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ORIGINAL ARTICLE

Isolation and characterization of a bisphenol A-degrading strain, *Pseudomonas aeruginosa* DU2, from soil containing decaying plants

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ABSTRACT

Background and Objectives: Bisphenol A (BPA) is a toxic compound with broad applications in the plastics industry. BPA has harmful effects on various organisms and its efficient removal is necessary. The microbial degradation of BPA is a safe and economical approach. In this research, soil samples containing decaying plants were screened to isolate a BPA-degradable bacterial strain.

Materials and Methods: Soil samples were collected from different locations in Damghan, Semnan province, Iran. To enrich BPA-degrading bacteria, the samples were cultured in a stepwise manner in a mineral medium containing increasing BPA concentrations (5 to 40 mg/L). The ability of isolated bacteria in degrading BPA was assayed by Folin-Ciocalteu and high-performance liquid chromatography methods. The biodegradation efficiency of the most efficient isolate was assayed under distinct conditions and it was identified through the sequencing of the 16S rRNA gene.

Results: Among the isolated bacteria, *Pseudomonas aeruginosa* DU2 (GenBank accession number: OP919484) showed the most BPA biodegradation ability. The highest BPA degradation (52.98%) was observed in the mineral medium containing 5 mg/L BPA and the inoculum size of 6×10^7 CFU/mL at pH 9 and in the presence of 0.05% (w/v) NaCl during 10 days.

Conclusion: These results offer soil containing decaying plants as a promising source for finding BPA-degrading bacteria. *P. aeruginosa* DU2 has basal BPA removal ability, which could be improved by optimization of medium components and growth conditions.

Keywords: Biodegradation; Bisphenol A; *Pseudomonas*; Soil

INTRODUCTION

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl) propane) is a synthetic chemical, which is broadly used in plastic and epoxy resin industries. Industrial wastewaters are the primary source of environmental BPA contamination (1-4). BPA is a toxic and endocrine disruptor compound that interferes with hormonal activity (4-6). The ingestion and inhalation of BPA through environmental exposure, and also dermal contact with products containing BPA are hazardous to human health (1, 5). Harmful effects of BPA on male and female reproductive organs and fertility, bone strength, skin, mental behavior, liver, cardiovascular function, and epigenome have been confirmed in human cases (7, 8). Also, several cancers can be related to BPA exposure (9). BPA endangers the life of aquatic species (10), and accumulation

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of BPA in their edible tissue threats human health (11). According to the prediction, BPA production will be increased during 2022-2027 (12). Moreover, the high concentration of BPA (14-1390 ng/L) in the surface water of Asian regions in recent decades is worrying (13). So, the removal of BPA from different ecosystems is necessary and has been considered by researchers (12). Photodegradation, oxidation, photo-electrocatalytic oxidation, and biodegradation methods can be applied for BPA degradation (1, 14), but the biodegradation method has more advantageous than the other techniques for BPA elimination from the environment (1, 15). Plants, algae, fungi, and bacteria are biocatalysts that are used for this purpose (3, 14). Bioremediation by microorganisms is an eco-friendly, inexpensive, and efficient approach, which does not produce organic pollutants (13).

Most of the isolated bacteria with BPA degradation capability belong to *Pseudomonas, Sphingomonas,* and *Bacillus* genera (12). Several BPA-degrading bacteria isolated from various sources were reported previously. Badiefar et al. (16) isolated *Enterobacter gergoviae* BYK-7 from petrochemical wastewater, which removed around 23 mg/L BPA in a basal medium within 8 h. *Bacillus megaterium* ISO-2 is another strain identified from the wastewater of the polycarbonate industry. This bacterium removed 5 mg/L BPA within 72 h (2). *Pseudomonas putida* YC-AE1, isolated from polluted soil in China, completely degraded 500 mg/L BPA within 72 h (6). Moreover, there are some studies showed that bacteria from unpolluted environments can degrade BPA. For example, *Streptomyces* sp. (17), *Sphingobium* sp. YC-JY1 (18), and *Pseudomonas palleroniana* GBPI_508 (19) with the bioremediation activity of BPA isolated from river water of Japan, a sludge sample of a river in China, and the high altitudes of Indian Himalayan region, respectively. *Streptomyces* sp. degraded > 90% of BPA (200 mg/L) for 10 days (17). *Sphingobium* sp. YC-JY1 removed 97.1% of BPA (100 mg/L) after 9 h (18), and *Pseudomonas palleroniana* GBPI_508 had 97% biodegradation ability at 270 mg/L BPA within 96 h (19).

