ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (4): 630-640, 2008
ISSN 1682-296X
© 2008 Asian Network for Scientific Information

Biodegradation of Monocyclic Aromatic Hydrocarbons by a Newly Isolated *Pseudomonas* strain

[1]Ranya A. Amer, [1]Mahmoud M. Nasier and [2]Ehab R. El-Helow
[1]Department of Environmental Biotechnology, GEBRI,
Mubarak City for Scientific Research and Technology Application, Alexandria, Egypt
[2]Department of Botany, Faculty of Science, Alexandria University, Egypt

Abstract: An aromatic hydrocarbon utilizing bacterium was isolated from Egyptian sewage treatment plant, it was identified as *Pseudomonas* sp. strain H12 and it showed 98% similarity to *16S rDNA* genes of *Pseudomonas meridiana* and *Pseudomonas antarctic*a. It has been never reported that these strains have any relation to hydrocarbon degradation. On the other hand, the isolated *Pseudomonas* sp. strain H12 has the ability to remove 85-90% of BTXHB mixture (benzene, toluene, xylene, hexyl-benzene and butyl-benzene) within 24 h. The results strongly suggest that the experimental bacterium has a TOL like-plasmid and carries *tmoA*, *xylM* and *xylA* involved in the expression of catechol-forming mono-oxygenases and *xylE1* which expresses a catechol dioxygenase. Thus, the catabolic pathway of an aromatic hydrocarbon by *Pseudomonas* sp. H12 is possibly initiated by only the activity of a mono-oxygenase system followed by a di- oxygenase catechol ring cleavage reaction.

Key words: *Pseudomonas*, aromatic hydrocarbons, bioremediation, catabolic genes, biodegradation

INTRODUCTION

Aromatic hydrocarbons such as benzene, toluene, ethyl-benzene and xylene (BTEX) are classified as environmental priority pollutants. They are volatile mono-aromatic hydrocarbons commonly found together in crude petroleum and petroleum products such as gasoline and diesel fuel (Budavari, 1996). BTEX enter the environment through processes associated with gasoline and petroleum fuels, leakage of underground petroleum storage tanks, spills at petroleum wells and also from industrial effluents. These hydrocarbon compounds are also produced on the large scale as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics, etc. (Budavari, 1996; Clavalca *et al*., 2000; Nicholson and Fathepure, 2004; Mattison *et al*., 2005; Andreoni and Gianfreda, 2007).

It has been reported by the US EPA (Environmental Protection Agency) that there are many sites polluted with BTEX (USEPA, 1997). BTEX are highly soluble and volatile toxic substances and they are the most soluble components of bulk gasoline. Due to their toxicity and ability to bioaccumulate through the food chain, their presence in the environment is hazardous to public health and has an ecological concern (Brigmon *et al*., 2002). It has been reported by Pruden *et al*. (2003) that benzene is a human carcinogen and Bielefedt and Stensel (1999a) suggested that aromatic hydrocarbons are present in nature as a mixture, which could cause human toxicity or ground water pollution. Thus, it is important to develop methods to accelerate the removal of these volatile components from contaminated environments. Bioremediation is one of the useful techniques that can be applied for such pollution problems (Taki *et al*., 2007).

The key steps of aerobic BTEX biodegradation begin with initial oxidative attack to the hydrocarbon mixture converting the compound to catechol structure followed by ring cleavage of the catechol (Hendrickx *et al*., 2006). The initial oxidative attack consists of a direct oxidation of the aromatic ring via a mono-oxygenase (Khan *et al*., 2001) or a dioxygenase (Zylstra and Gibso, 1989; Fruukawa *et al*., 1993) or the oxidation of the alkyl side chain, which is catalyzed by mono-oxygenases (Williams and Sayers, 1994; Gibson and Parales, 2000). Each of these pathways result in the formation of a catechol intermediate that undergoes ring cleavage by catechol

Corresponding Author: Ranya Aly Amer, Department of Environmental Biotechnology, GEBRI,
Mubarak City for Scientific Research and Technology Application, P.O. Box 21934, Alexandria, Egypt
Tel: (203) 4593420 Fax: (203)4593407

2,3-dioxygenase (C23O), after which the structure is further degraded into Krebs cycle intermediates (Harayama and Rekik, 1993; Reineke, 1998).

In the present study, we isolated and identified a bacterium from a polluted sewage treatment plant capable of *in situ* degradation of BTXHB (benzene, toluene, xylene, hexyl-benzene and butyl-benzene). Furthermore, the responsible catabolic enzymes were approached by examining the ability of specific primer sets to PCR amplify corresponding genes from the DNA of the new isolate.

MATERIALS AND METHODS

Media composition: Unless otherwise indicated, medium used through this work was mineral medium composed of the following salts (g L^{-1}): $MgSO_4.7H_2O$, 0.02; $(NH_4)_2SO_4$, 0.1; NaCl, 0.01; $CaCl_2$, 0.01; K_2HPO_4, 0.45; $FeCl_3$, 0.002. The initial pH was adjusted to 7.0. Agar was added in a concentration of 15 g L^{-1} to prepare plates. The medium was sterilized at 121°C for 15 min. After autoclaving, hydrocarbons were added in mixtures or individually according to the experiment as the sole carbon source(s) and inoculated with a 1% of overnight bacterial seed cultures growing on LB. The cultures were incubated as described below.

Chemicals and standards: Benzene, toluene, butyl-benzene, hexyl-benzene, m-xylene and n-pentane were purchased from Sigma-Aldrich. All medium components were purchased from Fluka Chemie or Sigma Aldrich Chemie.

