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Toluene Biodegradation by Novel Bacteria Isolated from Polluted Soil Surrounding Car Body Repair and Spray Painting Workshops

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Abstract

Toluene can enter the environment due to various industrial activities. Toluene exposure may cause serious health risks to human and other living organisms. Thus, removal of toluene from the environment is exceptionally important in toluene-contaminated habitats. The aim of present study was to isolate and characterize toluene-utilizing bacteria from contaminated soil surrounding car body repair and painting workshops in Irbid industrial city, Jordan. Therefore, polluted soil samples (10 g) were transferred to flasks containing 99 ml of Stanier's medium supplemented with 1% toluene and aerobically incubated at 30˚C for 72 h. Subsequently, four morphologically different toluene-utilizing bacteria, designated as T1, T2, T3, and T4, were isolated. The cells of T1, T2 and T3 were Gram-positive, rod-shaped, aerobic, and positive for oxidase and catalase. However, the cells of T4 were Gram-negative, round-shaped, aerobic, negative for oxidase, and positive for catalase. Based on 16S rDNA sequencing data, isolates T1, T2 and T3 had high homology (97% - 98%) with *Lysinibacillus boronitolerans*, *Bacillus subtilis* and *Rhodococcus pyridinivorans*, respectively, whereas the isolate T4 had a homology of 89% with *Acinetobacter schindleri*, and could represent a distinct lineage within the genus *Acinetobacter*. The generated 16S rDNA sequences were deposited in GenBank database. After testing different physicochemical conditions, the isolates appeared to grow best at 1% toluene, 30˚C and pH 6.8, with the generation times ranged between 8 - 11 h. In conclusion, we reported for the first time the identification of four novel soil bacterial species with the capacity to utilize toluene as sole source of carbon and energy from soil sites surrounding car painting workshops. The beneficial effect of the four isolates in the bioremediation of toluene from toluene polluted soil areas must be examined under *in situ* and *ex situ* conditions.

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Keywords

Toluene Biodegradation, Soil Pollution, *Lysinibacillus*, *Rhodococcus*, *Acinetobacter*

1. Introduction

Toluene is an aromatic compound that exhibits water-insolubility and low chemical reactivity due to the delocalized π electron system and the resonance energy of the aromatic ring [1]. It has an *n*-octanol:water partition coefficient (K_{ow}) of 2.69. Lipophilic aromatic compounds with K_{ow} of more than 1.5 are known to exert membrane-directed toxicity [2]. Due to this fact, toluene tends to accumulate into the lipophilic layer of the cell membrane and therefore disturbing its integrity [3]. As a result, toluene has multiple negative effects on living cells.

Worldwide, the annual production of toluene was estimated to be around 10 million tons [4]. Toluene is daily released in large quantities in the environment due to crude oil spills following oil tanker accidents and pipeline disruption. It can also be released into the environment due to extensive use in agricultural and industrial activities such as production paints, lacquers, thinners, pesticides, cigarette, polymers, as well as from automobile and car spray painting workshops and others [5] [6]. Consequently, toluene may accumulate in the surface and ground water, shoreline and soil. When toluene enters the soil, some of it will adhere to soil particles through the process of adsorption, and some of it will reach groundwater.

Exposure to toluene may cause various serious health hazards to human being as well as to the wide range of animals and plants [7]-[9]. For instance, continuous exposure to toluene can exert mutagenic effects on cells derived from human or other wildlife animals due to covalent binding to nucleic acids, mainly DNA [10]. It is also one of the well-known human neurotoxins and may cause leukoencephalopathy in human at the long exposure time [11]. Therefore, for these reasons, removal of toluene from soils, surface water and underground water have received a greater attention due to environmental issues as well as prerequisites for restoration of soil and water qualities.

In the last two decades, an increasing interest has been observed for utilizing microorganisms such as bacteria for removal of various aromatic compounds from the environment [9] [12] [13]. This was due to the fact that bacteria can adapt quickly to various conditions, reproduce quickly, and have simple growth and storing requirements. Several studies demonstrated that bacteria such as *Arthrobacter* sp., *Acinetobacter calcoaceticus*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Ewingella Americana*, *Kleblsiella oxytoca*, *Actinobacillus* and *Enterobacter aerogenes* were capable to grow on some selected aromatic compounds as their sole carbon and energy sources or degrading them as co-metabolic substrates [13]-[17].

Toluene was shown to be degraded by a variety of aerobic bacterial species and at least five different degradation pathways have been identified [15]. Examples of frequently isolated toluene-degrading aerobic bacteria include genera such as *Acinetobacter* and *Rhodococcus* [14], *Pseudomonas* [18] and *Bacillus* [19]. These four genera were found to have the ability to produce wide range of enzymes that help in metabolism of toluene [7] [12]. In presence of air, bacterial cells can use the highly reactive oxygen species (derived from O_2) via mono- or di-oxygenase-catalysed reactions to overcome the resonance stability of the ring structure [20] [21]. Besides, toluene biodegradation under anaerobic conditions was also described. Anaerobic toluene degraders belong to groups of iron-(III)-reducing bacteria, sulfate-reducing bacteria and denitrifying bacteria were identified and characterized [22]-[24].