Despite the identification of several BPA-degrading bacteria from various sources, more studies should be done to isolate and identify autochthonous isolates from different ecosystems. These studies can demonstrate the wide distribution of BPA-degrading microorganisms. Since there are diverse phenolic compounds in plants, bacteria with the ability to degrade them can be found in soils containing decaying plants (20). In this regard, the main novelty of the current study was isolation and identification a BPA-degrading bacterium from soil samples containing decaying plants with no history of contamination with petroleum derivatives. The current study found a new *P. aeruginosa* strain DU2 with 52.98% BPA biodegradation ability at alkaline pH within 10 days, and its capability in BPA removal was examined under distinct conditions.

MATERIALS AND METHODS

Chemicals and growth medium. Bisphenol A (> 99% purity) was purchased from Sigma-Aldrich (St. Louis, United States). Mueller-Hinton (MH) broth and MH agar were provided by Ibresco (Zist Kavosh Iranian Company, Iran). Yeast extract was bought from Ghatran Shimi (Tehran, Iran). Methanol and acetonitrile (HPLC grade) were provided by Mojallali (Tehran, Iran). Other chemicals were purchased from Merck KGaA (Darmstadt, Germany).

Mineral salts medium (MSM) containing BPA (5, 10, 20, and 40 mg/L) was applied for the enrichment and isolation of BPA-degrading bacteria. The composition of MSM was as follows (g/L distilled water): KH_2PO_4 (0.3), K_2HPO_4 (0.3), NaCl (0.5), KCl (0.1), $CaCl_2$ (0.1), $MgSO_4.7H_2O$ (0.1), NH_4Cl (0.1), $CuSO_4$, H_2O (0.038), $ZnCl_2$ (0.115), $FeCl_3$ (0.01), and yeast extract (1.0) (2, 3). MH medium was used for bacterial cultivation. The pH of the media was adjusted to 7.0, and they were autoclaved at 121°C for 15 min.

Sample collection and isolation of BPA-degrading bacteria. Four soil samples were collected from different parts of Damghan (Semnan Province, Iran). The selected locations contained the remains of decaying plants. Samples were taken from a depth of 1 to 10 cm of the land and brought immediately to the laboratory by sterile falcon tubes. The pH of soil samples was determined. To isolate the most potent bacteria, 1.0 g of each sample was separately suspended in 100 mL of MSM supplemented by 5 mg/L BPA as the sole carbon source. The inoculated media were incubated at 30°C and 150 rpm shaking in a dark condition (AQUA LYTIC, AL 186, Germany). After appropriate incubation time (3-5 days) and the presence of noticeable turbidity in the medium, 1% (v/v) of the culture was inoculated in an MSM containing

10 mg/L BPA under the mentioned incubation conditions. Bacterial culture in MSM with higher BPA concentration (20 and 40 mg/L) was continued in the same way. Finally, 20 µL of the suspension of MSM containing 40 mg/L BPA was cultured on MH agar (at 30°C) by the streak method to isolate various bacterial colonies.