Sample collection and isolation of hydrocarbon degrading bacteria: For isolation of hydrocarbon-degrading bacterial strains, sludge samples were collected in summer of 2005 from western Alexandria sewage treatment plant, Egypt. The enrichment culture technique was carried out in a 250 mL conical flask by using 0.5 mL sludge to inoculate 50 mL mineral medium supplemented with 250 μL BTXHB, which is equivalent to approximately 4.3 g L^{-1} from each of the five aromatic hydrocarbons (benzene, toluene, hexyl-benzene, xylene and butyl-benzene) individually. The flasks were incubated at 30°C for 24 h at 200 rpm. Mineral agar plates were inoculated with 150 μL inoculum size from flasks that showed turbidity. Hydrocarbons were individually added on the inner-side of the lids in a concentration of 2.6 g L^{-1} of BTXHB (Junca and Pieper, 2003). Plates were incubated at 30°C for 5 days in an inverted position to allow the diffusion of the vapor upwards through the media. Growing colonies were picked up for further purification and screening process.

Cultivation and growth monitoring: Unless otherwise indicated, 50 mL mineral medium supplemented with hydrocarbon(s) as a sole carbon source at the desired concentration, as indicated in each experiment, was inoculated with 0.5 mL overnight culture. The culture was incubated at 30°C with agitation at 200 rpm on a rotary shaker. The growth was monitored by counting the cfu on LB plates using suitable dilutions or by measuring the optical density of the culture (Plaza *et al*., 2007) using PerkinElmer UV/V spectrometer Lambda EZ 201 at 600 nm.

Biochemical identification of the isolated strain: The isolate, which grew well in the presence of the mixture of aromatic hydrocarbons, was identified biochemically following Bergy's Manual determinative bacteriology (Krieg and Holt, 1984). Hemolytic activity was carried out (Carrillo *et al*., 1996) using blood agar plates containing 5% v/v blood with an incubation period of 24-48 h at 30°C. β-Hemolytic activity was detected by formation of a clear zone around the colony.

Plasmid curing and plasmid mini-prep: Plasmid curing was carried out as described by Rusanky *et al.* (1987) using 25-250 μg mL^{-1} Acridine Orange as a curing agent. Plasmid mini-prep was performed (Sambrook *et al*., 1989) for wild type and cured strains to insure the efficiency of curing experiment.

PCR amplification of 16S rDNA and catabolic genes: Total genomic DNA was extracted from cells of 5 mL LB overnight bacterial culture as described by Sambrook *et al.* (1989). PCR was performed in a light cycler Eppendorf PCR machine. The primers used in this study are shown in Table 1. A 1300 bp fragment was obtained by PCR amplification of the *16S rDNA* gene in a 50 μL reaction mixture containing around 100 ng of purified strain DNA (Ausubel *et al*., 1999). The methods used for amplification of *xylM* (Baldwin *et al*., 2003), *tmoA*, *xylA*, *xylE1*, *todC1* and *todE* (Hendrickx *et al*., 2006) were applied with some modifications. The reactions were carried out as follow: 5 min at 95°C, 35 cycles for 1 min at 94°C, 2 min at the temperatures indicated in Table 1, 2 min at 72°C and 10 min at 72°C. To amplify *tmoA*, *todE* and *todC1*, the annealing temperature was reduced to 1 min.

Amplicons of *16S rDNA*, *xylM* and *xylE1* were purified using PCR purification kit (Quigen). Each of these purified products was sequenced by the chain terminator method (ABI 3130XL system, DNA technology, Denmark) using the two corresponding PCR primers separately. The resulted DNA sequences of *16S rDNA*, *xylM* and *xylE1* were phylogenetically analyzed using the BLAST search

Table 1: The primer sets used

Primer pair		Sequence	Target	Annealing temperature	Amplicon size (bp)	Reference
Bact16-	F	5'-AGAGTTTGATCMTGGCTCAG'	Eubacterial *16S rDNA* gene	55	1300	Ausubel *et al.* (1999)
	R	5'-TACGGYACCTTGTTACGACTT'				
TMOA-	F	5'-CGAAACCGGCTT(C/T)ACCAA(C/T)ATG	(*tmoA*) gene coding for subfamily 2 of α-subunits of hydroxylase component of multi-component mono-oxygenases	61.2	505	Hendrickx *et al.* (2006)
	R	5'-ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA				
TOL-	F	5'-TGAGGCTGAAACTTTACGTAGA	(*xylM*) subfamily 5 of hydroxylase component of two-component side chain mono-oxygenases	55	475	Baldwin *et al.*
	R	5'-CTCACCTGGAGTTGCGTAC				
XYLA-	F	5'-CCAGGTGGAATTTTCAGTGGTTGG	(*xylA*) gene coding for electron transfer component of two component side chain mono-oxygenases	64	291	Hendrickx *et al.* (2006)
	R	5'-AATTAACTCGAAGCGCCCACCCCA				
TODC1-	F	5'-CAGTGCCGCCA(C/T)CGTGG(C/T)ATG	(*todC*) gene coding for subfamilies D.1.B+D.1.C+D.2.A+D.2.B+D.2.C of α-subunits of type D iron-sulfur multi-component aromatic di-oxygenases	66	510	Hendrickx *et al.* (2006)
	R	5'-GCCACTTCCATG(C/T)CC(A/G)CCCCA				
XYLE1-	F	5'-CCGCCGACCTGATC(A/T)(C/G)CATG	(*xylE1*) gene coding for subfamily I.2.A of catechol extradiol di-oxygenases	61.5	242	Hendrickx *et al.* (2006)
	R	5'-TCAGGTCA(G/T)CACGGTCA(G/T)GA				
TODE-	F	5'-GGATTTCAAACTGGAGACCAG	(*todE*) gene coding for subfamily I.3.B of catechol extradiol Di-oxygenases	58	246	Hendrickx *et al.* (2006)
	R	5'-GCCATTAGCTTGCAGCATGAA				

program and sequences are deposited in GeneBank under accession numbers EU258472, EU258473 and EU304454, respectively.

