Detection of the cytochrome C gene in bisphenol A-degrading Bacteria isolated from contaminated soil in some areas of Karbala Province, Iraq

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Abstract

Bisphenol A (BPA) is determined as an organic material, with municipal and industrial wastewater being the primary sources of contamination with BPA in the environment. This study aimed to enhance understanding of BPA removal by investigating the biodegradation capacity of bacteria found in contaminated soil. Molecular detection of specific genes that may be responsible for biodegradation of BPA. So, 32 swabs were taken from polluted soils from different areas in Kerbala province/ Iraq. These isolates were subjected to examine their ability to degrade bisphenol by using MSM with BPA as the sole carbon source for bacterial growth. By using a specific primer for the Cytochrome C gene (bisAB operon), the detection of this gene was done, and the current study found that, out of 20 isolates that could grow on the media containing BPA as a sole carbon source15 isolates harbouring this gene that suggested degraded BPA. The current study concluded that using local isolates of bacteria isolated from polluted soils could effectively remediate BPA from the media containing it as only a carbon source. The operon of the bisAB gene is detected in most isolates, and this gene is suggested to be involved in BPA degradation.

Keywords: BPA, *bisAB*, operon, BPA-degrading bacteria, environment pollution, water pollution.

Introduction

Bisphenol A (BPA), also chemically denoted as 2,2-bis(hydroxyphenyl) propane, is a synthetically derived substance widely employed in various plastics production, notably resins of epoxy and polycarbonates; its worldwide production surpassed 5.0 million tons in the year 2010 [1]. Over several decades, there has been extensive and pervasive introduction of BPA into terrestrial soils, sedimentary deposits, and both

subterranean and surface aquatic environments [2]. Wastewater in municipal and industrial sectors represented the predominant sources of contamination with BPA within aquatic ecosystems [3]. Besides natural settings, confirmed exposure to BPA had been observed among both adults and juveniles, encompassing both human populations and various animal species [4]. Indeed, BPA had been identified in the serum, urine, tissue, and blood samples of individuals exposed to it either through occupational activities or environmental factors [5]. The pervasive distribution of plastics throughout the environment encompassed the atmosphere, soil, and water sources; this widespread presence raises concerns about the potential entry of microplastics into the food chain, thereby posing significant risks to both human and animal health [6]. So, BPA exhibited acute toxicity towards aquatic organisms [7]. BPA had been verified as an endocrine-disrupting compound (EDC). Its estrogenic effects were first observed by the researchers Dodds and Lawson, in 1936 and have since been confirmed by various studies [8]. Certainly, BPA impacts reproduction, growth, and the development of organisms by functions interfering with the different hormones of endocrine, specific pathways of intracellular signalling, and regulators epigenetic [9]. Recent findings also suggested that, exposure to BPA could trigger hepatotoxic, immune-toxic, mutagenic, and carcinogenic effects [10]. According to Son *et al.*, [11], BPA had a significant impact on the viability of keratinocytes, triggered apoptotic processes, and increased the DNA damage activity of marker proteins in both human and animal models.

Moreover, a growing number of bacteria with BPA-degrading ability had been identified from diverse environmental reservoirs, including water bodies, sediment, soil, and wastewater treatment facilities. These bacteria comprise both Gram-negative and Gram-positive strains [12]. The scientific literature presented several proposed pathways for BPA degradation, which were based on the intermediates that detected during the process of biodegradation of specific strains of bacteria [13].

To date, the predominant focus of BPA biodegradation research had focused on one strain organisms or consortia of bacteria, sourced from sedimentary or habitats of aquatic environment, with limited investigation into the biodegradative capacities of microbial populations inhabiting desert and arid locales [14]. The biodegradation of microorganisms on BPA had been demonstrated as a reliable and cost-effective method for BPA removal from different types of the environments [15].

Many putative genes studied as genes responsible of BPA-biodegradation; and most important of which was Cytochrome P450 (CYP) monooxygenase gene, which included in the biodegradation of BPA; in other words, researchers have discovered the involvement of the *bisdAB* operon, which encodes cytochrome P450 (*bisdAB*) and ferredoxin (*bisdFd*) components of the system of cytochrome C P450,

monooxygenase, in the breakdown of BPA [16]. While significant attention has been devoted to studying the degradation pathways of BPA, the understanding of the metabolic mechanisms involved, including the catalysts and genes implicated, remains constrained, as noted by [17].

