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Application of Multi Walled Carbon Nanotubes (MWCNTs) in Phenol Biosensor based on Bacterial Cells

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Abstract

Introduction: In recent years, electrochemical detection techniques have proved quite promising since they are simple, fast and cost effective. To date, some electrochemical biosensors based on enzymes and microorganisms have been fabricated for the detection of phenol as a priority pollutant listed by the United States Environmental Protection Agency (USEPA). MWCNTs have been widely considered as attractive materials due to their high electrical conductivity, chemical stability and extremely high mechanical strength. The presented work includes the development of a fast, sensitive and miniaturized microbial conductometric biosensor for the determination of phenol based on the cells of *Pseudomonas* sp. (GSN23) and modified microelectrodes with MWCNTs.

Materials and methods: Cells of *Pseudomonas* sp. (GSN23) were grown in the presence of phenol as the sole source of organic carbon and adapted cells were immobilized on the surface of gold interdigitated microelectrodes. Carbon nanotube modified microelectrodes were also prepared to test nanoparticle effect on the efficiency of biosensor performance.

Results: From the results obtained by conductometric measurement, sensitive detection of phenol from 1 to 300 $mg.L^{-1}$ (10-3187 μM), was estimated. Furthermore, substrate specificity and operational stability were investigated.

Discussion and conclusion: The proposed system does not require any complex immobilization procedures and shows the linearity and repeatability with a high operational stability. The use of optimum amounts of MWCNTs and phenol adapted bacteria provide better sensor sensitivity by promoting the ion transfer within the structure of the biosensor

Key words: Carbon Nanotube, Interdigitated Microelectrodes, Phenol, *Pseudomonas* sp. (GSN23), Whole Cell Biosensor

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Introduction

Phenol is regarded as a common environmental waste material due to its increasing production and implementation in industrial activities such as refineries, coking operations, coal process, and petrochemical manufactures (1).

A large number of aquatic organisms such as microorganisms, plants and fishes can enhance the role of mutagenic, teratogenic and carcinogenic impacts in the environments including phenol pollutants. Therefore, phenol was considered as a remarkably hazardous chemical (2).

Some methods such as spectrophotometric, gas chromatography and liquid chromatography are costly and time-consuming, although they are normally used to detect phenol (3). Therefore, more attention has been paid to develop a simple, sensitive, specific, correct and portable system like biosensor in order to determine phenolic compounds.

Biosensing is conducted by utilizing a large number of techniques, among which electrochemical detection techniques are more advantageous, compared to other techniques. In addition, different microorganisms may be used for degrading and detecting phenolic compounds like *Pseudomonas, Arthrobacter, Rhodococcus, Trichosporon* and *Moraxella* (4, 21, 22, 23, 24, 36).

In applied electrochemical detection techniques, few studies have been conducted by focusing on conductometric systems, compared to amperometric biosensors. Several reports addressed the development of amperometric biosensor devices for phenol by using *Rhodococcus sp.*(39), *Trichosporon beigelii*(4), *Pseudomonas putida* DSM 50026 (17), *Arthrobacter* (40), *Moraxella spp.* (41), *Pseudomonas putida* DSM 50026 (17), *Pseudomonas putida GFS-8* (38).

Conductometric biosensors are more advantageous compared to alternative sorts of transducers like amperoetric (5). Conductance measurements involve the resistance determination of a sample solution between two parallel electrodes. The conductometric biosensors present a number of advantages: (a) the planar conductometric electrodes are simple and relatively cheap which suit for miniaturization and large-scale production, and therefore are promising for practical use, (b) they do not require a reference electrode, (c) the applied voltage can be sufficiently small to minimize substantially the sensor's power consumption, (d) large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms.

For electrochemical detection, membrane bound enzymes are of particular interest since enzymatic reactions occur on cell surface. Their activities can then lead to a global change in the ionic composition of the sample.