The morphologically distinct bacterial isolates were cultured overnight in MH broth and stored at -80°C with a final concentration of 40% (v/v) glycerol. Subsequently, the isolates were pre-cultured in MH broth at 30°C for 18 h, and bacterial cells were harvested by centrifugation (5000 rpm, 5 min) (Eppendorf 5415 R, Hamburg, Germany). After twice washing with fresh MSM, bacterial cells were resuspended in MSM to reach an absorbance of 0.08-0.1 at 625 nm (equivalent to 1.5×10^8 CFU/mL) (Biotek, PowerWave XS microplate reader, USA). Finally, bacterial cells were cultured in MSM containing 20 mg/L BPA with an inoculum size of 1.5×10^7 CFU/mL for 10 days (150 rpm shaking at 30°C in a dark condition). In the primary screening, the residual of BPA in the supernatants was determined using the Folin–Ciocalteu (F-C) method (21). For the assay, 50 µL of the supernatants were mixed with 25 µL of F-C reagent in a 96-well plate and incubated at room temperature for 4 min. Then, 125 µL of 20% Na_2CO_3 (w/v) was added to each sample and after being in a dark place for 60 min, the absorbance was measured at 750 nm. The best samples were selected for high-performance liquid chromatography (HPLC) analysis. A non-inoculated medium was used also as an abiotic control.

HPLC analysis. The concentration of BPA in the supernatant from the experiments was determined with application of an HPLC (DanChrom, Kianshar Danesh, Iran). The stationary phase was a revers phase analytical column (Reprospher 100 C18, 4.6 × 150 mm, 5 µm) in an auto adjusted oven at the 30°C. The mobile phase was a mixture of methanol, acetonitrile, and water (20:60:20, (v/v) %) with a flow rate of 1.0 mL/min during the 5 min for each examination time. Twenty microliter of sample was injected and BPA was detected with a UV-Visible detector at 285 nm (22). The peak area was used for the quantitative calculation of BPA using a multi-point calibration curve of BPA. Blank and control samples were applied for each set of the samples. The limit of detection (LOD), the limit of quantification (LOQ), standard error (SE), and root-mean-square deviation (RMSD) were obtained from the calibration curve. The calibration curve of BPA displayed appropriate linearity ($y= 4.2259x + 25.339$) with a coefficient of determination (R^2) of 0.99. LOD and LOQ were calculated based on the standard error (SE) and the slope (m) of the calibration curve ($LOD = 3.3 \times SE/m$, $LOQ = 10 \times SE/m$) (23). From the calibration curve, LOD and LOQ were 1.26 and 3.78 mg/L, respectively. The results for SE and RMSD values were 2.17 and 2.27 mg/L, respectively.

Factors affecting BPA biodegradation. The effect of BPA concentration on biodegradation was studied in an MSM (pH 7) containing 5, 10, and 20 mg/L BPA. BPA biodegradation was also examined at different inoculum sizes (1.5×10^7, 3×10^7, 6×10^7 CFU/mL) of the most efficient strain in MSM (pH 7) with 5 mg/L BPA concentration. To find the best pH for effective BPA biodegradation, MSM with a pH ranging from 6 to 9 was prepared. The influence of NaCl concentration on BPA biodegradation efficiency was assayed in MSM (pH 7) containing different NaCl concentrations (0, 0.05, 0.1, and 0.2%, w/v). The inoculum size of 6×10^7 CFU/mL and 5 mg/L BPA concentration were used in these experiments. All the assays were performed at 30°C and 150 rpm shaking in a dark incubator, and the remaining BPA in media was detected by HPLC analysis after 10 days.

Effect of BPA on bacterial growth. The growth of the bacterial strain was monitored in MSM (pH 7) with a final concentration of 5 mg/L BPA and 6×10^7 CFU/mL inoculum size by reading absorbance at 600 nm every day over a period of 10 days. The bacterial culture was placed in a dark incubator at 30°C and 150 rpm shaking for 10 days.

