International Biodeterioration & Biodegradation 99 (2015) 85–94

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Insights into the metabolic basis of the halotolerant *Pseudomonas aeruginosa* strain LVD-10 during toluene biodegradation

Efi-Maria Drakou [a], Michalis Koutinas [a], Iakovos Pantelides [b], Maria Tsolakidou [b], Ioannis Vyrides [a, *]

[a] Department of Environmental Science and Technology, Cyprus University of Technology, 30 Archbishop Kyprianos, 3036 Lemesos, Cyprus
[b] Department of Agriculture Sciences, Biotechnology and Food Science, Cyprus University of Technology, 30 Archbishop Kyprianos, 3036 Lemesos, Cyprus

ARTICLE INFO

Article history:
Received 3 May 2014
Received in revised form
19 October 2014
Accepted 20 October 2014
Available online 28 January 2015

Keywords:
Toluene
Diesel biodegradation
Pseudomonas aeruginosa
Toluene catabolic genes
Salinity

ABSTRACT

In this work a *Pseudomonas aeruginosa* strain (LVD-10) has been isolated from activated sludge, based on its capability to biodegrade toluene under extreme conditions. In 0, 20 and 40 g L^{-1} NaCl the percentage of toluene removal reached 86%, 98% and 89% within 24 h, respectively. Interestingly, the removal of toluene occurred significantly faster compared to biomass growth, while the strain achieved 79.1% and 91.7% of 1 mL L^{-1} diesel removal after 7 days at 0 and 30 g L^{-1} NaCl, respectively. The pathways used by LVD-10 were determined through PCR amplification of genes that encode key enzymes involved in aromatics degradation and genes of the Quorum Sensing (QS) system. The genes *XylE1*, *1,2-CTD*, *2,3-CTD*, *rhlR1* and *rhlR2* were detected in LVD-10 whereas *tbmD*, *TodC1* and *Rmo* were not found. The transcription level of genes measured by Q-PCR did not show any significant variation in gene expression of cells stimulated with salinity conditions of 30 g L^{-1} NaCl. However, when NaCl was increased to 50 g L^{-1} the genes were significantly down-regulated 48 h after toluene induction, while the expression of genes was restored to normal levels at 120 h. Furthermore, the expression of genes *RhlI* and *RhlR2*, which have been proposed to be involved in the Quorum Sensing (QS) system of *P. aeruginosa*, indicates that the strain has great ability to tolerate toxic environments as well as to perform efficient degradation of aromatic hydrocarbons.

Introduction

There are many applications of hydrocarbons in industry, with their main role in fuelling. Due to the widespread use of hydrocarbons, spillages and contamination of soil and groundwater have been common for years. Petroleum can be classified into six main operationally defined groups of chemicals: saturated hydrocarbons, aromatic hydrocarbons, more polar, non-hydrocarbon components, resins and asphaltenes (Head et al., 2006; Ward et al., 2013). Toluene is a volatile and soluble in water pollutant, which belongs to the mono-aromatic petroleum hydrocarbons, exhibiting a methyl substitution on the aromatic benzene ring (Hamzah et al., 2011). It constitutes a major environmental issue, as it is highly toxic, carcinogenic, and stable in water and it is used in a wide range of manufacturing processes (Mukherjee et al., 2010; Saghafi et al., 2010). The U.S. Environmental Protection Agency (US EPA), the American Conference of Governmental Industrial Hygienists (ACGIH) and the International Agency for Research on Cancer (IARC) pay particular interest to the removal of toluene from polluted environments and categorize it, among others, as an environmental priority pollutant and human carcinogen (Kim and Jeon, 2009; Saghafi et al., 2010; Bahrami et al., 2011; Pratheesh and Jayachandran, 2012). Although the maximum contaminant level (MCL) of toluene as per EPA is 1 mg L^{-1}, its concentration in industrial wastewaters often varies between 7 and 753 mg L^{-1} depending on the type of manufacture (Enright et al., 2007), while groundwater and sea are often polluted with toluene, following leakage from petroleum fuel tanks or tankers (Enright et al., 2007; Di Martino et al., 2012).

Hydrocarbons can be removed from polluted environments using several 'natural' methods, such as evaporation and dissolution of the volatile and soluble components, photochemical decomposition, and microbial biodegradation (Ward et al., 2013). Microbial biodegradation is a cost-effective methodology and therefore several researchers have studied the microbial

* Corresponding author. Tel.: +357 25002218.
E-mail address: Ioannis.vyrides@cut.ac.cy (I. Vyrides).

http://dx.doi.org/10.1016/j.ibiod.2014.10.012
0964-8305/

decomposition of petroleum products demonstrating that biodegradation can serve as a promising technological alternative as compared to other physical or chemical approaches (Koutinas et al., 2007). Hydrocarbons in the environment are biodegraded mainly by bacteria, yeast and fungi. The efficiency of hydrocarbon biodegradation reported by Das and Chandran (2011), ranges between 6 and 82% for soil fungi, 0.13–50% for soil bacteria and 0.003–100% for marine bacteria, which appear to be the most active agents in petroleum degradation acting as primary degraders of spilled oil in the environment. In line with the above, several bacterial strains, such as *Arthrobacter*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas* and *Rhodococcus* are known to feed exclusively on hydrocarbons (Das and Chandran, 2011), while the aerobic degradation of simple aromatic compounds proceeds with different metabolic pathways, based on the enzyme system present in each microorganism (Cao et al., 2009; Hamzah et al., 2011).

There are three major initial routes for the catabolism of toluene, the oxidative initial attack on the aromatic ring by (a) monooxygenases, and (b) dioxygenases, or (c) attack on the alkyl side chain of the aromatic ring by monooxygenases (Hendrickx et al., 2006; Andreoni and Gianfreda, 2007). Following the initial oxidative reaction on the aromatic ring, the ring-cleavage pathway occurs with the action of catechol 1,2-dioxygenase (*ortho*-cleavage) or catechol 2,3-dioxygenase (*meta*-cleavage). The *ortho*- and *meta*-cleavage pathways are highly important for the metabolism of toluene, since the intermediate products formed undergo mineralization through the TCA cycle (Baldwin et al., 2003; Junca and Pieper, 2003; Hendrickx et al., 2006; Yong and Zhong, 2013). The initial oxidative attack, converting toluene into catechol, as well as the consequent ring cleavage constitute key steps in the aerobic degradation of toluene. Thus, the activities of both pathways are of direct interest as monitoring objects of the degrader's metabolic properties (Hendrickx et al., 2006). Furthermore, although the modification steps are variable for different aromatics, the catechol cleavage pathway can be considered as the common path leading to the TCA cycle for all aromatic compounds metabolised (Yong and Zhong, 2013).

As shown in Table 1, several studies have investigated the biodegradation of benzene, toluene, ethyl benzene, xylene (abbreviated as BTEX) and diesel; and only few investigated their biodegradation under high salinity. Moreover, monitoring of genes that encode key-enzymes for the metabolism of toluene is essential for characterisation of the strain's metabolic potential. Important genes for the adaptation of *Pseudomonas aeruginosa* in toxic environments comprise the Quorum Sensing (QS) system (as it is referred by Yong and Zhong, 2013), the *rhl* QS system (comprised of the transcriptional activator *RhlR*) as well as the N-butyryl homoserine lactone (BHL) synthase (*RhlI*), controlling the expression of a wide line of genes responsible for the production of rhamnolipids, pyocyanin, lectins, and others (Reis et al., 2011; Yong and Zhong, 2013). In this work, the genes were selected according to their presence in other *Pseudomonas* sp., while their relative expression levels were monitored under saline and non-saline conditions through the quantitative Real-Time PCR method.

Therefore, this study aims to isolate a microbial strain capable of degrading: i) toluene under extreme culture conditions (e.g. high salinity, extreme temperatures, absence of nutrients and a wide range of pH values), ii) diesel under normal and saline conditions and iii) to identify pathways involved in the catabolism of toluene.