During recent years, more attention has been paid to the use of nanomaterials, especially gold nanoparticles, magnetic beads and carbon nanotubes (CNTs) in order to develop electrochemical biosensors. In general, the chemical, structural, mechanical, and electronic properties of CNTs are exploited for developing some microbial biosensors. CNTs are able to eliminate the drawbacks related to conventional electrodes such as large response times, low reproducibility, poor sensitivity and stability, as well as a high over potential for demonstrating electron transfer reactions (6-9). By considering all the above-mentioned studies, the present study focused on the results of CNT modified gold interdigitated electrodes (IDE)s with bacterial cells for detecting phenol. Four

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Pseudomonas strains such as GSN13, GSN22, GSN23 and GSN28 were adapted to phenol with high concentration. Further, the whole viable cells were immobilized on CNT modified gold interdigitated electrodes, through glutaraldehyde (GA) cross linking by involving bovine serum albumin.

The different *Pseudomonas* sp. (GSN13, GSN22, GSN23 and GSN28) were isolated from oil contaminated soils in Tehran-Iran and recognized by conducting a metagenomics study (10). The present study emphasized the first step in developing a conductometric biosensor. To this aim, the response features, stabilities and substrate specificities of the designed conductometric biosensor, along with the effect of the CNT as a modifier in the biosensing system were evaluated after analyzing the phenol consumption in four *Pseudomonas* strains.

Materials and Methods

Reagents: In the present study, all chemicals were related to the analytical grade, which are commercially available from Fluka and Sigma Aldrich. In addition, phenol, bisphenol A and 4-chlorophenol, purity ≥ 99.5 % were purchased from Sigma-Aldrich company (Saint-Quentin-Fallavier, France). Multi-walled carbon nanotubes were obtained from Aldrich (diameter; 110-170 nm, lengh; 5-9 µm 90 %).

Growth Media and Microorganisms: Mineral salts medium (MSM) with the subsequent composition was implemented as a growth medium; $(NH_4)_2SO_4$ 325 $mg.L^{-1}$, $MgSO_4.7H_2O$ 133.07 $mg.L^{-1}$, K_2HPO_4 2627 $mg.L^{-1}$, KH_2PO_4 1436 mg. L^{-1} $CaCl_2.2H_2O$ 11.91 $mg.L^{-1}$ and $MnSO_4.H_2O$ 9.45 $mg.L^{-1}$. In all cases, the pH was adjusted to 7.5 and phenol was added at different final concentrations as the sole carbon source. Further, medium base and phenol solution were completely mixed after sterilization in order to prepare the MSM/phenol. The MSM was solidified as MSM phenol agar by adding 2 % washed agar-agar when it was necessary (11).

In the next stage, four *Pseudomonas* strains (GSN13, GSN22, GSN23 and GSN28) were obtained from the NLIM Collection of Microorganisms (National Laboratory of Industrial Microbiology, Tehran-Iran). The strains were isolated from oil refinery contaminated soils in the southern part of Tehran province -Iran (10) and were sub-cultured in MSM/phenol Agar (100 $mg.L^{-1}$).

Then, the daily prepared electrodes with fresh cells were implemented in all experimental steps. The cells were washed from the plates with 50 mM phosphate buffer pH 7.0 and the cells were counted by plating 1ml of successive serial dilutions of *Pseudomonas* sp. (GSN23) suspension on the MSM agar standard-sized plates and were correlated to turbidity measurements.

Adaptation method to Phenol: The cells were adapted to phenol as follows. First, every microorganism was grown in MSM with 100 $mg.L^{-1}$ *phenol*. Then, the cell mass was centrifuged and inoculated into MSM at 200 $mg.L^{-1}$. In the next stage, phenol was progressively added to MSM increased concentration to 1000 $mg.L^{-1}$ (incubation at 30 ± 0.1°C and 130rpm). In addition, the role of temperature on growth and phenol degradation was evaluated. All tests were conducted in triplicate. Hence, the results are regarded as the arithmetic means related to three independent experiments.

In the next procedure, the bacteria were transferred and grown on MSM phenol agar (1000 $mg.L^{-1}$) and preserved at 4°C for other studies after completing the adaptation (11).

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Analytical Procedure: First, the samples were centrifuged at 8000 rpm for 20 min in order to evaluate the biomass. Then, the supernatant was utilized for determining phenol. In addition, the biomass was suspended again in distilled water and accordingly the absorbance was evaluated against distilled water as reference at 600 nm (12).

