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Original Research

Isolation of Novel Toluene Degrading Bacteria from Waste Water Treatment Plants and Determination of their Toluene Tolerance and other Biotechnological Potential

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Abstract

Toluene is an aromatic hydrocarbon and spreads to the environment by the release of petroleum products, agricultural and industrial activities and toluene can cause serious social and health problems. Since the removal in toluene is considered an important environmental issue, there is an increasing interest of toluene biodegradation. In this comprehensive study, novel toluene degrading bacteria were isolated and identified both from municipal and industrial wastewater treatment plants. Isolates' toluene tolerance, enzyme production, benzene, ethyl benzene, xylene and phenol degradation potentials and biofilm formation were examined. Totally 109 bacterial isolates were obtained and most of them were determined as belong to *Stenotrophomonas*, *Acinetobacter* and *Pseudomonas* species.

Isolates' toluene tolerance, enzyme production, benzene, ethyl benzene, xylene and phenol degradation potentials and biofilm formation were examined. Most of isolates showed lipase, DNase and protease activity. Most of isolates showed growth in 300 mg/l^{-1} concentrations of benzene, ethyl benzene, xylene and phenol. Strains E4T16, 3AT2 and 3ET5 showed maximum growth in high toluene concentrations and also strain E4T16 showed growth up to 2100mg/l^{-1} toluene. Examination of biofilm formation at different toluene concentrations at the end of the 96 hours incubation revealed that strains E4T16, 4ET21 and 3ET5 had highly biofilm production potential. In conclusion, these bacteria and results could be considered as a powerful new approach for the removal of hydrocarbons from wastewater.

Keywords: toluene, biodegradation, biofilm, enzyme, wastewater

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Introduction

Toluene is an aromatic hydrocarbon, also is a natural component of petroleum and coal and is an important component of gasoline. It's IUPAC name is Methylbenzene [1, 2]. Toluene is produced industrially in the production of various chemicals and also as a solvent. The major factors in the spread of toluene to the environment are the release of petroleum products, agricultural and industrial activities such as herbicides, pesticides, production paints, thinners, cigarette, polymers, automobile and car sprays and paintings [3-6].

Toluene can cause serious social and health problems [7]. It is moderately toxic when inhaled [8]. Continuous exposure to toluene may show mutagenic effects on cells [9]. Toluene is a well-known human neurotoxin and can cause leukoencephalopathy and also is known as carcinogens. It is also a threat to drinking water [7, 10].

For these reasons, the removal of toluene has become an important environmental issue. So, there is an increasing interest for utilizing microorganisms such as bacteria for removal of various aromatic compounds from the environment. Biodegradation with bacteria may play an important role in the reduction of such compounds. This technique is considered to be an effective technology for the treatment of pollution [11, 12].

Bacteria and fungi have developed several interesting pathways for toluene degradation. Under aerobic conditions, oxygen is used directly as a substrate and generally catechol and derivatives act as central metabolites. In the absence of oxygen, anaerobic toluene gradients carry out a unique reaction which adds fumarate to toluene [7]. Examples of toluene-degrading aerobic bacteria include genera such as *Pseudomonas* [13], *Acinetobacter* and *Rhodococcus* [14] and *Bacillus* [15]. Anaerobic degradation of toluene has been observed in denitrifying strains such as in some *Thauera aromatica* strains [16].

The aim of this study was to isolate and identify novel toluene-degrading bacteria from waste water treatment plants, and determination of their toluene tolerance with modified method and also other potential biotechnological properties of them such as enzyme production, other substances' degradation and biofilm formation. This information can serve to support further development, design and operation of new processes.

Materials and Methods

Sample Collection

Wastewater samples were taken from fat/sand removal unit, primary clarifiers unit, activated sludge, secondary clarifiers and return activated sludge pump units of a municipal wastewater treatment plant and an industrial wastewater treatment plant for 4 times in a year. All samples were obtained manually, placed in labelled plastic bottles and quickly transported to the laboratory.

Determination of BOD_5 and pH Values of Samples

BOD_5 values of the samples were measured manometrically (Oxitop) and pH was measured with pH meter (Mettler Toledo).

Isolation of Toluene Degrading Bacteria

Mineral salt (MS) medium was used to cultivate toluene degrading microorganisms from the samples. Mineral salt media includes; K_2HPO_4 2.75 g, KH_2PO_4 2.25 g, $(NH_4)_2SO_4$ 1.0 g, $MgCI_2.6H_2O$ 0.2 g, NaCI 0.1 g, $FeCI_3.6H_2O$ 0.02 g, $CaCI_2$ 0.01 g per liter distilled water.1 ml of each sample was taken separately and inoculated to liquid media. For toluene degradation, 3 mM toluene was added as the sole carbon and energy sources. At the end of 7 days incubation at 37ºC, 150 rpm, MS solid media were prepared with toluene and 15 g/l^{-1} agar and spread plate method was applied. The growing colonies were taken into pure culture and were kept at -85ºC in 20% glycerol stocks [17].