Materials and methods

Mixed culture enrichment and isolation of pure strain

An activated sludge sample was collected from the Wastewater Treatment Plant situated in Moni (Limassol, Cyprus), which was screened for bacteria capable of tolerating and degrading toluene efficiently. The sample was used as an inoculum for the enrichment culture, which was cultivated in a sterilized mineral salts medium (MSM) modified as follows: (0.5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgCl_2$, 0.007 g L^{-1} $CaCl_2.H_2O$, 0.5 g L^{-1} NaCl). Enrichment of microorganisms was carried out in 100 mL flasks with 25 mL working volume, which were supplemented with 1 mM of toluene (Sigma–Aldrich anhydrous, 99.8%) and inoculated with 5 mL of mixed culture under sterile conditions for the initial activation of the pollutant degraders. The pH of the culture was adjusted to 7 and the flasks were tightly sealed with screw caps. A control flask, which was not inoculated with activated sludge, was incubated in parallel to the enrichment cultures. The flasks were incubated for a period of 10 days, during which the growth was being observed and the cultures were maintained at 30 °C and 100 rpm shaking.

The isolation and characterization of toluene degrading bacteria was performed by the removal of 0.1 mL samples from each flask that exhibited high turbidity, which were serially diluted from a factor of 10^{-1} to 10^{-10}. The diluted samples were plated by the streak method on the MSM containing agar and 1 mM of toluene.

Table 1
Isolated microorganisms capable of degrading toluene and diesel-oil.

Microorganism	Culture conditions	Substrate	% Removal	Reference
Janibacter spp. SB2	Aerobically, slurry system, 28.04 mM NH_4Cl and 0.6 mM NaH_2PO_4	BTEX (120 mg L^{-1})	62.8% removal in 60 h	Jin et al. (2013)
Marinobacter spp.	20 °C, 10% NaCl, pH 9.5	BTEX	100% removal of toluene in 50 h	Sorokin et al. (2012)
Mycobacterium cosmeticum byf-4	30 °C, 150 rpm	BTEX (150 mg L^{-1})	99.9% removal	Zhang et al. (2013)
Planococcus spp. ZD22	Aerobically, pH 7.5–9.5, 8–37 °C, 0–20% NaCl	BTEX (1 mM)	100% removal of toluene in 38 h	Li et al. (2006)
Pseudomonas aeruginosa DQ8	30 °C, 180 rpm	2% v/v diesel oil	83 ± 1.0% of TPHs in 32 h	Abu Hamed et al. (2004)
Pseudomonas aeruginosa PTz-5	Aerobically, 30–42 °C	Toluene	–	Mukherjee et al. (2010)
Pseudomonas aeruginosa WatG	pH 5.5–8.0 (optimum 6.5), temperatures 4–40 °C (optimum 30 °C)	Diesel oil (7% v/v)	90.8% ± 6.2	Wongsa et al. (2004)
Pseudomonas putida F1	32 °C, 200 rpm	Toluene (2–250 mg L^{-1})		Bordel et al. (2007)
Pseudomonas putida mt-2	6–30 °C (optimal 15 °C)	BTEX		Farrell et al. (2003)
Pseudomonas sp. SBCT-17	Aerobically, 35 °C, pH 7	Toluene (1 mM)	100% of toluene removal in 32 h.	Pratheesh and Jayachandran (2012)
Serratia marcescens HokM	pH 7.5, 30 °C	Diesel oil (7% v/v)	67% ± 17.6	Wongsa et al. (2004)
Staphylococcos aureus DRY11	pH 7.5–8.0, 27–37 °C	Diesel	Almost complete removal after 5 days	Shukor et al. (2009)
Stenotrophomonas maltophilia T3-c	pH 5–8 (optimal pH 7), temperatures 20–40 °C (optimal 30 °C)	Toluene	Degradation rate 2.38 μmol/g-DCW/h	Lee et al. (2002)

The inoculated Petri dishes were sealed with parafilm and incubated for 10 days at 30 °C. Following the incubation period, single colonies were picked and re-cultivated in MSM agar media following the same procedure as stated above. This procedure was repeated several times until pure identical colonies were observed. Only a single strain was capable of tolerating a toluene concentration of 2 mM. Therefore, the specific strain was subsequently characterised and used for the experiments presented below. In every condition (pH, salinity, temperature, nutrient, oil) the experiment was done in triplicates and standard error was calculated.

Strain characterization by 16S rRNA sequence analysis

Liquid cultures of the isolated pure strain were prepared in serum bottles with 100 mL total volume (30 mL culture volume) and incubated in a shaking incubator at 100 rpm in 30 °C for 48 h. Samples 1.5 mL of liquid culture were transferred into eppendorf tubes (1.5 mL total volume) and centrifuged twice at 13,500 rpm for 4 min each time. The formed pellet was resuspended in 1% Sodium Dodecyl Sulphate (SDS) and 0.2 M NaOH solutions for DNA extraction. Polymerase Chain Reaction (PCR) purification was performed to obtain the 16S rRNA. After the extraction, a PCR took place using the following primers: i) 8f: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and ii) 1542R: 5′-AAG GAG GTG ATC CAG CCG CA 3′. The reaction was carried out according to the following protocol: 94 °C (2 min) followed by 33 cycles consisting of 94 °C (1 min), 56 °C (1 min), 72 °C (2 min) and 72 °C (7 min). For each reaction 50 μL PCR mixtures were prepared containing 50 ng of template DNA, deoxynucleoside triphosphate at a concentration of 200 μM, 3 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 1 U Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific Inc.) and 0.5 mM of the PCR buffer. PCR-amplified 16S rRNA genes were purified using the NucleoFast® 96 PCRCleanup Kit (Macherey-Nagel, Düren, Germany). Sequencing alignment was performed by Macrogene, Netherlands and the resulting alignment of the 16S rRNA was compared for homology in the NCBI database by BLASTn nucleotide tool analysis. The partial nucleotide sequence of the 16S rRNA gene was characterised as a *P. aeruginosa* and deposited in GenBank with accession number KF728673.1 as *P. aeruginosa* strain LVD-10.

PCR for the detection of toluene catabolism and QS system genes

The isolation of DNA was performed from pure liquid cultures of LVD-10 as described above. Culture conditions were: 30 °C, 0 g L^{-1} NaCl and orbital shaking of 100 rpm. The DNA isolated from LVD-10 was screened for the presence of genes encoding enzymes involved in aromatics degradation (such as *TBMD*, *TMOA*, *TODC1*, *1,2-CTD* and *2,3-CTD*), as well as additional genes of great importance for hydrocarbon degradation, such as *xylE1*. For the detection of genes involved in the QS system, the *rhlR2* and *RhlI* primer sets were tested. For each reaction 10 μL PCR mixtures were prepared containing 1 μL of template DNA (10 ng), 0.2 μL dNTPs (0.2 mM), 0.8 μL of each primer set (10 μM), 0.04 μL (1 U) KAPA Taq DNA polymerase, 1 μL of PCR buffer B (0.5 mM) and 7.16 μL of dH_2O. PCR-amplification was carried out with the use of KAPA Biosystems kit (Massachusetts, USA).

The sequences amplified by the primer sets are listed in Table 2. Annealing temperatures were calculated according to the melting temperature of each primer set (Macrogen, Amsterdam, Netherlands) and gradient PCR was performed for 34 cycles. The initial denaturation step was set at 95 °C (3 min), the denaturation step at 95 °C (30 s). For the first four primer sets (Table 2) the annealing temperature was set to 60 °C, while for the rest of the primers temperature was set to 55 °C. The extension was carried out at 72 °C (60 s) and the final extension at 72 °C (60 s).

Isolation of total RNA, cDNA synthesis and quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) was performed to determine the expression of genes detected by DNA PCR amplification. The genes monitored during the course of the experiments were *xylE1*, *RhlI*, *RhlR2*, *1,2-CTD*, *2,3-CTD* and *proC*, which served as the housekeeping gene. 1 mL biomedium samples containing different concentrations of NaCl (0, 30 and 50 g L^{-1}) were centrifuged for

Table 2
Primers used in the present study.

