Desalination and Water Treatment
www.deswater.com

doi: 10.1080/19443994.2015.1086896

57 (2016) 17579–17584
August

Taylor & Francis
Taylor & Francis Group

Benzene degradation with bacterial strains isolated from rhizosphere of *Cannabis sativa* being irrigated with petroleum refinery wastewater

Muhammad Saad Bin Zahid, Aneela Iqbal, Muhammad Arshad*

School of Civil and Environmental Engineering, Institute of Environmental Sciences and Engineering, National University of Sciences and Technology, Sector H-12, Islamabad 44000, Pakistan, emails: saadzahidrandhawa@gmail.com (M.S.B. Zahid), aneela@iese.nust.edu.pk (A. Iqbal), Tel. +92 51 9085 4309; Fax: +92 51 9085 4202; email: marshad@iese.nust.edu.pk (M. Arshad)

Received 19 January 2015; Accepted 12 August 2015

ABSTRACT

Rhizoremediation is considered as an environmentally friendly technique to deal with the contamination of petroleum hydrocarbons. The current study was aimed at checking the potential of indigenous micro-organisms isolated from rhizosphere soil being irrigated by the wastewater of petroleum refinery to degrade benzene efficiently. For this study, soil samples were taken from the discharge point of oil refinery located in Rawalpindi, Pakistan, by brushing off the soil from roots of *Cannabis sativa*. The soil was processed for isolation of indigenous microbial strains. Sixteen bacterial strains isolated from the soil were screened for their potential to grow using benzene as sole carbon source *in vitro*. These were exposed initially to 250 mg L^{-1} followed by 500 and 750 mg L^{-1} of benzene, where only seven strains were able to survive with optical density (OD_{600}) of ≥1.1. Three better performing strains with $OD_{600} > 1.6$ were further subjected to degrade 1,000 mg L^{-1} benzene (sole carbon source) using M9 media. The community diversity analysis for the best performing strains was carried out using 16S ribosomal RNA. *Pseudomonas aeruginosa* (SAR-1) was the best degrader with 85% benzene degradation in 72 hours followed by *Bacillus cereus* (SAR-2) and *Acinetobacter junii* (SAR-3) with 83 and 70% efficiency, respectively. To the best of our knowledge, *A. junii* (SAR-3) is being reported as a benzene degrader for the first time.

Keywords: Rhizoremediation; Benzene; Refinery wastewater; *Pseudomonas aeruginosa*; *Bacillus cereus*; *Acinetobacter junii*

1. Introduction

Crude oil is produced from plants and animals that were buried deep below the earth's surface over the millions of years ago [1]. Crude oil is converted into various fractions like diesel, gas oil, kerosene, and gasoline during refining [2]. Wastewater is produced in large quantities during the process of petroleum refining. Petroleum refinery wastewater contains dissolved and dispersed oil with highest concentrations of BTEX (benzene, xylene, ethyl benzene, and toluene), phenols, and polycyclic aromatic hydrocarbons [3]. Benzene is a mono-aromatic hydrocarbon and its concentration varies in wastewater from 1 to 100 mg L^{-1} [2,4] and the levels within this range were recorded in the present study as well. The maximum contaminant level of benzene in drinking water is 0.005 mg L^{-1} [5].

Different physical and chemical treatment methods have been reported to remove benzene from

*Corresponding author.

petroleum refinery wastewater, but these methods are not cost effective [6], though biological methods are less expensive and eco-friendly [7]. In the biological processes, micro-organisms and/or their products are best known to break organic content into simple products under aerobic or anaerobic conditions [8–12]. Plant associated bacteria, i.e. endophytic (inside tissues of plant) and rhizophytic (root surface of plant) are well known for the biodegradation of toxic pollutants from soil [13,14]. Endophytes and rhizospheric bacteria are responsible for the promotion of plant growth by mobilization of nutrients, production of phytohormones, and provide protection against plant pathogens [14].