Nucleic Acid Extraction from Isolates

For extraction, Cifuentes et al., 2000; Nogales et al., 1999 and Singka et al., 2012 protocols were modified [18-20]. 200 milliliter of each sample was used for nucleic acid extraction. Samples were filtered through a 0.22-µm pore size GV filter (Millipore). 0.5 mm glass beads in equal volume and extraction buffer (100 mM Tris- HCl, 100 mM EDTA pH 8.0) were mixed. Then, lysozyme (3 mg mL^{-1}), and Proteinase K (150 mg mL^{-1}) were added and incubated at 37 °C for 15 minutes. After these steps, 10 % sodium dodecyl sulfate added 0.7M NaCl and 10 % CTAB (Cetyltrimethyl-ammonium bromide) solution were added and vortexed. Then, nucleic acid extraction and purification were performed using phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) followed by ethanol precipitation. After the ethanol was removed by pipette, the pellet was dried at room temperature and resuspended with 50 µl of diethylpyrrocarbonate (DEPC) water, and kept at -85ºC. 0.5 µl of samples were pippetted to NanoDrop ND-1000 Spectrophotometer for detection of DNA concentrations. A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA. Other values show that a sample is contaminated by residual phenol, guanidine, or other extraction reagents.

16S rRNA Gene Targeted PCR Analysis

PCR reactions were set up using Bacteria specific primers from the qualified DNAs. For the PCR reaction,

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27F (5'AGAGTTTGATCATGGCTCAG-3') for Bacteria was used as the forward primer, and 1492R (5'-GGTTACCTTGTTACGACTT3') was used as the reverse primer. Amplification was applied in 25 µl PCR reaction mixture containing 22 µl of 1X master mix which was prepared from 2X Master Mix (Biolabs OneTaq® Quick-Load®2X Master Mix (M0486S)), 0.4 µM forward primer, 0.4 µM reverse primer and 1 µl template. PCR was performed in Techne TC-5000 Thermal Cycler at programme: a cycle of 94ºC for 3 min, 30 cycles of 94ºC for 15 s, 55ºC for 30 s, and 72ºC for 2 min; plus an extension step of 7 min at 72ºC [21].

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Hinf I enzyme was used for restriction analysis of PCR products obtained for 16S rRNA gene. For each reaction 1 µl 5 U enzyme (Biolabs), 10 µl PCR product, 2 µl restriction enzyme buffer (Biolabs) and 7 µl DNase-RNase-free ultra-pure distilled water (Gibco) (total volume of 20µl) were used. The mixture was incubated overnight at 37 ° C for restriction. For analysis, samples were loaded on a 2% agarose gel for 4 hours in 1X TBE (Tris-Boric acid-EDTA) buffer [22].

DNA Sequence Analysis

Different profiles were determined by ARDRA and were purified. Then sequencing reactions were set up separately using primers 27F and 1492R. Then 16S rRNA gene sequences were compared with reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) by using BLAST. Finally, representative sequences were determined and stored in GenBank with the accession numbers by using Sequin system.

Phylogenetic analysis was performed with these sequences and sequences from analysis were added to the tree using MEGA X. [23-25].

Determination of the Toluene Monooxygenase Gene

PCR reaction was established using primary subunit targeted primers of toluene monooxygenase gene. RMOF (5' TCTC(A/C/G)AGCAT(C/T)CAGAC (A/C/G)GACG-3') was used as the forward primer and RMOR (5'–TT(G/T)TCGATGAT(C/G/T)AC(A/G) TCCCA-3') was used as the reverse primer to establish PCR reaction. Amplification was applied in 25 µl PCR reaction mixture as before. PCR was performed at programme: a cycle of 94ºC for 3 min, 30 cycles of 94ºC for 15 s, 53 °C for 1 min, and 72ºC for 1 min; plus an extension step of 10 min at 72ºC [26].

Benzene, Ethyl Benzene, Xylene and Phenol Degradation Potential of Isolates

For evaluation degradation potential of other substances, modified method was tried [17]. Mineral salt (MS) medium was used and 3 mM benzene, ethyl benzene, xylene and phenol was added separately to medium for each isolates. Then after three days incubation time, growth rate determined for each bacteria.