Gene	Primers sequence	Expected size (bp)	Observed size (bp)	Role	Reference
tbmD	f-5′-GCCTGACCATGGATGC(C/G)TACTGG-3′ r-5′-CGCCAGAACCACTTGTC(A/G) (A/G)TCCA-3′	640	N/D	Subfamily 1 of a-subunit of hydroxylase component hydroxylase component of multicomponent mono-oxygenases	Hendrickx et al., 2006; Kim and Jeon, 2009
tmoA	f-5′-CGAAACCGGCTT(C/T)ACCAA(C/T)ATG-3′ r-5′-ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA-3′	505	900		
Rmo	F-5′-TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG-3′ R-5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA-3′	466	N/D	Large subunit of Toluene Ring hydroxylating monoxygenase	Baldwin et al., 2003
todC1	f-5′-CAGTGCCGCCA(C/T)CGTGG(C/T)ATG-3′ r-5′-GCCACTTCCATG(C/T)CC(A/G)CCCCA-3′	510	N/D	TodC1 or toluene 1,2-dioxygenase (TOD pathway)	Hendrickx et al., 2006; Di Martino et al., 2012
xylE1	f-5′-CCGCCGACCTGATC(A/T) (C/G)CATG-3′ r-5′-TCAGGTCA(G/T)CACGGTCA(G/T)GA-3′	237	237	Subfamily I.2.A of catechol extradiol dioxygenases	
1,2-CTD	f-5′-ACCATCGARGGYCCSCTSTAY-3′ r-5′-GTTRATCTGGGTGGTSAG-3′	411	411	Catechol 1,2-dioxygenase	Li et al., 2012
2,3-CTD	f-5′-GARCTSTAYGCSGAYAAGGAR-3′ r-5′-RCCGCTSGGRTCGAAGAARTA-3′	411	411	Catechol 2,3-dioxygenase	
rhlI	f-5′-TTCATCCTCCTTTAGTCTTCCC-3′ r-5′-TTCCAGCGATTCAGAGAGC-3′	606	200	Auto-inducer synthase	Perfumo et al., 2013 Yong and Zhong, 2013
rhlR2	f-5′-CGGTGCTGGCATAACAGATA-3′ r-5′-GCTCGAAGCTGGAGATGTTC-3′	726	600	Transcriptional regulator	
proC	f-5′-CAGGCCGGGCAGTTGCTGTC-3′ r-5′-GGTCAGGCGCGAGGCTGTCT-3′	226	226	Housekeeping gene: encodes the pyrroline-5-carboxylate reductase	Salvi et al., 2003

N/D: not detected.

1 min at 11,000 g and RNA extraction from the cells was performed using the protocol provided by the suppliers of the NucleoSpin® RNA II kit (Macherey–Nagel, Düren). Total RNA was eluted with 15 μL RNase-free water and it was used immediately for cDNA synthesis. cDNA was synthesized using PrimeScript RT Reagent Kit (Takara, Japan) using random and oligo-dT primers.

Q-PCR assays were performed using KAPA SYBR® FAST qPCR Kit (KAPA Biosystems, Massachusetts, USA). For each reaction, 1 μL of cDNA was mixed with 10 μL of the PCR solution, which contained 5 μL Kapa SYBR Green fast mix, 0.2 μL of forward primer (10 μM) and 0.2 μL of reverse primer (Macrogen, Amsterdam, Netherlands) at a concentration of 10 μM, and 4.6 μL of sterile water. The primer sequences used are listed in Table 2. The PCR was carried out with enzyme activation at 95 °C (3 min), followed by 40 cycles of denaturation at 95 °C (3 s) and annealing/extension at 60 °C (20 s).

Threshold cycle values (C^T) were calculated using the iQ5 Bio-Rad Software, Singapore. The reference gene was *proC* and was used to normalize the C^T values of *XylE1*, *1,2-CTD*, *RhlI* and *RhlR2*. A non-template sample for each primer set was used as negative control. The normalization and *t*-test ($p < 0.05$) were carried out through the Relative Expression Software Tool (REST-XL©) using the Pair Wise Fix Reallocation Randomisation Test.

Microbial growth conditions and biomass dry cell weight determination

Subcultures of *P. aeruginosa* were pre-grown overnight at 30 °C in MSM supplemented with 2 mM toluene as a carbon source. Duplicate cultures were prepared and the incubation was performed using serum bottles with 100 mL total volume (30 mL working volume), in a shaking incubator (Stuart SI500) stirred at 100 rpm. The growth rates were compared according to four factors, namely: temperature, pH, salinity and absence of nitrate and phosphorus. The rest of the culture conditions were maintained constant during the performed experiments. Therefore, the pH of the culture was adjusted at 7, temperature was set to 30 °C and the stirring speed was maintained at 100 rpm. Liquid cultures of *P. aeruginosa* were also prepared in MSM supplemented with 1 mL L^{-1} diesel. The strain was cultured in non-saline and saline conditions by the addition of 30 g L^{-1} NaCl in MSM. The incubation of the cultures was performed in serum bottles of 500 mL total volume (200 mL working volume) in a shaking incubator (Stuart SI500) which was stirred at 100 rpm for 7 days. All chemicals used were obtained from the Sigma–Aldrich Company (UK) and were of analytical grade.

Biomass concentration was determined by absorbance at 600 nm on a UV/VIS spectrophotometer (PerkinElmer Lambda 25), using a previously established dry cell weight calibration curve. The coefficient of variation for 4 samples was 0.25% at a concentration level of 0.6 $g_{biomass}$ L^{-1}.

Analytical techniques

Gas chromatography (GC) was used to determine the concentration of toluene in aqueous samples. A Shimadzu GC-2014 equipped with a Flame Ionisation Detector (FID), an AOC-20i auto-injector and a column with ZB-5 (Zebron, Phenomenex, USA) stationary phase (30 m × 0.25 mm × 0.25 μm) and nitrogen as a mobile phase were used. Liquid samples of 2 mL were first centrifuged for 4 min at 13,500 rpm, the supernatant solution was filtered through 0.2 μm filters to remove any remaining solids and it was mixed with 2.5 mL of n-Decane for toluene extraction. The mixture was stirred on vortex for 1 min and 1.5 mL of the organic phase was transferred into a GC vial. The injection volume was 1 μL of the sample and the temperature program was run at 80 °C for 3 min which was then increased to 160 °C at a rate of 20 °C min^{-1}. The SPL temperature was at 250 °C, splitless injection mode, the pressure at 111.8 kPa and the injection volume 1 μL. The SFID temperature was set at 280 °C. The coefficient variation for two samples was 0.2% at a concentration level of 2 mM toluene.

GC analysis was used for the determination of diesel concentration in the aqueous samples using the same chromatograph as described above. Samples were collected using the same methodology as for toluene measurements, whereas dichloromethane (Sigma–Aldrich Company, UK) was used as the organic solvent and 2 μL of the sample was injected into the GC. The temperature program was run at 40 °C for 1 min, then increased to 260 °C at a rate of 5 °C min^{-1} and it was held at 260 °C for 15 min.

Results and discussion

Isolation and characterization of LVD-10

A sample from activated sludge was first enriched with toluene as the sole source of carbon. Sixteen bacterial strains were isolated, of which only one was capable of growing on toluene at a concentration level of 2 mM in MSM. For the identification of the isolated toluene degrader, 16S rRNA gene sequencing of the strain was performed and the nucleotide sequence was generated and aligned by Macrogen (Netherlands). The selected nucleotide sequence of 1075 bp was analysed for bacterial species homology through the NCBI database by BLASTn tool analysis. Following a comparison of the strain's homology with that of other microorganisms in the NCBI database, a 99% homology with *P. aeruginosa* strain PCP29 and *P. aeruginosa* strain HCB5 was confirmed. The isolate has been deposited in GenBank as *P. aeruginosa* strain LVD-10 with accession number KF728673.1.