Finally, phenol analysis was conducted by assessing the absorbance at a wavelength of 500 nm by utilizing a spectrophotometric method, after developing color by 4-aminoantipyrine, based on the standard methods for evaluating water and wastewater (13). The phenolic solution was mixed with 4-aminoantipyrine in the presence of potassium ferricyanide (as alkaline oxidizing agent), at high pH, formed a red quinone dye that its absorbance was read at 500 nm.

Sensor Design and Experimental Setup: The conductometric transducers were fabricated at the Institute of Analytical Sciences and Nanotechnology (Villeurbanne, Lyon, France). Two pairs of gold interdigitated thin film electrodes (150 nm thick) and the sensitive surface was about 2.9mm for each electrode pair ^{2}. Regarding size, the related electrodes were 5 mm × 30 mm and were fabricated into a glass substrate by vapor deposition of gold (14) As shown in Fig.1. The sensitive part of each electrode was about 1 mm^2.

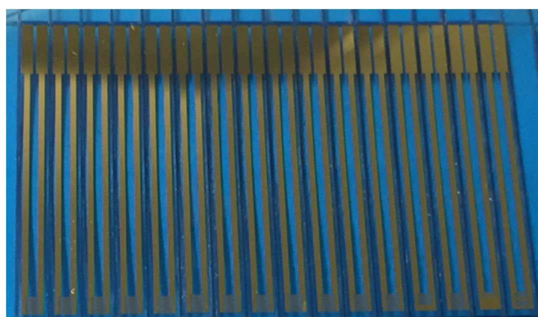

Fig. 1- Gold Interdigitated Thin Film Electrodes

The electrodes were sonicated at 28°C for 10 min in the acetone ultrasonic bath before using and accordingly rinsed with ultrapure water and dried under nitrogen flow. In the next procedure, the electrode surfaces were cleansed for 1 min with a freshly prepared "piranha" mixture (H_2O_2/H_2SO_4, 3:7, v/v) and were rinsed rigorously with a lot of deionized water (15).

In addition, the active membrane related to the sensitive space of the electrode was established by cross-linking bacterium, which was combined with CNT- bovine serum albumin and exposed to saturated GA vapors (16-18).

Then, different amounts of MWCNTs were completely dispersed in 5% BSA in 100 µL of 50 mM phosphate buffer (pH 7.0) for the purpose of preparing CNT-Bovine serum solution. Accordingly, the blends were treated under an ultrasonic field for 10 min and stirred for 2 h. In order to disperse the tubes effectively, a complete preparation of ultrasonification is essential, as throughout preparation CNTs are insoluble in most solvents. Further, *Pseudomonas* sp. cells with 12×10^8 cell titer (100 µL) were added to the current mixture at 28 °C and accordingly drop technique was implemented to deposit the 0.8 µL of the combined solution into the sensitive space of the interdigitated electrodes.

Regarding the second series of biosensors, a mixture including *Pseudomonas* sp.(GSN23) cells with 12×10^8 cell titer (100 µL), as well as 5 % BSA in 100 µL of 50 mM phosphate buffer (pH 7.0) were produced at 28 °C. Then, drop method was used for depositing 0.8 µL of this solution into the sensitive area of the interdigitated electrodes.

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Additionally, an equivalent procedure was performed to prepare the reference sensors, without using microorganism cells. Finally, sensors were put into saturated GA vapors for 10 min and then electrodes were dried at 28 °C for 30 min and kept at 4 °C before conducting the experiments.

Scanning Electronic Microscopy (SEM) Observations: To this aim, a Quanta 250 microscope was used to capture the SEM images related to the dispersion of the bacteria on the sensing element surface. The modified biosensors related to microorganism were immersed in 3 % glutaraldehyde solution, which was purchased from Sigma-Aldrich, and particularly refined for using as microscopy fixative for 45 min. Then, a series of dehydration in the solutions related to successive absolute ethanol were implemented for 10 min by employing increased concentrations of 20 %, 40 %, 60 %, 80 %, and 100 % ethanol (18).