Materials and Methods

A) Isolation of bacterial strains

1) 32 samples were taken from polluted soil with plastic wastes of different sites in Holy Kerbala province.

2) These samples were prepared and cultured on different media such as (MacConkey agar, blood agar, nutrient agar). For identification of these types of bacteria, the isolates identified by VITEK 2 System.

B) Preparation of Minimal salts media (MSM)

Minimal salts media (MSM) was employed to assess the bacterial isolates' capability to degrade BPA, consisting of different types of salts and minerals dissolved in 1000 ml of distilled water (DW); furthermore, sterilized by using autoclave equipment at 121ºC for 15 minutes in 15 bar. Subsequently cooled to 50ºC, BPA was added into the mixture as only a carbon source provided at a final Conc. of 200 mg/L. This medium was utilized for the detection of BPA-degrading activity [18].

C) High-Performance Liquid Chromatography (HPLC) analysis

Following the fourth-day incubation period at 37°C, one ml of each bacterial broth culture underwent centrifugation for 10 minutes at 12,000 rpm. The resultant supernatant was then filtered using a 0.22 µm Millipore filter to eliminate potentially insoluble components; the resulting filtrate was employed for the residual concentration and quantification of BPA [19].

Bisphenol A residual concentration within the culture media was determined using (HPLC) on a column of C18 type, employing (Shimadzu system/Japan). The mobile phase consisted of water and/or acetonitrile, with totally 25 minutes running time, and a flow rate set at one ml/min. Elution occurred at a rate of one ml/min, and detection was performed at an absorption wavelength of 220nm. The retention time for BPA was observed between 3.9 to 6 minutes [20].

D) Detection of *bisdAB* operon

Forward primer or F 5'GGAAGCTTGGCCTCCGCACAGC3', **reverse primer or R** 5'AGCTGCAGGCCTACCTCTGACTGC3'. PCR amplification was carried out as follows

Following a 5-minute denaturation at 94°C, two sets of amplification cycles were conducted the first set consisted of 20 cycles and the second of 10 cycles. For the first set (loop 1), each cycle involved denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 2 minutes, and a final incubation at 72°C for 10 minutes. The resulting amplicon size is approximately 3717 base pairs [18].

Results and Discussion

32 swabs were taken from polluted soils from different areas in Kerbala province/ Iraq. These isolates were subjected to examine their ability to degrade bisphenol by using MSM with BPA as the sole carbon source for bacterial growth. Table (1).

Table (1): Positive bacterial growth grown in Bisphenol-a-containing media

No. of isolates	Positive bacterial growth	Negative bacterial growth
32	20 (62%)	12 (38%)

After inoculation the bacterial isolates to the MSM with bisphenol A. Each isolate that could grow in the Bisphenol A-containing media was subjected to identification by VITEK 2 equipment. The results obtained are revealed in the following table. Out of 50 samples taken from different sites of polluted soils.

Table (2): The bacterial isolates that grow in Bisphenol a-containing media according to identification by VITEK 2

No.	**Bacterial isolates**	**Number of isolates**
1	*Serratia plymuthica*	**3**
2	*Pantoea spp*	**2**
3	*Shingomonas paucimobilis*	**3**
4	*Acinetobacter haemolyticus*	**1**
5	*Acinetobacter lwoffii*	**1**
6	*Pseudomonas aeruginosa*	**3**
7	*Escherichia coli*	**3**
8	*Bacillus spp.*	**1**
9	*Bacillus licheniformis*	**1**
10	*Brevibacillus borstelensis*	**2**
Total number		**20**

The results of the capacity of biodegradation of BPA of all isolates are illustrated in table (3), as compared to the control tube.

