International Journal of Engineering

Journal Homepage: www.ije.ir

# Biodegradation of Polychlorinated Biphenyls by *Lysinibacillus macrolides* and *Bacillus firmus* Isolated from Contaminated Soil

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*PAPER INFO*

*Paper history:*
Received 16 January 2019
Received in revised form 13 April 2019
Accepted 21 April 2019

*Keywords*:
*Bioremediation*
*Polychlorinated Biphenyls*
*Isolation*
*16S rRNA*
*GC/MS*

*ABSTRACT*

Polychlorinated biphenyls (PCBs) cause many significant ecological problems because of their low degradability, high harmfulness, and solid bioaccumulation.Two bacterial strains were isolated from soil that had been polluted with electrical transformer liquid for more than 40 years. The isolates were distinguished as *Lysinibacillus macrolides* DSM54Tand *Bacillus firmus* NBRC15306Tthrough 16S rRNA sequencing analysis.This is the primary report of an agent of the genus Bacillus firmus equipped for the removal of PCBs. The strains could develop broadly on 2-Chlorobiphenyl and 2,4-dichlorobiphenyl. GC/MS analysis of individual congeners revealed up to 80% degradation of the xenobiotics in 96h, attendant with cell proliferation of up to two orders of extent. More extensive investigation of the isolates may be helpful in surveying detoxification forms and in planning bioremediation and bioaugmentation strategies.

***doi***: *10.5829/ije.2019.32.05b.01*

## 1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are resistant organic compounds which were broadly utilized amid the 1940s. They were created industrially by chlorination of biphenyls in catalytic reactions. PCBs consist of a blend of various isomers with various numbers of chlorine atoms result to 209 items called congeners [1]. They were utilized in a broad span of industrial applications since they possess incredible physical and chemical attributes. As they are extremely persistent to degradation, they stay in soils and waters for a long period even years. PCBs are also lipophilic in environment, and thus, they accumulate in organisms and enter the food chain [2]. The outcomes of researchers' investigations suggest that PCBs are carcinogens [3]. High concentrations of PCBs exposure can result in various damages, counting skin ailment and liver harm [4].

Several methods have been proposed for the removal of PCBs from the environment including chemical and physical processes. However, burning as a regular transfer strategy with high effectiveness produces unfortunate results, for example, polychlorinated dibenzofurans and polychlorinated dibenzodioxins because of inadequate ignition [5]. Biological degradation is considered as one of the most efficient ways for cleaning organic pollutants from the environment [6, 7]. Numerous PCB-degrading bacteria have been isolated from pollutant areas, for example, Gram-negative strains having a place with the genera *Pseudomonas*, *Alcaligenes*, *Sphingomonas* and *Ralstonia*, just as Gram-positive strains having a place with the genera *Arthrobacter*, *Corynebacterium*, *Rhodococcus* and *Bacillus* [8, 9]. These bacteria generally use biphenyl as carbon and vitality source. They also biodegrade PCBs into chlorinated benzoic acids (CBAs) through oxidative pathways [10]. Investigations on the biodegradation of PCBs demonstrate that the initial substrate can either be altered to less dangerous substances or mineralized. There are two biological procedures for the debasement of PCBs: anaerobic reductive dechlorination and oxygen consuming oxidative corruption [11]. Exceedingly chlorinated PCB congeners are dechlorinated under

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Please cite this article as: A. Samadi, H. Sharifi, Z. Ghobadi Nejad, S. Yaghmaei, Biodegradation of Polychlorinated Biphenyls by *Lysinibacillus macrolides* and *Bacillus firmus* Isolated from Contaminated Soil, International Journal of Engineering (IJE), IJE TRANSACTIONS B: Applications Vol. 32, No. 5, (May 2019) 628-633

anaerobic conditions making lower chlorinated PCBs. Lower chlorinated congeners are degraded through an aerobically pathway [12].