Simple Screening for Enzyme Activities of Isolates

Amylase, DNase (DNA), lipase on trybuthyrin and protease activities were tested at 37ºC. Prepoured plates were inoculated with bacteria. To avoid any interference from nearby colonies, only one isolate was inoculated onto each plate as a line form. For amylase activity the bacterial strains were inoculated media supplemented with 0.25% of starch. After incubation the zone of clearance was observed by adding Gram's iodine as detecting agent. Isolates were inoculated for protease production on milk nutrient medium. The zone of clearance was observed around the colonies indicated the proteolytic activity of that isolates [27]. For lipase, bacterial isolates were inoculated on tributyrin agar medium. After incubation development of a clear halo zone around the colony indicated lipase activity [28]. DNase Test Agar (Himedia) was used to differentiate between microorganisms based on their ability to produce the enzyme deoxyribonuclease (DNase). DNase producing organisms exhibited clear zone around growth against green background.

Toluene Tolerance and Biofilm Production of Isolates

Eight bacteria were then selected for the toluene degradation tests and allowed to grow in different toluene concentrations (300-600-900-1200-1500-1800-2100 mg/L^{-1}) in 96-well microtiter plates. 150 µL Minimal Salt medium in different toluene concentrations was inoculated with 50 µL of culture which was McFarland 0.5 standard was provided. Tolerance and growth capacity was measured in O.D.(Optical Density) 600 nm at 24 h interval for 96h [29].

Biofilm formation was assessed using same 96-well microtiter plates. After end of the 96 hours incubation, the wells washed three times with 200 µL sterile PBS (Phosphate buffer saline) to remove suspended cells, dried at room temperature, and the amounts of forming biofilms were quantified using crystal violet method. Briefly, the wells were stained with 150 µL/well 1% (w/v) crystal violet aqueous solution for 30 min. Microtiter plates were placed in shaking for 30 min. After removing the stain, the wells were washed three times by 200 µL PBS and air dried. Finally, 200 µL ethanol-acetone (80:20 v/v) was added to each well and the absorbance measured at 590 nm wavelength [30, 31].

Results and Discussion

Experimental planning aimed to determine the potential of biodegradation because this is a subject of

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great interest in wastewater treatment. Biodegradation is an extremely ecological method, as it usually involves the breakdown of organic compounds by microorganisms into more cell biomass and less complex compounds and water. Especially hydrocarbon contamination in soil and water is still a major concern and considered as a serious environmental issue to various natural habitats.

Thus, the discovery of new species capable of more efficient biodegradation can bring great innovation to this field. In addition, biological treatment methods have less environmental impact, eco-friendly and are less costly than other methods, have greater capacity to remove organic pollutants [32, 33].

BTEX (benzene, toluene, ethylbenzene and xylene) are simple aromatic compounds and that are highly toxic. Many chemical and physical methods can be used for degradation and breakup into nontoxic products. But, use of microorganism is considered to be the best suitable way for the degradation of BTEX [6, 34, 35]. Use of microorganisms and their enzymes for degradation can overcome the difficulties with other conventional methods.

Growth of bacteria in the presence of aromatic compounds shows their bioremediation ability of contaminated sites. Studies on biodegradation of toluene have been reported worldwide. However, biodegradation studies under aerobic conditions are lesser than anaerobic ones [36]. So, the present study aimed the identification of toluene degrading bacteria and determination of BTEX-degrading and biofilm-forming potentials of bacteria which was developed in wastewater.

The pH and BOD_5 measurement results of the samples were given in Tables 1 and 2. High rates of BOD_5 values were obtained from industrial waste water treatment plant. pH values were average 7-8 in both plants, but it could be seen slightly higher in municipal waste water treatment plant.

Morphologically different and distinct a total of 48 isolates were determined from toluene medium from municipal wastewater treatment plant. A total of 61 isolates were isolated from industrial wastewater treatment plant. After isolation, DNA extraction was applied and DNA concentrations were measured as a minimum 55.8 ng per microliter, maximum 183.7 ng per microliter and average concentration value approximately 50-60 ng per microliter for templates. 260/280 ratios were measured nearly 1.8. Then extracts were used as PCR templates. Approximately 1500 base pairs of bacterial 16S rRNA fragments were amplified from PCR analysis for isolates. Isolates were grouped with ARDRA. 8 different profiles of 16S rRNA products were detected. These representative profiles were sequenced. Sequences were compared with reference sequences at NCBI using BLAST. Most of the isolates were determined as belong to *Stenotrophomonas* species *Acinetobacter baumanni* and *Pseudomanas* species. Their accesion numbers and closest relatives were shown in Table 3. In addition, their status in the phylogenetic tree was Fig. 1.