Conductometric Transducers and Experimental Setup: First, measurements were conducted at 28°C in a 5 ml glass cell, which was filled with a 5 mM phosphate buffer pH 7 under constant stirring. Then, the electrodes were submerged in sample solution and were measured with an alternating voltage (10 mV amplitude, 100 kHz frequency) created by a generator (SR830 Lock-in amplifier from Stanford Research System).

After the signaling was stabilized, different concentrations of stock solution related to substrates such as phenol, 4-chlorophenol, 2, 3, 6-Trichlorophenol, 2, 4, 6-Trichlorophenol *p*-Nitrophenol, and Bisphenol-A were added into the vessel. In addition, the differential signaling was computed for every compound. In the next procedure, a relationship was established between biosensor response and different substrate in the range of 0.01-3 mM (1-300ppm) and accordingly the limit of detection (LOD) was computed. Then, the electrode was rinsed with phosphate buffer (5 mM, pH 7) when the measurement was conducted. Finally, every experiment was replicated at least for three times.

Regarding control experiments, only electrode was implemented in the presence of different substrates including phenol, 4-chlorophenol, 2, 3, 6-Trichlorophenol, 2, 4, 6-Trichlorophenol, *p*-Nitrophenol, and Bisphenol-A although no response signal was observed without bacterium.

Results

Biodegradation Capability and Growth of Microorganism after Adaptation: Fig.2 illustrates the most degradation capability, as well as the influence of the adaptation to the substrate (Phenol) in batch mode among flasks. The well-acclimatized culture of *Pseudomonas* sp.GSN23 was degraded initial phenol concentration of 1000 $mg.L^{-1}$ completely in less than 56 h. Then, an optimal number of this bacterium (*Pseudomonas* sp. GSN23) with this proper degradation was immobilized on the surface of the electrodes and conductometric measurements were done at 28 °C.

Microscopic Characterizations: An optimal number of bacteria should be immobilized on the surface of electrodes for the purpose of obtaining a correct response. Based on the results, the best microorganism cell concentration for max response was 12×10^8 with the optical density of 1.8 at 600 nm. In addition, as illustrated in Fig. 3, SEM image indicated the microorganism cells, along with their attachment on the surface related to the conductometric sensing element.

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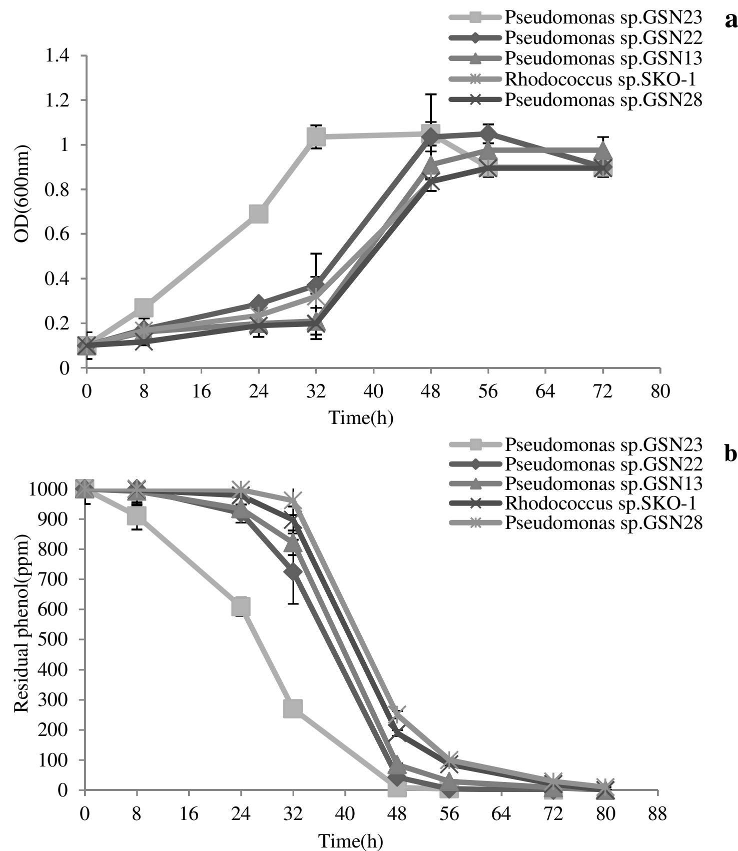