GC analysis of aromatic hydrocarbons: Aromatic hydrocarbons were extracted from cultures by n-pentane:media (1:5) (Bielefeldt and Stensel, 1999a, b). The extracted hydrocarbons were injected with standards to a GC Shimadzu GC-17A, Autosampler Shimadzu C-20i using RTX-5 column (length 15 m, internal diameter 0.25 mm and film thickness 0.26 μm), linear velocity was 49 cm sec^{-1} and nitrogen was used as a carrier gas at a flow rate of 2 mL min^{-1}. The detector and injector temperatures were 250°C and the oven temperature was maintained at 150°C.

RESULTS AND DISCUSSION

Screening and selection of bacterial isolate capable of BTXHB degradation: A total of 58 hydrocarbon degrading bacterial isolates were picked up from enrichment of a sludge sample in liquid medium. Bacterial isolation and purification were then carried out on mineral medium plates containing approximately 2.6 g L^{-1} of BTXHB and incubated for 5 days. The highest number of isolates was obtained on the two alkyl-benzene compounds including hexyl-benzene followed by butyl-benzene (16 and 13 isolates, respectively), while only 5 isolates were obtained on toluene. Secondary and tertiary screening procedures were performed to select the most potent isolate among grown colonies.

The selected isolates were allowed to grow in the liquid mineral medium containing equal amounts of hydrocarbons (benzene, toluene, xylene hexyl-benzene and butyl-benzene) as sole carbon source at a total concentration of 4.3 g L^{-1}. The Optical Density (OD) was recorded every 24 h for three days. As shown in Fig. 1, most of the cultures achieved their maximum growth within the first 24 h. Afterwards, the ODs decreased as the incubation time increased, possibly as a result of the bacteria entering the decline phase. As shown in Fig. 1, the experimental isolate H12 achieved the highest optical density (0.28) within 24 h of incubation in the presence of the introduced hydrocarbon mixture. Therefore, H12 was selected for further investigations.

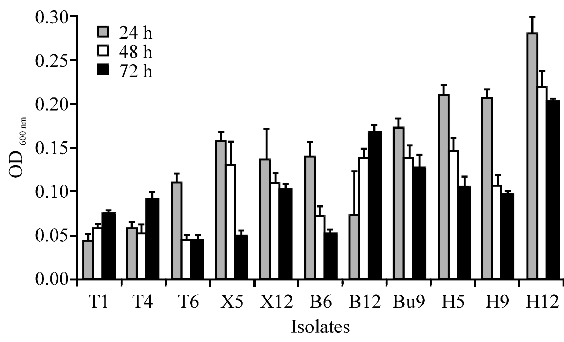


Fig. 1: Growth (OD at 600 nm) of the selected isolates on mineral media containing 4.3 g L^{-1} of BTXHB after incubation periods of 24, 48 and 72 h

Characterization and identification of the selected hydrocarbon degrader: The most potent BTXHB degrader isolate, H12, was subjected to morphological, biochemical as well as molecular characterization studies. According to Bergy's Manual for biochemical identification (Krieg

and Holt, 1984), H12 was characterized as a gram-negative, rod-shaped and expressed glucose and mannitol fermentation, nitrate reduction and catalase activity phenotypes. The isolate did not show any growth on starch or gelatin. No clear zones were observed around isolate colonies grown on blood agar plates reflecting the absence of α- as well as β-haemolytic activities. An association between bacterial hemolytic activity and surfactant production has been previously reported (Carrillo *et al*., 1996; Youssef *et al*., 2004) and thus, blood agar lysis was recommended as a primary method to screen for biosurfactant activities. Accordingly, it can be concluded that H12 does not express biosurfactants.

Since biochemical characterization studies are not enough for species identification (Rainey *et al*., 1996), a *16S rDNA*-based molecular characterization step was performed. A fragment of 1300 bp of the *16S rDNA* gene was PCR amplified from the chromosomal DNA extract of H12 using bacterial universal primers. The PCR product was then purified and sequenced.

The obtained sequencing data were aligned against the *16S rDNA* sequences of the ribosomal database project (http://www.cme.msu.edu/RDP/html/index/html, Maidak *et al*., 1994; Rainey *et al*., 1996). The phylogenetic relationship between the experimental isolate and the closely related species were analyzed by using the multi-sequence alignment program (BioEdit Sequence Alignment Editor). The resulted phylogenetic tree, in which branch lengths were considered, is presented in Fig. 2. Based on this taxonomic relationship, the closest *16S rDNA* gene sequences are those of the genotypes *Pseudomonas meridiana* and *Pseudomonas antarctica* with 98% similarities. Therefore, the isolate H12 was designated as *Pseudomonas* sp. H12. Future molecular analyses of other evolutionary chronometers are required for full identification of this new isolate.

Evaluation of the potency of *Pseudomonas* sp. H12 in biodegradation of BTXHB individually: *Pseudomonas* sp. H12 was cultivated in mineral medium amended by BTXHB, individually and in a mixture, at 5 different concentrations (1.7, 2.1, 2.6, 3.0 and 4.3 g L^{-1}). The biodegradation efficiency of this strain was monitored by measuring the concentration of residual hydrocarbons extracted from the media with pentane. Figure 3 shows the bacterial growth efficiency in the presence of hydrocarbons introduced individually and in mixture. In the majority of the cases, bacterial biomass slightly increased with increasing hydrocarbon concentration. As