Bacilus cereus was helpful to degrade 150 mg L^{-1} benzene in 25 d [15]. Bacterial consortium obtained from rhizosphere soil of *Cyperus* sp. grown in petroleum contaminated area degraded BTX in 14 d [16]. BTEX biodegradation was achieved by bacteria from effluents of petroleum refinery in 20 d [17]. Soil bacteria were used to achieve benzene degradation in 5 d [18]. Current study aimed to isolate indigenous rhizospheric bacteria from petroleum contaminated soil and to check the potential of those bacteria to degrade benzene, followed by their identification through 16S ribosomal RNA (rRNA) gene using phylogenetic markers.

2. Materials and methods

2.1. Soil sampling

Soil sampling was carried out from discharge point of the oil refinery located in Rawalpindi, Pakistan by brushing off the soil from the roots of *C. sativa* for the isolation of rhizospheric bacteria. Soil pH was 6.9. Electrical conductivity was 1.5 mS m^{-1} and soil texture was sandy loam. Soil organic matter contents were 0.46%. Nitrogen, phosphorus, and potassium levels in soil were 200, 12, and 135 mg kg^{-1}, respectively.

2.2. Isolation and screening of bacterial isolates

Sixteen bacterial strains were isolated from soil samples on the basis of colony texture and morphology using the standard dilution technique followed by plating method. The bacterial isolates having different morphologies were subcultured thrice to ensure purity. In order to analyze the potential of bacterial isolates to survive on benzene, different concentrations of benzene (250, 500 and 750 mg L^{-1}) were used along with M9 media (64 g $Na_2HPO_4{\cdot}7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl and 5.0 g NH_4Cl at pH 7.0).

Acclimatization of bacterial strains was done in 250-mL flasks containing distilled water, M9 media, and different concentrations of benzene (250, 500 and 750 mg L^{-1}) as a sole carbon source to study the growth pattern of bacterial strains by measuring OD at 600 nm [19]. Surviving bacterial isolates were used for biodegradation studies. All experiments were carried out in triplicate.

2.3. Biodegradation studies

Benzene degradation potential of different bacterial strains was determined in batch experiments (3 d). Benzene was provided through an artificial source. Analytical grade benzene was used for the study, having density of 876.50 kg/m. Flasks were maintained at flask shaker at an ambient temperature within an orbital shaker at 120 rpm. Control samples without bacterial inoculum were also maintained. To prevent benzene volatilization, all the flasks were wrapped with aluminum foil. This was confirmed through the controls without bacterial cultures where no decrease in the concentration of benzene was recorded. Samples were extracted from each experiment flask after 0, 2, 4, 6, 24, 48, and 72 h under sterilized conditions of laminar flow hood. Benzene residual concentration after degradation was checked on UV spectrophotometer at 254 nm. The instrument can quantify benzene in a sample up to 5,000 mg L^{-1} with an absorbance of 0.948. Its limit of detection (LoD) is 0.1 mg L^{-1} with an absorbance of 0.005. LoD and limit of quantification (LoQ) for the method were determined by following the procedure described by Ambruster and Pry [20]. LoD for the method was 0.23 mg L^{-1} and LoQ was 0.3 mg L^{-1}.

2.4. Identification of best performing bacterial isolates

The bacterial strains with best degradation capabilities were preserved in glycerol and stored at −20°C. The isolates were sequenced from Genome Analysis Department Macrogen Inc. Korea. Sequences obtained were analyzed using BLAST search from National Center for Biotechnology Information (NCBI) databases revealing up to 99 or 100% similarity to different bacterial species. Multiple sequence alignments were carried out using CLUSTALW after complete deletion of the mismatch sequences. A phylogenetic tree, constructed using the TREEVIEW program illustrates the phylogenetic relatedness of identified strains. 16S rRNA sequences of organisms related to benzene degradation were submitted to GenBank and have been under accession numbers KM520129, KM520130, and KM520131.