Fig. 2- a. The cell growth of (■) *Pseudomonas* sp.(GSN23), (♦)*Pseudomonas* sp.(GSN22), (▲)*Pseudomonas* sp.(GSN13) and (×)*Pseudomonas* sp.(GSN28) in the minimal salt medium containing initial 1000 mg L^{-1} Phenol after acclimatization process (600nm). b. Phenol degradation of (■) *Pseudomonas* sp.(GSN23), (♦)*Pseudomonas* sp.(GSN22), (▲)*Pseudomonas* sp.(GSN13) and (×)*Pseudomonas* sp.(GSN28)– By using 4-aminoantipyrine following the standard methods- in the minimal salt medium containing initial 1000 mg L^{-1} Phenol after acclimatization process

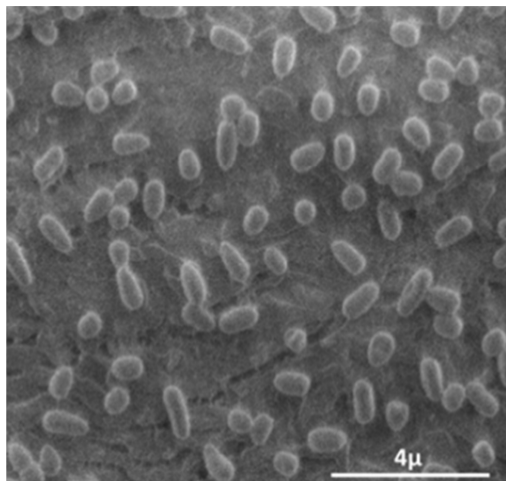

Fig. 3- SEM and Optical Microscopic Images of the Microorganism

Analytical Characteristics of Biosensor supported *Pseudomonas* sp. (GSN23): First, different biosensors including MWCNT (diameter; 110-170 nm, length; 5-9 µm) and *Pseudomonas* sp. GSN23 (12×10^{8} cell titer) were prepared and rather high responses for phenol were determined with 0.1 mg/ml MWCNT, compared to MWCNT- free biosensors.

As illustrated in Fig. 4, the maximum response with MWCNT is 98.3 µS, in comparison to 48 µS for MWCNT- free biosensors in the presence of 300 mg.L^{-1} (3187 µM) phenol. A linear relationship was observed between biosensor response (y) and phenol concentration (x) in the

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range of 1-300 mg.L^{-1} phenol in both of the electrodes. The inter-sensor RSD% (n=7) of 9.34 % and 11.2 % was obtained for CNT-free and modified *Pseudomonas* sp. GSN23 biosensors, respectively.

In addition, the results indicated that the CNT had a higher sensitivity (0.324 µS/ mg.L^{-1}), compared to CNT free biosensors (0.133 µS/ mg.L^{-1}), which may be related to special features of CNT which can accelerate the electron transfer.