Biodegradation of toluene by P. aeruginosa LVD-10 under extreme conditions

As shown in Fig. 1, LVD-10 exhibited significant tolerance in salinity presenting an optimal concentration of 20 g L^{-1} NaCl for growth on toluene. LVD-10 was capable of growing in salinity as high as 50 g L^{-1}. However, there was a slow growth rate requiring more than 15 days of incubation for complete biodegradation of toluene. The maximum salinity that growth was observed in 65 g L^{-1} of NaCl, where negligible turbidity was formed after 15 days of incubation (data not shown). The biodegradation of toluene was achieved in all tested salinities, which ranged from 20 g L^{-1} to 40 g L^{-1}. In 0 g L^{-1} and 20 g L^{-1} NaCl the percentage of removal reached 86% and 98% within 24 h respectively. Although LVD-10 showed a slow rate of growth in 40 g L^{-1} of NaCl, nonetheless, the biodegradation of toluene was 89% after 24 h, which was increased to 98% within less than 48 h (Fig. 1). Therefore, although toluene is almost completely biodegraded within the first 24 h, most of the biomass production takes place between 24 and 72 h. This effect could be due to the utilization of the substrate mainly for energy production and cell maintenance during the first few hours of cultivation, followed by the anabolic processes leading to biomass growth at a later stage. At 40 g L^{-1} the yield of biomass (1.2 $g_{dry\ weight}/g_{toluene}$) of LVD-10 was substantially lower compared to the yield of 0, 20 g L^{-1} (4 and 4.2 $g_{dry\ weight}/g_{toluene}$, respectively). This could be due to the energy that is consumed in order to adapt to high salinity such as the production of compatible solutes.

Only limited studies have led to the isolation of strains capable of biodegrading toluene under extreme conditions. Li et al. (2006) reported that 1 mM of toluene was 100% removed within 32 h by *Planococcus* spp. under 200 g L^{-1} of NaCl. Furthermore, Sorokin et al. (2012) isolated a *Marinobacter* spp. that was psychrophilic

and was capable of mineralising toluene after 50 h under saline conditions of 100 g L^{-1} NaCl, a pH value of 9.5 and temperature of 20 °C (Table 1).

LVD-10 was capable of using toluene for growth in pH values ranging from 6 to 9, where the optimum pH was shown to be 7. The growth was higher in alkali conditions pH 9 than under more acidic conditions (pH 6) and this is also was showed from the yield that were produced, where it was lower at pH 6 (2 $g_{dry\ weight}/g_{toluene}$) compared to the yield at pH 9 (4 $g_{dry\ weight}/g_{toluene}$), The cultures performed at pH 9 exhibited a prolonged lag phase, with the exponential growth phase starting at 48 h, this could be also due to the energy that is consumed in order to adapt to acidic conditions. At pH 7 the LVD-10 showed the highest growth with the exponential phase starting at 16 h and reaching the stationary phase after 72 h (Fig. 2). The yield at pH 7 was similar (4.2 $g_{dry\ weight}/g_{toluene}$) compared to the yield at pH 9. The biodegradation of toluene achieved in cultures performed at different pH values was also determined. The percentage of toluene removal reached 90% for the culture performed at pH 7 after 24 h and 74.5% for the cultures performed at pH 6 and 9 after 120 h (Fig. 2).

Previous studies conducted with *P. aeruginosa* sp. strains have also shown that an optimal growth rate occurs for pH 7 (Abu Hamed et al., 2004; Mukherjee et al., 2010; Pratheesh and Jayachandran, 2012). In general, various studies demonstrate that *P. aeruginosa* sp. is capable of easily adapting to a wide range of pH values (Abu Hamed et al., 2004; Wongsa et al., 2004; Hamzah et al., 2011). Pratheesh and Jayachandran (2012) isolated *Pseudomonas* sp. SBCT-17, which showed a steady increase in the degradation of toluene with incubation time, achieving 100% degradation after 32 h with optimum culture conditions of pH 7 and 35 °C (Table 1). The optimal culture conditions for SBCT-17 were similar to those of the present study (Fig. 2), since the degradation of toluene reached 99% removal after 32 h at pH 7.

Another factor that varied was the temperature of the culture, where LVD-10 also showed to be capable of adapting to a wide range of conditions (tested temperatures ranged between 20 and 40 °C). The optimum temperature for bacterial growth was 30 °C (Fig. 3). Although the variation of the temperature from the optimum point appeared to decrease biomass growth, toluene removal was substantial for 20 °C and 40 °C reaching 60 and 71% after 48 h for the two cultures, respectively (Fig. 3). In addition LVD-10 was capable of growing on toluene even at temperatures as low as 10 °C (data not shown). Several studies demonstrate that *P. aeruginosa* sp. is capable of surviving in a wide range of temperatures, while most of the strains present their optimum growth between 30 and 35 °C (Abu Hamed et al., 2004; Wongsa et al., 2004; Mukherjee et al., 2010; Pratheesh and Jayachandran, 2012). Additionally, Farrell et al. (2003) showed that *Pseudomonas putida* mt-2 was capable of biodegrading toluene at an optimum temperature of 15 °C (Table 1). Other *P. aeruginosa* strains are capable of degrading hydrocarbons at several temperatures. The SBCT strain (Table 1), studied by Pratheesh and Jayachandran (2012) in a range of temperatures, between 25 and 45 °C, was capable of degrading toluene in all temperatures tested. However, the rate of toluene degradation decreased for temperatures different than the optimum value (35 °C). Another study of *P. aeruginosa* strain PTz-5 (Table 1) conducted by Mukherjee et al. (2010), showed that the strain exhibited the highest growth rate at temperatures between 30 and 42 °C. Furthermore, Wongsa et al.

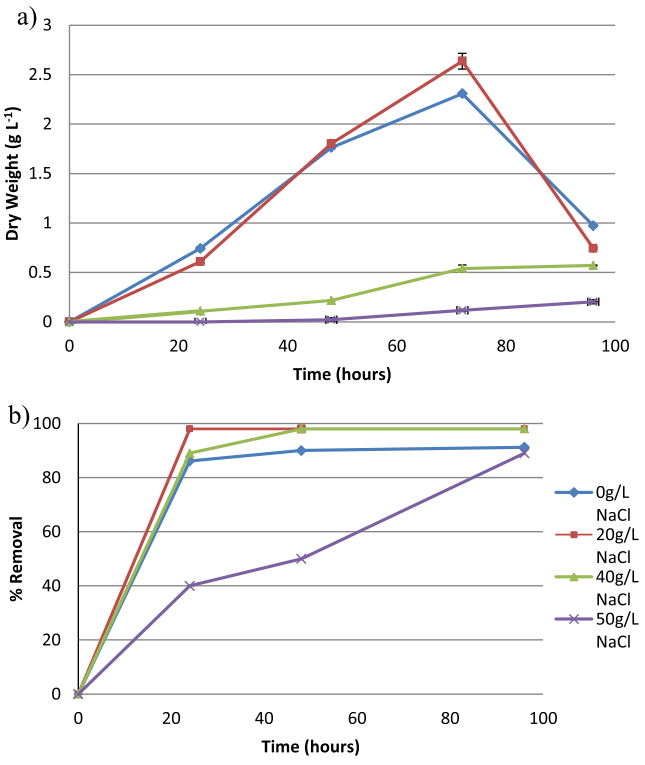

Fig. 1. Dry weight and toluene biodegradation under saline conditions. (a) Bacterial growth (expressed as dry weight in g L^{-1}) in fermentations performed with 2 mM of toluene in MSM. The bacterial growth was determined under saline conditions (0, 20, 40 and 50 g L^{-1} NaCl) for 96 h. (b) The same culture was examined for toluene biodegradation (expressed as % removal of toluene).

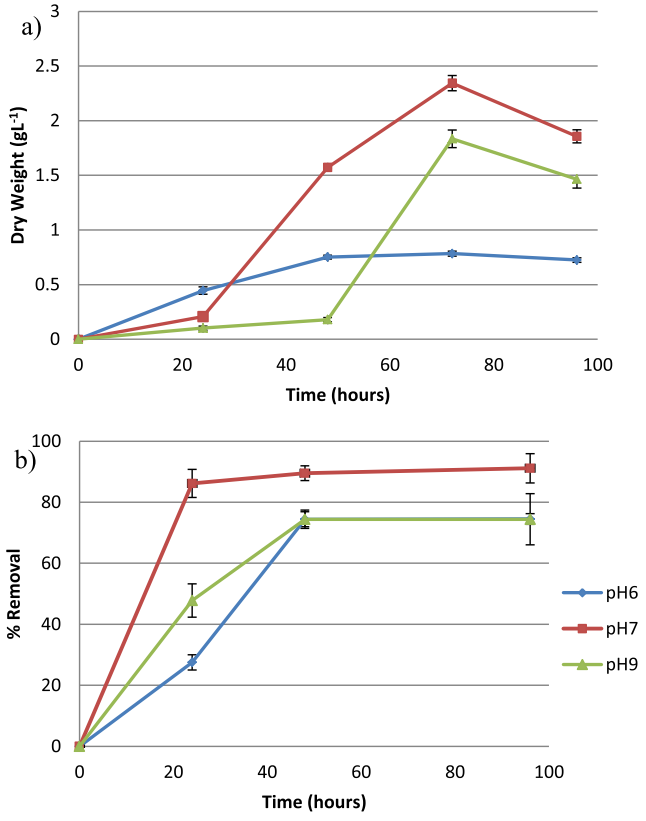

Fig. 2. Dry weight and toluene biodegradation in different pH. (a) *Pseudomonas aeruginosa* LVD-10 growth (expressed as dry weight in g L^{-1}) in fermentations performed with 2 mM of toluene in MSM. The bacterial growth was examined in a range of pH values and in cultures incubated for 96 h. (b) The culture was also examined for toluene biodegradation (expressed as % removal of toluene).