3. Results and discussion

3.1. Obtaining screened bacterial cultures acclimatized to benzene

Continuous exposure to increasing concentrations of the targeted compounds to the micro-organisms which can efficiently degrade those compounds is a commonly used enrichment technique [19]. Rhizospheric bacteria were acclimatized using this technique. In preliminary studies, out of 16 isolated bacterial strains in response to exposure of 250 mg L^{-1} benzene, 4 strains were unable to grow (data not shown). However, all other strains had optical density greater than 0.5. These strains were then cultured on media containing 500 mg L^{-1} of benzene and results are shown in Fig. 1. Out of these 12 strains, only 7 had optical density greater than 0.7 and other 5 strains namely SAR-8 to SAR-12 were unable to grow using benzene as sole carbon source. These seven strains (SAR-1 to SAR-7) were acclimatized at 750 mg L^{-1} of benzene and results are shown in Fig. 2. All the seven strains were able to grow and an increase in optical density was observed for all the seven bacterial strains. SAR-1 had maximum optical density, i.e. 2.1, while that for SAR-2 was 1.7 and SAR-3 had an optical density of 1.6. These seven

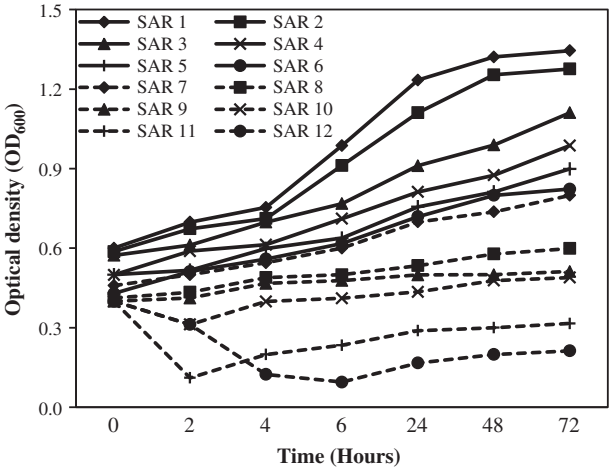

Fig. 1. Growth of different bacterial strains cultured with 500 mg L^{-1} of Benzene in M9 minimal media. Growth was measured in terms of optical density at 600 nm (OD_{600}).

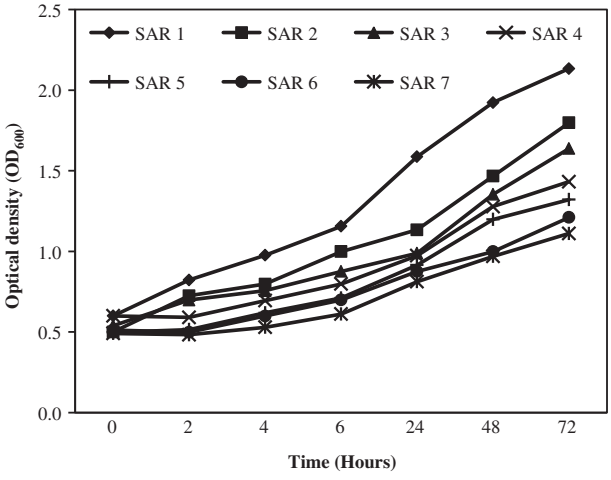

Fig. 2. Growth of seven bacterial strains previously screened. All the strains were able to have OD_{600} more than 1.0 when cultured at 750 mg L^{-1} of Benzene in the media.

