

Effect of Bacterial Isolates from Soil Samples on Bisphenol A

Neihaya H. Zaki*

Department of Biology, College of Science, Mustansiriyah University, Baghdad, IRAQ

*Author email: neihaya_2008@yahoo.com

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ABSTRACT

Bisphenol A is known as one of endocrine disruptors compounds and has an acute toxicity to aquatic organisms in freshwater and marine species. Twenty five samples were collected from the soil around the Tigris River from different locations in Iraqi cities, and 45 bacterial isolates were obtained. Three of these isolates were further tested for their degrading capacity of Bisphenol A (BPA) in Basal Mineral Medium, included: *Pseudomonas orizohibtanis*, *Escherishia coli* and *Proteus penneri*. The optimal temperature for the removal of BPA was tested at 20°C, 37° and 45°C for 1, 5, and 15 days, and the degradation increased up to a temperature of 37°C. Growth test was performed on isolated bacteria with BisPhenol A as the sole carbon source, and with increasing incubation time, the culture grew almost linearly to 24 hours. BPA decreased after 1days after incubating with tested bacterial isolates, and almost broken after 5 days, while it disappeared after 15 days at 37C, and *Pseudomonas orizohibtanis* exhibited the best degradation of BPA. The absorbance peaks in the UV region appeared at 222 and 276 nm and attributed to the benzene ring and triazine ring respectively. The end products of BPA degradation were analyzed by GCMS after 15 days of incubation. The chromatogram for *Pseudomonas orizohibtanis* showed three peaks at retention times of 70, 210 and 280 min, and referred to hexasiloxane, heptasiloxane, and Octasiloxane respectively. The bacterial isolates from the soil around Tigris River had the ability to degrade Bisphenol-A, and it could grow in different environmental conditions, also this degradation process produced many of active materials which it is useful in many applications.

KEYWORDS: Bacterial degrading isolates, Bisphenol-A, degradation, Optimization, GC-MS.

الخلاصة

Bisphenol-A يعرف كأحد المركبات التي تسبب اختلال الغدد الصماء ويكون ذو سمية حادة للكائنات المائية في المياه العذبة والانواع البحرية تم جمع ٢٥ عينة من التربة المحيطة بنهر دجلة من مواقع مختلفة في المدن العراقية، وتم الحصول على ٤٥ عزلة بكتيرية. تم اختبار ثلاث من هذه العزلات بشكل أكبر بسبب قدرتها المحللة لمادة Bisphenol A (BPA) في وسط المعادن الاساسي، وشملت الاجناس: *Pseudomonas orizohibtanis* و *Escherishia coli* و *Proteus penneri*. تم اختبار درجة الحرارة المثلى لإزالة BPA عند ٢٠ درجة مئوية و ٣٧ درجة و ٤٥ درجة مئوية لمدة ١ و ٥ و ١٥ يوماً، وزاد التحلل البايولوجي بدرجة حرارة ٣٧ درجة مئوية وبعد ١٥ يوم. بعدها تم إجراء اختبار النمو للبكتيريا المعزولة مع BisPhenol A كمصدر وحيد للكربون، ومع زيادة وقت الحضانة، زاد منحنى النمو خطياً تقريباً إلى ٢٤ ساعة. انخفض وجود BPA بعد ١ يوم بعد الحضانة مع العزلات البكتيرية المختبرة، وتكسر بعد ٥ أيام تقريباً، في حين اختفى بعد ١٥ يوماً بدرجة ٣٧، وكان جنس *Pseudomonas orizohibtanis* أفضل محلل للـ BPA. ظهرت قمم الامتصاص في منطقة الأشعة فوق البنفسجية في ٢٢٢ و ٢٧٦ نانومتر ويعزى ذلك إلى وجود حلقة البنزين وحلقة التريازين على التوالي. تم تحليل المنتجات النهائية من تحلل BPA بواسطة جهاز GC-MS بعد ١٥ يوماً من الحضانة. أظهر المنحنى لـ *Pseudomonas orizohibtanis* ثلاثة قمم في أوقات الاحتفاظ بـ ٧٠ و ٢١٠ و ٢٨٠ دقيقة، وأشار إلى hexasiloxane، heptasiloxane و Octasiloxane على التوالي. البكتيريا المعزولة من التربة حول نهر دجلة لديها القدرة على تحليل مركب Bisphenol-A، ولها القابلية على النمو في الظروف البيئية المختلفة، وأيضاً انتجت عملية التحلل هذه العديد من المواد الفعالة والتي هي مفيدة في العديد من التطبيقات.