Toluene biodegradation has been reported and reviewed by numerous studies with different strain and culture condition in laboratory experimentation. When the studies on toluene degradation are examined, *Pseudomonas* and *Bacillus* species were generally isolated and identified [37-39]. Specifically, *Pseudomonas putida* and *Pseudomonas aeroginosa* species have been described in many studies. In our study, microorganisms was isolated from the toluene medium which was containing 300 mg/liter toluene and their number was quite high. Toluene degrading species

Table 1. BOD_5 measurement results of waste water treatment plants.

	BOD mg/L June 2015		BOD mg/L October 2015		BOD mg/L January 2016		BOD mg/L April 2016	
SAMPLES	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant
Fat/Sand Removal Unit	420	1000	500	900	340	900	380	1000
Primary Clarifiers Unit	60	840	360	1000	200	1000	380	1000
Activated Sludge Unit	920	1000	1000	180	1000	180	1000	820
Return-Activated Sludge Pump	820	980	1000	1000	1000	1000	960	1000
Secondary Clarifiers Unit	60	160	100	120	40	120	40	280

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Table 2. pH values of waste water treatment plants.

	pH June 2015		pH October 2015		pH January 2016		pH April 2016	
SAMPLES	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant	Municipal waste water treatment plant	Industrial waste water treatment plant
Fat/Sand Removal Unit	7.81	5.4	8.06	6.79	8.12	7.24	7.56	6.69
Primary Clarifiers Unit	8.19	6.8	8.03	6.67	8.32	6.92	7.77	6.75
Activated Sludge Unit	7.49	7.1	7.46	7.33	7.21	7.33	7.25	7.39
Return -Activated Sludge Pump	7.27	7.1	7.37	7.43	7.13	7.65	7.16	7.46
Secondary Clarifiers Unit	7.01	8.04	7.66	7.74	7.32	7.72	7.56	7.60

were identified as *Stenotrophomonas acidaminiphila*, *Acinetobacter baumannii*, *Pseudomonas balearica*, *Pseudomonas otitidis*, *Streptococcus mitis* and *Stenotrophomonas maltophila*. Many studies had been done on *Stenotrophomonas maltophila* and the number of studies on *Stenotrophomonas acidaminiphila* is extremely low. [40, 41]. Another species which makes toluene degradation was *Pseudomonas balearica*. *Pseudomonas balearica* was identified as a naphthalene degrader [42, 43]. In another study, it was isolated from biological wastes as oil degraders [44]. One of the species that degrades toluene was *Pseudomonas*

Table 3. Isolates, their accession numbers and their closest matches in GenBank.

Isolate number	Species	Accesion Number (GenBank) Number	Closest Relative in Gene Bank	Similarity %
E5T32	*Stenotrophomonas sp.* strain E5T32 16S ribosomal RNA gene, partial sequence	KX682240	Stenotrophomonas sp. strain JJ63 16S ribosomal RNA gene, partial sequence (KU375545.1)	1161/1177(99%)
E4T16	*Pseudomonas balearica strain E4T16 16S ribosomal RNA gene, partial sequence*	KX682241	Pseudomonas balearica DSM 6083, complete genome (CP007511.1)	1337/1341(99%)
4ET21	*Stenotrophomonas acidaminiphila strain 4ET21 16S ribosomal RNA gene, partial sequence*	KX682242	Stenotrophomonas acidaminiphila partial 16S rRNA gene, strain Marseille-P2028 (LT223687.1)	575/588(98%)
4ET29	*Stenotrophomonas acidaminiphila strain 4ET29 16S ribosomal RNA gene, partial sequence*	KX682243	Stenotrophomonas acidaminiphila strain KP2 16S ribosomal RNA gene, partial sequence (KU850955.1)	435/445(98%)
3AT1	*Stenotrophomonas acidaminiphila strain 3AT1 16S ribosomal RNA gene, partial sequence*	KX682244	*Stenotrophomonas acidaminiphila strain SR50-5 16S ribosomal RNA gene, partial sequence (KF279369.1)*	*1275/1280(99%)*
3AT24	*Acinetobacter baumannii strain 3AT24 16S ribosomal RNA gene, partial sequence*	KX682245	*Acinetobacter baumannii OIFC189 clone 1061064264506 16S ribosomal RNA gene, partial sequence (JN668944.1)*	*684/695(98%)*
3ET4	*Pseudomonas otitidis strain 3ET4 16S ribosomal RNA gene, partial sequence*	KX682246	*Pseudomonas otitidis gene for 16S ribosomal RNA, partial sequence, strain: 81f (AB698739.1)*	*1264/1280(99%)*
3ET5	*Streptococcus mitis strain 3ET5 16S ribosomal RNA gene, partial sequence*	KX682247	*Streptococcus mitis strain KCOM 1350 (= ChDC B183), complete genome (CP012646.1)*	*1248/1263(99%)*

