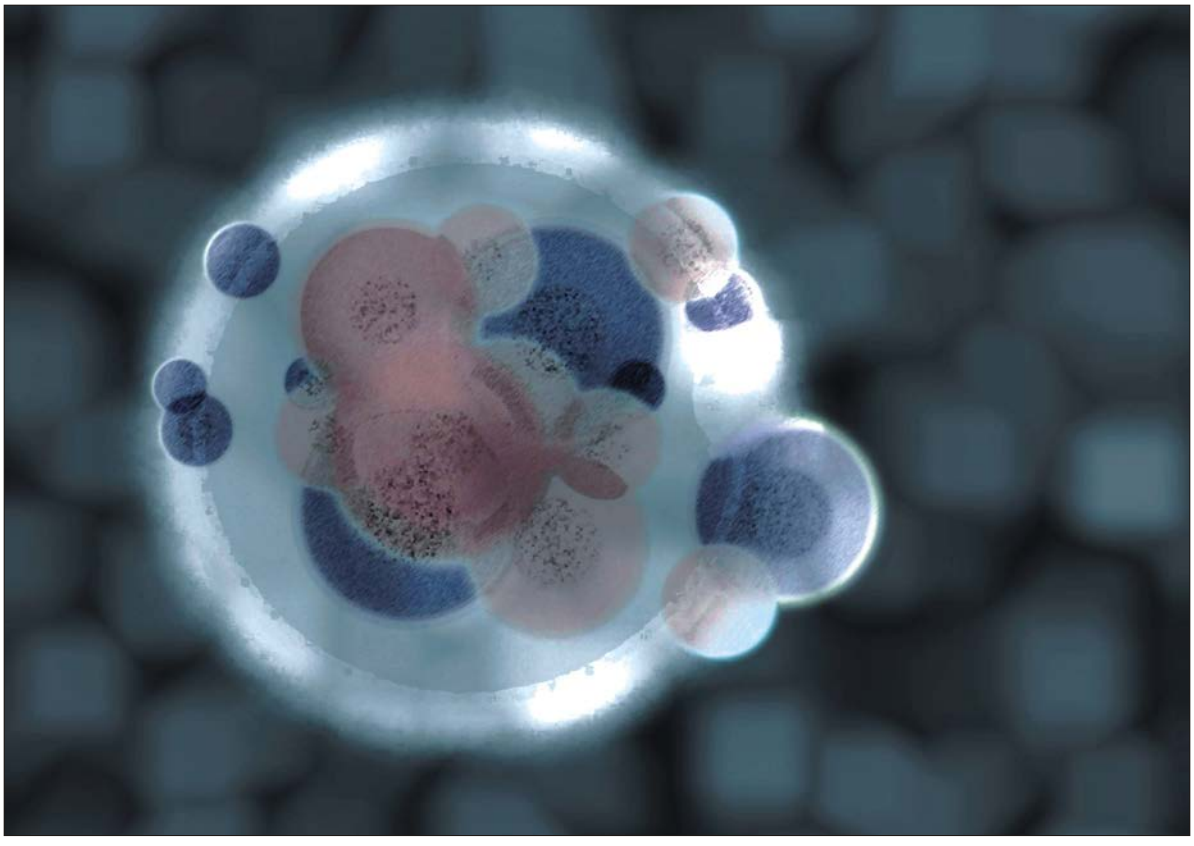

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Screening and Characterization of Aerobic Xylene-Degrading Bacteria from Gasoline Contaminated Soil Sites Around Gas Stations in Northern Jordan

Fawzi I. Irshaid and Jacob H. Jacob
Department of Biological Sciences, Al Al-Bayt University, P.O. Box 130040, Al-Mafraq, 25113, Jordan

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Corresponding Author:
Fawzi I. Irshaid
Department of Biological Sciences,
Al Al-Bayt University,
P.O. Box 130040,
Al-Mafraq, 25113, Jordan
Tel : 00962776948182
Fax : 0096265151261

ABSTRACT
Xylene is frequently released into the environment from biomass. As a consequence of this, its bioaccumulation can cause adverse health effects in humans. The purpose of this study was to screen for aerobic xylene-degrading bacteria from gasoline contaminated soil sites located around gas stations in the city of Al-Mafraq, Jordan. The effects of some physicochemical factors were examined. The 10 g of soil sample were transferred to Stanier's mineral medium supplemented with 1% m-xylene and incubated at 30°C for 72 h. At least 4 aerobic m-xylene degrading isolates, designated as X1-X4 were identified using biochemical and molecular biology techniques. Isolates X1 and X2 were rod-shape Gram negative, oxidase and catalase positive bacteria. Isolate X3 was a rod-shape and Gram negative bacterium that was catalase positive and oxidase negative. Isolate X4 was a rod-shape, spore forming and Gram positive bacterium that was oxidase and catalase positive. Isolates X1, X2 and X4 showed high similarity to *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Bacillus firmus*, respectively, whereas X3 was a novel species of the genus *Citrobacter*, similar to *Citrobacter amalonaticus*. The growth rates of these isolates were slower at 2% m-xylene than at 1% m-xylene. The growth rate was less when the temperature was reduced from 30-25°C, whereas, at 45°C, the growth rate almost completely ceased. The growth rate was higher at pH 6.5 than at pH 5.5 or 8.5. The shortest generation times were found to be 8 h for *Bacillus firmus*, followed by 9 h for *Pseudomonas stutzeri*, 10 h for *Citrobacter amalonaticus* and 11 h for *Pseudomonas aeruginosa* under 1% m-xylene at 30°C and pH 6.8. In conclusion, we reported for the first time the isolation of four bacterial species with the ability to utilize m-xylene as a growth substrate.