Identification of bacterial strain. More over Gram staining and microscopic observation, the strain was molecularly identified by 16S rRNA gene sequencing. Genomic DNA was extracted by using Pouya Gene Azma kit (Iran) according to the manufacturer's instructions. The PCR reaction in a final volume of 25 µL contained: 0.5 µL (250 ng DNA) of the DNA template, 1 µL (10 pmol) from fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC) primers (24), 1 µL dNTP Mix (10 mM; SinaClon, Iran), 0.8 µL Taq DNA polymerase (10 U/µL) (SinaClon, Iran), 2.5 µL 10 × Buffer, and 0.75 µL $MgCl_2$ (50 mM). The thermal cycling program was as fol-

lows: initial denaturation of 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 51°C for 30 s and 72°C for 1 min, with a final elongation of 72°C for 10 min. PCR product, which was around 1500 bp, was purified using Dena Zist Asia gel extraction kit (Iran), and cloned in pUCM-T vector (Bio Basic, Canada) according to the manufacturer's protocols. Sanger sequencing was performed in two directions using M13 primers, and the assembled sequence was BLAST against NCBI 16S rRNA database. The most similar sequences were used to construct a phylogenetic tree by the neighbor-joining method with a bootstrap value of 500 (MEGA 11) (25).

Statistical analysis. The SPSS (v.16) and Minitab (v.21.4) were applied to perform the statistical analysis in this research. The normal distribution analysis of Kolmogorov-Smirnov was also carried out and the further parametric or non-parametric analyses were based on the results of the normality test. Pearson and Spearman's rho correlation coefficient were applied to determine the significant effects of the factors on degradation of BPA. Mann-Whitney U test and Kruskal-Wallis H test were used to analyze the data between the groups. All the analyses were in the confidence interval of 95% and the error value of 5%.

RESULTS

Isolation of BPA-degrading strain from soil. The soil samples had a pH range of 7.8-8. Among eight bacteria isolated from MSM with the highest BPA concentration (40 mg/L), three isolates showed better BPA biodegradation activity by primary screening with F-C assay (Table 1). These isolates were designated as DU1 to DU3. HPLC analysis indicated that these strains respectively had 14.60, 35.00, and 9.39% biodegradation activity in MSM containing 20 mg/L BPA as the sole carbon source within 10 days. So, strain DU2 was selected as a candidate for further study. This bacterial strain was isolated from the soil sample at the following GPS location: 36°10'04.3" N 54°19'23.5" E.

Table 1. Biodegradation of BPA by Folin–Ciocalteu method.

	Bacterial strains							
	DU1	**DU2**	**DU3**	**DU4**	**DU5**	**DU6**	**DU7**	**DU8**
BPA degradation (%)	18.00	35.00	19.10	6.74	9.34	6.41	9.61	1.92

Effect of BPA concentration, inoculum size, pH, and NaCl on BPA biodegradation. The initial BPA concentration and inoculum size of the bacterium affect BPA biodegradation. By increasing BPA concentration, the biodegradation process was decreased (Fig. 1A). There was a significant negative correlation between the concentration of BPA and the degradation rate (Pearson's r = -0.81, P-value < 0.05). The maximum BPA removal (48.71%) was observed after 10 days of culture of strain DU2 (6×10^7 CFU/mL) in MSM containing 5 mg/L BPA. Fig. 1B shows the influence of inoculum size on the degradation efficiencies of BPA. The results showed that by increasing the initial bacterial inoculum size from 1.5×10^7 to 6×10^7 CFU/mL, the BPA degradation efficiencies were enhanced from 41.56 to 50.71% after 10 days. There was not a significant correlation between the inoculum size and degradation rate of the BPA (Spearman's rho = 0.6, P-value > 0.05) and there was no significant difference between the groups of inoculum size for degradation of BPA (P-value > 0.05). Since the greater degradation of BPA was seen at a concentration of 5 mg/L BPA and inoculum size of 6×10^7 CFU/mL, these conditions were selected for the remaining assays.

