10^{-4}$	1	$4.061*10^{-4}$	4.21	0.0674^{ns}
X_2X_3	$1.081*10^{-3}$	1	$1.081*10^{-3}$	11.20	0.0074**
Residual	$9.653*10^{-4}$	10	$9.653*10^{-5}$		
Lack of Fit	$7.165*10^{-4}$	5	$1.433*10^{-4}$	2.88	0.1353^{ns}
Pure Error	$2.488*10^{-4}$	5	$4.977*10^{-5}$		
Cor Total	0.024	19			
Std. Dev.= $9.825*10^{-3}$		R-Squared=0.9603			
Mean=0.59		Adj R-Squared=0.9246			
C.V.= 1.67		Pred R-Squared=0.7229			
PRESS=$6.737*10^{-3}$		Adeq Precision=15.910			

X_1: pH; X_2: Temperature (ºC); X_3: Toluene concentration (mg.l^{-1})
*Values of "Probability>F value" less than 0.05 indicate model terms are significant
Source: The Author

SEM Observations and FT-IR Analysis

The bacterial adhesion on the surface of MWCNTs in the presence of 100 mg.l^{-1} toluene was observed using SEM. Fig. 2 demonstrates that bacteria cells are trapped among the bundles of MWCNTs arrays. It can be due to the interactions of bacteria cells with the external surfaces of MWCNTs arrays. In addition, Fig. 2 indicates no major changes in the morphology of the bacteria cells after incubating with MWCNTs arrays. These SEM images reveal that MWCNTs clusters only capture the bacteria cells due to sieving mechanisms without any damage to the cell wall.

Figure 02. Scanning electron microscopy imagary of immobilized cells of Staphylococcus gallinarum ATHH41 with MWCNTs

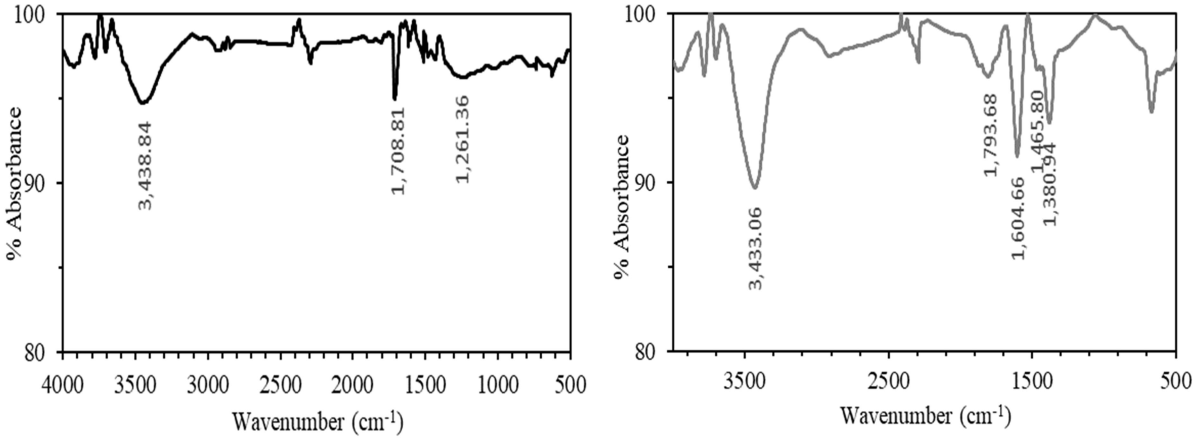


Source: The Author

The whole spectrum of MWCNTs and MWCNTs/*Staphylococcus gallinarum* are compared in Fig. 3. The peak appearance in the areas about 1708.81 cm^{-1} can be ascribed to functional groups containing C=O stretching bond and the peak observed near 3438.84 cm^{-1} is attributed to the band vibration of O-H (Fig. 3A).

The peak appearance in the areas about 3433.06 cm^{-1} is attributed to the band vibration of O-H and the peak observed near 1604.66 cm^{-1} can be ascribed to functional groups containing C=O stretching bond. The peak appearance in the areas about 1380.94 and 1465.80 cm^{-1} is attributed to the band vibration of C-O and C-N stretching mode. In addition, the peak appearance near 1793.68 cm^{-1} can be ascribed to functional groups containing C=O stretching bond (Fig. 3B). This peak is revealing of the presence of the functional groups and bacterial strain on the MWCNTs surface that have designed duration the formation MWCNTs/*Staphylococcus gallinarum* and purification processes. The peak observed near 1798.68 cm^{-1} can be ascribed to functional groups containing C=O single bond.