Key words: *Bacillus firmus*, bioremediation, *Citrobacter amalonaticus*, growth rate, *Pseudomonas* species, 16S rDNA

INTRODUCTION

Petroleum is a mixture of several of aromatic hydrocarbon compounds, including four alkylbenzenes (benzene, toluene, ethylbenzene and xylene) collectively known as BTEX (Jahn *et al*., 2005; Otenio *et al*., 2005; Marshall and Rodgers, 2008). The BTEX are stable aromatic hydrocarbons and represent major components of gasoline constituents. The BTEX are also volatile compounds and tend to be more water soluble than other components of gasoline such as alkanes and polyaromatic hydrocarbons (Applegate *et al*., 1998; Kao and Wang, 2000).

One of the alkylbenzenes that is gaining more access to nature is xylene. Xylene consists of a benzene ring with two methyl substituent arranged in three different positions to yield three different isomers (m-, o-, p-xylene) (Nersesian *et al*., 1985; Uchida *et al*., 1993; Otenio *et al*., 2005; Marshall and Rodgers, 2008; Kandyala *et al*., 2010). It occurs naturally in

petroleum and coal tar and is a constituent of smoke from most combustion sources. It can be used in industry as a solvent in the manufacturing of chemicals, agricultural sprays, adhesives and coatings and as an ingredient in aviation fuel and gasoline. Xylene also serves as a feedstock in manufacturing various polymers, including phathalic anhydride, isophathalic acid, terephthalic acid and dimethyl terephthalate (Marshall and Rodgers, 2008; Kandyala *et al*., 2010; Alrumman *et al*., 2015).

Xylene is frequently released into the environment from biomass. It can enter surface water, ground water and soil through natural seeps and accidental oil spills from underground crude oil tanks or gasoline pipelines, wells and other accidental spills (Uchida *et al*., 1993; Otenio *et al*., 2005; Vieira *et al*., 2007; Andreoni and Gianfreda, 2007; Kandyala *et al*., 2010; Atlas and Hazen, 2011; Alrumman *et al*., 2015). It can also be release into the environment due to crude oil spillages during transportation, loading and storage as well as from deliberate oil waste disposal from petroleum refining operations and petrochemical manufacturing industries, from runoff from roads and from water discharge from offshore and onshore oil installations. As a consequence of these various activities and processes, the accumulation of xylene in soil and ground water can pose a tremendous threat to the soil and ground water resources (Kao and Wang, 2000; Kandyala *et al*., 2010; Das and Chandran, 2011; Atlas and Hazen, 2011; Alrumman *et al*., 2015).

The occurrence and abundance of xylene contamination in the soil and aquatic environments can have a major health impacts on humans through consumption of food contaminated with this compound (Nersesian *et al*., 1985; Martinez *et al*., 1989; Uchida *et al*., 1993; Andreoni and Gianfreda, 2007; Kandyala *et al*., 2010). Some of the effects of xylene include nausea, vomiting, gastric discomfort and irritation of the nose, eye and throat (Nersesian *et al*., 1985; Uchida *et al*., 1993). In addition, acute exposure to high levels of xylene can cause hepatic toxicity (Uchida *et al*., 1993). Unconsciousness, amnesia, brain hemorrhage and epileptic seizure have also been linked to acute inhalation exposure to solutions containing xylene (Martinez *et al*., 1989).

Monitoring and removing xylene from the environment has been viewed as a high priority around the world (Kao and Wang, 2000; Andreoni and Gianfreda, 2007; Kandyala *et al*., 2010; Das and Chandran, 2011; Atlas and Hazen, 2011). Physical, chemical and biological approaches can be used to remove xylene and other petroleum hydrocarbons from contaminated sites. Microbial biodegradation is less expensive and hence considered one of the most promising alternative methods for cleaning up the environment from petroleum hydrocarbons (Kovalick, 1992; Vidali, 2001; Jahn *et al*., 2005; Vieira *et al*., 2007; Das and Chandran, 2011; Alrumman *et al*., 2015).

In recent decades, the presence of petroleum utilizing bacteria in oil and gas fields has been reported in several regions of the world (Das and Chandran, 2011; Atlas and Hazen, 2011; Thamer *et al*., 2013; Alrumman *et al*., 2015). These include xylene degrading microorganisms such as *Bacillus stearothermophilus*, *Arthrobacter*, *Acinetobacter calcoaceticus*, *Pseudomonas putida*, *Pantoea agglomerans* and *Enterobacter cloacae* (Worsey and Williams, 1975; Leahy and Colwell, 1990; Evans *et al*., 1992; Sorkhoh *et al*., 1995; Brusseau, 1998; Applegate *et al*., 1998; Jahn *et al*., 2005; Das and Mukherjee, 2007). Several studies have reported that the growth rates of aromatic hydrocarbon degrading bacteria were affected by the concentration of xylene compounds, temperature, pH of the medium and other factors (Brusseau, 1998; Das and Chandran, 2011; Alrumman *et al*., 2015).