INTRODUCTION

Bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl propane); is an organic compound composed of two phenol rings connected by a methyl bridge, with two methyl functional groups attached to the bridge. BPA is known as one of endocrine

disruptors and has an acute toxicity to aquatic organisms in freshwater and marine species [1].

The considerable production and application of phenol in industrial activities make it a major environmental pollutant in most wastewater various facilities, such as oil refineries, pharmaceuticals and plastic industries. Thus,

phenol has been classified as a highly hazardous chemical [2].

BPA widely used as a material for the production of epoxy resins, phenol resins, polycarbonates, polyacrylates, polyesters and lacquer coatings on food cans. BPA can be leached from these plastic products, food- and drink-packaging [3].

Bacteria distributed in river water can degrade BPA. Many studies reported that BPA had a short half-life and was rapidly biodegraded in river water. In addition, BPA is readily biodegraded in waste treatment using bacteria [4]. Based on the theory that a few microorganisms can utilize phenol as the sole source of carbon and energy, numerous bacterial species have been isolated and characterized as phenol-degrading microorganisms. These bacterial species include *Pseudomonas putida* [5], *Bacillus subtilis*, *Bacillus brevis* [6], *Serratia marcescens* [7], *Arthrobacter citreus* [8] etc. Native microbial species have been reported to be more adaptive and capable of out-competing non-indigenous microorganisms in the remediation of special polluted environments [9]. Therefore, identifying new phenol-degrading bacteria are necessary for the bioremediation of the phenol-contaminated environments in various regions.

The aim of this study to isolate and identify bacteria from the soil around Tigris river, and examined the ability of these isolated bacteria to degrade Bisphenol A, then characterized the environmental conditions of their growth in BMM medium. In addition, use GCMS to analysis the end products of the biodegradation.

MATERIALS & METHODOLOGIES

Chemicals

Bisphenol A (bis-(4-hydroxyphenyl) methane) was tested for the ability to support the growth of phenol degrading bacterial isolates as the sole carbon source. It was obtained from the Chemistry department/ College of Science- Mustansiriyah University, Baghdad, Iraq.

Collection of samples

Twenty five samples were collected from the soil around Tigris River from different locations of Iraqi cities in sterile plastic containers and stored at 4°C until use.

Growth of testing bacteria in Basel Mineral Medium (BMM)

The ability of these bacteria to use Bisphenol as a nutrient was determined by growing cultures in BMM mineral salts medium, Table 1 with 2 g/l of Bisphenol A, and the initial pH value was 7.2 [10].

The inoculated flasks were agitated on an orbital shaker, 120 rpm at 30°C for 72 h. then 2 ml of the culture medium was transferred to another 50 ml of fresh culture medium, and cultivation was carried out under the same condition for 2 to 3 times. The obtained suspensions were streaked on the nutrient agar plates. All the plates were incubated at 37°C for 48 h. The morphologically distinct bacterial isolates, showing different shape, color and size of colonies were selected and preserved in nutrient agar slant at 4°C [11].

Table 1: The composition of BMM medium

Material	Weight (g)
K ₂ HPO ₄	5.17
KH ₂ PO ₄	1.70
NH ₄ CL	1.63
MgSO ₄	8.5
MnSO ₄	5
FeSO ₄	5
CaCl ₂	0.3

Isolation and characterization of bacteria

Each bacterial isolates were characterized by its growth characteristics, standard staining and biochemical reactions according to [12]. APi 20 system, chromagar test used for further diagnosis of isolated bacteria [13].