Bioremediation of PCB-polluted sites needs insemination with bacterial strains which have exhibited capacity of PCBs biodegradation in the bench-scale treatability tests. Nevertheless, a few factors such as availability of the substrate and the nearness of different microorganisms can affect the capability of the isolated microorganisms to consume PCBs in polluted sites [13]. Some processes such as adding biphenyl and frequent insemination of the site have been reported as aids to PCBs removal [14]. Unfortunately, biphenyl is not an appropriate additive because of its harmful health effects, great expense and very low solubility in water. Therefore, discovering new chemicals which can be utilized as alternatives of biphenyl will help the expansion of a modern method for the bioremediation of polluted sites [15].

The amount of PCBs released in Iran is unknown since PCBs amounts used in different industries are unknown [16]. In this study, two new PCB-degrading strains, *Lysinibacillus macrolides* DSM54T and *Bacillus firmus* NBRC15306T, are reported which were isolated from soils of an electrical power station from south of Iran. The bacteria were assessed for their potential to degrade mono and di-chlorobiphenyls. This study contains the earliest report of a strain of the genus *Bacillus firmus* which is able to utilize 2-Chlorobiphenyl and 2,4-Dichlorobiphenyl as its only substrate.

## 2. MATERIALS AND METHODS

**2. 1. Chemicals** 2-Chlorobiphenyl, 2,4-dichlorobiphenyl and 4-chlorobenzoic acid were purchased from Sigma–Aldrich Co. (Germany). Seven soil samples were gathered (profundity of 10 cm) from various fields near a power plant in Hormozgan province (Iran). Transformer liquid was generously supplied by the power plant of Tehran (Iran). All other required chemicals were purchased by Merck and Sigma-Aldrich.

**2. 2. Bacterial Growth** Isolation and removal tests carried out in two mineral salts (MS) media (A and B). MS medium A contained 0.2 g/L $MgSO_4$, 0.1 g/L NaCl, 0.1 g/L $CaCl_2$, 1.0 g/L $KNO_3$, 0.02 g/L $FeCl_3$, 1.0 g/L $K_2HPO_4$, and enriched with yeast extract (0.05 g/L). The pH of medium was adjusted to 6.5 using diluted HCl and NaOH. MS medium B contained 5 mM phosphate buffer ($K_2HPO_4$, $KH_2PO_4$; pH 7.3), 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 0.076 g $Ca(NO_3)_2 \cdot 4H_2O$ in one liter distilled water enriched with 0.1 mL trace minerals and vitamins [17].

**2. 3. Isolation of the PCB-degrading Strains** The microorganisms used in this research were insulated via enrichment method using 0.1% v/v Transformer oil (an industrial mixture of polychlorinated biphenyls) in two MS media. Then, the bacteria strains purified on nutrient agar [17]. Details of the processes are presented below. One gram of contaminated soil samples, 20 mL of MS media and 0.1% (v/v) transformer oil, which was the only substrate, were blended in 100 mL flasks. Then, the flasks were incubated at 200 rpm and 25°C for 20 days. Consumption of the chemicals in the media was proved by increase in optical density, tubidity and color variation. Afterward, 10% of the inoculums were transmitted to new 100 mL flasks including 20 mL of MS media enriched with transformer oil and 200ppm 4-chlorobenzoic acid. The new flasks were incubated for 20 days under the similar situation. The same method was used for five successful times. After the end of the fifth period, the remaining strains were transferred to plates containing nutrient agars via pour plate method. Then, bacteria strains were purified using streak plate method for several times. The morphological characteristics of the purified strains were investigated after gram staining.

**2. 4. Selection of Degradative Bacterial Strains and Degradation Studies** Bacteria strains utilized in the removal experiments were grown with enough air at 25-30°C in nutrient broth. Cells were separated from media by centrifugation and washed with serum physiology. Then, they were suspended in their specific MS medium (A or B) to reach to an absorbance of 0.2 at 600 nm ($10^7$ cells/mL) [18]. Experiments were conducted in 100 mL flasks including 20 mL of media inoculated with bacterial strains at 200 ppm of the intended chemicals as an only substrate. The 100 mL flasks were incubated at 30°C for 96h. The removal was stopped by adding 20 mL of hexane and shaking constantly on a tubular shaker for 24h. The organic and aqueous phases were separated and preserved at refrigerator for further experiments. Bacterial strains which were able to remove the test compounds significantly were picked up for subsequent inspection. The selected strains were stored in nutrient broth and serum physiology enriched with yeast extract and glycerin at -80°C. Degradation experiments were performed for each test compound and selected strain. In experiments, two samples were examined at every time point.