Unfortunately, monitoring xylene bioaccumulation and its environmental consequences are understudied in the Middle East. In Jordan, xylene is not subjected to any regulation, which is partly due to limited economic resources. More importantly, with rapid growth of industrialization and urbanization in Jordan, increases in production, use, discharge and bioaccumulation of xylene in soil and groundwater are anticipated. As a consequence of this, the potential health risk for human and animals due to xylene exposure will increase, especially with the majority of the Jordanian population depends upon groundwater as a major source of drinking water. These facts prompted us to search for the presence of petroleum degrading microorganisms in our environment, in particular for xylene. In this present study, aerobic m-xylene eating microorganisms were isolated and identified from contaminated soil sites around gas stations located in northern Jordan. Additionally, the effects of some environmental factors (e.g., temperature, concentration and pH) on growth rates of these potential isolates were investigated.

MATERIALS AND METHODS

Sample collection: Soil samples of about 100 g each were randomly collected from gasoline-contaminated sites located around gas stations in the Al-Mafraq city of Jordan. The samples were taken from a depth of about 1-10 cm and placed in labeled sterile plastic bags. During the collection, all stones, plant residues, animal debris and other materials were removed from each sample. The soil samples were immediately transported to the lab for further analysis and isolation of bacteria. The soil samples were collected in September, 2014.

Media preparation: For cultivation and isolation of m-xylene degrader bacteria, vitamin-free sterile Stanier's medium was prepared as described previously (Stanier *et al*., 1966). For Stanier's agar medium, 2% agar was added to the medium and then boiled to dissolve agar. The pH of the medium was adjusted using either NaOH or HCI. The medium was sterilized by autoclaving at 121°C for 15 min.

The m-xylene with 99% purity was purchased from Sigma-Aldrich Co. (USA). It was added into the Stanier's medium as the sole source of energy and carbon to a final concentration of 1 or 2% as required under laminar flow hood. The m-xylene was sterilized separately by filtration using nitrocellulose membrane with 0.2 mm pore size and then added aseptically to the autoclaved Stanier's medium to appropriate final concentration. Deionized water was utilized in all working solutions for all experiments, under aseptic conditions.

Isolation of m-xylene degrading bacteria: To screen for the presence of xylene degrading bacteria in the collected soil sample, ten grams of soil were transferred to 90 mL of sterile Stanier's medium supplemented with 1% m-xylene as enrichment medium in Erlenmeyer flasks (250 mL capacity). The inoculated flasks were aerobically kept in orbital shaking incubator (JS Research Inc., Korea) at 180 rpm and 30ºC (close to *in situ* temperature) for 72 h. The inoculated flasks were crimp sealed with Teflon-coated stoppers to decrease any losses from volatilization and sorption.

After 72 h of incubation, the culture medium was allowed to settle and then 5 mL from the supernatant was transferred to new fresh enrichment medium flasks and aerobically incubated again at 30°C while being shaken at 180 rpm for 24 h. This procedure was repeated for three successive times. After incubation, 1 mL of the culture medium was spread onto Stanier's agar plates containing 1% m-xylene and incubated at 30°C until colonies appeared. Unique colonies were reinoculated into a new fresh Stanier's liquid medium containing 1% m-xylene to confirm their ability to grow on m-xylene. The colonies collected from agar medium were evaluated based on their morphology (size, color, edges and elevation). Finally, a 0.7 mL of each pure culture isolate was dissolved in 0.3 mL sterile glycerol in small eppendorf tubes and stored at -20°C.

Morphological and biochemical characterization of m-xylene degrading isolates: Four morphologically different colonies utilizing xylene as the sole carbon and energy source were selected based on their colony appearance on enrichment medium agar plates. They were designated as X1-X4. These four isolates were further purified by streak plate method on new enrichment medium agar plates and incubated at 30°C for 48 h to obtain substantial growth. Then, the four isolates were subjected to analyses for characterization and identification as well as for physicochemical studies.

Gram stained technique was performed to classify these isolate as Gram positive or negative. Briefly, fresh pure isolates were prepared by growing in agar plates aerobically at 30°C for 24 h. The bacteria were then stained and observed under the light microscope as previously described (Cappuccino and Sherman, 2008).

The oxidase and catalase tests were performed on the isolates as described previously (Cappuccino and Sherman, 2008). Briefly, few drops of the oxidase test reagent (2% of N, N, N', N', tetra-methyl-p-phenylenediamine dihydrochloride) were added to a viable culture from each strain. Change of color of the reagent to purple was considered as a positive result. The catalase test was also carried out by adding few drops of H_2O_2 on fresh colonies of the isolated strains and observing the bubble formation.