Interest in the use of bacteria to get rid of crude oil and aromatic compounds from contaminated soil has been continued. There are several studies on aerobic bacterial biodegradation of aromatic compounds at the international level, however, at the local level, *i.e.* in Jordan, they are very scarce. In Jordan, Mrayyan and Battikhi (2004) have documented aerobic biodegradation of crude oil by bacterial consortia enriched from oily sludge from Jordanian oil refinery plant [25]. Another study was also carried out to enumerated and identified bacteria in contaminated soil with crude oil in the Jordanian Badia region [26]. Recently, biodegradation of crude oil by bacterial species from different sites in the Gulf of Aqaba has been reported [27]. Very recently, our lab reported the isolation and identification of four bacteria species (*Pseudomonas aeruginos*, *Pseudomonas stutzeri*, *Bacillus firmus* and *Citrobacter amalonaticus*) from soil contaminated with gasoline collected from sites located around

gas stations in Al-Mafraq city, Jordan. These species showed the capability to grow in Stanier's medium supplemented with 1% m-xylene [28]. Since screening studies of environmental bacteria in different areas of Jordan are limited, particularly in industrial sites, it is likely that additional environmental bacteria with novel metabolic capabilities maybe present in our soil. Therefore, the present work was carried out to isolate and characterize aerobic toluene-degrading bacteria from contaminated soil samples obtained from sites surrounding vehicle body repair and spray painting workshops in Irbid industrial city, Jordan.

2. Materials and Methods

2.1. Sample Collection and Soil Analysis

Soil samples of approximately 100 grams each were randomly collected from the top 10 cm of surface of polluted soil sites located around auto body repair and spray painting workshops. The selected workshops were very busy and located in the industrial city of Irbid, Jordan. Sampling was performed in June, 2014. During the collection, soil samples were cleaned up from all debris such as stones, plant and animal residues and other materials. Soil samples were placed in sterile plastic bags and immediately transported to the laboratory for further analysis and tested within 1 h. The chemical and physical properties of the soil were determined by standard protocol [29].

2.2. Preparation of Enrichment Medium

Vitamin-free sterile Stanier's mineral medium (SMM) was used for isolation and cultivation [30]. The following components were used to prepare one liter of liquid SMM for growing soil bacteria: 50 ml of stock metal solution, 20 ml of Hunter's vitamin-free mineral base solution, 40 ml of mineral base buffer and 890 ml deionized water. Stock metal solution contained per 100 ml of deionized water: 387 mg EDTA, 1.1 g $ZnSO_4 \cdot 7H_2O$, 914 mg $FeSO_4 \cdot 7H_2O$, 154 mg $MnSO_4 \cdot 7H_2O$, 39.2 mg $CuSO_4 \cdot 5H_2O$, 24.8 mg $Co(NO_4) \cdot 6H_2O$, 17.7 mg $Na_2B_4O_7 \cdot 19H_2O$ and 1.0 g $(NH_4)_2SO_4$. Several drops of 2N H_2SO_4 were also added to neutralize the metal solution and to retard precipitation. Hunter's vitamin-free mineral base solution was prepared by dissolving the following constituents in one liter of deionized water: 10 g nitrilotriacetic acid, 6.0 g KOH, 14.45 g $MgSO_4$, 3.335 g $CaCl_2 \cdot 2H_2O$, 99 mg $FeSO_4 \cdot 7H_2O$ and 9.25 mg $(NH_2)_6Mo_2O_{24} \cdot 4H_2O$. Finally, mineral base buffer contained per liter the following: 1 M Na_2HPO_4 and 1 M KH_2PO_4 buffer (pH 6.8).

When Stanier's agar medium was needed, 15 g/L of bacteriological agar were added to the medium and boiled to dissolve agar prior to autoclaving. The pH of the media was then adjusted to 6.8. If the pH has to be changed, drops of 1M HCl and/or NaOH were used to control the pH of the medium. Liquid SMM (or agar SMM) were then autoclaved and dispensed into the required volumes in sterile flasks or sterile tubes or Petri dishes in case of agar SMM.

Toluene was obtained from Sigma-Aldrich Co. (USA) and had a purity of 99.0%. It was used as the sole source of carbon and energy and to enrich the degraders. It was added into the SMM to a final concentration of 1% or 2%. It was sterilized separately by filtration using nitrocellulose membrane with 0.2 µm pore size and then added aseptically to the autoclaved SMM to appropriate final concentration. Deionized water was used to prepare the working solutions for all experiments, under aseptic conditions.

2.3. Cultivation, Isolation and Maintenance of Bacterial Strains

Ten grams of soil were transferred to Erlenmeyer flasks (250 ml capacity) containing 99 ml of growth SMM supplemented with 1% toluene as enrichment medium. The inoculated flasks were crimp-sealed with Teflon-coated stoppers to reduce losses from volatilization and sorption. The flasks were incubated at 30˚C with shaking using a shaking incubator (JS Research Inc., Korea) at 180 rpm for 72 h. Uninoculated flask was also prepared and used as a control.

After 72 h of incubation in the enrichment culture, one ml from the enrichment culture was transferred to new Erlenmeyer flasks containing 99 ml of fresh enrichment medium and incubation was continued at 30˚C with shaking at 180 rpm for 72 h. This procedure was also repeated for two successive times. Then, the appropriate dilutions of the culture enrichment medium were spread onto enrichment agar medium plates by streak plate method. These plates were incubated aerobically at 30˚C until the colonies appeared.

Four unique colonies were selected from agar plates based on their morphological appearance (Shape, surface, color, texture, margins and elevation) using a single colony isolation procedure. The unique colonies were inoculated into fresh liquid enrichment medium to confirm their capability to grow on toluene as the sole source of carbon and energy. The selected colonies were designated as isolates T1, T2, T3, and T4. Later, these isolates were transferred to new agar plates and incubated at 30˚C to obtain heavy growth. To preserve these four bacterial isolates, 0.7 ml of fresh pure culture for each isolate was obtained and mixed with 0.3 ml sterile glycerol in small sterile Eppendorf tubes. These tubes were then stored at −20˚C for further use. The selected isolates were subjected to analyses for characterization, identification and physicochemical studies.