Figure 03. FT-IR analysis of the A) carboxylate multi-walled carbon nanotubes, B) MWCNTS/*Staphylococcus gallinarum*

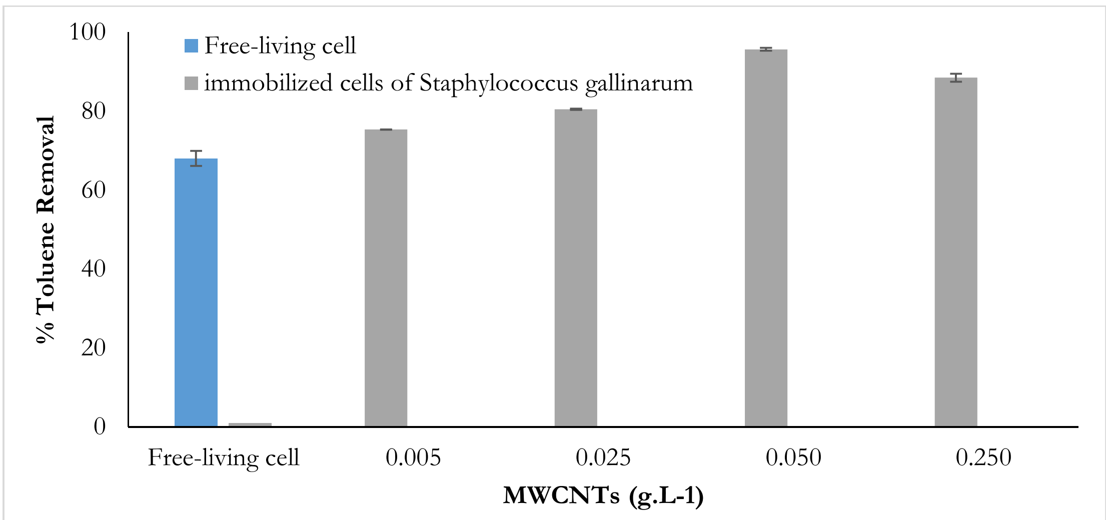


Source: The Author

Adsorption Performance of Free-Living and Immobilized Cells

The removal of 636.04 mg.l^{-1} toluene by the free and immobilized cells of *Staphylococcus gallinarum* ATHH41 with different concentrations of MWCNTs under an initial pH of 7.68 and the temperature of 31.73ºC during 24 h, and shaking at 150 rpm were studied (Fig. 4). In addition, a higher toluene removal percentage was achieved by immobilized cells by 0.05 g.l^{-1} MWCNTs. Another thing about the effect of carbon nanotubes was that carbon nanotubes at low concentrations had reverse effect on a high concentration. In this study, toluene, at pH of 7.68, temperature of 31.73ºC, and an initial concentration of 636.04 mg.l^{-1} was considerably degraded by 68.01% by the free-living cells and up to 95.68% by immobilized cells of *Staphylococcus gallinarum* ATHH41 (Fig. 4).

Figure 04. The comparison of toluene removal percentage by free-living cells and immobilized cells of *Staphylococcus gallinarum* ATHH41 with MWCNTs