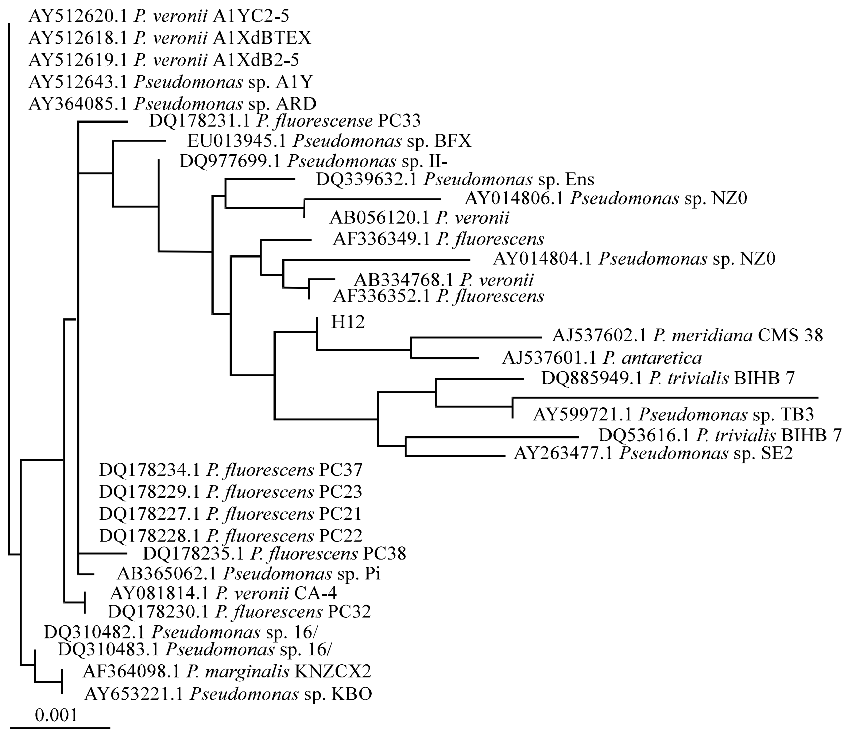


Fig. 2: Phylogenetic position of the isolate H12 based on partial sequencing of the *16S rDNA* gene

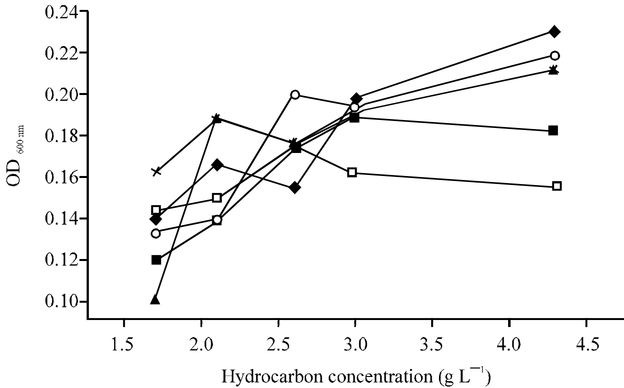


Fig. 3: Growth profile (OD at 600 nm after 24 h) of *Pseudomonas* sp. H12 in liquid minimal media containing different concentrations of benzene (■), toluene (♦), xylene (▲), hexyl-benzene (×) and butyl-benzene (○) individually or as a mixture (□) (BTXHB), each as a single carbon source

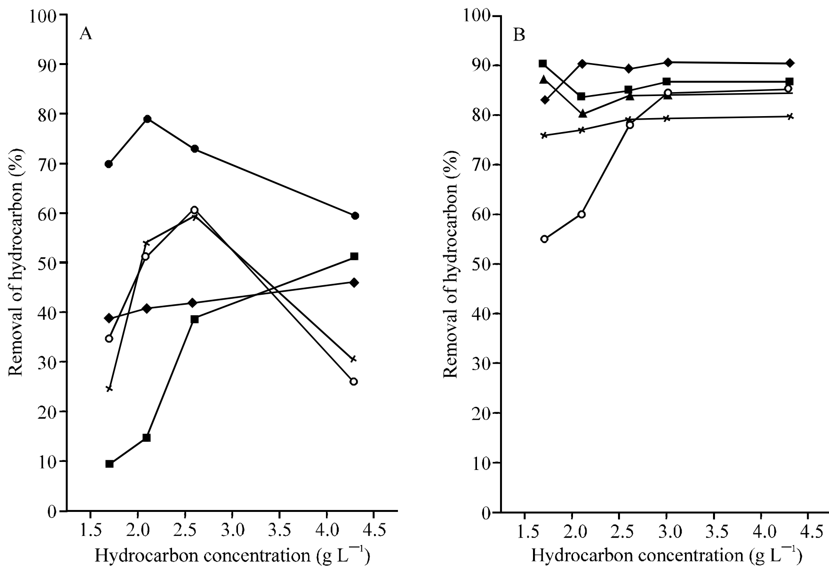


Fig. 4: Efficiency of hydrocarbon removal by *Pseudomonas* sp. H12 from liquid minimal media containing different concentrations of benzene (■), toluene (♦), xylene (▲), hexyl-benzene (×) and butyl-benzene (○) individually (A) or as a mixture (B) (BTXHB), each as a single carbon source. Cultures were incubated for 24 h

shown in Fig. 4a, the percentage of hydrocarbon removal was also affected by changing substrate concentration, nevertheless, with different patterns. The highest removal efficiency was achieved in the case of xylene (approximately 80% removal) at a concentration of 2.1 g L^{-1}. Similar patterns, but with lower removal efficiencies, were recorded by cultures growing on butyl-benzene and hexyl-benzene. The degradative response of the bacterium towards these two alkyl benzene compounds is almost the same, possibly due to their close structural similarity. Benzene removal was clearly slower than the other experimental hydrocarbons and approximately 50% removal was achieved at the highest examined concentration (4.3 g L^{-1}). On the other hand, increasing toluene concentration and the percentage of its removal showed almost a linear relationship with a slight positive effect.

In general, the observed order of degradation of individual BTXHB hydrocarbons by *Pseudomonas* sp. H12 was as follows: xylene> alkyl-benzene> toluene> benzene. To a great extent, these data are similar to those achieved by *Alcaligenes* BTEX cultures, which degraded benzene slower than xylene and toluene (Plaza *et al.*, 2007). Most of the enzymes involved in the degradation of BTXHB hydrocarbons pathways are the same (Hendrickx *et al.*, 2006); they convert substrates to

catechol as a common intermediate. Thus, it is possible that these observed variations in biodegradation efficiency are the result of diverse enzyme substrates.