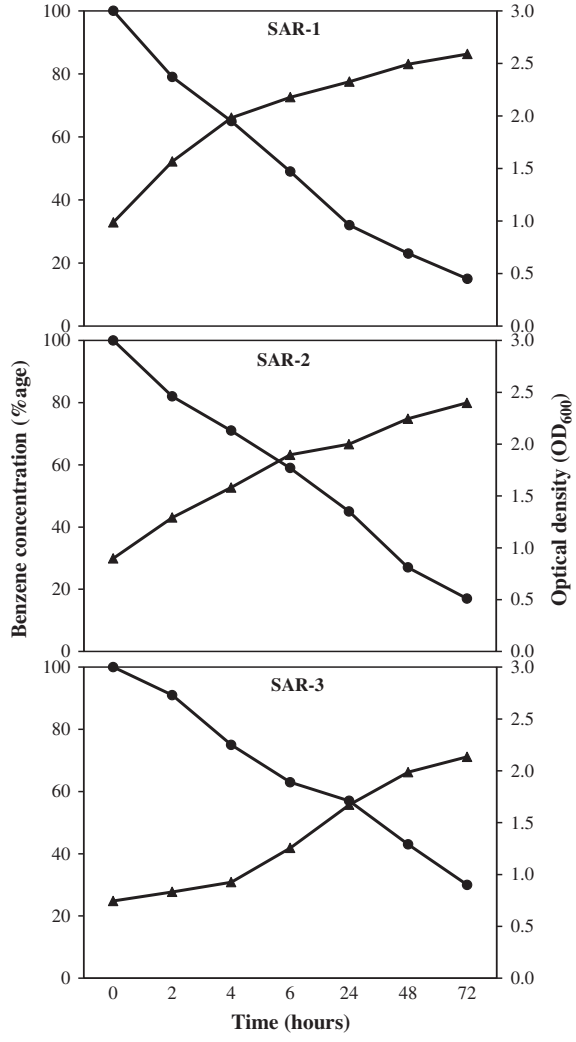

Fig. 3. Benzene degradation studies at 1,000 mg L^{-1} of contaminant in the media with three potential degraders, i.e. SAR-1, SAR-2, and SAR-3. Optical density (—▲—) increased with the passage of time while benzene concentration (—●—) decreased in the presence of all the three bacterial strains.