(2004) reported that a *P. aeruginosa* strain (Table 1) was capable of growing in a range of temperatures between 4 °C and 40 °C, while the optimum temperature was 30 °C.

The degradation of toluene by LVD-10 was also tested in the absence of important nutrients, such as phosphorus and nitrogen (Fig. 4). The absence of phosphorus had a negligible effect on bacterial growth and removal of toluene, since the removal was measured as 90% within 24 h in cultures conducted with the addition of all nutrients and in cultures where phosphorus was not added (Fig. 4). The culture of LVD-10 in MSM without nitrogen showed low turbidity and the percentage of toluene removal reached only 50%, indicating that nitrogen is a limiting factor for toluene biodegradation. Jin et al. (2013) have previously shown that the addition of nitrogen has a greater effect on BTEX biodegradation compared to the supplementation of phosphorus for *Janibacter* spp. (Table 1).

Biodegradation of other aromatic compounds by Pseudomonas aeruginosa strain LVD-10

LVD-10 was studied for growth in MSM containing other aromatic hydrocarbons than toluene, such as 500 mg L^{-1} phenol, 500 mg L^{-1} *p*-cresol, 100 mg L^{-1} phenanthrene, 300 mg L^{-1} 4-nitrophenol and 200 mg L^{-1} indole. High turbidity was observed for all aromatic compounds, indicating that the bacterial strain was capable of metabolising all the tested aromatics. A similar performance was reported by Hamzah et al. (2011) and other researchers (Farrell et al., 2003; Di Martino et al., 2012) demonstrating that *Pseudomonas* spp. strains are versatile and capable of degrading BTEX and other aromatic hydrocarbons, such as phenanthrene, phenol, cresol, naphthalenes and asphaltenes.

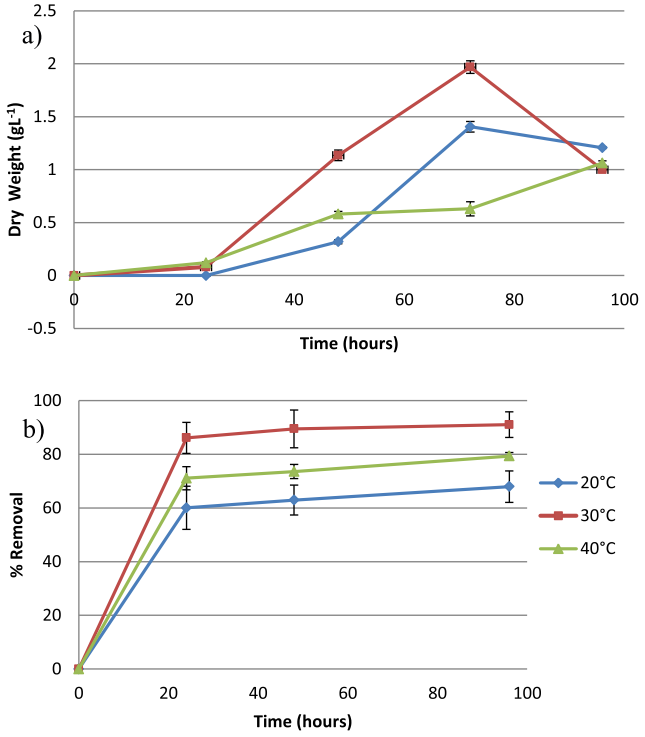

Fig. 3. Dry weight and biodegradation of toluene in different temperatures. (a) *Pseudomonas aeruginosa* LVD-10 growth (expressed as dry weight in g L^{-1}) in fermentations performed with 2 mM of toluene in MSM and under a range of temperatures for 96 h. (b) The cultures were examined for toluene biodegradation (expressed as % removal of toluene).

Diesel biodegradation under saline and non saline conditions

LVD-10 was additionally studied in MSM that contained 1 mL L^{-1} of diesel and in cultures incubated at 100 rpm in 30 °C for 7 days. GC analysis showed that diesel was substantially biodegraded by LVD-10 under non saline and saline conditions. The initial concentration of diesel in 100 mL of MSM was 1109 mg L^{-1} (Fig. 5a), while after 7 days of incubation the concentration of diesel was 232 mg L^{-1} (Fig. 5b), which corresponded to a 79.1% of removal. Since LVD-10 showed a remarkable adaptation in high salinity, the same concentration of petroleum (1 mL L^{-1}) was added in MSM containing 30 g L^{-1} of NaCl. The results showed higher Total Petroleum Hydrocarbons (TPHs) removal after 7 days of incubation under salinity, indicating that LVD-10 is capable of metabolising the substrate at a higher rate under saline conditions. The concentration of diesel in 100 mL of liquid culture after 7 days was 91.6 mg L^{-1}, which corresponded to 91.7% of removal. Zhang et al. (2011) reported removal of 83 ± 1.0% by a *P. aeruginosa* sp. (strain DQ8) within 10 days (Table 1), indicating a similar performance to this work as well as the great potential of *P. aeruginosa* to degrade diesel at high rates. Strain LVD-10 showed a slightly higher degradation of diesel under salinity (Fig. 5c) which indicated the potential for diesel biodegradation in a spillage in the sea.

Screening of genes involved in the metabolism of aromatics by Pseudomonas aeruginosa LVD-10

Information regarding the pathways involved in toluene degradation by LVD-10 was determined through the detection of genes encoding important enzymes used in the catabolism of aromatics, while the effect of saline conditions on gene expression was also determined. Previous studies have demonstrated that the microbial degradation of toluene proceeds through five metabolic pathways (Harayama et al., 1999; Hendrickx et al., 2006; Di Martino et al., 2012). In the first pathway toluene is converted into a catechol intermediate through the benzyl alcohol route. This pathway was identified in *P. putida mt-2*, known as the TOD pathway (encoding *xyl* genes) (Harayama et al., 1999). In the second pathway toluene is catabolised via the toluene-*cis* diol pathway into 3-methylcatechol through the action of the enzymes toluene 1,2-dioxygenase and *cis*-toluene dihydrodiol dehydrogenase, encoded in *tod genes* (Zylstra and Gibson, 1989; Hendrickx et al., 2006; Di Martino et al., 2012). The toluene-*cis* diol pathway was identified in *P. putida* F1. In the third pathway toluene is degraded via *p*-cresol to protocatechuate, which is further oxidised in the TCA cycle. This pathway proceeds through *ortho*-cleavage of the aromatic ring, where a group of monooxygenase enzymes are involved, such as

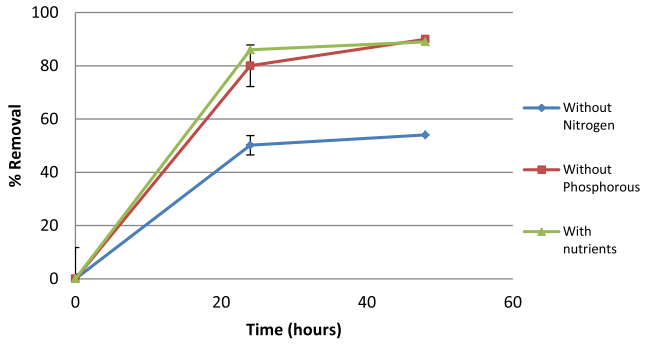

Fig. 4. Toluene biodegradation in the absence of nutrients. *Pseudomonas aeruginosa* LVD-10 was capable of biodegrading toluene (2 mM concentration) in the absence of nitrogen and phosphorus in separate cultures. The graph shows the percentage of toluene removal in cultures performed in the presence or absence of key nutrients.