Optimization of growth of bacteria

About 1ml of bacterial suspension mixed with 0.001ml of BPA and incubated for different interval times (0, 1, 5 and 15 days) [14], at different temperature degrees (20, 37, and 45 °C) to identify effects of the incubation temperature and times on the ability of bacteria to degrade Bisphenol A compound using the UV-vis spectrophotometer [15].

Bacterial viability on agar media

Agar well diffusion method was used to identify the effect of BPA compound on bacterial viability [10]. Briefly, 1 ml of each bacterial culture was

spreading on Nutrient agar, and five wells were made on agar surface, then 0.001 ml of BPA put into each well and incubated for 24hrs. at 37°C.

Bacterial Growth test with BPA

The bacteria were suspended in the isolation medium to make up a bacterial suspension (OD = 1.0 at 600nm). About 1000 µl of suspensions was inoculated into a fresh medium (100 ml) with 0.2 ml of BPA, and the medium was agitated at 120 rpm and incubated at 37°C. Bacterial growths was monitored by the measurement of the optical density of the medium at 600 nm [14].

Bacterial Degradation test

The bacterial degradation was performed in broth medium by inoculating 15% of overnight cultures of degrading bacteria to fresh 100 ml of BMM medium containing phenol in 250 ml flask. Flasks without inoculation was kept as control. After incubation, 10 ml of each sample was analyzed at a range of wavelengths (200-400) nm [1].

GC-MS Analysis of biodegradation products

Bis Phenol A compound was degraded by using bacterial isolate at optimal environmental conditions, and the end products were analyzed by GCMS after 15 days of incubation. A GCMS analysis was conducted by using the culture medium (q500 ml) and extracted with an equal volume of ethyl acetate, and the ethyl acetate layer (1 ml) was analyzed by GC-MS (SHIMADZU-Japan) [16].

RESULTS AND DISCUSSION

Bacterial Isolation & Identification

A total of 45 bacterial isolates were obtained after enrichment on agar media Figure 1. All isolates showed different culturing and biochemical characteristics showed in Table 2.

Three types of phenol degrading bacteria (Which have the ability to use Bisphenol A as a nutrient in BMM) diagnosed and chose from soil samples by culture characteristic, biochemical tests, Chromagar test Figure 2, and APi 20 system. These bacteria included: *Pseudomonas orizohibtanis*, *Escherishia coli* and *Proteus penneri*.



Figure 1: Different types of bacteria isolated from soil on Nutrient agar medium

Table 2. Biochemical and morphological characteristics of degrading bacteria.

Soil Samples	Gram Stain	Microscopic Characteristics	Culture Characteristics	Catalase	Oxidase
Diyala bridge 1	-ve	Cocci	Large colonies White color	-ve	-ve
Diyala bridge 2	-ve	Diplococci	Small mucoid colonies	-ve	-ve
Diyala bridge 3	-ve	Rod	Very large milky colonies	-ve	+ve
Diyala bridge 4	-ve	Clusters cocci	Large colonies White color	+ve	-ve
Diyala bridge 5	-ve	Clusters cocci	Pale colonies	+ve	-ve
Latifiya 1	-ve	Rod	Small colonies	+ve	+ve
Latifiya 2	-ve	Rod.	white colonies	-ve	-ve
Latifiya 3	+ve	Cocci.	Milky colonies	+ve	-ve
Shorja 1	-ve	Cocci.	Small red colonies	-ve	+ve
Shorja 2	-ve	Rod	Mucoid colonies	-ve	+ve
Shorja 3	-ve	Rod	Pale colonies	-ve	+ve
Shorja 4	-ve	Cocci	Large colonies	-ve	-ve
Shorja 5	-ve	Rod	Orange small colonies	+ve	+ve
Dewaniya 1	-ve	Cocci.	Pink mucoid colonies	-ve	-ve
Dewaniya 2	-ve	Clusters cocci.	Red mucoid colonies	+ve	-ve
Yosifiya 1	-ve	Diplococcic.	Yellow colonies	+ve	+ve
Yosifiya 2	+ve	Diplococcic.	Orange colonies	+ve	-ve
Yosifiya 3	-ve	Rod	Pink mucoid colonies	+ve	-ve
Yosifiya 4	-ve	Rod	Red mucoid colonies	+ve	+ve
Yosifiya 5	+ve	Bacilli	Milky colonies	+ve	-ve