Table (3): The Percentage of area, height and biodegradation capacity of bacterial isolates according to HPLC results

No.	Bacteria	Area %	Height %	Biodegradation capacity
1	*Serratia plymuthica*	0.680	1.396	99.3%
2	*Pantoea spp*	2.068	1.457	97.9%
3	*Shingomonas paucimobilis*	6.131	5.237	93.8%
4	*Acinetobacter haemolyticus*	**67.868**	91.164	32.1%
5	*Acinetobacter lwoffii*	**74.160**	93.420	25.8%
	Pseudomonas aeruginosa	19.459	28.122	80.5%
6	*Escherichia coli*	**75.458**	77.999	24.5%
7	*Bacillus spp.*	6.178	5.883	93.8%
8	*Bacillus licheniformis*	**66.573**	90.122	33.4%
	Brevibacillus borstelensis	**60.352**	82.295	35.6%

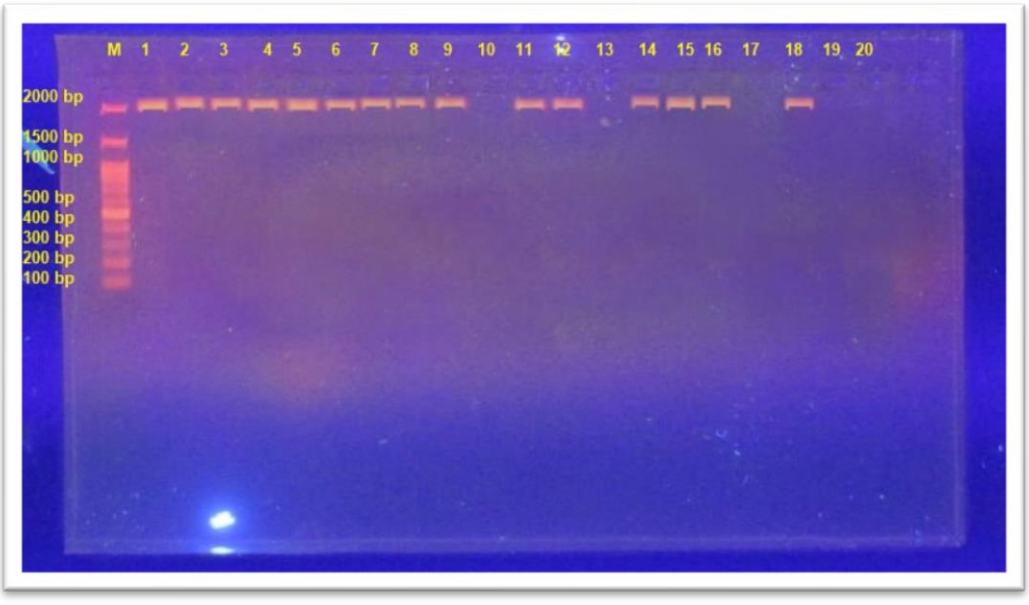

Figure (1): Gel electrophoresis of *bisd AB* operon, the isolates no. (1-9, 11-12, 14-16 and 18) had positive results, and isolates (10, 13, 19 and 20) had negative results.

Serratia plymuthica line 1-3 had positive results, *Pantoea spp* line 4-5 had positive results, *Shingomonas paucimobilis* line 6-8 had positive results, *Acinetobacter*

haemolyticus line 9 had positive results, *Pseudomonas aeruginosa* line 10 had negative results while line 11-12 had positive results, *Acinetobacter lwoffii* line 13 had negative results, *Bacillus spp.*line 14 had positive results, *Escherichia coli* line 15-16 had positive results while line 17 had negative results, Bacillus licheniformis line 18 had positive results and finally *Brevibacillus borstelensis* line 19-20 had negative results.

Bisphenol A (BPA), is an organic chemical substance with industrial significance that serves as a crucial raw material in the synthesis of food containers, polycarbonates, the industry of thermal paper, epoxy resins, and also, various other products; the extensive utilization of these chemical compounds results in substantial amounts of BPA being released directly into terrestrial and aquatic environments, posing a severe toxicity threat to numerous types of organisms [21].