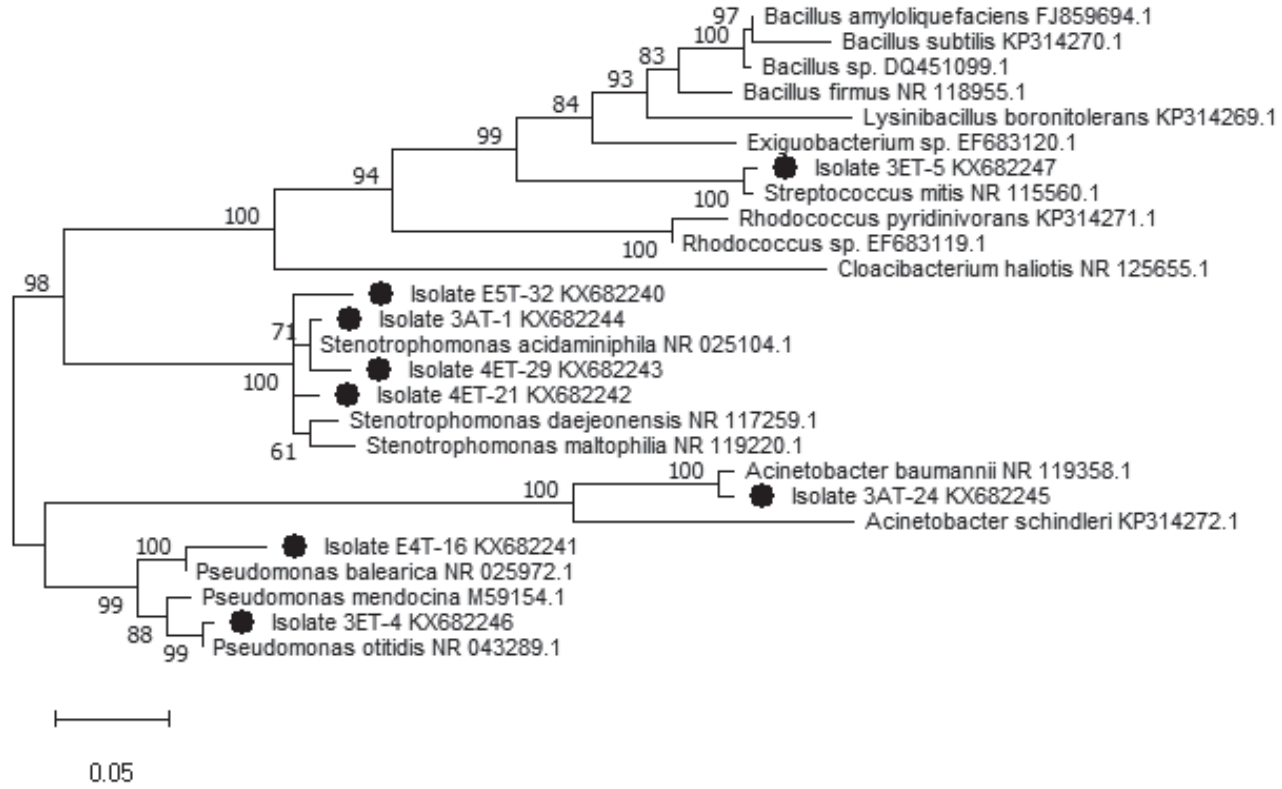

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences from isolates belonging to bacteria (indicated by black circles) was constructed by maximum likelihood. The horizontal scale bar represents the number of substitutions per site.

otitidis. The number of studies related to this species is quite low. In a study on biosurfactant production, it was isolated and thought to have bioremediation potential [45]. Up to now several studies have identified many bacteria that degraded toluene (Table 6) [33, 46-51]. But, as a result, when compared with Table 6, in our study new species that degraded toluene were identified.