Source: The Author

Discussion

This study investigated the biodegradation of toluene by free and immobilized *Staphylococcus gallinarum* strain ATHH41 and the following conclusions were drawn: Toluene degrading bacterium with high biodegradation activity and high tolerance of toluene, *Staphylococcus gallinarum* strain ATHH41, was isolated from the oil-contaminated soils. The genus *Staphylococcus* is gram positive with a thick peptidoglycan layer in the cell wall, and it shows tolerance to organic solvents, such as toluene, benzene, and xylene (Torres et al. 2011). This strain was capable of removing toluene from liquid mineral salt medium by 68.011% in 24 h. Multi-walled carbon nanotubes (MWCNTs) were used to immobilize the strain ATHH41. No changes have been observed in other studies in the structures of the carbon nanotubes after bacterial immobilizing, which is the benefit of the method. Using non-array CNTs have shown that CNTs rupture cell wall-membrane due to toxicity mechanisms, such as oxidative stress and physical damage (Kolangikhah et al. 2012) while this observation has not been observed here. The immobilized cells possess better storage stability and could remove toluene by 95.68% in 0.05 $g.l^{-1}$ MWCNTs during 24 h. Based on the results, it is evident that the toluene degradation by immobilized bacteria is higher than by bacteria alone. The interesting point was that in spite of the increase in nanotubes concentration and the degradation effect, it was not linear and regular. The adsorption mechanism of toluene on MWCNTs is essentially ascribed to the π–π electron donor-acceptor interaction among the aromatic ring of toluene and the surface carboxylic groups of MWCNTs. Positively charged toluene molecules attract the negatively charged molecules such as carbon nanotubes. Carbon nanotubes are effective adsorbent of BTX compounds and have a good potential for the removal of BTX compounds from the wastewater (Bina et al. 2012). Pang et al. (2011) showed that immobilized *Pseudomonas aeruginosa* with multi-walled carbon nanotubes (MWCNTs) were able to increase the absorption of Cr(VI) and the repeated operation of them. The MWCNTs show better toluene adsorption efficiency in 0.05 $g.l^{-1}$ MWCNT. When the MWCNTs contents were more than 0.05 $g.l^{-1}$, toluene degradation would be decreased because of the toxicity of MWCNTs. Also, high MWCNTs contents cause a certain degree of inhibition to the microbial cells. The antimicrobial nature of CNTs depends on multiple variables related to their physical structure and composition. The exposure of microbes to CNTs induces severe oxidative stress in microbes pursued by cell membrane hurt and the release of internal cell contents (Kolangikhah et al. 2012). Thus, the establishment of proficient contact between the CNTs and bacterial cell surface determines the biocidal action of CNTs. However, this effort depends on a variety of factors, such as: (i) physical and structural properties of

CNTs (size and length); (ii) physical condition of CNTs (aggregated or dispersed); (iii) type and concentration of infections associated with CNTs and their availability to bacteria (heavy metal impurities); and (iv) number of layers (single or multi-walled) of CNTs. Normally, loosely-packed, debund-led, highly-dispersed, and shorter length tubes can easily penetrate through the cell membrane and display higher cell cytotoxicity (Al-Jumaili et al. 2017).

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Degradação De Tolueno Por Staphylococcus Gallinarum Livre E Imobilizado Em Nanotubos De Carbono Multi-Carregados

Resumo

A poluição por hidrocarbonetos é uma preocupação ambiental e de saúde mais importante. Usar bactérias livres e imobilizadas pode ser uma atitude adequada para encontrar um agente de bioaumentação adequado. Uma bactéria degradadora de tolueno foi isolada de ambientes contaminados com óleo (localizado em Bandar-Anzali, Guilan, Irã). A cepa foi identificada molecularmente como Staphylococcus gallinarum ATHH41 (número de acesso: KX344723) por sequenciamento parcial do gene 16SrDNA. A metodologia de superfície de resposta (RSM) foi empregada para biodegradação do tolueno por ATHH41. O projeto composto central (CCD) foi utilizado para otimizar o pH, a temperatura e a concentração de tolueno por ATHH41. De acordo com o propósito de otimização do software Design-Expert, as condições ótimas de degradação do tolueno foram obtidas quando o pH, a temperatura e a concentração de tolueno foram ajustados para 7.68, 31.73 ° C e 630,04 mg.l-1 , respectivamente. Nanotubos de carbono de paredes múltiplas (MWCNTs) foram usados para imobilizar a cepa. A espectroscopia de infravermelho e a microscopia eletrônica de varredura mostraram que as células aderiram à superfície MWCNT e desenvolveram um biofilme. Os resultados revelaram que as células livres foram capazes de degradar 68.01% do tolueno como única fonte de carbono e energia em 24 horas sob condições otimizadas. As células imobilizadas atingiram 95.68%.

Palavras-chave: Nanotubo de carbono; Metodologia de superfície de resposta; Staphylococcus gallinarum ATHH41; Tolueno

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