**2. 5. Identification of the Bacterial Strains** Identification of the selected strains was performed based on 16S rRNA analysis technique. DNAs were extracted based on altered Marmur method [19]. PCR reactions were conducted utilizing forward primer 27F and reverse primer 1492R. Approximately 1400-bp long fragments were purified with the following sequencing details: Applied Biosystems 3730/3730xl DNA Analyzers Sequencing, Bioneer (Korea), Sanger method.

**2. 6. Analytical Methods** The amounts of polychlorinated biphenyls in hexane solutions were

specified through gas chromatography mass spectrometry (Agilent 5975C) with a Triple-Axis HED-EM Detector and a Combi-Pal autosampler. Then, 2µL of samples was injected into the GC/MS column (30m × 250µm × 0.25 µm). The injector was kept at 275°C. The temperature schedule was set at the primary temperature of 60°C for 5 minutes, which was elevated by 8°C/min to 300°C and kept at that temperature for 10 min. The polychlorinated biphenyl content was obtained by matching it against the standard diagram of concentrations. This standard diagram was achieved through determining the concentration of the pollutant which was expected no to degrade at four sections. GC/MS yield and precision were continually examined via injections of standard solutions before sample examination.

## 3. RESULTS AND DISCUSSION

**3. 1. Isolation, Identification and Purification of the PCB-Degrading Bacterial Strains** The isolation process resulted in eight isolated strains capable of utilizing 2-CB and 2,4-diCB in batch culture. Figure 1 demonstrates the microscopic photos of isolated strains after gram staining. The cultural and morphological characteristics of bacterial isolates and degradation percentage of each test compound are presented in Table 1. Two isolates, S1 and S2, were selected for further studies given their capability to significantly remove the test compounds. The two selected organisms were classified based on 16S rRNA method by comparing the acquired sequence data with the GenBanks [20-22]. DNA analysis of 1413 nucleotides revealed that S1 is much similar (99.2%) to the strain of *Lysinibacillus macrolides* DSM54T with accession number LGC101000008. S2 (1417 nucleotides) had 99.5% homology with *Bacillus firmus* NBRC15306T with accession number BCUY01000205. Note that *Lysinibacillus macrolides* is a short rod, gram-positive and immobile strain. The living cells have an estimated 2.5-3.0 micrometers length and an estimated 0.5-1 micrometers width. On the other hand, *Bacillus firmus* is a species of bacteria within the genus Bacillus which is very alkaline-tolerant and may grow in environments with pH as high as 11 [23].

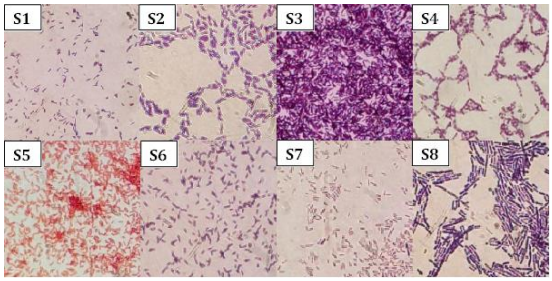


**Figure 1.** Microscopic photos of isolated strains after gram staining

**3. 2. Substrate Variety of the Selected Strains** Growth of microorganisms has been defined as an increase in the numbers of cells accompanying with removal of the test compound relative to control samples lacking the test compounds. A summary of the growth of S1 and S2 isolates consuming PCB congeners and PAHs as only substrate is reported in Table 2. The S1and S2 isolates were able to utilize 2-CB, 2,4-CB, and 4-CBA. The strains also showed visible signs of growth on nonchlorinated organic compounds (e.g., benzoate and naphthalene). The ability of these microorganisms to utilize various compounds, including PCBs and PAHs, suggests that they are promising microorganisms for bioremediation.