Evaluation of the potency of *Pseudomonas* sp. H12 in biodegradation of BTXHB mixture: The introduction of hydrocarbons in the form of a BTXHB mixture to the *Pseudomonas* sp. H12 MM culture did not support bacterial growth more than their introduction individually. The BTXHB mixture resulted in an optimum bacterial growth at a concentration of 2.6 g L^{-1} (Fig. 3). However, according to the data presented in Fig. 4a, b, the removal percentages of all experimental hydrocarbons were markedly increased by their introduction as a BTXHB mixture. On contrary to these results, a mixture of bacteria isolated from petroleum contaminated soils degraded the components of BTEX individually faster than in a combined mixture (Plaza *et al*., 2007). The results shown in Fig. 4b suggested also that the removal efficiencies of most of the mixed BTXHB components were not markedly affected by varying the concentration. For instance, almost all the examined BTXHB concentrations showed toluene, xylene and benzene, removal efficiencies within the range 90-85%. These results, especially in the case of benzene, demonstrated that the order of hydrocarbon removal efficiency is altered compared to the order when each was introduced individually. Therefore, it could be suggested that variation in the behavior of *Pseudomonas* sp. H12 with various hydrocarbons is not mainly a result of different enzyme-substrate interactions but also due to the presence of related substrates at the same time, which could enhance the enzymatic system involved in the biodegradation. Therefore, simultaneous presence of the whole mixture of the substrates in the surrounding environment generally encouraged their corresponding catabolic pathways in the bacterium.

The influence of environmental factors and plasmid curing on BTXHB degradation by *Pseudomonas* sp. H12: The results of complimentary degradation experiments revealed that pH 7 and a temperature of 30°C are optimum for the growth of *Pseudomonas* sp. H12 on BTXHB (data not shown). It has been found also that within an examined range of shaking-speed (100-200 rpm), the more the rounds per minute the more the biodegradation efficiency.

H12 cells were subjected to plasmid curing by Acridine Orange, no growth was observed in the presence of Acridine Orange higher than 150 μg mL^{-1}. Colonies were picked out from plates appeared in the presence of 150 μg mL^{-1} Acridine Orange and examined in comparison to wild type on mineral medium containing

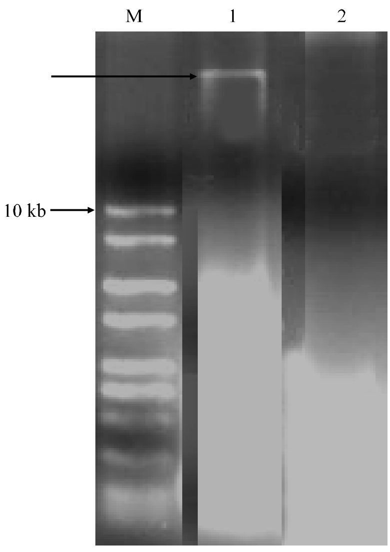


Fig. 5: Agarose gel electrophoresis (1.5 %) of plasmid DNA isolated from *Pseudomonas* sp. H12. in Lane 1, high molecular weight marker; Lane 2, wild type strain and lane 3, the cured strain

4.3 g L^{-1} hydrocarbons mixture. After incubation for 48 h under optimum conditions, low optical densities were recorded for cured cells (0.0013±0.00025), on the other hand, wild type showed good growth expressed by 0.158±0.0052 OD. In addition, a plasmid mini-prep experiment was carried out to verify the plasmid loss in the cured strain. Figure 5 demonstrated the loss of plasmids in the cured strain after exposure to 150 μg mL^{-1} Acridine Orange, while wild type strain showed the presence of high molecular weight plasmid. Jussila *et al*. (2007) previously reported that *Pseudomonas* strains harbor different types of TOL-plasmids with varying molecular weights ranging from 20-200 kb, were responsible for catabolic pathways of aromatic hydrocarbons. Present results suggest that plasmid borne-genes are essential for BTXHB degradation by *Pseudomonas* sp. H12.

Detection of genes involved in the BTXHB degradation by *Pseudomonas* sp. H12: A broad range of oxygenases, differing in structure, mechanism and cofactor requirements, were distributed among microorganisms (Andreoni and Gianfreda, 2007). For instance, the toluene biphenyl family include enzymes that interact with toluene (*tod* genes), benzene (*bed* genes), isopropyl benzene (*bph* genes), chlorobenzene (*tcb* genes) and biphenyl (*bph* genes) and are expressed by both Gram-negative and Gram-positive bacteria (Gibson and Parales, 2000; Baldwin *et al*., 2003). Phylogenetic studies of amino acid

sequences suggested that the proteins involved could be divided into specific families and subfamilies showing significant sequence homology and indicating a common ancestry. This allowed the design of group-specific primer sets for detection of their corresponding genes by PCR (Baldwin *et al*., 2003). The distribution of these genes in a set of BTEX degrading bacterial isolates and total community DNA from subsurface soil samples of a BTEX contaminated site was explored by Hendrickx *et al*. (2006). Aerobic BTEX biodegradation involves an initial oxidative attack of the aromatic hydrocarbon converting it to a catechol structure followed by ring cleavage of the catechol (Hendrickx *et al*., 2006). We examined the DNA extract of *Pseudomonas* sp. H12 for PCR amplification using primer sets recently reported for PCR detection of genes responsible for these two key steps of biodegradation (Hendrickx *et al*., 2006). Thus, the first group-specific primers included the primer pairs TMOA, TOL, XYLA and TODC1, which target genes encoding catechol-forming mono-and di-oxygenases; while the second group included XYLE1 and TODE, which are specific for genes encoding catechol di-oxygenases (Table 1).