2.4. Biochemical Characterization of Isolated Bacteria

The selected isolates were first Gram-stained by the standard technique [31]. Briefly, agar plate containing enrichment medium was prepared for each isolate. Then, cells for each isolate were stained and observed under the microscope (Euromax, Novex, 86.099LED, Holland) at the highest magnification (1000X) and photographed. The oxidase and catalase tests were carried out for the four isolates as described previously [31]. The oxidase test was conducted by using an aqueous solution of 2% N, N, N', N'-tetramethyl-p-phenylenediamine. The change of color of this oxidizing reagent to purple was considered as a positive result. The catalase activities were performed by adding few drops of fresh catalase reagent on fresh colonies of the isolated bacteria. For catalase test, the formation of gas bubbles was indicative of a positive catalase activity.

2.5. Molecular Identification of Isolated Bacteria

To identify and classify the four bacterial isolates to species levels, DNA extraction, 16S rDNA PCR and 16S rDNA sequencing techniques were performed as previously described [32]. Briefly, fresh culture for each isolate was prepared by inoculation of each isolate in liquid enrichment medium and incubated overnight, at 30˚C with shaking at 180 rpm. For each isolate, genomic DNA was extracted from freshly prepared culture and purified using EZ-10 Spin Column Genomic DNA Isolation Kit according to the manufacturer's instruction (Biobasic, Ontario, Canada). The 16S rRNA gene for each isolate was PCR amplified and sequenced by GENEWIZ sequencing service facility (GENEWIZ, Inc., USA).

To determine the closest relatives of the four isolates on the basis of 16S rRNA gene sequence, the resulting 16S rDNA sequences of the isolates were probed against the nucleotide collection in NCBI database (National Center for Biotechnology Information) using BLASTN 2.2.31+ Program (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). The identification at the species level for the purpose of this study was considered acceptable and final.

The resulting 16S rDNA sequences of the four isolates were deposited in GenBank database (www.ncbi.nlm.nih.gov/genbank). The following accession numbers: KP314269, KP314270. KP314271 and KP314272 were given for isolates T1, T2, T3 and T4, respectively.

2.6. Measurement of Growth Rate and Generation Time

Growth rate and generation time were monitored and calculated for the four selected isolates under various physicochemical conditions by measuring the increase in optical density (OD) at 600 nm using a UV-Vis Spectrophotometer (JENWAY, UK) as described previously [28].

2.7. Determining the Optimal Biodegradation Physicochemical Conditions

Experiments were carried out to determine the optimal physicochemical conditions using one factor at a time method. For experimental works, the growth rates and generation times of the cells from each isolate were determined for each factor in an aerobic culture tubes containing enrichment medium. Tubes were initially prepared by inoculation with freshly prepared culture pre-grown for several times with enrichment medium, and was used for each experiment under aseptic conditions. Tubes were crimp-sealed with Teflon-coated stoppers to prevent loss from volatilization. A control tube with SMM medium containing no toluene was performed for each case.

To determine the optimal initial toluene concentration, isolates were cultivated with three different toluene concentrations (0%, 1% and 2%; v:v, toluene: medium). The pH of growth medium was adjusted to 6.8. Tubes,

a set of three replicate tubes, were freshly prepared for each concentration and inoculated as described above. Then, inoculated tubes were incubated at 30˚C with shaking at 180 rpm for 120 h. Aliquot of 1 ml was taken regularly from each tube at various time points after inoculation (time zero). The growth rate and generation time for each identified isolate were monitored and determined as described above at each time point for each selected toluene concentration. The optimal toluene concentration is concentration that yields the shortest generation time.

The optimal growth temperature of each isolate was also determined by cultivating the cells of each isolate in tubes containing enrichment medium (SMM supplemented with 1% toluene and pH 6.8) at three different incubation temperatures: 25˚C (as room temperature), 30˚C (close *to in situ* temperature), and 45˚C (as relatively high temperature). Inoculated tubes were incubated at three selected temperatures with shaking at 180 rpm for 120 h as described above. One ml from each tube was taken at various intervals of time after inoculation. The growth rate and generation time for all isolates were monitored and determined as described above by a set of three replicate tubes at each time point for each selected temperature. The optimal growth temperature is temperature that yields the shortest generation time.

Each isolate was also cultivated in three enrichment media differ in pH values (5.5, 6.8, and 8.5) to determine the optimal pH. Inoculated tubes for each isolate were incubated at 30˚C with shaking at 180 rpm for 120 h as explained above. One ml from each tube was collected at various intervals of time after inoculation. The growth rate and generation time were estimated for each isolate by a set of three replicate tubes at each time point for each selected pH value as mentioned above. Optimal initial pH is pH value that yields the shortest generation time.

2.8. Statistical Analysis

Standard deviation (SD) and standard error of the mean (SEM) were calculated using Microsoft Excel 2010.

3. Results

3.1. The Physical and Chemical Properties of the Polluted Soil

The physical and chemical properties of the soil samples taken from sites surrounding car body repair and spray painting workshops located in Irbid industrial city, Jordan are shown in **Table 1**. The data revealed that the pH, total phosphorus, total nitrogen, total organic matter, and moisture content of polluted soil samples were 6.3, 40 $mg \cdot Kg^{-1}$, 60 $mg \cdot Kg^{-1}$, 5.5%, and 14%, respectively.