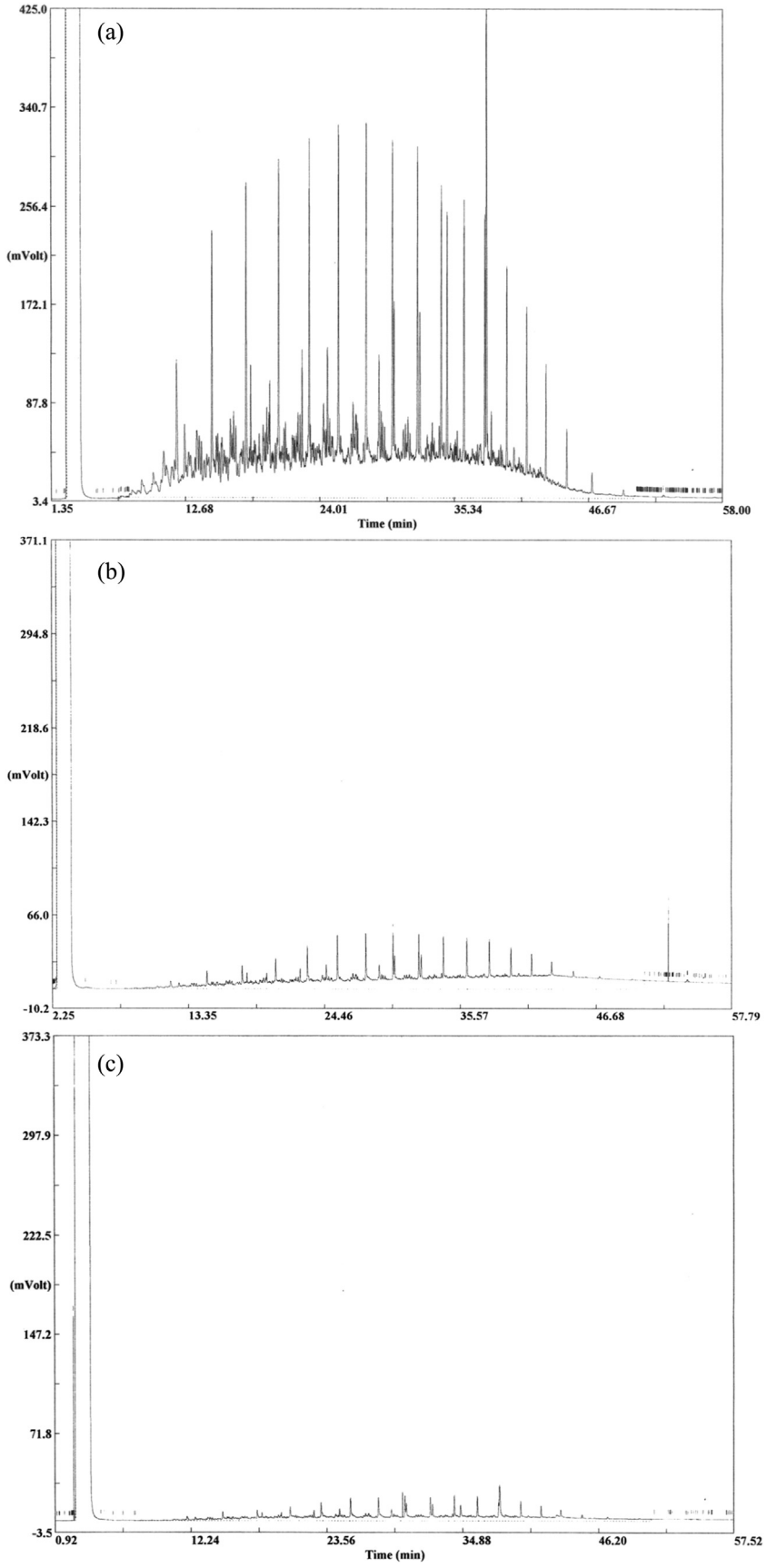

Fig. 5. The chromatograph of diesel and its removal by LVD-10 under saline and non-saline conditions. (a) The first chromatograph shows the concentration of diesel in the control flask (not inoculated with the microorganism). The major peaks correspond to *n*-alkanes, minor peaks include *iso*-alkanes and *cyclo*-alkanes, while minor peaks in the area on the left hand side of the chromatograph correspond to aromatic hydrocarbons. (b) The second chromatograph displays the biodegradation of diesel by LVD-10 in MSM under normal conditions and (c) the last chromatograph shows the biodegradation of diesel by LVD-10 under salinity, where the MSM was supplemented with 30 g L^{-1} of sodium chloride. Samples were analysed following seven days of incubation in 30 °C in cultures stirred at 100 rpm.

toluene 4-monoxygenase encoded by the *tmo* genes (Whited and Gibson, 1991). The pathway was identified in *Pseudomonas mendocina* KR1 and it is known as the TOL pathway (pWW0) (Harayama and Rekik, 1990). The fourth and fifth pathways convert toluene in 3-methylcatechol via *4*-methylcatechol and *o*-cresol (*ortho*-metabolic pathways). The pathway proceeding via *o*-cresol was identified in *Burkholteria cepacia* G4, while the enzyme that catalyses the first reaction is toluene *ortho*-monoxygenase (*tmoA* genes) (Shields et al., 1995; Harayama et al., 1999). The pathway proceeding via 4-methylcatechol was identified in *Ralstonia pickettii* PK01 where *tbu* genes encode the toluene-3-monoxygenase (Olsen et al., 1994; Harayama et al., 1999).

In this study six genes were selected to investigate the metabolic pathways involved in the metabolism of aromatics in LVD-10. The genes tested encode the most important enzymes involved in aromatics biodegradation, according to Li et al. (2012) and Hendrickx et al. (2006), while two genes that regulate the expression of genes involved in the QS system were also examined (Perfumo et al., 2013; Yong and Zhong, 2013). *TmoA*, *TodC1* and *tbmD* encode enzymes participating in the initial attack on the aromatic ring (Table 2). *1,2-CTD* (encoding catechol 1,2-dioxygenase) is also involved in aromatics catabolism through the *ortho*-cleavage pathway, while *xylE1* and *2,3-CTD* (encoding catechol 2,3-dioxygenases) are expressed in the *meta*-cleavage pathway of aromatics degradation (Li et al., 2012). Therefore, monitoring the expression of these genes may serve as an indicator of the presence of the enzymes involved in relevant metabolic pathways, thus providing important information about the toluene catabolic pathways of LVD-10. Six out of the nine genes found to be present in LVD-10 (Table 2), which were *XylE1*, *tmoA*, *1,2-CTD*, *2,3-CTD*, *rhlR1* and *rhlR2* (Fig. 6). However, *rhlI and tmoA*, were detected at different size that the expected size (Table 2). The negative results for *TodC1* indicate that the genes involved in the TOD pathway of *P. putida* are not present in LVD-10. *XylE1* is part of the *meta*-cleavage pathway of the TOL (pWW0) plasmid of *P. putida* mt-2, encoding a catechol extradiol dioxygenase enzyme (catechol 2,3-dioxygenase) responsible for the conversion of catechol to (2E,4E)-2-hydroxy-6-oxohexa-2,4-dienoate with the insertion of two atoms of molecular oxygen (Burlage et al., 1989; Hendrickx et al., 2006; Koutinas et al., 2011). *XylE1* was positively expressed in the samples tested indicating the presence of a *meta*-cleavage pathway for the metabolism of aromatics in LVD-10.