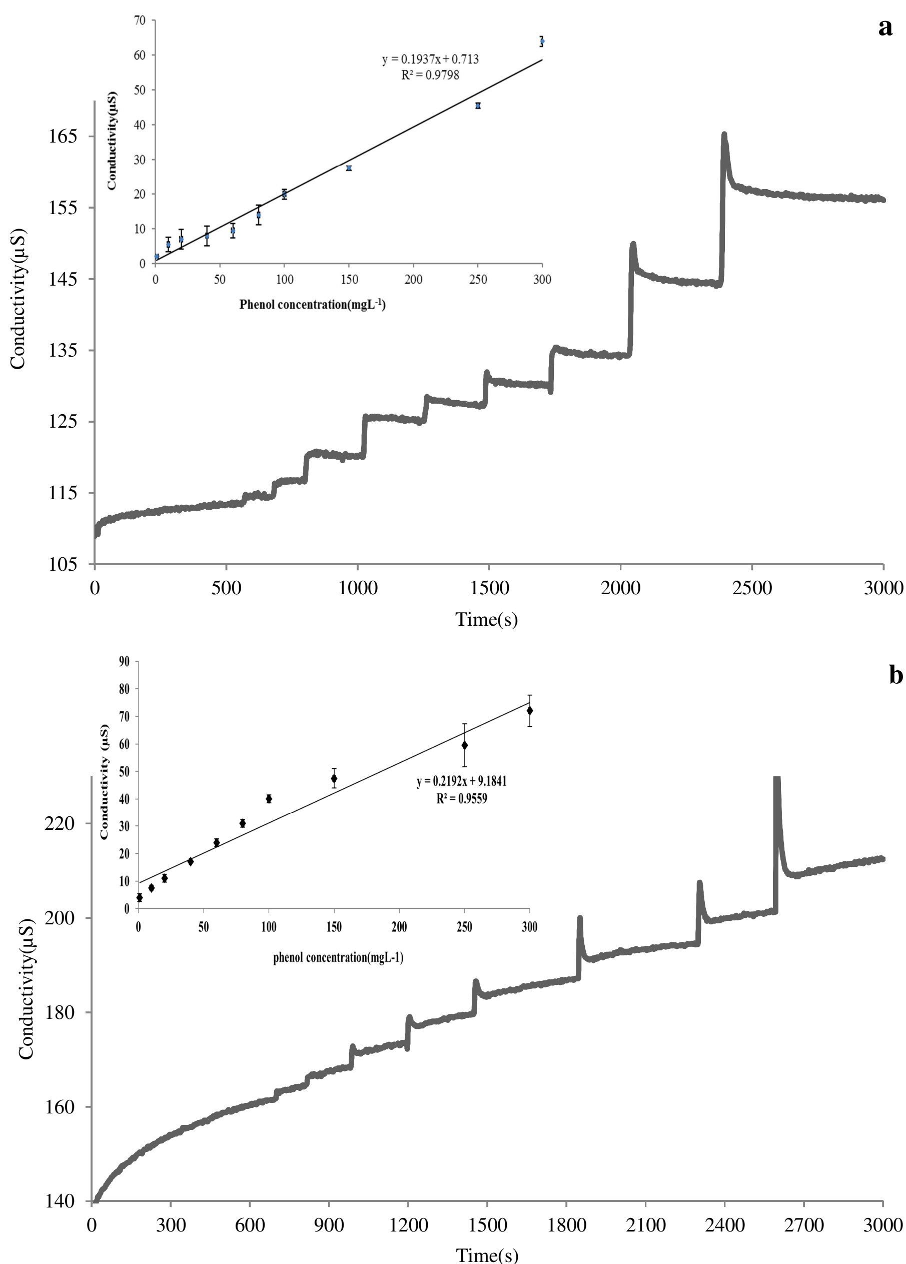

Fig. 4- comparison of the biosensor responses recorded for phenol (1-300 mg L^{-1}) in absence (a) and presence (b) of MWCNT (diameter; 110-170 nm, lengh; 5-9 µm). The inserted graph presents electrochemical response of conductometric *Pseudomonas* sp. GSN23 biosensors to repeated addition of phenol.

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The present biosensor indicated a good analytical performance, compared to the previously-reported ones (4, 7, 17, 38, 39, 40, 41), due to a large linear range from 1 to 300 mg.L^{-1} (10-3187 µM), as well as the detection limit of 0.5 mg.L^{-1} (5 µM) obtained with a signal to noise ratio of 3- and response time near 2 minutes.

Further, the sensor response was measured for ten days in order to evaluate the stability of the biosensors in presence of 300 mg.L^{-1} (3187 µM) phenol. Then, the biosensors were stored at 4 °C in the presence of phenol vapor based on the bacterium with MWCNT. In the next procedure, the signals remained stable during the five days of storage while a sharp decrease in sensitivity was observed for the electrodes (Fig. 5).