3.2. Isolation and Characterization of Novel Toluene-Degrading Bacteria

In this current study, soil samples were collected from sites surrounding car body repair and painting workshops in Irbid industrial city, Jordan. Four morphologically different bacteria were recovered from soil samples after enrichment in liquid SMM containing 1% toluene at 30˚C and pH 6.8. The selected isolates were designated as isolates T1, T2, T3, and T4. They were found to utilize toluene as the sole source of carbon and energy under aerobic condition. Utilization of toluene in enrichment cultures and growth of these four isolates were clearly evident by visual increase in turbidity and microscopic observation.

Table 1. The physical and biochemical properties of soil collected from the studied area.

Parameter	Mean Determination
Physical Appearance	Dark and Stick
pH	6.3
Total Phosphorus ($mg \cdot Kg^{-1}$)	40
Total Nitrogen ($mg \cdot Kg^{-1}$)	60
Total Organic Matter (%)	5.5
Moisture Content (%)	14

As can be seen in **Figure 1**, examination of the cells under light microscope revealed that the four isolates were different in cellular morphology and Gram-staining reaction. The cells of T1 were seen under the microscope as long single, double or multiple, rod-shaped and Gram-positive bacteria. The cells of T2 and T3 were seen as small single, double or multiple rod-shaped and Gram-positive bacteria. However, the cells of T4 were seen as small single, double or multiple Gram-negative round bacteria. Biochemical tests revealed that all isolates were positive for oxidase and catalase activities, except the cells of T4 were oxidase negative (**Table 2**).

The 16S rDNA analysis was employed for the identification and classification of the four isolates to species levels. The resulting 16S rDNA sequences of the four isolates were deposited in GenBank database, and reported in **Table 3**. Based on DNA analysis of 1070 nucleotides, isolate T1 (given accession number: KP314269) was found to be highly homologous (98% identity) to the *Lysinibacillus boronitolerans* 16S rDNA sequence (accession number: FJ237498.1). Isolate T2 (885 nucleotides, given accession number: KP314270) had 97% homology with the *Bacillus subtilis* 16S rDNA sequence (accession number: KF054916.1). Isolate T3 (1108 nucleotides, given accession number: KP314271) was shown to be highly homologous (98% identity) to the *Rhodococcus pyridinivorans* 16S rDNA sequence (accession number: CP006996.1). Isolate T4 (1051 nucleotides, given accession number: KP314272) had 89% homology with *Acinetobacter schindleri* 16S rDNA sequence (accession number: KC609744.1), suggesting the most possible taxonomic placement was at this species level.

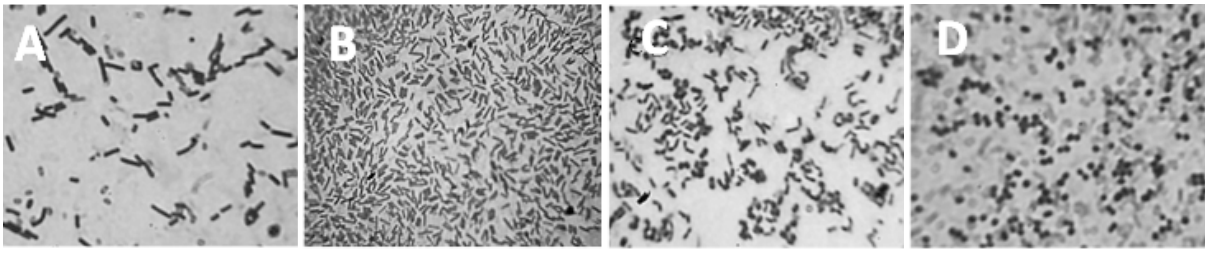

Figure 1. Light micrographs of the four bacterial isolates T1 (A), T2 (B), T3 (C), and T4 (D). Cells were magnified 1000×.

Table 2. Morphological and biochemical characteristics of the four bacterial isolates recovered from soil sites located around car spray painting workshops in Irbid industrial city, Jordan.

Isolate	Morphology			Biochemical tests		
	Shape	Size (μm)	Spore-forming	Gram stain	Catalase	Oxidase
T1	Rod	2 × 4	Yes	+	+	+
T2	Rod	1 × 2	Yes	+	+	+
T3	Rod	1 × 2	No	+	+	+
T4	Round	2	No	−	+	−

Legend: +: positive; −: negative.

Table 3. Percentage 16S rRNA gene sequence similarity and the closest relative species for the four bacterial isolates recovered from soil sample collected from sites surrounding car spray painting workshops and their given accession numbers.

Strain Code	Closest Relative Species	Identity Percentage	GenBank Accession Number
T1	*Lysinibacillus boronitolerans*	98%	KP314269
T2	*Bacillus subtilis*	97%	KP314270
T3	*Rhodococcus pyridinivorans*	98%	KP314271
T4	*Acinetobacter schindleri*	89%	KP314272

3.3. Optimizing the Growth Physicochemical Conditions

Growth curves under different growth conditions were determined for each isolate. The growth curves of isolate T1 with toluene at different growth conditions are illustrated in **Figure 2**. Two different concentrations of toluene (0% (control), 1% and 2% toluene) were used to determine the effect of toluene concentration on growth rate of isolate T1. The best growth rate of isolate T1 appeared to be at 1% toluene as compared to 2%, especially in the first 90 h of incubation, while no growth was observed in 0% toluene (**Figure 2(A)**). In the case of incubation at 25˚C, slight decrease in growth of isolate T1 was observed when compared to incubation at 30˚C. On other hand, incubation at 45˚C resulted in an appreciably lower rate of growth (**Figure 2(B)**). Along the same line, the growth rate of isolate T1 appeared to be better at pH 6.8 than at pH 5.5 and 8.5, especially in the first 90 h of incubation at 1% toluene and 30˚C, as illustrated in **Figure 2(C)**.