Figure 2. Results of Chromagar test: Green color (*E.coli*), yellow color (other coliforms).

Optimization of Biodegradation of bacteria

The optimal temperature for the removal of BPA was determined for testing bacteria. The reactions were incubated at 20°C, 37° and 45°C for 1, 5, and 15 days.

Figure 3 (a, b, and c) was used to Determine the compounds which resulting from phenol degradation by *P. orizohibtanis*, and exhibited three peaks at wave length 276nm (x-axis), and showed that the values of O.D (Y- axis) at 20°C were (1.307, 1.305, 1.301) for 1, 5, and 15 days respectively, while at 37°C were (1.338, 1.65, 1.89) for 1, 5, and 15 days respectively, and at 45°C were (1.301, 1.302, 1.303). From these results, biodegradation of Bis phenol A was increased up to a temperature of 37°C, and then further increase in temperature (to 45°C) was caused decrease in the degradation.

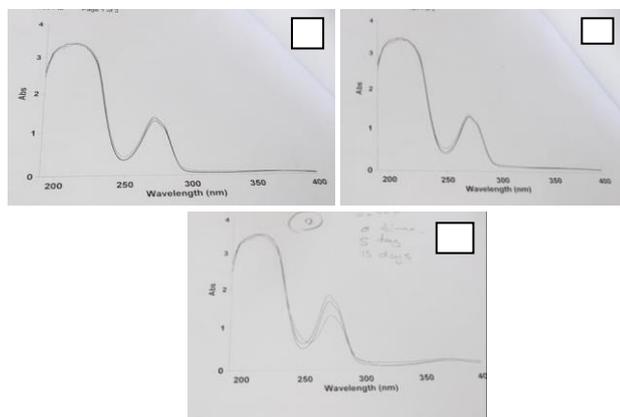


Figure 3. Effect of Temperature on bacterial degradation by *P.orizohibtanis* after 1, 5 and 15 days. (a) At 20C (b) 37C (c) 45C.

Bacterial viability on agar media

E.coli, *Proteus penneri* and *Pseudomonas orizohibtanis* were showed a capacity to growing in the presence of phenolic compounds in solid

medium, and *Pseudomonas orizohibtanis* showed high resistance to phenolic compounds Figure 4.

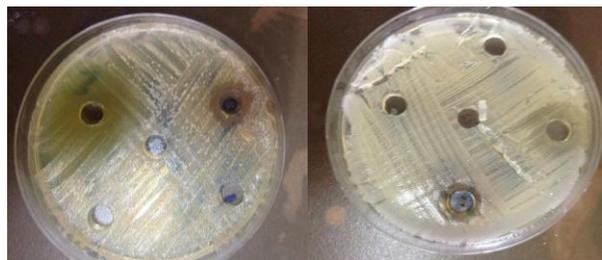


Figure 4. Viability test showed the resistant of degrading bacterial isolates to phenol on Nutrient agar for 24hrs. at 37°C.

Bacterial growth test

Growth test was performed on isolated bacteria (*E. coli*, *Proteus penneri* and *Pseudomonas orizohibtanis*) with Phenol compound as the sole carbon source as shown in Figure-5. *Pseudomonas orizohibtanis* exhibited the best growth rate compared to the other types of bacteria. With increasing incubation time, the culture grew almost linearly until 24 h., and then reached stationary phase.