Molecular identification of m-xylene degrading isolates: For further identification and classification of the m-xylene degrading isolates to species level, DNA extraction, 16S rDNA PCR and 16S rDNA sequencing techniques were carried out as previously described (Jacob and Irshaid, 2012). Briefly, genomic DNA from each pure isolate was extracted from freshly prepared culture and purified by EZ-10 Spin Column Genomic DNA Isolation Kit (Biobasic, Ontario, Canada) as recommended by the manufacturer's instruction. For all obtained pure DNA samples from the 4 isolates, 16S rRNA gene was PCR amplified and sequenced by 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 searched against the nucleotide collection in NCBI genetic sequence database using Web BLAST Service (http://blast.ncbi.nlm.nih.gov/blast/ Blast.cgi).

Nucleotide sequence accession numbers: All the resulting DNA sequences of the partial 16S rRNA genes of the 4 isolates (X1-X4) reported in this study were deposited in GenBank database under the following accession numbers: KP314273, KP314274, KP314275 and KP314276, respectively, (www.ncbi.nlm.nih.gov/genbank).

Molecular phylogenetic analysis: Molecular phylogenetic analysis was performed for the m-xylene degrading bacteria as described by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) and a phylogenetic tree was constructed by MEGA6 (Tamura *et al*., 2013).

Growth of isolates at different m-xylene, pH and temperature values: The growth rates for the 4 isolates were monitored by measuring the increase in Optical Density (OD) at 600 nm (Koch, 1970) using a UV-Vis spectrophotometer (JENWAY, UK). The OD values were then converted to cell number (colony-forming units per mL (CFU mL^{-1})). Uninoculated enrichment medium was used as blank in the absorbance measurements.

To estimate the generation times for the isolates, the spread plate technique was used for counting bacterial colonies. Aliquots of 0.1 mL of aerobic fresh liquid culture for

each isolate were place on agar plates containing enrichment Stanier's medium and incubated for 48 h at 30°C. After incubation, the developed bacterial colonies were manually counted using colony counter and reported in CFU mL^{-1}. All counts were performed in triplicate. The generation time was calculated by using the following equation (Solomon and Viswalingam, 2013):

$$g = t/n,\ n = 3.3\ (\log N - \log N_o)$$

where, g is the generation time, t is time interval in hours, n is the number of generations during the period of exponential growth, N is the final cell number and N_o is the initial cell number.

The effects of different m-xylene concentrations, pH and temperatures on the growth rate and generation time of each isolate were examined. For each experiment, aerobic culture tubes were prepared and crimp-sealed with Teflon coated stoppers to prevent loss from volatilization. These tubes were inoculated with freshly prepared culture pre-grown in liquid enrichment medium under aseptic conditions. As control experiments, inoculated tubes with enrichment medium containing no m-xylene were performed for each case.

To determining the effect of m-xylene concentration, cells from each isolate were grown in tubes containing enrichment medium supplemented with 0, 1 or 2% m-xylene. Incubation was aerobically conducted at pH 6.8 and 30°C with shaking at 180 rpm for 100 h. At periodic intervals after inoculation (time zero), the samples were drawn aseptically from triplicate inoculated tubes. The growth rates and generation times of these isolates were measured under these conditions as described above. Enrichment medium supplemented with 1% m-xylene was used for subsequent steps of the investigation.

The effects of pH on growth were studied at pH 5.5, 6.8 and 8.5. Three enrichment media supplemented with 1% m-xylene and with different pH values were prepared. Fresh cells from each isolate were cultivated for each of the three enrichment media in crimp-sealed tubes. Tubes were incubated, in triplicate, at 30°C with shaking at 180 rpm for 100 h. An aliquot of 1 mL was obtained from each tube at various time points after inoculation and growth rates and generation times were determined as described above.

Three growth temperatures 25°C (considered as room temperature), 30°C (close *to in situ* temperature) and 45°C (as relatively high temperature) were used to study the growth rate of the 4 isolates for 100 h. The isolates were cultivated in enrichment medium containing 1% m-xylene and at pH 6.8 and incubated at the above selected temperatures with shaking at 180 rpm for 100 h. An aliquot of 1 mL was obtained from each tube at various time points after inoculation and growth rates and generation times were measured as described above.

RESULTS

Four strains of bacteria capable of utilizing m-xylene as the sole carbon and energy source were isolated from soil samples of gasoline polluted site in Al-Mafraq district, Jordan. The four isolates were designated as isolates X1-X4. To place these isolates to the closest relative species, the 16S rRNA gene sequences for these isolates were first determined and compared to those in the GenBank Database (http://blast.ncbi.nlm.nih. gov/blast /Blast.cgi).