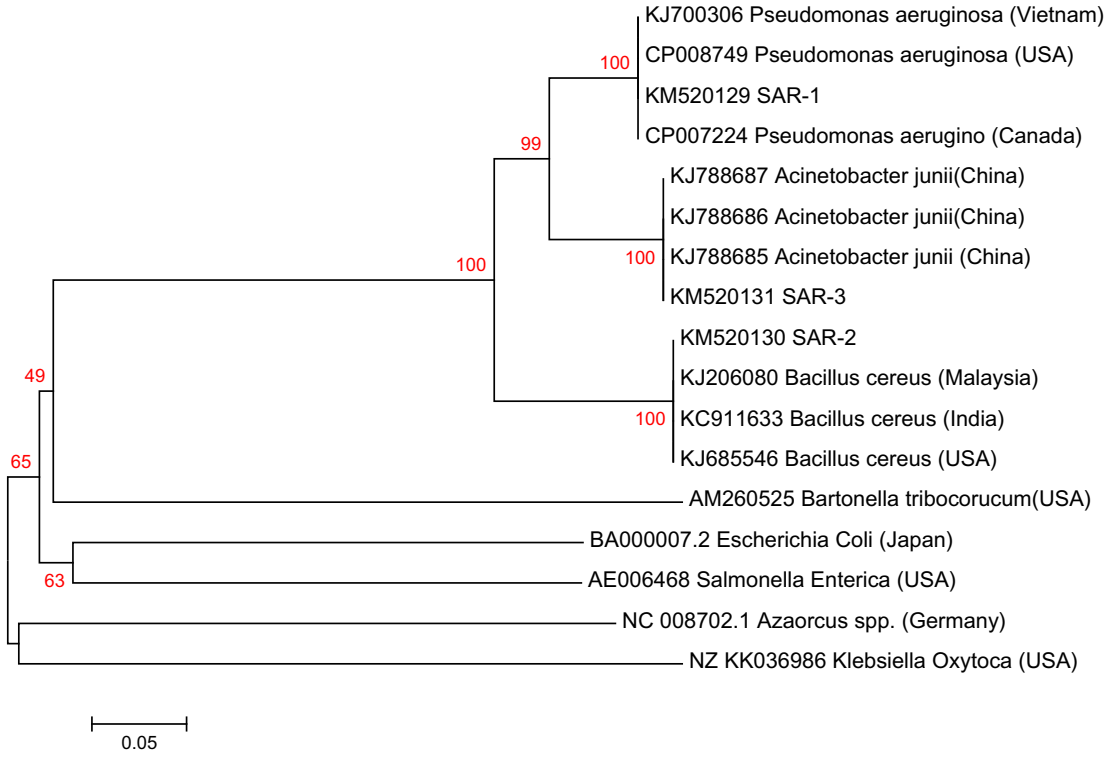

Fig. 4. Phylogenetic tree showing the relatedness of bacterial strains. Alignments of all 16S rRNA sequences were performed using CLUSTALW. Tree was constructed using TREEVIEW program. Genus names were identified as *Pseudomonas, Bacillus,* and *Acinetobacter*. Sequences submitted to NCBI GenBank can be accessed with accession numbers KM520129-KM520131.

acclimatized bacterial strains were further used for the biodegradation of benzene.

3.2. Estimation of benzene degradation

The residual concentration of benzene was determined on UV spectrophotometer in order to estimate the biodegradation of benzene. It was confirmed that decrease in the concentration of benzene is the result of biodegradation because controlled cultures showed negligible reduction (0.56%) in the concentration of benzene that was statistically nonsignificant. Comparison of profiles of different cultures to degrade benzene showed that the degradation occurred slowest with SAR-3 (Fig. 3). For the biodegradation of 1,000 mg L^{-1} of benzene, SAR-1 degraded 68% benzene within first 24 h, and 77% degradation was achieved at 48th h, ultimately 85% degradation was achieved in 72 h, while optical density had a maximum value of 2.5 during the same time frame (Fig. 3). SAR-2 degraded 55% benzene in first 24 h, 73% in 48 h and finally degrading 83% benzene in 72 h and optical density for SAR-2 was 2.3. SAR-3 degraded 43, 57, and 70% benzene in 24, 48 and 72 h, respectively, and optical density was 2.1.

Lin et al. [21] demonstrated that 75% phenanthrene was degraded by *Pseudomonas* sp. BZ-3 of petroleum hydrocarbon at initial concentration of 50 mg L^{-1} in 7 d. Three strains were isolated by Zhang et al. [22] which were able to degrade n-alkanes up to n-C40. Removal efficiency of each strain increased up to 10% within 7 d, under aerobic conditions. The three strains were identified as *Pseudomonas aeruginosa*. In our case, *P. aeruginosa* degraded 85% of 1,000 mg L^{-1} benzene in 72 h. AL-Saleh and Obuekwe [23] achieved degradation of aromatic hydrocarbons including benzene and phenanthrene by *Cupriavidus gilardii, Pseudomonas* sp., *B. cereus, and Paenibacillus ehimensis*. When the concentration of benzene was 1.62 μg L^{-1}, they achieved 22.2% degradation. According to Sharma et al. [24] *P. aeruginosa* degraded 66% of diesel hydrocarbons including benzene in 30 d. Dou et al. [15] degraded 150 mg L^{-1} benzene completely within 25 d with the help of *B. cereus*. In the present study, *B. cereus* was able to degrade 83% of 1,000 mg L^{-1} benzene in 72 h.