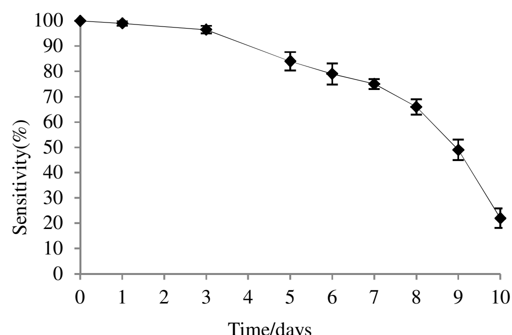

Fig. 5- Representation of designed biosensor long term storage stability. The electrodes were stored at 4°C in presence of phenol vapor. Three measurements were performed at room temperature for 300 mg L^{-1}(3187 µM)

Substrate Specificities: Table 1 demonstrates the biosensor responses to various phenolic substrates with respect to the response to phenol (100%). The results indicted the highest sensitivity to phenol for all types of biosensors. Additionally, relative responses for p-nitrophenol and bisphenol-A were 19% and 16 %, respectively, although no response was specified within the higher concentration of these compounds (more than 80 mg.L^{-1}, 850 µM).

Further, the presence of 4-chlorophenol, 2, 3,6-Trichlorophenol and 2,4,6-Trichlorophenol could establish different types of detection including 1 to 300 mg.L^{-1}, 10-3187 µM) with 26 %, 22% and 20% response, compared to that of phenol, respectively. However, as shown in Table 1, the substrate sensitivity was considerably lower (Table 1) than that of phenol (0.5423 µS ppm^{-1}).

Table 1- Substrate Specificity of *Pseudomonas*-based Biosensor

Phenolic substrates	Reponse(%)	Linear range(ppm)	Sensitivity (µS ppm^{-1})
p-Nitrophenol	19%	1-100	0.0582
Bisphenol-A	16%	1-100	0.0394
4-chlorophenol	26%	1-300	0.0554
2,3,6-Trichlorophenol	22%	1-300	0.0577
2,4,6-Trichlorophenol	20%	1-300	0.0489

Discussion and Conclusions

Primarily, phenol biodegradation has been evaluated well with *Pseudomonas* species (12,19,25). In addition, some other microorganisms like *Phanerochaete chrysosporium* (26), *Rhodococcus* spp. (27), *Candida tropicalis* (28), *Candida* sp. (29), *Bacillus* spp. (30-32), *Penicillium chrysogenum* (33), *Acinetobacter* spp. (34) and *Alcaligenes* sp. (35) were reported to degrade phenol and phenolic compounds.

For example, Gonzáles *et al*. (20) implemented only two adaptation steps in order to assess the degradation capacity of more than 90% of 500 mg.L^{-1} phenol in 25 h by immobilized cells of *Pseudomonas putida* ATCC 17484.

As shown in Table 1, different types of microorganisms were implemented in biosensor systems for detecting phenol in previous studies. A large body of research used some bacteria, such as *Pseudomonas* sp. and *Rhodococcus* sp. in order to improve whole-cell biosensors for detecting the phenol. For example, Timur *et al*. (8) developed a biosensor by focusing on CNT

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with associate osmium polymer and *Pseudomonas putida* DSM 50026. Based on the results, phenol adapted cells were implemented as the biological component and a linear relationship was reported between the sensor response (nA/cm^2) and phenol concentration in the range of 500-4000 µM. Accordingly, Kirgoz *et al.* (7) designed a microbial biosensor by CNT and phenol degrading bacteria in order to measure glucose. Then, the results indicated higher current values, compared to conventional electrodes (2 or 3 folds). By promoting the electron transfer at intervals in the structure of biosensor, higher device sensitivity was obtained with optimum amounts of the CNT.

Although the electrocatalytic features of the CNT have not been identified yet, the open ends of nanotubes may be evident based on this attractive behavior. Further, the high electrical conductivity of these nanoparticles allows them to be used as an electrode material. Furthermore, it can offer the ability to mediate electron transfer reactions in combination with its strong electro catalytic activity. The protection of the microorganism cells by the MWCNT could help improve the stability of the biosensors since CNTs with huge surface areas can create efficient matrices to maintain a number of cells on the electrode in order to set the ground for shielding the enzymes against degradation or outpouring by keeping them safe within the membrane.