The maximum O.D. (Optical Density) results indicates the maximum growth of those bacteria in different toluene concentrations. Fig. 2. showed maximum growth efficiency of identified strains with increased toluene concentrations in 24 hours. From Figs 3, 4, 5 it was found out that, 3ET4 (*Pseudomonas otitidis*) showed only growth in 300 mg/l^{-1} toluene. Other isolates showed moderate results. Increasing toluene concentration negatively affects biodegradation by making toxic effects on some bacterial cells [52]. However, there were also bacteria that grow at very high concentrations of toluene. Isolates 3ET5 (*Streptococcus mitis*) and 4ET21 (*Stenotrophomonas acidaminiphila*) tolerated toluene concentration up to 1500 mg/l^{-1}. E4T16 (*Pseudomonas balearica*) showed growth up to 2100mg/l^{-1} toluene. Compared to the literature, these isolates can tolerate extremely high concentrations. The examined bacterial species showed varying degrees of toluene degradability. For example, *Acinetobacter junii* was able to degrade 69, 73 and 80% of 150, 100, and 50 ppm toluene, respectively, within 72 h at 37ºC [36]. In another study, it was concluded that three bacteria, *Pseudomonas aeruginosa TJB01*, *Pseudomonas sp. TJB05* and *Acinetobacter sp. SBG05* isolated from ferry dock water contaminated with toluene. *P. aeruginosa TJB01* produced a higher toluene degradation with an initial concentration of 173.10 ppm of toluene [33].

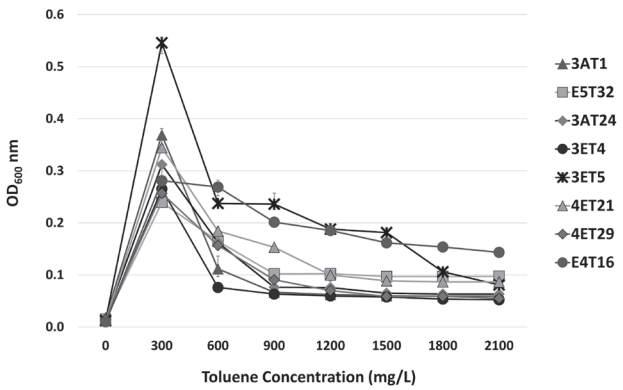

Fig. 2. Effect of toluene concentration on specific growth rate of bacterial strains after incubation at 37ºC for 24 hours.

For determination of the toluene monooxygenase gene, the products obtained after PCRs made with toluene monooxygenase gene targeted primers were carried out on 1% agarose gels. The presence of toluene monooxygenase gene was determined by the presence of 466 bp product. Toluene monooxygenase-specific PCR was established for 48 isolates with positive results in municipal wastewater treatment plant and

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only 2 positive results were obtained. The specific PCR of toluene monoxygenase gene was established in 61 isolates from industrial waste water treatment plant with positive results and only 5 positive results were obtained. The number of isolates containing the toluene monooxygenase gene is very small. This is because the isolates have different mechanisms and genes for using or degrading toluene.

Previous studies have reported that toluene-degrading bacteria play an important role in degradation

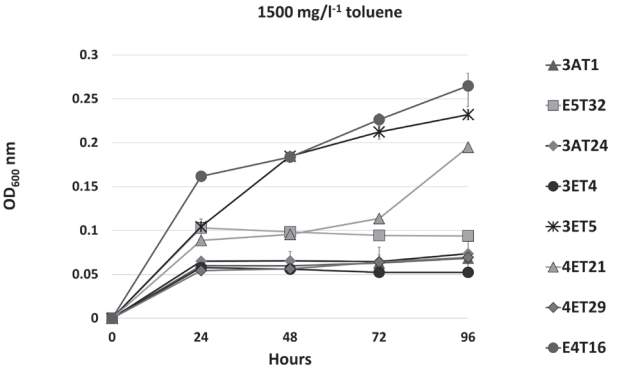

Fig. 3. Isolates' growth curve in 1500 mg/l^{-1} toluene concentration as a carbon resource.

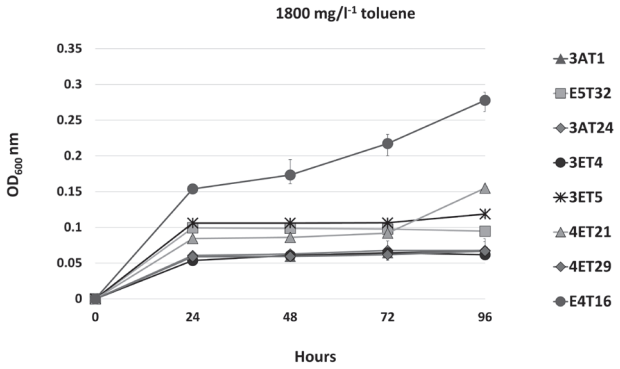

Fig. 4. Isolates' growth curve in 1800 mg/l^{-1} toluene concentration as a carbon resource.