Except for the TODC1 primer pair, all primer sets from the first group detected the corresponding catabolic gene sequences in the DNA of H12 isolate (Fig. 6). These results suggest that the H12 isolate lacks a *P. putida todC* counterpart, which encodes the α-subunit of a multi-component aromatic dioxygenase (Hendrickx *et al*., 2006). However, the PCR amplifications performed in the presence of the primer pairs TMOA, TOL and XYLA yielded products of the expected sizes (505, 475 and 291 bp, respectively). These observations demonstrated that the experimental bacterium carries the genes *tmoA*,

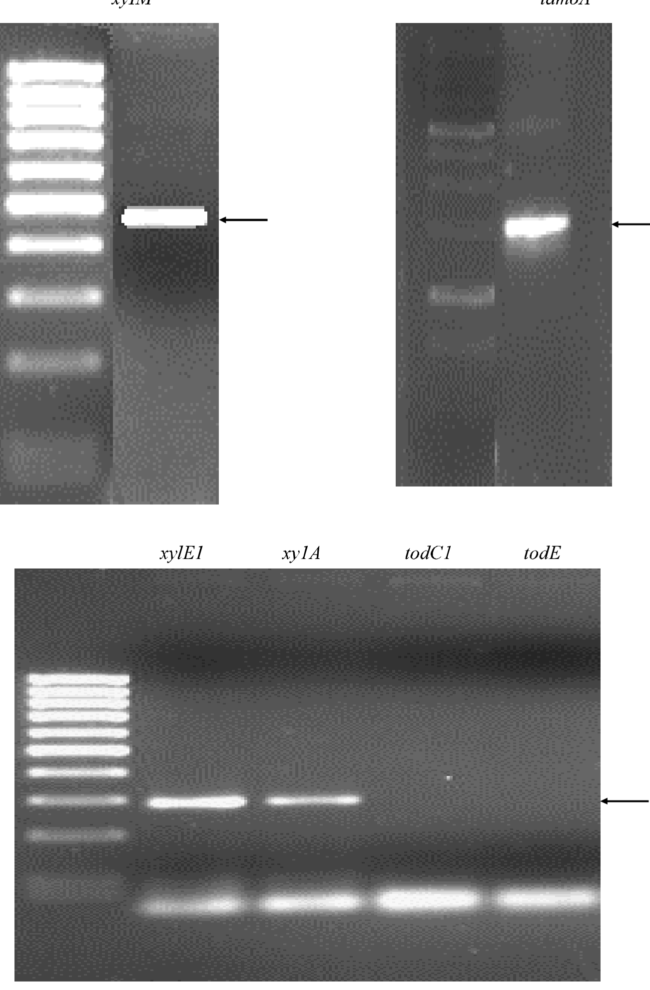
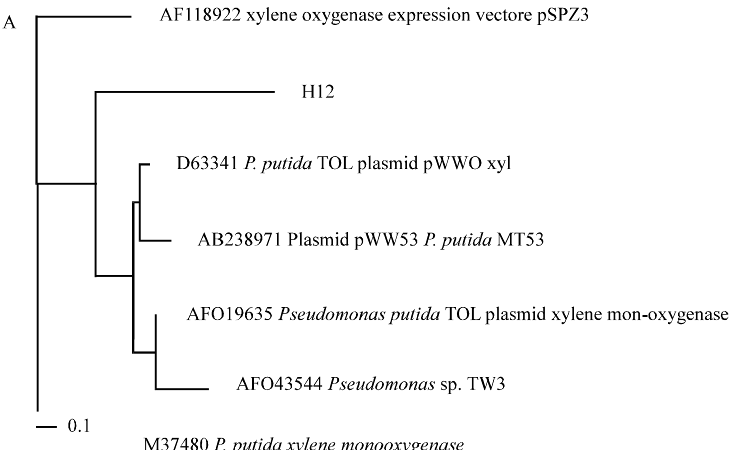


Fig. 6: PCR products of the indicated catabolic genes obtained from DNA extract of *Pseudomonas* sp. H12 using the primer sets specific for hydrocarbon oxygenases and presented in Table 1. The fist lane in each gel has a 100 bp ladder. The arrows indicate expected size fragments (Table 1)



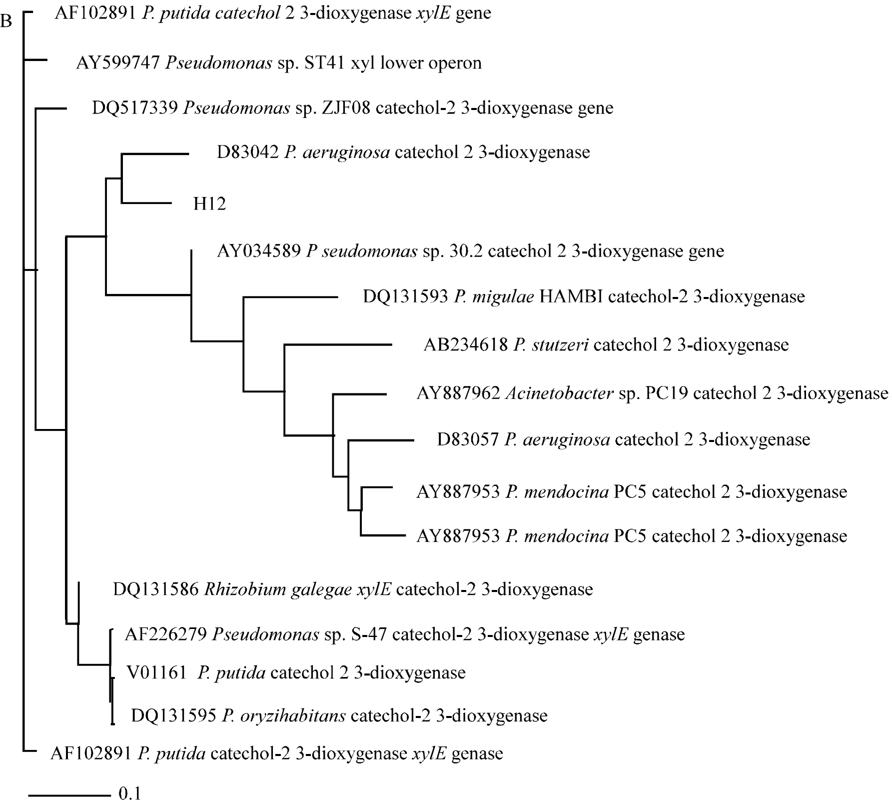


Fig. 7: Phylogenetic analyses of the aromatic mono-oxygenase gene *xylM* (A) and the large subunit of ring-hyroxylating catechol 2,3-dioxygenase gene *xylE1*(B)