The closest relative species, sequence identity values and accession numbers for the four bacterial isolates are presented in Table 1. The 16S rRNA gene sequence analysis of both isolates X1 and X2 revealed high similarity (97 and 96%) with species of *Pseudomonas aeruginosa* (accession number: LK391633.1) and *Pseudomonas stutzeri* (accession number: KM278988.1), respectively. Based on biochemical analysis, these two species were Gram negative, catalase and oxidase positive aerobic bacteria (Table 2). Morphologically, the cells of these two species were rod-shape bacteria. The 16S rRNA gene sequence for isolate X3 revealed 88%

Table 1: Percentage 16S rRNA gene sequence similarity and the closest relative for the four bacterial isolates obtained from soil sample contaminated with gasoline capable of utilizing m-xylene for growth as well as their given GenBank accession number

Isolate code	Closest relative	Coverage (%)	Identity (%)	GenBank® Accession Number
X1	*Pseudomonas aeruginosa*	92	97	KP314273
X2	*Pseudomonas stutzeri*	92	96	KP314274
X3	*Citrobacter amalonaticus*	86	88	KP314275
X4	*Bacillus firmus*	87	98	KP314276

Table 2: Morphological and biochemical characteristics of the four bacterial isolates capable of utilizing m-xylene as the sole source of carbon and energy for growth isolated from gasoline contaminated soils

			Biochemical test	
Isolate name and code	Morphology	Gram stain	Catalase	Oxidase
Pseudomonas aeruginosa (X1)	Rod-shape	-	+	+
Pseudomonas stutzeri (X2)	Rod-shape	-	+	+
Citrobacter amalonaticus (X3)	Rod-shape	-	+	-
Bacillus firmus (X4)	Rod-shape	+	+	+

+: Positive, -: Negative

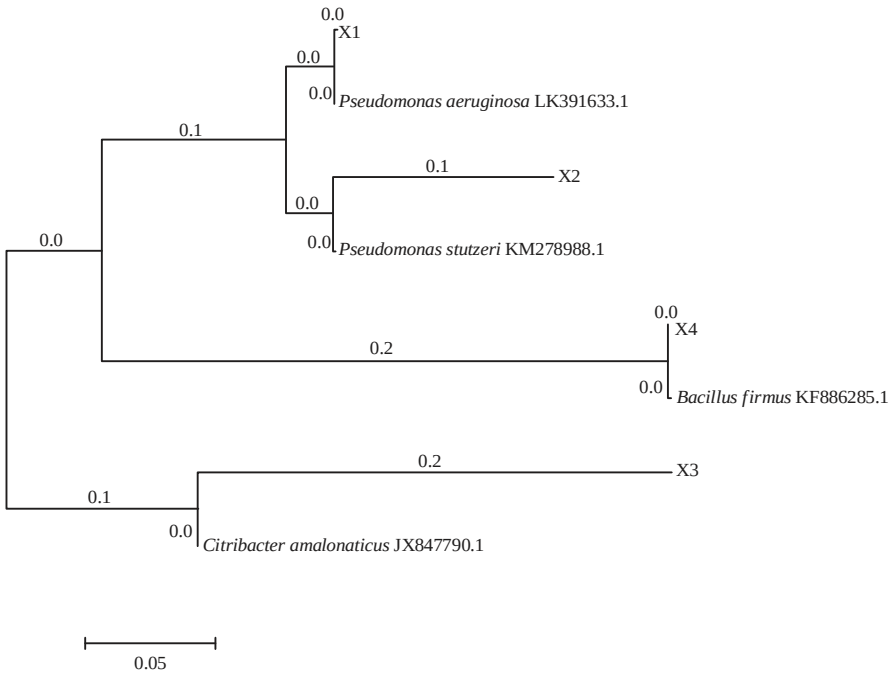

Fig. 1: Molecular phylogenetic tree; this tree is based on 16S rRNA gene sequences, showing the position of the four bacterial isolates capable of using m-xylene as a growth substrate (X1-X4) isolated from soil sample contaminated with gasoline and their closely related species of bacteria. Accession numbers of these species are given between brackets. The scale bar on bottom represents genetic distances in substitutions per nucleotide. The branch lengths measured in the number of substitutions per site (above the branches). This evolutionary history was inferred by the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) and tree was constructed by MEGA6 (Tamura *et al*., 2013)

Table 3: Mean generation times in hours for the 4 bacterial species isolated from gasoline contaminated soil samples in liquid Stanier's medium supplemented with m-xylene under different growth conditions

	Growth conditions							
	Xylene concentration (%)		Temperature (°C)			pH		
Isolate name and code	1	2	25	30	45	5.5	6.8	8.5
Pseudomonas aeruginosa (X1)	11	34	13	11	24	28	11	23
Pseudomonas stutzeri (X2)	9	25	11	9	47	20	9	18
Citrobacter amalonaticus (X3)	10	31	14	10	39	22	10	31
Bacillus firmus (X4)	8	19	10	8	31	45	8	21

homology with *Citrobacter amalonaticus* (accession number: JX847790.1), indicating the most probable taxonomic placement at species level. This species was a rod-shaped and Gram negative aerobic bacterium that was catalase positive and oxidase negative. Isolate X4 showed 98% sequence similarity with *Bacillus firmus* (accession number: KF886285.1). Morphologically, the cells of isolate X4 were spore forming and rod-shaped. Biochemically, this strain was found to be a Gram positive aerobic bacterium that was both oxidase and catalase positive. Analysis of these strains by both the maximum likelihood method and the phylogenetic tree analysis support the placement of these isolates to above mentioned species as shown in Fig. 1.