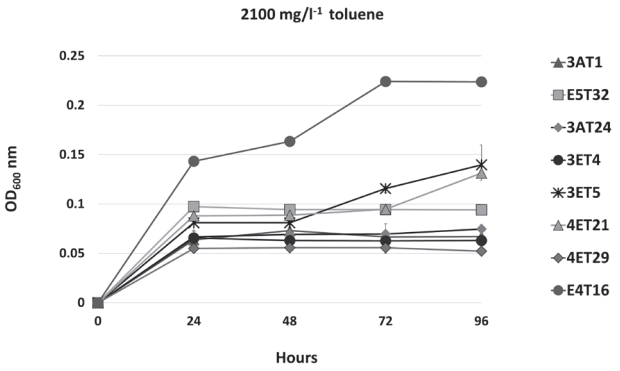

Fig. 5. Isolates' growth curve in 2100 mg/l^{-1} toluene concentration as a carbon resource.

Table 4. Results of benzene, ethyl benzene,xylene and phenol degradation of toluene degrading bacteria (Growth rate was shown by the symbols "+", "++, "+++"and "++++". The symbols were designed to compare both chemicals and bacteria with each other).

Strain number	Benzene	Ethyl benzene	Xylene	Phenol
4ET21	+++	++	+	+++
4ET29	+++	+++	++	+++
3AT1	+	+++	++	-
3AT24	++++	+++	++++	++++
3ET4	+++	+++	+	+++
3ET5	+++	+++	++	+++
E4T16	++++	++++	++++	++++
E5T32	++++	+++	++++	++++

of BTEX compounds in nature. Hydrocarbon-degrading microorganisms can be found many different environments. Among microorganisms, bacteria have been reported as common in hydrocarbon degradation [53]. For example, *Acinetobacter*, *Pseudomonas*, *Gordonia*, *Rhodococcus*, *Cobetia*, *Halomonas*, *Alcanivorax*, *Marinobacter*, *Microbacterium*, *Corynebacterium*, *Ochrobactrum*, *Stenotrophomonas* and *Hahella* [53-57]. In addition to potential for use in biological treatment of toluene removal, our bacteria can also be involved in the treatment of different substances such as benzene, toluene, xylene and phenol and in different processes as like enzymatic activities. So we tested BTEX degredation and enzyme production potentials of toluene degrading bacteria with simple methods. Bacterial isolates 4ET16 (*Pseudomonas balearica*), E5T32 (*Stenotrophomonas* sp.), 3AT24 (*Acinetobacter baumannii*), 4ET29 (*Stenotrophomonas acidaminiphila*) and 3ET5 (*Streptococcus mitis*) showed

Table 5. Level of enzyme activity for toluene degrading bacteria (Activity was scored by the symbols "+", "++" and "+++". These symbols showed the level of halos.).

Strain number	Lipase	Protease	Amylase	Car-boxymeth-ylcelullase	DNAase
4ET21	+	++	-	-	++
4ET29	+	+++	-	-	+++
3AT1	+	++	-	+	++
3AT24	-	-	+	+	-
3ET4	+	++	-	-	++
3ET5	++	-	-	-	++
E4T16	+++	-	-	++	-
E5T32	+	-	+	-	-

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Table 6. Toluene-degrading bacteria and isolation sources.

Isolation Source	Species	References
Contaminated sea water	*Bacillus amyloliquefaciens, Sporosarcina halophile, Bacillus firmus*	[46]
Soil sample from area contaminated with petroleum products	*Baciluus badius* *Brevibacterium liguefaciens* *Pseudomonas mendonica* *Pseudoxanthomonas mexicana*	[47]
Surface sediment from oil contaminated area	*Bacillus cereus, Micrococcus* spp, *Pseudomonas aeruginosa E. coli*,	[48]
Sample of soil in automobile repair and spray paint areas	*Lysinibacillus boronitolerans, Bacillus subtilis, Rhodococcus pyridinivorans, Acinetobacter schindleri*	[49]
Different contaminated soil from the Caspian Sea (Bandar-Anzali, Guilan, Iran).	*Bacillus cereus*	[50]
Ferry dock water contaminated with toluene	*Pseudomonas aeruginosa, Pseudomonas* sp., *Acinetobacter* sp.	[33]
Soil, mud, river sediment, and sewage sludge samples	*Bacillus subtilis, Comamonas testosteroni*	[51]