Vijayalakshmi *et al.* reported that the bacteria *Pseudomonas aeruginosa* displayed the ability to grow in a nutritional broth medium with different amounts of BPA, spanning from 5 mM to 35 mM. [22]. The analytical study conducted by Yue et al. revealed that, while Sphingomonas spp. exhibited the capability to fully degrade BPA, the process was deemed inefficient because intermediates tended to accumulate; in contrast, Pseudomonas sp. demonstrated more rapid utilization of these intermediates, thereby facilitating the overall mineralization of BPA within the microbial community; this observation cleared the intricate dynamics of microbial interactions and the significance [23]. Furthermore, Bacillus spp. had the capability of utilizing BPA as the only carbon source; isolated from the creek sediment located within recycling electronic-waste sites, these bacteria demonstrated remarkable efficiency by completely removing 100 percent of 5 mg/L BPA under optimal aerobic conditions; this underscores the efficiency of Bacillus spp. in serving as effective agents for BPA biodegradation, especially in environments associated with electronic-waste conversion [24].

The *bisdAB* operon from *Sphingomonas spp*. was cloned into the bacteria *E. gergoviae*, and the impact of its expression and degradation activity of BPA had been investigated; so, strains carrying the *bisdAB* operon were successfully isolated on media with 25 µg/mL BPA as the only carbon source, confirming the presence of the *bisdAB* operon and its role in BPA degradation activity [25]. According to Sasaki *et al.*, expressing the *bisdAB* genes in various strains of *E. coli* grown on BPA-containing medium increased the degradation activity of BPA, from 10 mg/L to 30-90 mg/L within 18 hours; these engineered strain of *E. coli* exclusively converted BPA into 1,2-bis,4-hydroxyphenyl-2-propanol (byproduct1), a cytochrome P450 monooxygenase activity product; additionally, another product of BPA degradation by these modified cells was detected as byproduct II, which was not previously observed in degradation pathway of BPA in these strains [26]. The proposed degradation pathway of BPA outlined

specific enzymes as well as, encoded genes; further verification solidified the cytochrome C P450 (CYP450) role, in degradation of BPA; a notable reduction in BPA degradation was observed in the presence of a CYP450 inhibitor; subsequently, a CYP450 *bisdAB*-deficient strain exhibited a loss in its ability to transform BPA compared to the wild type strain; moreover, introducing *bisdAB* into *E. coli* enabled the degradation of 66 mg/L of BPA within 24 hours; these findings underscore the importance of CYP450 in the BPA biodegradation [25].

Bacteria carrying the *bisdAB* gene had been reported to acquire the capability to degrade BPA; conversely, the *bisdAB* gene knockout strain had no ability to remediate BPA, highlighting the crucial role of P450bisdB for BPA metabolism as an essential initiator in bacterial strains; therefore, for remediation BPA-polluted soil, strains containing this operon significantly enhanced the biodegradation of BPA in conjunction with the soil microbial community; these findings suggested that, such strains hold promise as effective microbes for BPA removal, showcasing significant application potential [16].

Some bacterial isolates yielded negative results for the *bisdAB* gene, indicating a potential absence of this specific metabolic pathway for BPA degradation in those bacteria; this observation suggested the possibility of an alternative BPA metabolic pathway existing in these bacterial strains.

Ethical Approval

The research was carried out according to ethical principles rooted in the Helsinki Declaration. The protocol of this study, along with the subject information and consent form, underwent scrutiny and received approval from a local committee ethics identified by the reference number (UOK. VET. HE.2023.067).

Bisphenol A (BPA) is a harmful chemical compound. Local isolates of bacteria isolated from polluted soils could effectively remediate BPA from the media containing it as the only carbon source. The operon of the bisAB gene was detected in most isolates, and this gene is suggested to be involved in BPA degradation. Therefore, we recommend the following:

a) Examine the bacteria's biodegradation activity at different concentrations of BPA and different concentrations of biomass to detect the most appropriate one.

b) Identify other genes that may be included in BPA-degradation activity

c) Manipulate the Cytochrome C gene (bisAB) in bacteria lacking it and detect its ability to remediate BPA.

d) Extract the enzyme cytochrome C dehydrogenase and apply it in BPA degradation.