Furthermore, *1,2-CTD* and *2,3-CTD* encode the enzymes catechol 1,2-dioxygenase and catechol 2,3-dioxygenase that follow the *ortho*- and *meta*-cleavage pathways of aromatics, respectively. Specifically, *xylE1* and *2,3-CTD* are essential for complete mineralisation of aromatics, since the intermediates formed through the *meta*-cleavage pathway are channelled into the TCA cycle (Baldwin et al., 2003; Junca and Pieper, 2003; Hendrickx et al., 2006; Yong and Zhong, 2013). The results obtained suggest that LVD-10 employs a pathway where toluene degradation proceeds through the formation of a catechol intermediate. The first reaction of the pathway is possibly catalysed by toluene/xylene monoxygenase, while the *meta*-cleavage pathway could be also involved with the conversion of the produced catechol into (2Z,4E)-2-hydroxy-6-oxohexa-2,4-dienoate through the action of catechol 2,3-dioxygenase encoded by the *xylE1* gene. Furthermore, *1,2-CTD* was also present in LVD-10 demonstrating that the strain is capable of mineralising toluene through the *ortho*-cleavage pathway, which catalyses the reaction of ring fission forming intermediates channelled to the TCA cycle.

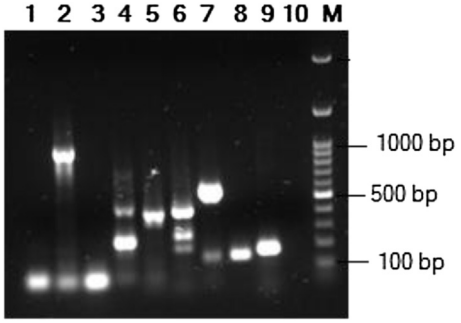

Fig. 6. PCR amplification of toluene catabolic genes and rhamnolipid production genes in *Pseudomonas aeruginosa* LVD-10. PCR amplification was performed with a non-template containing sample. Lanes: 1) *tbmD*, 2) *tmoA*, 3) *TodC1*, 4) *xylE1*, 5) *1,2-CTD*, 6) *2,3-CTD*, 7) *rhlI*, 8) *rhlR2*, 9) *proC* (housekeeping gene), 10) non-template control and M) 100–3000 bp markers.

Gene expression by quantitative RT-PCR

The expression level of the genes identified in LVD-10 was analysed by RT-PCR under saline and non-saline conditions (Fig. 7). The cells were tested at two different concentrations of NaCl (30 and 50 g L^{-1}) as well as in a control culture without salinity at different time points. The normalization process was carried out using *proC* as the housekeeping gene, which encodes the pyrroline-5-carboxylate reductase. *XylE1* and *1,2-CTD* were normally expressed at both time points tested for 30 g L^{-1} NaCl, since non-significant down- or up-regulation was observed compared to the control.

In 50 g L^{-1} NaCl *xylE1* was substantially down-regulated (29-fold in 48 h), whereas *1,2-CTD* was not expressed at that time point. The growth curve of LVD-10 in 50 g L^{-1} of salinity showed a prolonged lag phase and negligible biomass formation (Fig. 1) at 48 h, which is in agreement to the significant down-regulation of the catabolic genes monitored under high salinity conditions. Nevertheless, *1,2-CTD* was normally expressed at 120 h and did not exhibit any significant down- or up-regulation, while the growth of LVD-10 was at the exponential phase and the concentration of biomass was 0.4 g L^{-1} (data not shown). Furthermore, under 30 g L^{-1} NaCl *xylE1* was 5-fold down-regulated compared to the control, a reduction that was substantially smaller compared to the 30-fold down-regulation observed at 50 g L^{-1}.

The *RhlI* gene was suggested by Yong and Zhong (2013) as an important component of the QS system that contributed significantly towards the adaptation of bacteria in toxic conditions and

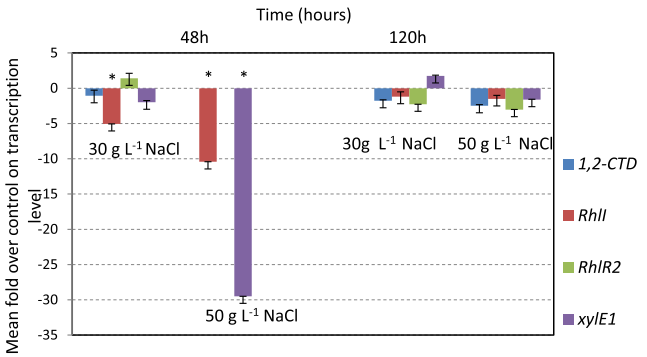

Fig. 7. Transcription levels of toluene metabolic genes. The transcription level obtained under non-saline conditions was set as 1.0 and was used as the control (data not shown). In the figure is shown the mean fold on gene transcription level at 48 h and 120 h in salinities of 30 and 50 g L^{-1} of NaCl. Negative values indicate gene down-regulation, whereas positive values indicate gene up-regulation. Statistical significance of differences between the tested samples was analysed by the *t*-test, $p < 0.05$ (Pair Wise Fixed Reallocation Randomisation Test©). Asterisks represent significant difference of the mean fold of the data points over the control where the *p* value was <0.05.

the improvement of their ability to degrade aromatic hydrocarbons in less- or non-toxic intermediates. They also proposed that the QS system positively regulates the *meta*-cleavage pathway. The present study demonstrated that the *RhlI* and *RhlR2* genes are encoded in the genome of LVD-10. These genes were expressed under saline and non-saline conditions and their expression level was affected when the concentration of NaCl was increased. A 5-fold and a 10-fold down-regulation of *rhlI* were monitored in 30 g L^{-1} and 50 g L^{-1} of NaCl (48 h) respectively. However, the expression of *rhlI* was normal at 120 h for both NaCl concentrations tested, suggesting that after the repression of toluene degradation pathways at early stages of the culture LVD-10 was capable of restoring the activity of key metabolic genes under salinity. Expression of *RhlR2* did not show any significant variation to the presence of salinity. However, at early stages of the culture performed at 50 g L^{-1} NaCl the gene was not expressed.

Conclusions

In the present work *P. aeruginosa* LVD-10 has been isolated and characterized (1075 bp, GenBank accession number KF728673.1) from an activated sludge sample based on its capability to biodegrade toluene under extreme conditions. Under 0, 20 and 40 g L^{-1} of NaCl, the percentage of toluene removal reached 86%, 98% and 89% within 24 h, respectively. The removal of toluene at pH 6 and 9 reached 75% within 24 h although no further increase was observed over time. The percentage of toluene removal for cultures performed at different temperatures ranged between 60% and 90%, while the highest removal was observed at 30 °C. LVD-10 was also tested for its capability to degrade other aromatic hydrocarbons (phenol, *p*-cresol, 4-nitrophenol, phenanthrene and indole), demonstrating that the strain was able to utilize them as sole carbon sources. The absence of phosphorus from the biomedium had a negligible effect on toluene biodegradation whereas, the absence of nitrogen source resulted in significant reduction of toluene biodegradation (50% overall toluene removal). *P. aeruginosa* LVD-10 was capable of removing diesel by a factor of 79.1% and 91.7% after 7 d in cultures containing of 1 mL L^{-1} of petroleum at salinity conditions of 0 and 30 g L^{-1} of NaCl respectively. It has been demonstrated that LVD-10 is equipped with the *xylE* gene, which is part of the TOL pathway used by *P. putida* mt-2 for toluene degradation, as well as with catechol 1,2 and 2,3-dioxygenases which are key enzymes involved in the *ortho*- and *meta*-cleavage pathways of aromatics responsible for the mineralisation of pollutants through the TCA cycle. The presence of genes (*xylE and 1,2-CTD)* that encode key enzymes for aromatics degradation and the fact that the expression levels of those genes was not substantially reduced under saline conditions of 30 g L^{-1} NaCl characterises LVD-10 as a halotolerant strain with great potential for toluene degradation. Furthermore, the presence of *RhlI* and *RhlR2*, which are involved in the regulation of aromatics degradation and biosurfactant production (QS system) constitute LVD-10 as a potential candidate for hydrocarbon degradation in contaminated sites.

Acknowledgements

The authors would like to thank Cyprus University of Technology for the award of an internal grant for the implementation of this work.

References

Abu Hamed, T., Bayraktar, E., Mehmetoglu, Ü., Mehmetoglu, T., 2004. The biodegradation of benzene, toluene and phenol in a two-phase system. Biochem. Eng. J. 19, 137–146.