Optimal growth conditions of the 4 isolated bacteria were examined. These conditions include initial concentration, temperature and pH. The growth rates and generation times under different conditions were calculated and compared. All four strains demonstrated the same conditions for optimal growth: incubation in 1% m-xylene at 30°C and at pH 6.8; as compared to 2% m-xylene concentration and 25 or 45°C as incubation temperature and pH 5.5 or 8.5. The calculated generation time under these optimal conditions was 11 h for *Pseudomonas aeruginosa* (X1), 9 h for *Pseudomonas stutzeri* (X2), 10 h for *Citrobacter amalonaticus* (X3) and 8 h for *Bacillus firmus* (X4) (Table 3).

Furthermore, under these optimal conditions, all isolates reached their maximum density between 60-80 h. The density

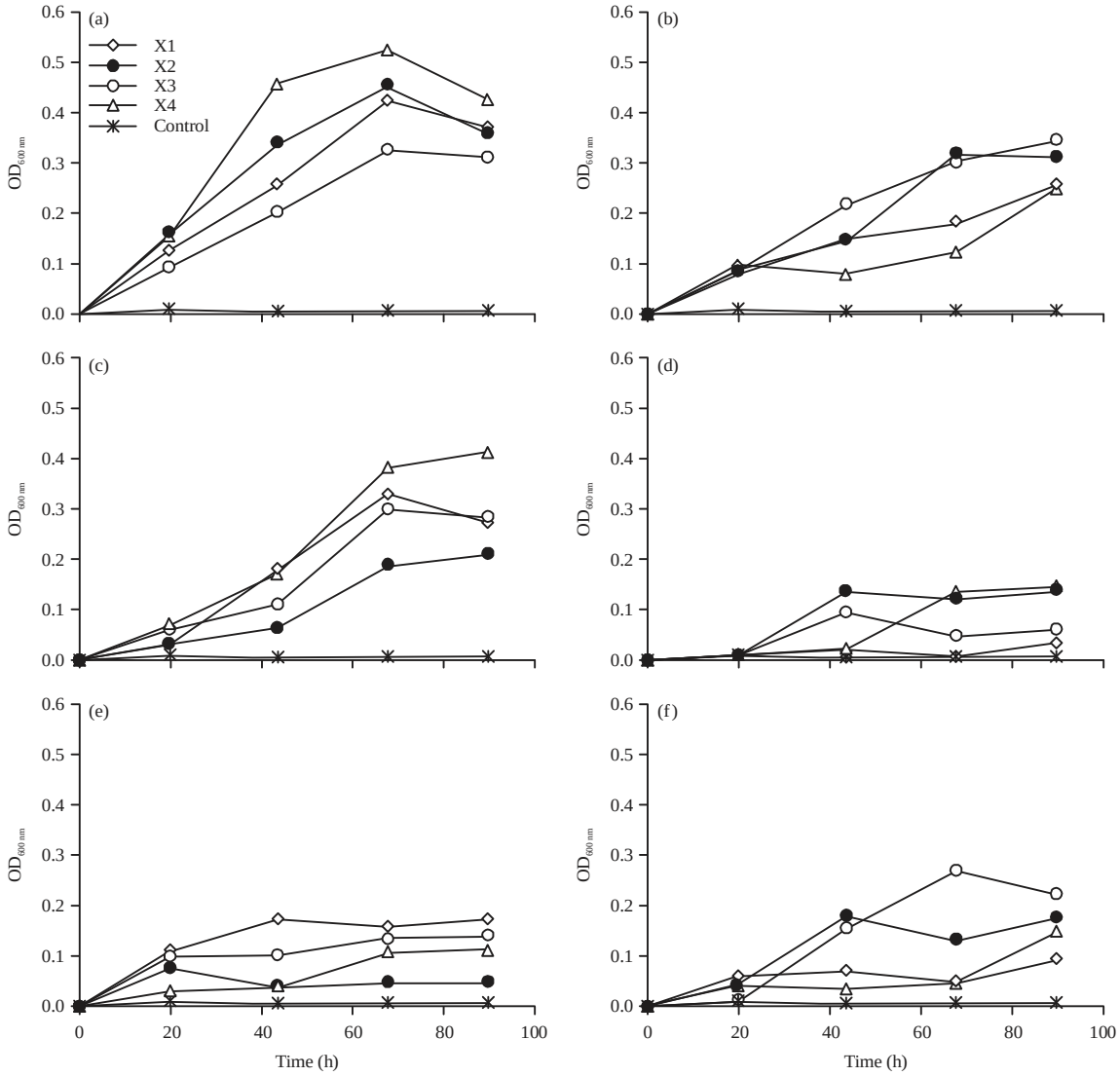

Fig. 2(a-f): Growth behavior of the four bacterial isolates (X1-X4) obtained from soil contaminated by gasoline in liquid Stanier's medium under different growth conditions, (a) 1% m-xylene, pH 6.8, 30°C, (b) 2% m-xylene pH 6.8, 30°C, (c) 25°C, 1% m-xylene, pH 6.8, (d) 45°C, 1% m-xylene, pH 6.8, (e) pH 5.5, 1% m-xylene, 30°C and (f) pH 8.5, 1% m-xylene, 30°C. Each point in graphs represents the mean of three measurements