References

1.Huang, Y. Q., Wong, C. K. C., Zheng, J. S., Bouwman, H., Barra, R., Wahlström, B., Neretin, L., & Wong, M. H. (2012). Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts. *Environment International*, *42*, 91-99. https://doi.org/10.1016/j.envint.2011.04.010

2. Careghini, A., Mastorgio, A. F., Saponaro, S., & Sezenna, E. (2015). Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: A review. *Environmental Science and Pollution Research International*, *22*(8), 5711-5741. https://doi.org/10.1007/s11356-014-3974-5

3.Mishra, A., Goel, D., & Shankar, S. (2023). Bisphenol A contamination in aquatic environments: A review of sources, environmental concerns, and microbial remediation. *Environmental Monitoring and Assessment*, *195*(11). https://doi.org/10.1007/s10661-023-11977-1

4. Lehmler, H., Liu, B., Gadogbe, M., & Bao, W. (2018). Exposure to Bisphenol A, Bisphenol F, and Bisphenol S in U.S. Adults and Children: The National Health and Nutrition Examination Survey 2013-2014. *ACS Omega*, *3*(6), 6523-6532. https://doi.org/10.1021/acsomega.8b00824

5.Ribeiro, E., Ladeira, C., & Viegas, S. (2017). Occupational Exposure to Bisphenol A (BPA): A Reality That Still Needs to Be Unveiled. *Toxics*, *5*(3), 22. https://doi.org/10.3390/toxics5030022

6.Dueñas-Moreno, J., Mora, A., Kumar, M., Meng, X., & Mahlknecht, J. (2023). Worldwide risk assessment of phthalates and bisphenol A in humans: The need for updating guidelines. *Environment International*, *181*, 108294. https://doi.org/10.1016/j.envint.2023.108294

7.Canesi, L., & Fabbri, E. (2015). Environmental Effects of BPA: Focus on Aquatic Species. *Dose-Response: An International Journal*, 1-14. https://doi.org/10.1177/1559325815598304

8. Faheem, M., & Bhandari, R. K. (2021). Detrimental Effects of Bisphenol Compounds on Physiology and Reproduction in Fish: A Literature Review. *Environmental Toxicology and Pharmacology*, *81*, 103497. https://doi.org/10.1016/j.etap.2020.10349

9. Booth, D. B., Scholz, J. G., Beechie, T. J., & Ralph, S. C. (2016). Integrating Limiting-Factors Analysis with Process-Based Restoration to Improve Recovery of Endangered Salmonids in the Pacific Northwest, USA. *Water*, *8*, 174. https://doi.org/10.3390/w8050174

10.Michałowicz, J. (2014). Bisphenol A--sources, toxicity and biotransformation. *Environmental Toxicology and Pharmacology*, *37*(2), 738-758. https://doi.org/10.1016/j.etap.2014.02.003

11.Son, S., Nam, K., Kim, H., Gye, M. C., & Shin, I. (2018). Cytotoxicity measurement of Bisphenol A (BPA) and its substitutes using human keratinocytes. *Environmental Research*, *164*, 655-659. https://doi.org/10.1016/j.envres.2018.03.043

12. Das, R., Li, G., Mai, B., & An, T. (2018). Spore cells from BPA degrading bacteria Bacillus sp. GZB displaying high laccase activity and stability for BPA degradation.

Science of The Total Environment, 640–641, 798-806. https://doi.org/10.1016/j.scitotenv.2018.05.379

13.Vijayalakshmi, V., Senthilkumar, P., Mophin-Kani, K., Sivamani, S., Sivarajasekar, N., & Vasantharaj, S. (2018). Bio-degradation of Bisphenol A by Pseudomonas aeruginosa PAb1 isolated from effluent of thermal paper industry: Kinetic modeling and process optimization. *Journal of Radiation Research and Applied Sciences, 11*(1), 56-65. https://doi.org/10.1016/j.jrras.2017.08.003

14.Moussavi, G., & Haddad, F. A. (2019). Bacterial peroxidase-mediated enhanced biodegradation and mineralization of bisphenol A in a batch bioreactor. *Chemosphere, 222*, 549-555. https://doi.org/10.1016/j.chemosphere.2019.01.190