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

Bahrami, A., Mahzub, H., Sadeghian, M., Golbabaei, F., 2011. Determination of benzene, toluene and xylene (BTX) concentrations in air using HPLC developed method compared to gas chromatography. Int. J. Occup. Hyg. (IJOH) 3, 12–17.

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

Bordel, S., Muñoz, R., Díaz, L.F., 2007. New insights on toluene biodegradation by *Pseudomonas putida* F1: influence of pollutant concentration and excreted metabolites. Appl. Microbiol. Biotechnol. 74, 857–866.

Burlage, R.S., Hooper, S.W., Sayler, G.S., 1989. The TOL (pWWO) catabolic plasmid. Appl. Environ. Microbiol. 55, 1323–1328.

Cao, B., Nagarajan, K., Loh, K.-C., 2009. Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl. Microbiol. Biotechnol. 85, 207–228.

Das, N., Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int. 2011, 941810–941823.

Di Martino, C., López, N.I., Raiger Iustman, L.J., 2012. Isolation and characterization of benzene, toluene and xylene degrading *Pseudomonas* sp. selected as candidates for bioremediation. Int. Biodeterior. Biodegrad. 67, 15–20.

Enright, A.-M., Collins, G., O'Flaherty, V., 2007. Low-temperature anaerobic biological treatment of toluene-containing wastewater. Water Res. 41, 1465–1472.

Farrell, R.L., Rhodes, P.L., Aislabie, J., 2003. Toluene-degrading Antarctic *Pseudomonas* strains from fuel-contaminated soil, 312, 235–240.

Hamzah, A., Tavakoli, A., Rabu, A., 2011. Detection of toluene degradation in bacteria isolated from oil contaminated soils. Sains Malays. 40, 1231–1235.

Harayama, S., Rekik, M., 1990. The meta cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Mol. Genet. Genomics 1, 113–120.

Harayama, S., Kishira, H., Kasai, Y., Shutsubo, K., 1999. Petroleum biodegradation in Marine environments. J. Microbiol. Biotechnol. 1, 63–70.

Head, I.M., Jones, D.M., Röling, W.F.M., 2006. Marine microorganisms make a meal of oil. Nat. Rev. Microbiol. 4, 173–182.

Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Rüegg, I., Bucheli-Witsche, M., Faber, F., Egli, T., Mau, M., Schlömann, M., Brennerova, M., Brenner, V., Pieper, D.H., Top, E.M., Dejonghe, W., Bastiaens, L., Springael, D., 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.

Jin, H.M., Choi, E.J., Jeon, C.O., 2013. Isolation of a BTEX-degrading bacterium, *Janibacter* sp. SB2, from a sea-tidal flat and optimization of biodegradation conditions. Bioresour. Technol. 145, 57–64.

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

Kim, J.M., Jeon, C.O., 2009. Isolation and characterization of a new benzene, toluene, and ethylbenzene degrading bacterium, *Acinetobacter* sp. B113. Curr. Microbiol. 58, 70–75.

Koutinas, M., Baptista, I.I.R., Meniconi, A., Peeva, L.G., Mantalaris, A., Castro, P.M.L., Livingston, A.G., 2007. The use of an oil-absorber-bioscrubber system during biodegradation of sequentially alternating loadings of 1,2-dichloroethane and fluorobenzene in a waste gas. Chem. Eng. Sci. 62, 5989–6001.

Koutinas, M., Kiparissides, A., Lam, M.-C., Silva-Rocha, R., Godinho, M., de Lorenzo, V., Martins dos Santos, V.A.P., Pistikopoulos, E.N., Mantalaris, A., 2011. Bridging the gap between genetic circuits and microbial growth kinetic models. Biochem. Eng. J. 55, 108–118.

Lee, E.Y., Jun, Y.S., Cho, K., 2002. Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. Air Waste Manag. Assoc. 52, 400–406.

Li, H., Liu, Y.h., Luo, N., Zhang, X.Y., Luan, T.g., Hu, J.M., Wang, Z.Y., Wu, P.C., Chen, M.J., Lu, J.Q., 2006. Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic *Planococcus* sp. strain ZD22. Res. Microbiol. 157, 629–636.

Li, H., Zhang, Q., Wang, X.-L., Ma, X.-Y., Lin, K.-F., Liu, Y.-D., Gu, J.-D., Lu, S.-G., Shi, L., Lu, Q., Shen, T.-T., 2012. Biodegradation of benzene homologues in contaminated sediment of the East China Sea. Bioresour. Technol. 124, 129–136.

Mukherjee, S., Bardolui, N.K., Karim, S., Patnaik, V.V., Nandy, R.K., Bag, P.K., 2010. Isolation and characterization of a monoaromatic hydrocarbon-degrading bacterium, *Pseudomonas aeruginosa* from crude oil. J. Environ. Sci. Health. A, Toxic/hazardous Subst. Environ. Eng. 45, 1048–1053.

Olsen, R.H., Kukor, J.J., Kaphammer, B., 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. 12, 3749–5376.

Pratheesh, P.T., Jayachandran, K., 2012. Biodegradation of toluene hydrocarbon by a *Pseudomonas* sp. isolated from gasoline contaminated soil. Int. J. Plant Anim. Environ. Sci. 2, 210–216.

Perfumo, A., Rudden, M., Smyth, T.J.P., Marchant, R., Stevenson, P.S., Parry, N.J., Banat, I.M., 2013. Rhamnolipids are conserved biosurfactants molecules: implications for their biotechnological potential. Appl. Genet. Mol. Biotechnol. 97, 7297–7306.

Reis, R.S., Pereira, A.G., Neves, B.C., Freire, D.M.G., 2011. Gene regulation of rhamnolipid production in *Pseudomonas aeruginosa*—a review. Bioresour. Technol. 102, 6377–6384.

Saghafi, S., Bakhshi, Z., Najafpour, G.D., Kariminezhad, E., Rad, H.A., 2010. Biodegradation of toluene and xylene in an UAPB bioreactor with fixed film of *Pseudomonas putida*. American-Eurasian J. Agric. Environ. Sci. 9, 1–7.

Salvi, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., Vahaboglu, H., 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. J. Med. Microbiol. 52, 403–408.

Shukor, M.Y., Dahalan, F.A., Jusoh, A.Z., Muse, R., Shamaan, N.A., Syed, M.A., 2009. Characterization of a diesel-degrading strain isolated from a hydrocarbon-contaminated site. J. Environ. Biol./Acad. Environ. Biol. India 30, 145–150.

Shields, M.S., Reaqin, M.J., Gerger, R.R., Campbell, R., Somerville, C., 1995. TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl. Environ. Microbiol. 4, 1352–1356.

Sorokin, D.Y., Janssen, A.J.H., Muyzer, G., 2012. Biodegradation potential of Halo(alkali)philic Prokaryotes. Crit. Rev. Environ. Sci. Technol. 42, 811–856.

Ward, D., Atlas, R.M., Boehm, P.D., Calder, J.A., 2013. Microbial biodegradation and chemical evolution of oil from the Amoco Spill. R. Swed. Acad. Sci. Ambio 9, 277–283.

Whited, G.M., Gibson, D.T., 1991. Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J. Bacteriol. 9, 3017–3020.

Wongsa, P., Tanaka, M., Ueno, A., Hasanuzzaman, M., Yumoto, I., Okuyama, H., 2004. Isolation and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescenss* possessing high efficiency to degrade gasoline, kerosene, diesel oil and lubricating oil. Curr. Microbiol. 49, 415–422.

Yong, Y.-C., Zhong, J.-J., 2013. Regulation of aromatics biodegradation by *rhl* quorum sensing system through of catechol meta-cleavage pathway. Bioresour. Technol. 136, 761–765.

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao, F., Xu, P., 2011. Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresour. Technol. 102, 4111–4116.

Zhang, L., Zhang, C., Cheng, Z., Yao, Y., Chen, J., 2013. Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by the bacterium *Mycobacterium cosmeticum* byf-4. Chemosphere 4, 1340–1347.

Zylstra, G.J., Gibson, D.T., 1989. Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the todC1C2BADE genes and their expression in *Escherichia coli*. J. Biol. Chem. 25, 14940–14946.