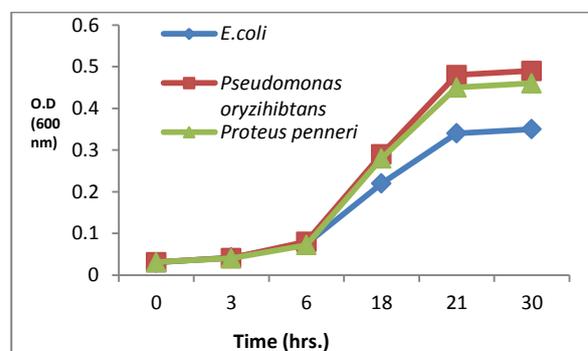


Figure 5. Growth rates of phenol degradation bacteria when incubated at different time intervals in Nutreint broth.

Bacterial degrading test

The main visible absorption peaks of *E.coli* appeared in Figure 6-a and O.D was 1.762, 3.907 at 276 and 222 nm respectively, while *Proteus penneri* in Figure 6-b showed o.d 2.03, 4.04 at 276 and 222 nm respectively, and O.D of *Pseudomonas orizohibtanis* in Figure 6-c was 2.794 , 4.936 at 276 and 222 nm respectively. They decreased after 1day and almost broken after 5 days, while it disappeared after 15 days at 37°C.

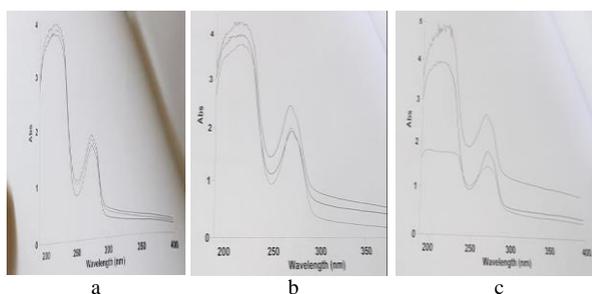


Figure 6. Degradation of Bisphenol A by a-*E.coli*, b-*Proteus penneri*, c- *Pseudomonas orizohibtanis*.

GCMS analysis

Figure 7 exhibited GC-MS analysis of BisPhenol A (BPA) before degradation, and it showed only one peak at 7.314 min, while Mass spectrum data showed BPA metabolites after degradation by tested bacteria, and its belong to hexasiloxane, heptasiloxane, and Octasiloxane, with retention time of 70, 210 and 280 nm respectively as shown in Figure 8.

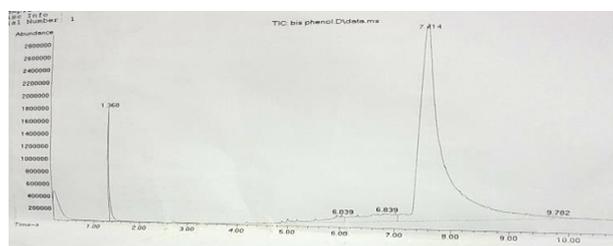


Figure 7. Standard spectrum of BisPhenol A, and showed one peak at 7.314 min.

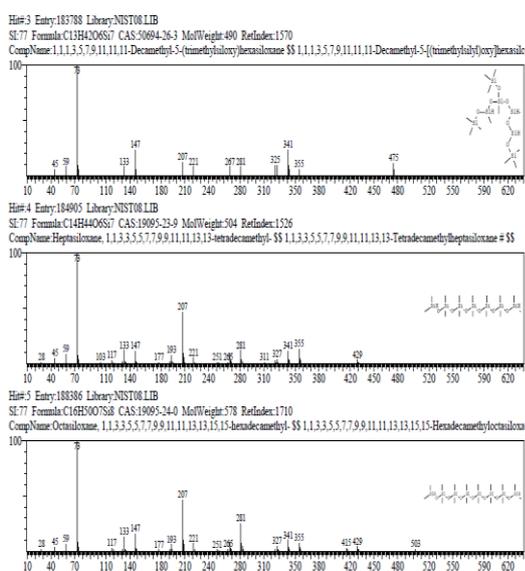


Figure 8. GC/MS spectra of BPA metabolites after degradation by *Pseudomonas orizohibtanis*, and its exhibited hexasiloxane, heptasiloxane, and Octasiloxane, with retention time of 70, 210 and 280 nm respectively.