xylM and *xylA*, which are involved in the expression of the initial mono-oxygenase system that attacks aromatic hydrocarbons. According to present results, it can be concluded that the catabolic pathway of BTXHB by *Pseudomonas* sp. H12 is possibly initiated by a mono-oxygenase system rather than by a di-oxygenase.

To further support the results achieved by PCR, the product amplified by the TOL primer set, which is likely a *xylM* allele, was arbitrary chosen for nucleotide sequencing. Phylogenetic analysis of the obtained sequence showed 99% identity with *Pseudomonas putida* mono-oxygenase genes. These included genes of a hydroxylase component of a mono-oxygenase carried by

TOL plasmid (*xylM*), a xylene mono-oxygenase electron transfer component (*xylA*) and other mono-oxygenase genes in. The obtained sequence was deposited in the GeneBank with the accession number EU258473 and its phylogenetic relationships with closely related sequences are presented in Fig. 7a.

It has been reported that BTEX side chain mono-oxygenases are two-component enzyme systems consisting of a terminal hydroxylase component encoded by the *xylM* gene and an electron transfer component encoded by the *xylA* gene (Hendrickx *et al*., 2006). Based on molecular plasmid diversity studies, it was found that *xylA* and *xylM* genes of some *Pseudomonas* strains are always linked on TOL plasmids (Shaw and Harayama, 1992; Sentchilo *et al*., 2000). Moreover, TOL-like *xyl* genes were previously detected in other *Pseudomonas* genomes (Greated *et al*., 2002; Keil *et al*., 1985). However, no *xylA* PCR amplicon was obtained from *P. putida* MT15, although it carries the pWW15 TOL plasmid and a *xylM* PCR amplicon was recovered using the TOL primer set (Hendrickx *et al*., 2006). The results of the present study demonstrated that plasmid curing reduced markedly the efficiency of the experimental strain to grow on a BTXHB-based medium. According to these results and the above mentioned previous reports, it is likely that the cells of the *Pseudomonas* H12 strain carry the two genes *xylA* and *xylM* linked on a TOL-like plasmid.

With respect to the second group of examined primer pairs, which target genes encoding catechol di-oxygenases, the sequence corresponding to XYLE1 (*xylE1*) was successfully amplified (Fig. 6). However, no amplicon was achieved in the PCR reaction with TODE primer pair suggesting the absence of a *todE* gene in the *Pseudomonas* H12 genome. The obtained PCR product was sequenced and deposited in the Genebank with the accession number EU304454. A phylogenetic tree of the sequence is shown in Fig. 7b. Computational analysis of this nucleotide sequence showed 97% identity with catechol 2,3 dioxygenase genes (*xylE1*) of some *Pseudomonas* sp. Similar to our results, it has been previously reported that *xylE1* is often combined with *xylM* in BTEX degrading isolates (Hendrickx *et al*., 2006).

CONCLUSION

A newly isolated strain from water treatment plant in Egypt was identified as *Pseudomonas* sp. H12. We demonstrated that this strain is highly efficient in degrading hydrocarbon mixture BTXHB The degradation efficiency was found to be higher when hydrocarbons are supplemented in mixture rather than individually. We also demonstrated that the biodegradation pathway is controlled by genes of a TOL-like plasmid. Possibly, the catabolic pathway is initiated by the activity of a mono-oxygenase system, which converts the aromatic hydrocarbon into a catechol structure followed by catechol ring cleavage by a di-oxygenase.

ACKNOWLEDGMENT

We would like to thank Dr. Hany Hussein for providing us with soil samples and Eng. Mohamed Fahmy for his contribution in all GC measurements.

REFERENCES

Andreoni, V. and L. Gianfreda, 2007. Bioremediation and monitoring of aromatic-polluted habitats. Applied Microbiol. Biotechnol., 76: 287-308.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidam, J.A. Smith and K. Struhl, 1999. Short Protocols in Molecular Biology. 4th Edn. John Wiley and Sons, Inc. New York .

Baldwin, B.R., C.H. Nakatsu and L. Nies, 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Applied Environ. Microbiol., 69: 3350-3358.

Bielefeldt, A.R. and H.D. Stensel, 1999a. Evaluation of biodegradation kinetic testing methods and long term variability in biokinetics for BTEX metabolism. Water Res., 33: 733-740.

Bielefeldt, A.R. and H.D. Stensel, 1999b. Modeling competitive inhibition effects during biodegradation of BTEX mixtures. Water Res., 33: 707-714.

Brigmon, R.L., D. Camper and F. Stutzenberger, 2002. Bioremediation of Compounds Hazardous to Health and the Environment-An Overview. In: Biotransformation: Bioremediation Technology for Health and Environment Protection, Singh, V.P. and R.D. Stapleton (Eds.). Elsevier Science Publishers, The Netherlands, pp: 1-28. ISBN 0-444-50997-6

Budavari, S., 1996. The Merck Index: An Encyclopedia of Chemicals, Drugs and Biologicals. 12th Edn. Merck and Co. Inc., Whitehous Station, NJ .

Carrillo, P.G., C. Mardaraz, S.J. Pitta-Alvarez and A.M. Giulietti, 1996. Isolation and selection of biosurfactant-producing bacteria. World J. Microbiol. Biotechnol., 12: 82-84.