The shortest mean generation time of 11 h for isolate T1 cells was observed at 1% toluene and incubated at 30˚C and pH 6.8. On other hand, the cells of isolate T1 grew with the mean generation time of 34 h when incubated at 2% toluene, 30˚C and pH 6.8 (**Table 3**).

Growth of the cells of isolate T2 in SMM containing 1% toluene showed an exponential curve during the first 90 h (**Figure 3**). Growth of the cells of isolate T2 in tubes containing 1% toluene and incubated at 30˚C resulted in T2 cells increase of approximately three orders of magnitude than that for the cells of T2 in tubes containing 2% toluene and incubated at 30˚C. No growth was detected with 0% toluene (control), during the course of the experiment. As shown in **Figure 3(B)**, there was an about three-folds greater increase in OD values for the cells of T2 in tubes containing 1% toluene and incubated at 30˚C and pH 6.8 than that for cells of T2 in tubes containing 1% toluene and incubated at 45˚C and pH 6.8. Along the same line, there was an increase in OD value by about two-folds for the cells of T2 incubated at 1% toluene, 25˚C and pH 6.8 as compared with T2 cells incubated at 1% toluene, 45˚C and pH 6.8. In addition, the OD value of isolate T2 seemed to be higher at pH 6.8 than at 5.5 and 8.5, especially in the first 90 h of incubation at 1% toluene and 30˚C, as presented in **Figure 3(C)**.

It can also be seen that the mean generation time of 9 h was obtained for the cells of isolate T2 incubated at 1% toluene and 30°C with a culture pH 6.8 (**Table 3**). However, the mean generation time of 34 h was obtained when the cells of T2 were incubated at 2% toluene and 30˚C with a culture pH 6.8. The longest generation time was 47 h when the cells of T2 were incubated at 1% toluene, 45˚C with a culture pH 6.8.

Growth of the cells of isolate T3 in enrichment medium followed an exponential curve until 70 h (**Figure 4(A)**). In 2% toluene, however, the process of growth was very slow (**Figure 4(A)**). For the cells of T3 incubated at 25˚C and 30˚C, the OD increased to maximum OD of 0.37 and 0.46, respectively (**Figure 4(B)**). After the second day no increases in OD values were detected. On other hand, growth rates for the cells of T3 were very slow at all tubes incubated at 45˚C, where the increase in the OD during the first 70 h was 0.06. Isolate T3 grew better at pH 6.8 than at pH 5.5 or 8.5, during the first 90 h of incubation, as presented in **Figure 4(C)**. No growth was observed in control tube containing 0% toluene during the period of the experiment.

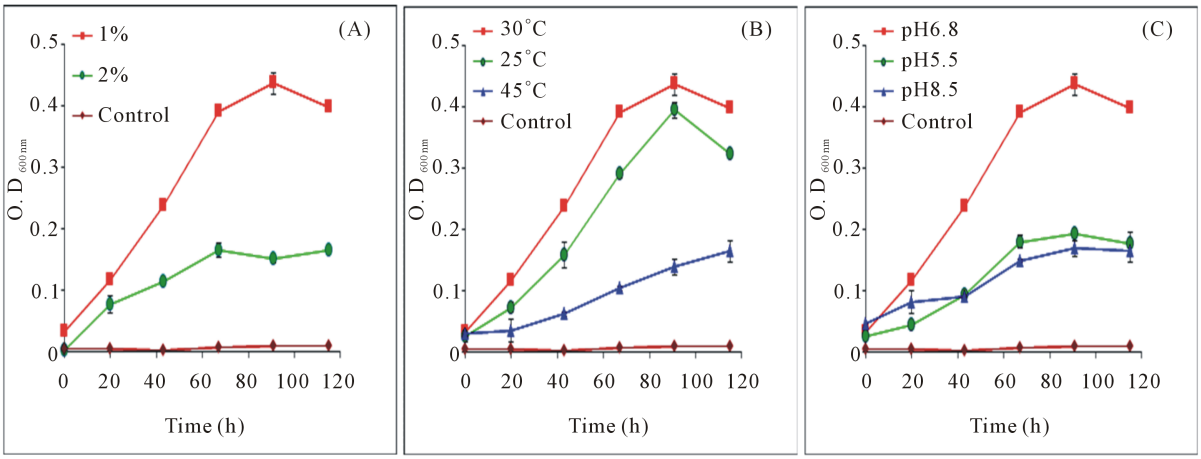

Figure 2. Growth profile of isolate T1 under different growth conditions: 1% and 2% toluene concentration (A); 25˚C, 30˚C, and 45˚C temperature (B); and pH values of 5.5, 6.8, and 8.5 (C). Data points represent the mean of three replicate tubes ± SEM.