3.3. Identification of best degraders

Best performing strains were sequenced for 16S rRNA gene from Genome Analysis Department Macrogen Inc. Korea. They were identified by doing a Basic Local Alignment Search Tool (BLAST) search from NCBI databases revealing ≥99% similarity to different bacterial species. SAR-1 through SAR-3 was identified as *P. aeruginosa*, *B. cereus* and *Acinetobacter junii*, respectively. Partial 16S rRNA sequences of these three bacterial isolates have been submitted to GenBank and accession numbers provided are KM520129 through KM520131. These bacteria are promising candidates for benzene removal and they can be used to remediate soil contaminated with petroleum hydrocarbons. To the best of our knowledge, *A. junii* is being reported as benzene degrader for the first time and it degraded 70% of 1,000 mg L^{-1} benzene in 72 h. The sequences were aligned using CLUSTALW after complete deletion of the mismatch sequences. A phylogenetic tree, constructed using the TREEVIEW program illustrates the phylogenetic relatedness of identified strains as shown in Fig. 4.

4. Conclusion

Sixteen bacterial strains were isolated from the rhizopshere of *C. sativa* being irrigated with petroleum refinery wastewater. After gradual screening at increasing concentration followed by degradation studies upon exposure to 1,000 mg L^{-1} benzene over a period of 72 h, three bacterial strains out-competed others in efficiency. These were identified as *P. aeruginosa*, *B. cereus* and *A. junii* which were coded as SAR-1, SAR-2, and SAR-3, respectively. All the three showed benzene biodegradation efficiency ≥70%. Partial sequences of these strains are available at NCBI with accession numbers KM520129, KM520130, and KM520131, respectively. Further studies are required to investigate the potential at gene level for better understanding of degradation mechanisms as Liu et al. [25] have mentioned that the *alkB* genes are involved in the degradation of aromatic hydrocarbons.

Acknowledgments

The authors would like to thank S&T Mega Fund, National University of Sciences and Technology (NUST), Islamabad, Pakistan for financial support. We are also thankful to Prof. Imran Hashmi and Dr Faraz Bhatti, NUST, Islamabad for their kind guidance and help during the study and to Ms Aisha Chaudhry, Walden University, Baltimore, USA for English revisions.

References

[1] D.H. Carlson, C.C. Plummer, L. Hammersley, Physical Geology: Earth Revealed, ninth ed., McGraw-Hill Companies Inc, New York, NY, 2011.
[2] S. Ishak, A. Malakahmad, M.H. Isa, Refinery wastewater biological treatment: A short review, J. Sci. Ind. Res. 71 (2012) 251–256.
[3] T.H. Khaing, J. Li, Y. Li, N. Wai, F. Wong, Feasibility study on petrochemical wastewater treatment and reuse using a novel submerged membrane distillation bioreactor, Sep. Purif. Technol. 74 (2010) 138–143.
[4] M.S. Jo, E.R. Rene, S.H. Kim, H.S. Park, An analysis of synergistic and antagonistic behavior during BTEX removal in batch system using response surface methodology, J. Hazard. Mater. 152 (2008) 1276–1284.
[5] US-EPA, United States—Environmental Protection Agency, Drinking Water Standards and Health Advisories, Office of Water, US-EPA, Washington, DC, 822-S-12-001 2012.
[6] L. Yan, H. Ma, B. Wang, Y. Wang, Y. Chen, Electrochemical treatment of petroleum refinery wastewater with three dimensional multiphase electrode, Desalination 276 (2011) 397–402.
[7] E. Dania, BTEX biodegradation by bacteria from effluents of petroleum refinery, Sci. Total Environ. 408 (2010) 4334–4340.
[8] A.F. Razi, A. Pendashteh, A.L. Chauh, D.A.B. Radiah, S.M. Syaed, Review of technologies from oil and gas produced water treatment, J. Hazard. Mater. 170 (2009) 530–551.
[9] F.Z. Mesbaiah, F. Mansour, K. Eddouaouda, A. Badis, Surfactant effects on biodegradation of polycyclic aromatic hydrocarbons, Desalin. Water Treat. (2014), doi: 10.1080/19443994.2014.988651.
[10] B.-J. Zhou, T.-H. Chen, Biodegradation of phenol with chromium(VI) reduction by the *Pseudomonas* sp. strain JF122, Desalin. Water Treat. (2014), doi: 10.1080/19443994.2014.987825.
[11] A. Hamitouche, Z. Bendjama, A. Amrane, F. Kaouah, Biodegradation of p-cresol by *Pseudomonas* spp., Desalin. Water Treat. (2014), doi: 10.1080/19443994.2014.982956.
[12] A. Popenda, M. Włodarczyk-Makuła, The application of biosurfactants into removal of selected micropollutants from soils and sediments, Desalin. Water Treat. (2015), doi: 10.1080/19443994.2014.996007.
[13] N. Weyens, D.V.D. Lelie, S. Taghavi, L. Newman, J. Vangronsveld, Exploiting plant-microbe partnerships to improve biomass production and remediation, Trends Biotechnol. 27 (2009) 591–598.
[14] B.R. Glick, Using soil bacteria to facilitate phytoremediation, Biotechnol. Adv. 28 (2010) 367–374.
[15] J. Dou, A. Ding, X. Liu, Y. Du, D. Deng, J. Wang, Anaerobic benzene biodegradation by a pure bacterial culture of *Bacillus cereus* under nitrate reducing conditions, J. Environ. Sci. 22 (2010) 709–715.
[16] D.K.O. González, D. Zaragoza, J.A. Garrido, H.R. Saad, C.S. Hernández-Rodríguez, J.J. Roblero, Degradation of benzene, toluene, and xylene isomers by a bacterial consortium obtained from rhizosphere soil of *Cyperus* sp. grown in a petroleum-contaminated area, Folia Microbiol. 58 (2013) 569–577.