Clavalca, L., P.D. Gennaro, M. Colombo, V. Andereoni, S. Bernasconi, I. Ronco and G. Bestetti, 2000. Distribution of catabolic pathways in some hydrocarbon-degrading bacteria from subsurface polluted soil. Res. J. Microbiol., 151: 877-887.

Fruukawa, K., J. Hirose, A. Suyama, T. Zaiki and S. Hayashida, 1993. Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). J. Bacteriol., 175: 5224-5232.

Gibson, T.G. and E.R. Parales, 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol., 11: 236-243.

Greated, A., L. Lambertsen, P.A. Williams and C.M. Thomas, 2002. Complete sequence of the IncP-9 TOl plasmid pWWO from *Pseudomonas putida*. Environ. Microbiol., 4: 856-871.

Harayama, S. and M. Rekik, 1993. Comparison of the nucleotide sequences of the meta-cleavage pathway genes of TOL plasmid pWWO from *Pseudomonas putida* with other meta-cleavage genes suggests that both single and multiple nucleotide substitutions contribute to enzyme evolution. Mol. Genet. Genomics, 239: 81-89.

Hendrickx, B., H. Junca, J. Vosahlova, A. Lindner, I. Röegg, M. Bucheli-Witschel, F. Faber, T. Egli, M. Mau, M. Schlömann, M. Brennerova, V. Brenner, D.H. Pieper, D.H., E.H. Top, W. Dejonghe, L. Bastiaens and D. Springael, 2006. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: Distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J. Microbiol. Methods, 64: 250-265.

Junca, H. and D.H. Pieper, 2003. Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. J. Microbiol. Methods, 55: 697-708.

Jussila, M.M., J. Zhao, L. Suominen and K. Lindström, 2007. TOL plasmid transfer during bacterial conjugation *in vitro* and rhizoremediation of oil compounds *in vivo*. Environ. Pollut., 46: 510-524.

Keil, H., S. Keil, R.W. Pickup and P.A. Williams, 1985. Evolutionary conservation of genes coding for meta pathway enzymes within TOL plasmids pWWO and pWW53. J. Bacteriol., 164: 887-895.

Khan, A.A., R.F. Wang, W.W. Cao, D.R. Doerge, D. Wennerstrom and C.E. Cerniglia, 2001. Molecular cloning, nucleotide sequence and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. strain PYR-1. Applied Environ. Microbiol., 67: 3577-3585.

Krieg, N.R. and J.G. Holt, 1984. Bergy's Manual of Systematic Bacteriology. Vol. 1, Williams and Wilkins Co., Baltimore. .

Maidak, B.L., N. Larsen, M.J. McCaughey, R. Overbeek, G.J. Olsen, K. Fogel, J. Blandy and C.R. Woese, 1994. The ribosomal database project. Nucleic Acids Res., 22: 3485-3487.

Mattison, R.G., H. Taki and S. Harayama, 2005. The soil flagellate *Heteromita globosa* accelerates bacterial degradation of alkyl-benzenes through grazing and acetate excretion in batch culture. Microb. Ecol., 49: 142-150.

Nicholson, C.A. and B.Z. Fathepure, 2007. Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. Applied Environ. Microbiol., 70: 1222-1225.

Plaza, G.A., J. Wypych, C. Berry and R.L. Brigmon, 2007. Utilization of monocyclic aromatic hydrocarbons individually and in mixture by bacteria isolated from petroleum contaminated soil. World J. Microbiol. Biotechnol., 23: 533-542.

Pruden, A., M.A. Sedran, M.T. Suidan and A.D. Venosa, 2003. Biodegradation of MTBE and BTEX in an aerobic fluidized bed reactor. Water Sci. Tech., 47: 123-128.

Rainey, F.A., N.W. Rainey, R.M. Kroppenstedt and E. Stackebrandt, 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage. Int. J. Syst. Bacteriol., 46: 1088-1092.

Reineke, W., 1998. 1998 Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. Ann. Rev. Microbiol., 52: 287-331.

Rusanky, S., R. Avigad, S. Michaeli and D. Gutnick, 1987. Involvement of a plasmid in growth on a dispersion of crude oil by *Acinetobacter calcoaciticus* RA57. Applied Environ. Microbiol., 53: 1918-1973.

Sambrook, J., E.F. Fritsch and T.A. Maniatis, 1989. Molecular Cloning: A laboratory Manual. 2nd Edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp: 136-147.

Sentchilo, V.S., A.N. Perebituk, A.J.B. Zehnder and J.R. van der Meer, 2000. Molecular diversity plasmids bearing genes that encode toluene and xylene metabolism in *Pseudomonas* strains isolated from different contaminated sites in Belarus. Applied Environ. Microbiol., 66: 2842-2852.

Shaw, J.P. and S. Harayama, 1992. Purification and characterization of NADH: Acceptor reductase component of xylene mono-oxygenase encoded by the TOL plasmid pWWO of *Pseudomonas putida* mt-2. Eur. J. Biochem., 209: 51-61.

Taki, H., K. Syutsubo, R.G. Mattison and S. Harayama, 2007. Identification and characterization of o-xylene-degrading *Rhodococcus* spp. Which were dominant species in the remediation of o-xylene-contaminated soils. Biodegradation, 18: 17-26.

USEPA., 1997. Cleaning up the Nation's Waste Sites, Market and Technology Trends, 1996 Edition. EPA 542-R-96-005.EPA, Office of Solid Waste and Emergency Response, Washington, DC.

Williams, P.A. and J.R. Sayers, 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation, 5: 195-217.

Youssef, N.H., K.E. Duncan, D.D. Nagle, K.H. Savage, R.M. Knapp and McInerney, 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods, 56: 339-347.

Zylstra, G.J. and D.T. Gibson, 1989. Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the todC1 C2BADE genes and their expression in *Escherichia coli*. J. Biol. Chem., 264: 14940-14945.