BPA degradation efficiency was studied in the presence of NaCl concentration from 0 to 0.2% (w/v). Maximum BPA biodegradation (49.30%) was obtained in the absence of NaCl in the medium within 10 days (Fig. 1C). There was not a significant correlation between the concentration of NaCl and degradation rate of the BPA (Spearman's rho = - 0.5, P-value > 0.05). While the results showed a fair decline of BPA degradation in NaCl of 0.1%, the comparison between the groups showed it was not significantly different between the NaCl of 0.1 and 0.05% nor 0.2% (P-value > 0.05). There was a strong correlation between the pH and degradation rate of the BPA (Spearman's rho = 0.78, P-value < 0.05) (Fig. 1D). The lowest removal level of BPA (30.59%) was found at pH 6.0. By increasing the pH of the medium, BPA degradation was significantly enhanced (P-value < 0.05). The optimum pH value for BPA removal by strain DU2 was 9.0 with 52.98% degradation efficiency.

Growth of strain DU2 in the presence of BPA. The growth curve of strain DU2 in the MSM con-

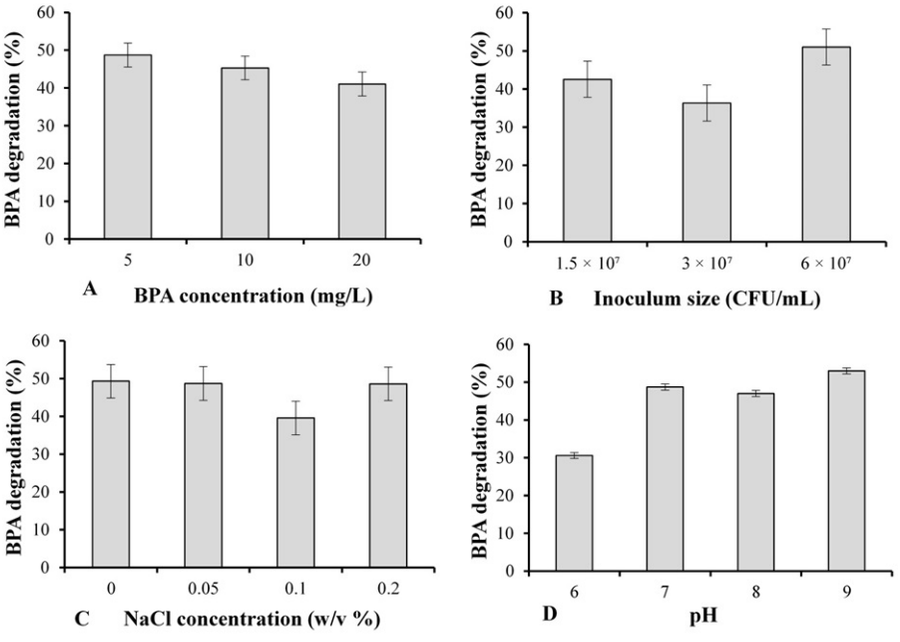

Fig. 1. Effect of BPA concentration (A); bacterial inoculum size (B); NaCl concentration (C); and pH value (D) on BPA removal by strain DU2 after 10 days of incubation in a dark incubator at 30°C and 150 rpm shaking.

taining 5 mg/L BPA as a sole carbon source within 10 days was shown in Fig. 2. No clear lag phase was detected in the growth of strain DU2, and the maximum growth of strain DU2 was observed on the first and second days. Cell growth was decreased after two days of incubation, and this trend of reduction continued up to the end of incubation time.

Identification of bacterial strain. The Gram staining showed that strain DU2 is Gram-negative bacterium and has rod-shaped morphology. Furthermore, the colony of the strain indicated a blue-green color and mucoid colony with a fruity odor, which is typically produced by *P. aeruginosa*. According to the BLAST analysis, the most similar sequences (≥ 98%) to the 16S rRNA sequence of the bacterial isolate belong to *P. aeruginosa*. The obtained 16S rRNA sequence (1528 bp) was submitted to NCBI under the accession number OP919484. According to these results, the strain was identified as *Pseudomonas aeruginosa* DU2, and it was clustered with other *P. aeruginosa* strains in a phylogenetic tree (Fig. 3).