[17] D.E. Mazzeo, C.E. Levy, D.F. de Angelis, M.A. Marin-Morales, BTEX biodegradation by bacteria from effluents of petroleum refinery, Sci. Total Environ. 408 (2010) 4334–4340.
[18] H. Xie, L. Zhu, L. Wang, S. Chen, D. Yang, L. Yang, H. Yuan, Photodegradation of benzene by TiO_2 nanoparticles prepared by flame CVD process, Particuology 9 (2011) 75–79.
[19] K. Masakorala, J. Yao, M. Cai, R. Chandankere, H. Yuan, H. Chen, Isolation and characterization of a novel phenanthrene (PHE) degrading strain *Pseudomonas* sp. USTB-RU from petroleum contaminated soil, J. Hazard. Mater. 263 (2013) 493–500.
[20] D.A. Ambruster, T. Pry, Limit of blank, limit of detection and limit of quantification, Clin. Biochem. Rev. 29 (2008) S49–S52.
[21] M. Lin, X. Hu, W. Chen, H. Wang, C. Wang, Biodegradation of phenanthrene by *Pseudomonas* sp. BZ-3, isolated from crude oil contaminated soil, Int. Biodeterior. Biodegrad. 94 (2014) 176–181.
[22] X.S. Zhang, D.J. Xu, C.Y. Zhu, T. Lundaa, K.E. Scherr, Isolation and identification of biosurfactant producing and crude oil degrading *Pseudomonas aeruginosa* strains, Chem. Eng. J. 209 (2012) 138–146.
[23] E. AL-Saleh, C. Obuekwe, Crude oil biodegradation activity in potable water. Int. Biodeterior. Biodegrad. 93 (2014) 18–24.
[24] A. Sharma, P. Kumar, M.B. Rehman, Biodegradation of diesel hydrocarbon in soil by bioaugmentation of *Pseudomonas aeruginosa*: A laboratory scale study, Int J. Environ Biorem. Biodegrad. 2 (2014) 202–212.
[25] H. Liu, J. Xu, R. Liang, J. Liu, Characterization of the medium- and long-chain n-alkanes degrading *Pseudomonas aeruginosa* strain SJTD-1 and its alkane hydroxylase genes, PLoS ONE 9(8) (2014) e105506, doi: 10.1371/journal.pone.0105506.