15.Fu, M., Xing, J., & Ge, Z. (2019). Preparation of laccase-loaded magnetic nanoflowers and their recycling for efficient degradation of bisphenol A. *Science of The Total Environment, 65*(2), 2857-2865. https://doi.org/10.1016/j.scitotenv.2018.10.145

16. Jia, J., Liu, D., Tian, J., Wang, W., Ni, J., & Wang, X. (2020). Visible-light-excited humic acid for peroxymonosulfate activation to degrade bisphenol A. *Chemical Engineering Journal, 400*, 125853. https://doi.org/10.1016/j.cej.2020.125853

17. Mtibaà, R., Olicón-Hernández, D. R., Pozo, C., & Nasri, M. (2018). Degradation of bisphenol A and acute toxicity reduction by different thermo-tolerant ascomycete strains isolated from arid soils. *Ecotoxicology and Environmental Safety, 156*. https://doi.org/10.1016/j.ecoenv.2018.02.077

18. Badiefar, L., Yakhchali, B., Rodriguez-Couto, S., Veloso, A., Garcia-Arenzana, J. M., Yoshinobu Matsumura, F. Y., & Khodabandeh, M. (2015). Biodegradation of bisphenol A by the newly isolated Enterobacter gergoviae strain BYK-7 enhanced using genetic manipulation. *RSC Advances, 5*, 29563–29572. https://doi.org/10.1039/C5RA01818H

19. Louati, I., Dammak, M., Nasri, R., Belbahri, L., Nasri, M., Abdelkafi, S., & Mechichi, T. (2019). Biodegradation and detoxification of bisphenol A by bacteria isolated from desert soils. *3 Biotech, 9*, 228.

20.Jia, Y., Eltoukhy, A., Wang, J., Li, X., Hlaing, T. S., Aung, M. M., New, M. T., Lamraoui, I., & Yan, Y. (2020). Biodegradation of Bisphenol A by Sphingobium sp. YC-JY1 and the Essential Role of Cytochrome P450 Monooxygenase. *International Journal of Molecular Sciences, 21*, 3588. https://doi.org/10.3390/ijms21103588

21.Inoue, D., Hara, S., Kahinhara, M., Murai, Y., Danzyl, E., Sei, K., et al. (2008). Degradation of Bis (4-hydroxyphenyl) methane Bisphenol F by Sphingobium yaboikuyae strain FM2 isolated from river. *Water and Applied Environmental Microbiology, 74*(2), 352-358.

22.Vijayalakshmi, V., Senthilkumar, P., Mophin-Kani, K., Sivamani, S., Sivarajasekar, N., & Vasantharaj, S. (2018). Bio-degradation of Bisphenol A by Pseudomonas aeruginosa PAb1 isolated from effluent of thermal paper industry: Kinetic modeling and process optimization. *Journal of Radiation Research and Applied Sciences, 11*, 56-65.

23.Yue, W., Yin, C., Sun, L., Zhang, J., Xu, Y., & Zhou, N. (2021). Biodegradation of bisphenol-A polycarbonate plastic by Pseudoxanthomonas sp. strain NyZ600. *Journal of Hazardous Materials, 416*, 125775. https://doi.org/10.1016/j.jhazmat.2021.125775

24. Zhang, W., Yin, K., & Chen, L. (2013). Bacteria-mediated bisphenol A degradation. *Applied Microbiology and Biotechnology, 97*(14), 5681-5689. https://doi.org/10.1007/s00253-013-4949-z

25. Eltoukhy, A., Jia, Y., Lamraoui, I., Abo Kadoum, M. A., Atta, O. M., Nahurira, R., Wang, J., & Yan, Y. (2022). Transcriptome analysis and cytochrome P450 monooxygenase reveal the molecular mechanism of Bisphenol A degradation by Pseudomonas putida strain YC-AE1. *BMC Microbiology, 22*, 294. https://doi.org/10.1186/s12866-022-02689-6

26. Sasaki, M., Maki, J., Oshiman, K., Matsumura, Y., & Tsuchido, T. (2005). Biodegradation of bisphenol A by cells and cell lysate from Sphingomonas sp. strain AO1. *Biodegradation, 16*, 449–459.