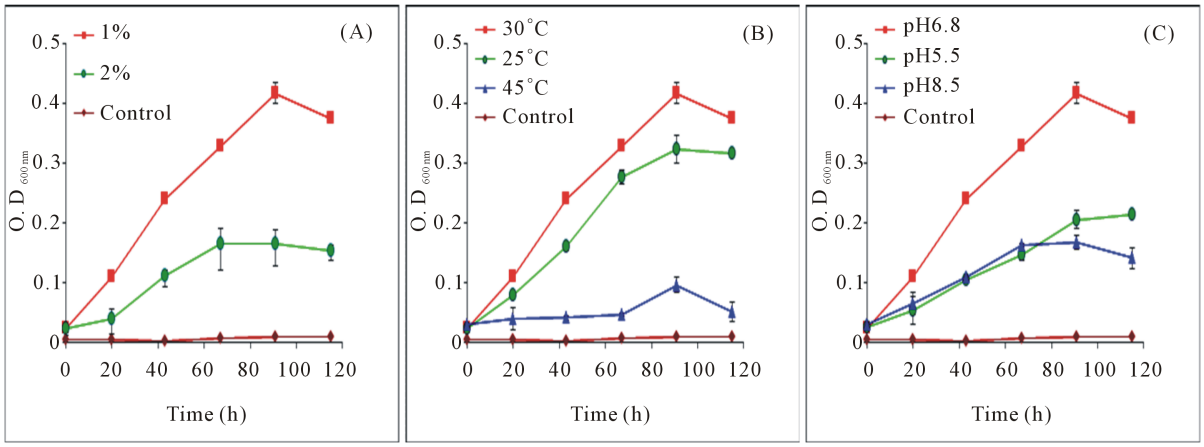

Figure 3. Growth profile of isolate T2 under different growth conditions: 1% and 2% toluene concentration (A); 25˚C, 30˚C, and 45˚C temperature (B); and pH values 5.5, 6.8, and 8.5 (C). Data points represent the mean of three replicate tubes ± SEM.

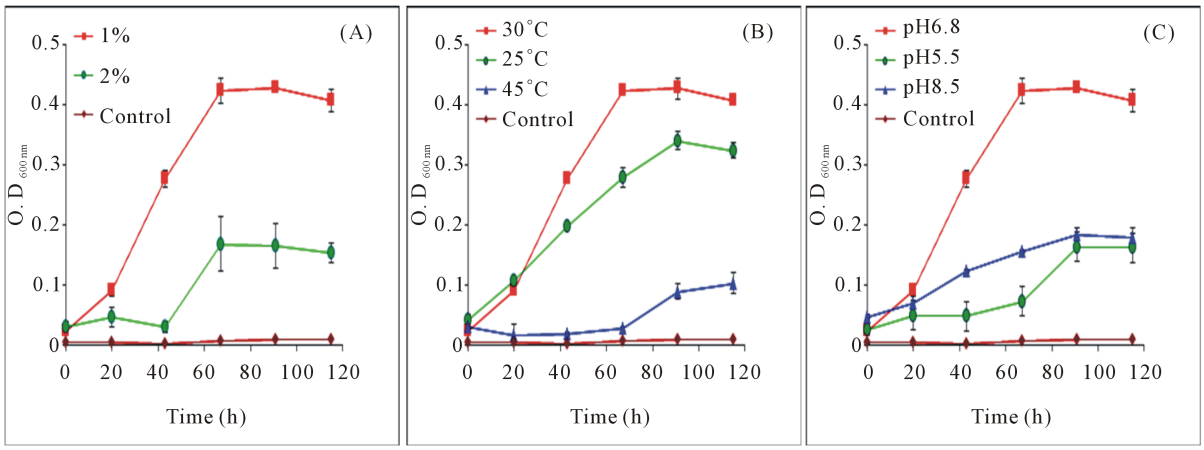

Figure 4. Growth profile of isolate T3 under different growth conditions: 1% and 2% toluene (A); 25˚C, 30˚C, and 45˚C temperature (B); and pH values 5.5, 6.8, and 8.5 (C). Data points represent the mean of three replicate tubes ± SEM.

The mean generation times for isolate T3 growing on 1% and 2% toluene were 10 h and 31 h, respectively (**Table 4**). The longest mean generation time for T3 was 39 h when the cells of T3 were grown at 1% toluene, 45˚C and pH 6.8. With respect to the effect of pH on the generation time, the lowest mean generation time was 10 h at pH 6.8. This was followed by pH 5.5 with the mean generation time of 22 h. For T4 cells grown in enrichment medium with pH 8.8, the mean generation time of 31 h was reported.

The growth rate of isolate T4 in tube containing 1% toluene and incubated at 30˚C and pH 6.8 was two-folds greater than that in tubes containing 2% toluene and incubated at 30˚C and pH 6.8, during the first 90 h of incubation (**Figure 5(A)**). In addition, no growth was observed in the control tubes (no toluene). The growth rate of the cells of isolate T4 reached a higher number of cells with OD of 0.45 at the end of 90 h, when the temperature was 30˚C (**Figure 5(B)**). When the temperature was reduced to 25˚C, the growth rate of T4 decreased slightly with OD of 0.35. On the other hand, the growth rate at 45˚C was very slow; the OD was about 0.10 at the end of 90 h. During the first 90 h, the growth rate of the cells of T4 in tubes containing enrichment medium with pH 6.8 was greater than in tubes containing enrichment medium with pH 5.5 and 8.5.

On the basis of the data presented in **Table 4**, the cells of isolate T4 grew with the mean generation time of 8h in 1% toluene, while the generation time of T4 cells was 19 h in 2% toluene. The cells of T4 grew with the mean generation times of 10 h at 25˚C, 8 h at 30˚C, and 31 h at 45˚C. Regarding the effect of pH on the generation time, the cells of T4 showed the shortest mean generation time of 8 h in enrichment medium with pH 6.8, this was followed by pH 8.5 with the mean generation time of 21 h. The longest mean generation time was 45 h for T4 cells grown in tubes with pH 5.5.