**3. 3. Bacterial Strain Growth on Mono-chlorobiphenyl and Di-chlorobiphenyl** The growth curves of isolates on 2-CB and 2,4-diCB are illustrated in Figure 2 Over the first 72h, S1 and S2 grew

**TABLE 1.** Cultural and morphological characteristics of bacteria isolates, and degradation percentage of each test compound

| Isolate Code | Soil Sample | MS medium | Morphology | Gram's reaction | Degradation Percentages (%) | |
|---|---|---|---|---|---|---|
| | | | | | 2-CB | 2,4-diCB |
| S1 | 4 | A | Growth moderate, mucoid and opaque | Gram positive short rods | 87.2 | 85.2 |
| S2 | 2 | B | Growth moderate, mucoid and opaque | Gram positive short rods | 86.4 | 79.1 |
| S3 | 7 | A | Growth abundant and translucent | Gram positive short rods | 62.1 | 53.4 |
| S4 | 5 | A | Growth abundant, and translucent | Gram positive short rods | 65.4 | 55.6 |
| S5 | 2 | A | Growth scanty and translucent | Gram negativ e rods | 70.5 | 61.1 |
| S6 | 4 | B | Growth moderate, mucoid and opaque | Gram positive rods | 54.6 | 43.1 |
| S7 | 6 | A | Growth scanty and translucent | Gram positive rods | 68.1 | 57.8 |
| S8 | 1 | B | Growth moderate and translucent | Gram positive long rods | 55.4 | 49.8 |

on 2-CB with mean generation times of 32 and 35 h, respectively. On the other hand, for growth on 2,4-diCB, these values increased to 36 and 39 h, respectively.

A little increase was clear in numbers of the cells in the control flasks lacking 2-CB and 2,4-diCB. In all cases, the rise in cell numbers was at least 2 times as large in flasks containing 2-CB or 2,4-diCB than in control flasks, clearly demonstrating growth on the congeners. The patterns of 2-CB and 2,4-diCB degradation were similar for the both isolates. By the end of the experiments, less than 20% of the initial test compounds were recovered in all cases.

**TABLE 2.** Substrate variety of the selected strains. Each chemical is enriched as the only substrate in strain- specific MS medium at 200 ppm

| Substrate | S1 | S2 |
|---|---|---|
| Biphenyl | + | + |
| Benzoate | + | + |
| Naphthalene | + | + |
| 2-Monochlorobiphenyl | + | + |
| 2,4-Dichlorobiphenyl | + | + |
| 4-Chlorobenzoic acid | + | + |
| Askarel Fluid | + | + |

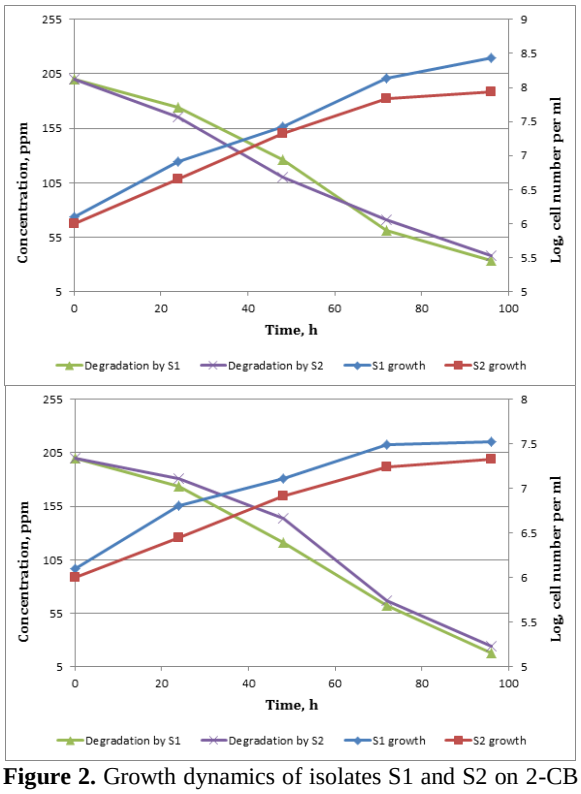


**Figure 2.** Growth dynamics of isolates S1 and S2 on 2-CB (chart top) and 2,4-diCB (chart below) and degradation patterns

**3. 4. Degradarion of 4-CBA** One of the most important issues in bioremediation is the ability of isolated microorganisms to remove intermediate toxic substances [24]. Biodegradation of the polychlorinated biphenyls (PCBs) by isolated or co-cultures of PCB-degrading bacteria were generally unfinished and terminates in the creation of chlorobenzoic acids [25]. The investigation of 4-chlorobenzodic acid removal by S1 and S2 strains revealed that the two isolates were able to degrade 4-chlorobenzoic acid by more than 90% (Figure 3).