started to decrease after 80 h and reached approximately 50% of the maximum density after 100 h (Fig. 2). The growth rate for *Bacillus firmus* (X4) was higher than any other isolate at 1% m-xylene concentration, 30°C and pH 6.8. *Pseudomonas aeruginosa* (X1) and *Pseudomonas stutzeri* (X2) showed moderate growth rate, while the lowest growth rate was observed with *Citrobacter amalonaticus* (X3). The shortest generation time was 8 h for *Bacillus firmus* (X4) followed by 9 h for *Pseudomonas stutzeri* (X2), 10 h for *Citrobacter amalonaticus* (X3) and 11 h for *Pseudomonas aeruginosa* (X1) under 1% m-xylene at 30°C and pH 6.8 (Table 3).

DISCUSSION

This present study was aimed at isolating bacterial m-xylene degrader from gasoline-contaminated soil sites around gas stations located in the northern part of Jordan. In this study, four bacterial isolates with the capacity to utilized m-xylene as the sole carbon and energy source were isolated from soil by the enrichment culture technique and were designated X1-X4. The identities of these isolates were determined by their morphology, biochemical and molecular properties. Based on these properties, isolates X1 and X2 were found to belong to the genus *Pseudomonas*, whereas,

isolate X4 belonged to the genus *Bacillus*. Isolate X3 showed a sequence similarity of 88% with *Citrobacter*, in particular to the species *amalonaticus*, suggesting a new potential species of *Citrobacter*. The phylogenetic analysis also supports these findings. It also supports the suggestion that isolate X3 represents a separate branch within the cluster of the genus *Citrobacter*.

The presence of aerobic m-xylene degrading bacteria in the examined soil samples taken from gasoline contaminated sites is perhaps expected. These findings are in accordance with previous reports which revealed that biodegradation of aromatic hydrocarbons has been commonly seen by aerobic bacteria, whereas anaerobic biodegradation of aromatic hydrocarbons is relatively uncommon (Evans *et al*., 1992; Edwards and Grbic-Galic, 1994; Das and Mukherjee, 2007). Moreover, degradation rates of aromatic compounds under anaerobic condition were comparatively slower and less efficient than aerobic one. It has also been mention that xylene degrading bacteria can easily grow in m-or p-xylene compared to o-xylene. However, few bacterial species, such *Nocardia* species have been isolated and shown to degrade o-xylene as the sole source of carbon and energy.

It is becoming increasingly clear that *Pseudomonas* species are perhaps the most intensively isolated and studied genera of bacteria in the field of biodegradation (Worsey and Williams, 1975; Van Hamme *et al*., 2003; Otenio *et al*., 2005; Das and Mukherjee, 2007). Degradation of m-xylene by *Pseudomonas* was documented earlier in the literature. Aerobic degradation of xylene by *Pseudomonas putida* has been shown to be genetically encoded by the TOL plasmid (Worsey and Williams, 1975). Later, a closely similar new strain of *Pseudomonas putida* CCMI 852 containing a TOL plasmid was identified and had the ability to metabolize xylene at a rate by 53% in a minimal liquid medium containing xylene (Otenio *et al*., 2005). Moreover, a metabolic pathway mainly dedicated to gasoline components such as xylene was identified in *Pseudomonas putida* CCMI 852. Taken together, our study and previous studies clearly demonstrated that *Pseudomonas* species can thrive in habitat contaminated with petroleum and its products. This is mainly because this genus can hydrolyze aromatic compounds such as xylene and others. Therefore, the recovery of *Pseudomonas* species from our gasoline contaminated soil samples was not surprising due to their frequent presence in soil and their ability to breakdown various organic compounds.

Bioremediation of crude oil by the action of *Bacillus* species has also been characterized from highly polluted soil samples (Das and Mukherjee, 2007; Thamer *et al*., 2013). *Bacillus* species are more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. Hence, *Bacillus* species represent the most predominant isolate of all the crude oil utilizing bacteria that are commonly occurring in extreme environments (Ghazali *et al*., 2004; Das and Mukherjee, 2007; Das and Chandran, 2011). Taken together, the findings of this present study combined with those of previous findings support the growing evidence that isolates belonging to both *Bacillus* and *Pseudomonas* species could be effective in clearing soil contaminated by gasoline and crude oil spills.

The role of *Citrobacter amalonaticus* in biodegradation of crude oil and its products had not been previously mentioned elsewhere. There was no related study available. However, a previous study by Obst *et al*. (2005) showed that *Citrobacter amalonaticus* utilized dipeptides released from cyanophycin granule polypeptide by another bacterium. Therefore, to the best of our knowledge, the present study documents, for the first time, the presence of *Citrobacter amalonaticus* in soil contaminated by gasoline and its ability to utilize m-xylene as the sole source of energy and carbon for growth. Furthermore, previous studies showed that the distribution and identities of crude oil degrading bacteria were not essentially similar in different parts of the world (Ghazali *et al*., 2004; Staats *et al*., 2011). It is therefore not surprising that unidentified bacterial species such as *Citrobacter amalonaticus* with the capability to use xylene as the sole source of carbon and energy are present in our area. The presence of *Citrobacter amalonaticus* in soil contaminated by gasoline may prove to be beneficial to our environment. It could also be speculated that this isolate might contain a unique set of candidate genes related with xylene degradation.