DISCUSSION

BPA was rapidly biodegraded in river water and readily biodegraded in waste treatment using bacteria as the sole source of carbon and energy [4]. Nishio *et al.* 2002, showed twelve bacterial isolates from the soil samples with OPPEO-degrading activity. These isolates were identified as *Pseudomonas putida* (10 isolates) and *Burkholderia cepacia*. While Kang and Kondo, 2002, found that 10 out of 11 bacterial isolates isolated from river waters had BPA biodegradability.

Temperature is an important factor affecting the performance of the cells, regulating the influence on the rate of metabolism. The influence of temperature on enzyme activity could be rationalized since the rate of enzyme reaction increases with increase in temperature and the rate of movement of molecules is slower at lower temperatures than at higher temperature. The temperature might play an equivalent or larger role than nutrient availability in the degradation of organic pollutants [17, 18].

Bacteria capable of doing complete BPA degradation or mineralization were isolated from the contaminated river waters with high bacteria counts, but bacteria incapable of degrading BPA were found in the fresh river water with low bacteria counts [19].

Maulin, 2014 showed that the biomass of bacteria and phenol degradation reached the maximal values at a temperature of 30°C, and both values showed only a slight change when the temperature was increased to 35°C. On the contrary, the phenol degradation declined sharply when the temperature reached 40°C and over. These bacteria with high BPA biodegradability may be useful for the fast purification of the aquatic environment contaminated by BPA. Many bacteria capable of biodegrading BPA have identified from soils and river waters [21, 22].

An *Arthrobacter* sp. was degrade acetophenones and chloroacetophenones via the formation of a phenyl acetate by an acetophenone oxygenase and then cleavage to phenol and acetate [5]. Moreover, *Streptomyces* sp. isolated from river water has high BPA biodegradability [4]. Lakshmi, *et al.* 2014 showed that *Pseudomonas spp.* produced laccase enzyme and completely degraded the higher concentration of bisphenol A within 5 hrs.

Spivack, 1994 found that the metabolism routes of BPA by bacteria by using a gram-negative bacteria strain MV1 utilized BPA as the sole carbon and energy source. They suggested from total carbon analysis for BPA that 60% of the carbon are mineralized to CO₂, 20% are associated with the bacterial cells and 20% are converted to soluble organic compounds. On the other hand, [25] suggested that the cytochrome P450 system is involved in BPA metabolism from a test by using *Sphingomonas* sp.

Hirano, *et al.* 2011 found that BPA by MnP was metabolized to phenol, 4-isopropenylphenol, 4-isopropylphenol and hexestrol. The polymerization of BPA for forming oligomers is included in the step of BPA metabolism by laccase, followed by either the addition of phenol moieties or the degradation of the oligomers to release 4-isopropenylphenol [27, 28].

CONCLUSIONS

Contaminate the environment with hazardous and toxic chemicals is one of the major problems faced today. In order to identify bacteria from environmental sources that able to tolerate a high levels of Bisphenol A, samples of soil were collected. From these samples, different bacterial isolates with moderate to high levels of phenol resistance were isolated. *Pseudomonas orizohibtanis* and other isolated bacteria from the soil samples can be a promising Bisphenol A degrades. Hence, this species has remarkable potential for application in the bioremediation and wastewater treatment, especially in detoxification of phenolic wastes. The present study mainly focused on bacterial isolates for its dynamics on Bisphenol A degradation as a part of developing an innovative Microbial Technology for cheaper and effective treatment of Phenol degradation.

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