Conductometric sensors are rather sensitive and their production utilizes the technology of inexpensive thin-film standard. In addition, low price of each device was guaranteed, along with the best strategies of immobilization related to biological materials (5).

In order to detect the phenol, Skladal *et al.* (38) reported whole-cell amperometric biosensors by implementing immobilized *Pseudomonas* sp. Three *Pseudomonas* strains could transfer electrons in combination with mediator-modified SPEs (Screen-printed electrodes), due to the specific oxidation of phenol to the electrode through mediators.

The lower limits for phenol detection were 1µM and 10µM for one of the strains including *Pseudomonas sp.* 74-III, and for the rest such as *Pseudomonas* sp. 83-IV and *Pseudominas* sp. 394, respectively. Furthermore, the sensor response was reported to different phenolic substrates. Based on the result, the sensitivity to chlorinated phenols in a selected concentration of these substrates was reported for all types of the sensors. In general, there are a few numbers of microorganism sensing element models which can possess the sensitivity just for one analyst. However, selectivity can be enhanced by cultivating the biomass by means of the expected analyst as a sole supply of carbon and energy (37).

In this study, a sensitive and simple electrochemical detection method has been proposed for the detection of phenol using pure cultures of *Pseudomonas* sp. (GSN23). The present phenol biosensor based on IDEs-MWCNTs-bacterium electrodes displayed a wide linear range from 10 to 3184 µM with a low detection limit of 5µM which is compared to others reported in the essay. Immobilization of bacteria instead of pure enzymes on the electrodes provides economic and practical disposable biosensor.

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Application of Multi Walled Carbon Nanotubes (MWCNTs) in Phenol Biosensor based on Bacterial Cells

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چکیده

مقدمه: در سالهای اخیرتکنیکهای تشخیصی الکتروشیمیایی به دلیل سادگی، سرعت و مقرون به صرفه بودن روشهایی نویدبخش به شمار می روند. تاکنون، تعدادی بیوسنسور الکتروشیمیایی، بر پایه آنزیم و یا میکروارگانیسمها برای تشخیص فنل، به عنوان یک آلاینده مقدماتی که در لیست آژانس حفاظت محیط زیست ایالات متحده قرار دارد، ساخته شده است. نانولوله های کربنی به دلیل خواصی چون هدایت الکتریکی بالا، ثبات شیمیایی و استحکام مکانیکی بالا به عنوان موادی موثر و جذاب در این مطالعات به شمار می روند. پژوهش حاضر شامل بررسی یک بیوسنسور هدایت سنجی حساس و سریع بر پایه سلولهای میکربی سودوموناس.GSN23 و نانولوله های کربنی می باشد.

مواد و روش‌ها: سلولهای میکربی سودوموناس.GSN23 در حضور فنل به عنوان تنها منبع کربن و انرژی رشد داده شده و سلولهای آداپته شده با این سوبسترا بر روی سطح میکروالکترودهای مرکب طلا تثبیت گردیدند. میکروالکترودهای اصلاح شده با نانو لوله های کربنی نیز تهیه گردیده تا میزان تاثیر آنها بر روی بیوستسور طراحی شده سنجیده شود..

نتایج: بنابر نتایج بدست آمده در بررسی های هدایت سنجی، سنجش حساس فنل در محدوده ۱ تا ۳۰۰ میلی گرم در لیتر(۱۰ تا ۳۱۸۷ میکرومولار) تخمین زده شد. اختصاصیت سوبسترا و پایداری بیوسنسور نیز موردبررسی قرار گرفت.

بحث و نتیجه‌گیری: سیستم پیشنهادی در این پژوهش نیازمند روشهای پیچیده تثبیت نبوده ، دارای نمودار خطی نسبت به افزایش غلظت فنل، تکرارپذیری و ثبات بالا را دارا می باشد. کاربرد میزان مناسب از نانولوله های کربنی و باکتری های آداپته شده به سوبسترای فنل حساسیت و انتقال مناسب تر یونها را در ساختار بیونسور فراهم می سازد.

واژه‌های کلیدی: نانو لوله کربنی، میکروالکترودهای مرکب، فنل، سودوموناس، بیوسنسور سلولی

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