Influences of physicochemical factors (temperature, concentration of m-xylene and pH) on the growth rates of these isolates were obvious. Our experimental results showed that these cells reached their maximum density between 60-80 h, under 1% m-xylene and at 30°C and pH 6.8 and decreased after that. The initial increase in density within the first 60 h can be attributed to the presence of sufficient amount of m-xylene, which provides a good substrate for the growth of these cells at this favorable pH and temperature. With the depletion in m-xylene as a substrate, the growth of these cells stalls, hence the subsequent decrease in the density of the cells that was observed after 100 h of incubation. The growth is almost completely inhibited at 45°C. This is consistent with fact that most bacteria do not grow well at temperatures much higher than 37°C, with the exception of thermophiles. Moreover, it can be seen that the cells of these isolates also grow best at a pH near neutral compared to acidic or basic pH (5.5 or 8.5). Our findings are similar to previous investigations which reported that these factors exhibit similar effects on growth rates of various bacterial species utilizing aromatic hydrocarbons (Leahy and Colwell, 1990; Brusseau, 1998; Andreoni and Gianfreda, 2007; Higashioka *et al*., 2011; Hassanshahian *et al*., 2010; Das and Chandran, 2011; Alrumman *et al*., 2015). Taken together, it can be inferred from these results that the cells of these four isolates adapted and grew very well under 1% m-xylene at 30°C and pH 6.8, indicating that these conditions are the most relevant and favorable parameters. These findings also suggest that these factors combined play a key role in diversity and enrichment of bacterial strains with enhanced efficiency at

degrading m-xylene. Therefore, for successful bioremoval of m-xylene from soil sites, these factors must be taken into consideration during the process of bioremediation under both *in situ* or *ex situ* conditions.

Globally, it has been documented in literature that variations and changing in the patterns of the microbial populations densities, quantity, microbial activities and heterogeneities have been observed among various petroleum polluted sites due to various physicochemical factors (MacNaughton *et al*., 1999; Ghazali *et al*., 2004; Yakimov *et al*., 2004; Hassanshahian *et al*., 2010; Das and Chandran, 2011; Staats *et al*., 2011; Higashioka *et al*., 2011; Andrade *et al*., 2012; Alrumman *et al*., 2015). According to these peer-reviewed articles, these variations in microbial population density and diversity were also affected by the levels of petroleum contamination, suggesting that highly polluted areas appear to have a greater quantity of these microbes. Variations could be attributed to adaptation of these bacterial species to utilize and flourish in the presence of crude oil or might be partly due to variations among several physicochemical environmental factors, including differing ambient environmental conditions, soil compositions and organic carbons in the soil. Moreover, the capabilities of degrading aromatic hydrocarbon by bacteria are showed some limitations and hence, no single species of bacteria can degrade all the components of petroleum (Ghazali *et al*., 2004; Andreoni and Gianfreda, 2007; Das and Chandran, 2011; Andrade *et al*., 2012; Alrumman *et al*., 2015). Many different bacterial species are usually needed for complete biodegradation of all the components of petroleum. Leahy and Colwell (1990) reported that the lighter fractions of the crude oil (short chain of alkanes) are the first to be degraded by bacteria. These lighter fractions are considered an easily accessible energy source and their presence or abundance might result in an increase in the number of the crude oil degrading bacteria. Later, these bacteria could develop the ability to utilize the more complex compounds, including aromatic compounds, like xylene, benzene, toluene and others.

Major limitations of this study are the lack of data about other environmental factors, including salinity of soil, availability of organic compounds and nutrients (especially those of nitrogen and phosphorus), presence of heavy metals and others. These factors are also important for the growth and utilization of m-xylene by these isolates. Nevertheless, the influence of these factors on the growth rate and extent of biodegradation cannot be easily predicted and sometimes are difficult to control.

CONCLUSION

In conclusion, to the best of our knowledge, we reported here in the Northern part of Jordan for the first time the isolation and identification of four novel bacterial isolates from gasoline-contaminated soil sites around gas stations with the capability to grow with m-xylene as the sole source of carbon and energy. These isolates were designated as isolates X1-X4. On the basis of biochemical and molecular analyses, these new isolates were identified as *Pseudomonas aeruginosa* (X1), *Pseudomonas stutzeri* (X2) and *Bacillus firmus* (X4). The isolate X3 seems to represent a novel species of the genus *Citrobacter* that closely resemble *Citrobacter amalonaticus.* These 4 species appeared to contribute to removal of m-xylene from soil contaminated by gasoline. The growth rates for these isolates were affected by concentration of m-xylene, the temperature and pH of the medium.

In the light of these findings more studies on the mechanisms of m-xylene biodegradation by these new isolates are required. More importantly, the beneficial effect of these four isolates in the bioremoval of m-xylene from gasoline contaminated areas must be examined under *in situ* conditions.

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