grown in BTEX media. Most of isolates showed growth in 300 mg/l^{-1} concentrations of each chemicals. 4ET16, E5T32 and 3AT24 growth rate was high in each hydrocarbon. 3ET4 and 4ET21 showed weakly growth in xylene and 3AT1 showed same result in benzene and there is no growth in phenol as seen in Table 4. In particular, members of the genus *Pseudomonas* have a wide variety of metabolisms that can survive in different environments and exhibit different features [58]. There were some studies for BTEX degradation. Twenty-three hydrocarbon-degrading bacteria strains were isolated from contaminated groundwater located in the Southern Amazon, Brazil. The amplification and sequencing of the 16S rRNA gene showed that isolates belonged to the genus Bacillus [59]. In a study *Bacillus pumilus MVSV3* was isolated from petroleum contaminated soil is utilized for BTEX degradation. At optimized conditions the isolate degraded 150 mg/L of BTEX completely within 48 h [35]. Also, Bujang et al. revealed in their study the *Bacillus* species have for high degradation capacity (up to 90 %) of total hydrocarbons in oily wastewater [60]. This is somewhat similar to our results, hydrocarbon-degrading bacteria have been characterized in two different soil. Bacterial

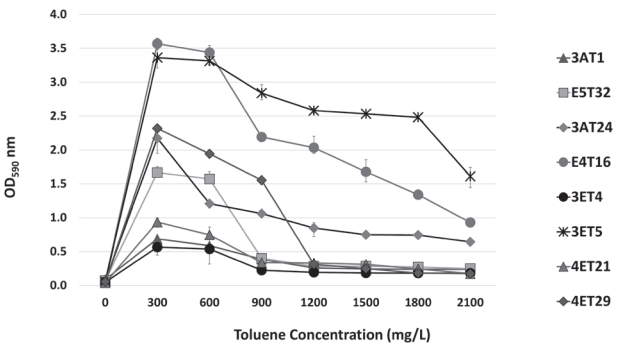

Fig. 6. Effect of toluene concentration on biofilm formation by bacterial strains in microtiter plates for 96 hours.

populations were exposed to the hydrocarbon substrate by the addition of toluene concentrations, ranging from 0.5% to 10% V/W and incubated. 16S rRNA analysis of the isolates revealed that some of them were members of the genera *Bacillus*, *Exiguobacterium*, *Enterobacter*, *Pseudomonas* and *Stenotrophomonas* [15].

When enzymatic activity results were examined, Table 5 highlights the isolates that could be considered as potential producers due to their apparent halos. Several strains showed good production of amylase, DNase and lipase. Most of isolates showed lipase, DNase and protease activity. 4ET21, 4ET29, 3AT1 had both three of enzymes activity. E4T16 had high lipase and cellulose activity.

Although there are many studies on characterization of bacterial biofilms growing in a wide variety of environments, there is still little information about bacterial biofilms developed under hydrocarbon contaminated conditions. For this reason, in addition to BTEX degradation and enzyme production ability, determination of biofilm formation features' of isolates were aimed in our study. Examination of biofilm formation at different toluene concentrations at the end of the 96 hours incubation revealed that isolates E4T16, 3ET5 and 4ET29 had highly biofilm production potential. Strongest biofilm formation was observed E4T16 and 3ET5 as seen in Fig. 6. Biofilm is preferred as a natural, economical and easy method to eliminate toxic chemicals [61]. Microorganisms can tolerate adverse conditions such as exposure to toxic chemicals so biofilm formation can serve as a tool for the design of new systems for municipal and industrial wastewater. There are many reports about biofilm production abilities of *Pseudomonas* on various surface types [62-64]. But, the studies and reports for E4T16 (*Pseudomonas balearica*) 3ET5 (*Streptococcus mitis*) and 4ET29 (*Stenotrophomonas acidaminiphila*) which were determined to produce high potential biofilms in our study, is very low.

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Conclusions

The results presented here may be useful for the design and development of new processes technologies in wastewater treatment. In our study, isolates were identified as belong to *Stenotrophomonas* species *Acinetobacter baumanni* and *Pseudomanas* species with molecular methods. Most of isolates had benzene, ethyl benzene, xylene and phenol degradation, amylase, DNase and lipase production and biofilm formation potentials. Especially for E4T16 (*Pseudomonas balearica*) and 3ET5 (*Streptococcus mitis*) by applying our datas and knowledge of the bacterial biotechnological potential on degradation, enzyme production and biofilm formation, new systems for the degradation of municipal and industrial wastewater could be designed.

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Conflict of Interest

There is no conflict of interest.

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