Table 4. Generation times (hours) of the isolated strains under different growth condtions.

Isolate	Concentration		Temperature (˚C)			pH		
	1%	2%	25	30	45	5.5	6.8	8.5
T1	11	34	13	11	24	28	11	23
T2	9	25	11	9	47	20	9	18
T3	10	31	14	10	39	22	10	31
T4	8	19	10	8	31	45	8	21

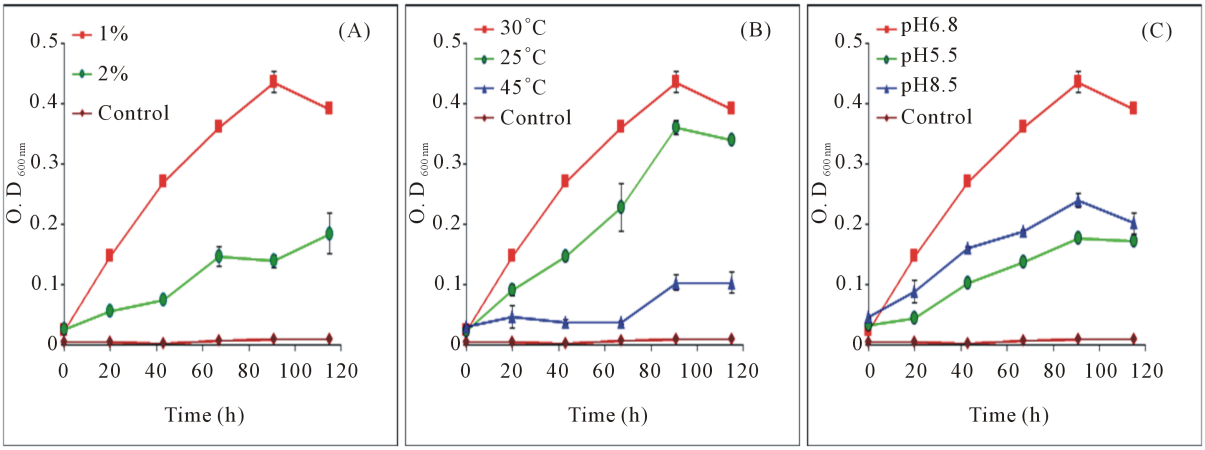

Figure 5. Growth profile of isolate T4 under different growth conditions: 1 and 2% toluene concentration (A); 25˚C, 30˚C, and 45˚C temperature (B); and pH values 5.5, 6.8, and 8.5 (C). Data points represent the mean of three replicate tubes ± SEM.

4. Discussion

This present study was interested in isolating toluene-degrading bacteria from contaminated soil collected from sites located near car body repair and spray painting workshops in industrial city of Irbid, Jordan. Bacterial growth was observed and confirmed in enrichment medium (SMM supplemented with 1% toluene) through the increase in the turbidity of the growth medium and OD measurements. Microscopic, morphological and Gram staining analyses revealed that there were at least four toluene-degrading isolates in the selected contaminated soil sites. All isolates appeared to utilized toluene as the sole source of carbon and energy at approximately equal rates, as illustrated in the growth curves.

Morphologically, the cells of isolates T1, T2 and T4 were rod-shaped Gram-positive aerobic bacteria and positive for catalase and oxidase activities. The cells of T4 were Gram-negative cocci, aerobic bacteria, and catalase positive and oxidase negative. Morphologically different colonies are supposed to be correlated to different species. Therefore, these four isolates were subjected to analyses for identification by 16S rDNA sequence analysis. The 16S rDNA sequences of the four isolates were deposited in GenBank database and accession numbers were given for each isolate. According to this analysis, isolates T1, T2 and T3 exhibited 97% to 98% sequence identity to the *Lysinibacillus boronitolerans*, *Bacillus subtilis* and *Rhodococcus pyridinivorans*, respectively. On other hand, isolate T4 exhibited a similarity score of lower than 90% sequence to *Acinetobacter schindleri*. Over the last two decades, 16S rDNA-based bacterial identification has been widely used to define identification at the genus and species levels [33] [34]. Furthermore, it has been proposed that individual isolate exhibiting a similarity score of less than 97% could be considered indicative of a new species within a known genus. Based on these data, isolate T4 may represent prototype isolate of a new species within the genus *Acinetobacter*.

Bacteria belonging to some genera of our identified isolates with the capability to degrade aromatic compounds were reported elsewhere in the literature. For instance, *Bacillus*, *Rhodococcus* and *Lysinibacillus* were detected in crude oil contaminated sites and found to possess the ability to degrade xenobiotic and aromatic compounds, including toluene [14] [19] [35]-[37]. Here in Jordan, *Bacillus*, *Acinetobacter*, *Rhodococcus*, *Pseu-*

domonas and *Lysinibacillus* were detected in soil contaminated with crude oil or gasoline, including the Badia area and the Gulf of Aqaba [26]-[28] [38]-[40]. However, this is the first report on isolation of bacteria with the ability to utilize toluene in Jordan from contaminated soil sites surrounding car body repair and spray painting workshops.