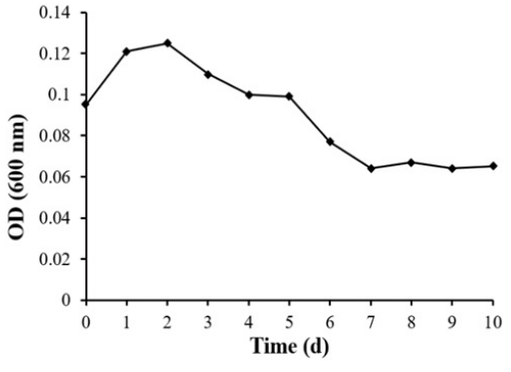

Fig. 2. Cell growth of strain DU2 in MSM containing 5 mg/L BPA under dark incubation at 30°C and 150 rpm shaking.

DISCUSSION

Environmental BPA contamination due to its hazardous properties for humans and animals is taken into consideration in recent years. The researches show the potential of various bacteria in the biological degradation of BPA (2, 6, 16, 18). In this study, we succeeded to isolate and identify the BPA-degrading bacterium, *P. aeruginosa* DU2, from soil containing decaying plants through stepwise culture in a medium with increasing concentrations of BPA. It seems that the long-time exposure of soil bacteria to phenolic compounds released from decaying plants develops their ability to degrade aromatic xenobiotic molecules. *Pseudomonas* genus has been widely identified as a potent bacterium in degrading aromatic compounds (26), and several BPA-degrading *Pseudomonas* strains reported previously (3, 6, 19, 27, 28).

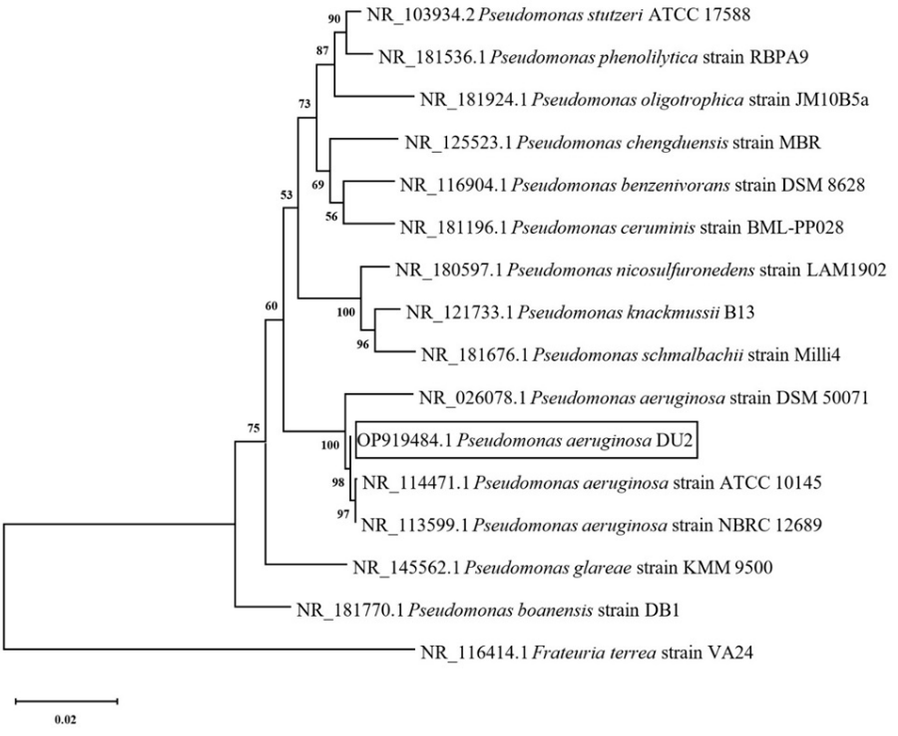

Fig. 3. Neighbor-joining tree based on the 16S rRNA sequences of *P. aeruginosa* DU2 with closely related bacteria. The scale bar indicates 0.02 changes per nucleotide.