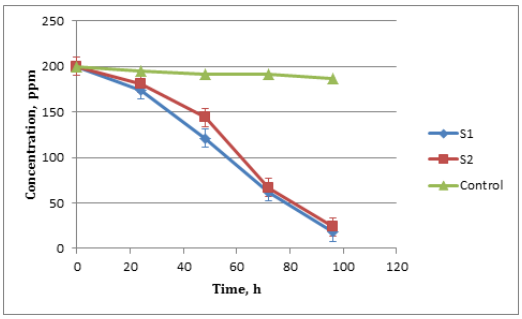


**Figure 3.** Degradation of 4-CBA by strains S1 and S2. Concentration in control without strain incubation stayed unchanged

## 4. CONCLUSION

In conclusion, isolation of *Bacillus firmus* and *Lysinibacillus macrolides* was noticeable with high capabilities to consume PCB and CBA as the only substrates. This study proposes that the genes for degradation of intended chemicals may be more extensively divided between bacteria genera than previously thought. In addition, the isolates are able to use different chlorinated and non-chlorinated aromatic chemicals which are often co-contaminants at some polluted sites. The wide substrate range of the isolated strains may aid in determining natural biodegradation procedures and designing remediation processes.

## 5. ACKNOWLEDGEMENTS

This research was supported by Vice President for research; Sharif University of Technology under Grant number QA 970713. The authors would like to acknowledge Biochemical and Bioenvironmental Research Center, Sharif University of Technology, for providing valuable help to accomplish the research work.

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# Biodegradation of Polychlorinated Biphenyls by *Lysinibacillus macrolides* and *Bacillus firmus* Isolated from Contaminated Soil

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*P A P E R I N F O*

*Paper history:*
Received 16 January 2019
Received in revised form 13 April 2019
Accepted 21 April 2019

*Keywords*:
*Bioremediation*
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*GC/MS*

**چکیده**

پلی‌کلروبی‌فنیل‌ها ترکیباتی بسیار سمی هستند که به علت حلالیت و تجزیه‌پذیری کم در محیط زیست باقی می‌مانند و با وارد شدن به زنجیره غذایی باعث بروز مشکلات زیادی می‌شوند. در این تحقیق، دو سویه بومی از خاک‌هایی که بیش از ٤٠ سال است آلوده به روغن ترانسفورماتور (آسکارل) هستند، جداسازی شد. شناسایی این دو سویه توسط تست 16S rRNA انجام شد که نتیجه تست تطابق سویه‌ها را با *Lysinibacillus macrolides* DSM54T و *Bacillus firmus* NBRC15306T نشان داد. در این تحقیق برای اولین بار توانایی حذف ترکیبات پلی‌کلروبی‌فنیل توسط یکی از سویه‌های گونه *Bacillus firmus* نشان داده شد. سویه‌های جداسازی شده توانایی رشد گسترده بر روی ترکیبات پلی‌کلروبی‌فنیل یک و دو کلره را نشان دادند. نتایج تست‌های GC/MS انجام گرفته نشان می‌داد که سویه‌های جداسازی شده توانستند در مدت ٢٤ ساعت بیش از ٨٠ درصد آلاینده‌های مورد نظر را حذف کنند. حذف این آلاینده‌ها با رشد همزمان سویه‌ها به تعداد بیش از دو واحد لگاریتمی از تعداد اولیه همراه بود. تحقیقات بیشتر بر روی حذف آلاینده‌های مرتبط می‌تواند به فهمیدن چگونگی فرایند حذف و طراحی فرایند زیست‌درمانی این آلاینده‌ها کمک کند.

***doi**: 10.5829/ije.2019.32.05b.01*