In the present investigation, the capabilities of these four isolated aerobic bacteria to degrade toluene under various physicochemical conditions were investigated. During the process of cultivation, the bacterial growth was very slow in the first few days, when there was only toluene as the source of carbon and energy in culture medium and no growth was observed especially when no toluene was added. After that, the bacterial growth rates increased. This is probably due to the fact that bacterial cells were greatly stressed in the first phase of cultivation. This was followed by the recovering of the bacterial cells from stress after few days, when the bacteria started to adapt to the enrichment liquid medium. It was also noticed that the four species grew faster during the incubation at 30˚C and pH 6.8 in enrichment liquid medium as compared to the experiments that have higher toluene level (2%) with different temperature or pH values. The observed inverse relationship between toluene concentration and growth were reported previously [41]. This inverse relationship can be attributed to the toxicity of toluene at higher concentrations.

Data generated from the estimation of the generation times for the four isolates revealed that the shortest generation times were obtained when 1% toluene was used at 30˚C as growth temperture and 6.8 as growth pH, and ranged between 9 to 11 h for all tested isolates. It is well known that temperature and pH play key roles in enhancing the growth of bacteria and metabolism of growth substrates. The best temperature for growth was 30˚C for all isolates, indicating that these isolates are mesophilic. Additionally, higher temperatures seem to have negative impact on growth rate of the isolated species. It has been reported that temperature can affect both the chemistry of the compound as well as the physiology of the degrading-microorganism [37] [41]. Lower growth temperatures seem to reduce the growth yield because lower temperatures usually reduce the enzymatic activity and ultimately lower the biodegradation rate and growth [41] [42]. On other hand, high temperatures may promote the volatilization of the toluene and thus lowering the available carbon source as well as may increase the toxicity of the hydrocarbon [41]. Our results also revealed that the isolated species appeared to grow best at pH 6.8, suggesting that they are neutrophilic. In general, neutral pH as optimal growth pH for some isolated bacteria with capability to degrade hydrocarbons was documented [41]. For example, most heterotrophic bacteria from the genus *Bacillus* species were found to favor a neutral pH [43] [44].

It has been reported that toluene can accumulate into the lipophilic layer of the cell membrane and exert membrane-directed toxicity and therefore disturbing membrane integrity [2] [3]. Subsequently, this could eventually lead to cell death. This could explains the presence of few isolates in this studied area because the selected site is very close to car body repair and spray painting workshops. Due to the motor vehicle repair activities such as body work, spray painting and others, several chemical substances might be released into the surrounding environment. Paints consist of three components: pigment, binding agent and solvent (www.sigmaaldrich.com). Pigments contain heavy metals, including lead, chromium and cadmium. Binding agents consist of acrylics, polyurethanes melamine resins and oil. The most commonly used solvents are toluene, benzene, xylene and ethylbenzene. The presence of such toxic compounds in paints might inhibit the growth of other bacterial species. In addition, elemental analysis of the contaminated soil used in this present study revealed that very low concentrations of nitrogen and phosphorous were found in the studied soil. These two elements are needed for efficient and normal growth and functioning of bacterial cells [33]. Bacterial cells also grow very slow in environment containing low concentrations of nitrogen and phosphorous. This added nutritional limitations for bacteria. Altogether, this may partially explain the presence of a small numbers of bacterial genera in our studied soil samples.

Our four isolates were recovered from a site with long history of pollution. Thus, it is possible to speculate that the presence of toluene may have selected the toluene-tolerant bacteria and those that are able to utilize it as the sole source of carbon and energy. It has been suggested that the lack of biodegradation capability of some aromatic hydrocarbons by bacteria has been attributed to the absence of exposure to these compounds during the course of evolution [21]. In support of this possibility, evidence was reported by Torsvik and Ovreas, (2002), who revealed that selection of substrates and shift in nutrient preference can be developed in some bacterial species due to competition for nutrients [45]. Based on these data, it is possible to suggest that our isolates might evolve in response to the selective pressure of long-term exposure adaptation to toluene compound in the selected sites. Moreover, the presence of few species in our soil samples can also be attributed to either wholly or in part, to the lack of ability of other species to utilize toluene and other aromatic hydrocarbons as the sole car-

bon and energy sources because these strains lack the genetic and enzymatic requirements for aromatic hydrocarbon-degradation. However, these suggestions are not entirely conclusive, and other possible interpretations may be equally valid.

Furthermore, several potential measures can be utilized to lower soil contamination in industrial sites. First, it is very important to encourage naturally occurring microbes in their native habitats to clean up contaminations. Second, among the potential measures are to enhancing the survival of bacteria and increase the bacterial population in the contaminated sites. Since the process of toluene utilization seems to be aerobic, this process can be improved by good aeration of soil in the contaminated sites. Third, soil analysis data also suggested that addition of enough amounts of nitrogen and phosphorous might promote the growth of bacterial populations in this type of soil and this may enhance the bioremediation process.

5. Conclusions

To the best of our knowledge, this is the first report of isolation and identification of four novel bacterial species from polluted soil samples collected from sites in close vicinity to auto body repair and spray painting workshops in industrial city of Irbid, Jordan. The four isolates were designated as T1, T2, T3, and T4. Isolates T1, T2, and T3 belong to Gram-positive aerobic bacteria and closely related to *Lysinibacillus boronitolerans*, *Bacillus subtilis* and *Rhodococcus pyridinivorans*, respectively. T4 was a Gram-negative aerobic bacterium, and its 16S rDNA sequence exhibited less than 90% sequence identity to *Acinetobacter schindleri*. T4 probably represented prototype isolate of new species within the genus *Acinetobacter*.

These four isolates were capable to utilize toluene as the sole source of carbon and energy. The best growth conditions for these isolates were at 1% toluene, 30˚C and pH 6.8. Most importantly, these species are one of the few bacterial species that have the potential to be used as bioremediation agents to remove toluene from toluene-polluted soil sites.

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