The HPLC analysis is more accurate than the colorimetric F-C assay, and the effect of various parameters on the biodegradation of BPA was measured by HPLC. Initial BPA concentration as a substrate has a significant impact on bacterial growth and BPA metabolism (2). Generally, BPA degradation efficiency by bacteria depends on their resistance to grow in the presence of BPA, their cell permeability to BPA, and their enzymatic profile involved in BPA metabolism (27). Besides the toxicity of BPA, some toxic intermediates may be produced during BPA degradation that adversely influences bacterial growth and degradation process (4). According to these facts, BPA biodegradation efficiency decrease at higher BPA concentrations (2). Similar results were seen in the current research, and the biodegradation of *P. aeruginosa* DU2 showed a significant negative correlation (P-value < 0.05) with increasing BPA concentration. According to the report of Suyamud et al. (2), *Bacillus megaterium* strain ISO-2 in the presence of 20 mg/L BPA lost around 33% of its potency in BPA removal in comparison to the medium containing 5 mg/L BPA after 72 h. The biodegradation activity of *P. aeruginosa* DU2 in the MSM containing 20 mg/L BPA reduced around 17% compared to the medium with 5 mg/L BPA. The low raise of bacterial cell density after 2 days in the presence of BPA as a sole carbon source also indicated the inhibitory effect of BPA on the growth of *P. aeruginosa* DU2.

The partial biodegradation of BPA was previously described. *Ralstonia eutropha* only degraded 10% of 20 mg/L BPA after 10 days in MSM (29). The degradation rate of *Acinetobacter* sp. K1MN and *Pseudomonas* sp. BG12 in a medium containing 100 mg/L BPA was 20% and 36% within 15 days, respectively (4). Disability of complete removal of BPA was similarly found in our study, and *P. aeruginosa* DU2 degrade 48.71% of 5 mg/L BPA within 10 days. This incomplete degradation can be related to the inhibitory impact of BPA on bacterial growth or enzymatic activity (29). Some studies exhibited that adding supplementations such as petrochemical wastewater (16) or other carbon sources in the mineral medium containing BPA can improve the biodegradation ability of bacteria (4, 27, 29).

By raising the initial number of *P. aeruginosa* DU2 in the culture medium from 1.5×10^7 to 6×10^7 CFU/mL, BPA biodegradation efficiency was enhanced around 1.2%, which was not significant (P-value > 0.05). Similar results were reported by Jia et al. (18).

NaCl concentration and the pH of a medium are important environmental factors, which are effective on the activity of enzymes contributing to BPA degradation (4, 18). The obtained results showed that NaCl concentration up to 0.2% (w/v) did not significantly (P-value > 0.05) affect the BPA biodegradation activity of *P. aeruginosa* DU2. Contrary to the findings of Kamaraj et al. (27) and Fouda et al. (30), in which the bacterial strains had optimum BPA biodegradation at neutral pH, the maximum BPA degradation rate of *P. aeruginosa* DU2 was specified at pH 9. The better biodegradation ability of *P. aeruginosa* DU2 at pH 9 may be related to its adaptation to the alkaline pH of the soil in Damghan (31).

CONCLUSION

Despite the accumulation of chemicals in various ecosystems, the isolation of native bacteria with the potency to degrade xenobiotic compounds from unpolluted sources is hopeful. These bacteria are beneficial for the removal of toxic compounds from various polluted ecosystems. This study, for the first time, offered soil containing decaying plants as an easily available source for the isolation of BPA-degrading bacteria. *P. aeruginosa* DU2 showed 52.98% BPA degradation at MSM containing 5 mg/L BPA and the inoculum size of 6×10^7 CFU/mL at pH 9 and 0.05% (w/v) NaCl. *P. aeruginosa* DU2 is a promising strain for the degradation of BPA at alkaline pH and the optimization of medium components and growth conditions by experimental design could improve its biodegradation efficiency.

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