Vol.64: e21200002, 2021
https://doi.org/10.1590/1678-4324-2021200002
ISSN 1678-4324 Online Edition

Article - Environmental Sciences

Biodegradation of Terephthalic Acid by Isolated Active Sludge Microorganisms and Monitoring of Bacteria in a Continuous Stirred Tank Reactor

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Ana Cláudia Barana

Received: 2020.01.29; Accepted: 2020.08.09.

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HIGHLIGHTS

- Terephthalic acid one of the phthalates has been known endocrine disrupting chemical and degraded by bacterial strains.
- Four bacterial isolates identified as *Arthrobacter* sp*., Chryseobacterium* sp. *Burkholderia* sp. and *Pseudomonas* sp are able to degrade 100 mgL^{-1} concentration terephthalic acid.
- In order to monitory bacteria in CSTR reactor, Real-time PCR was used.

Abstract: Terephthalic acid is extensively used as an important raw material in polyester fibers, as well as the production of polyethylene terephthalate bottles and textile industries. Especially, in the petrochemical industry, toxic chemicals are released to the atmosphere during the production of polyethylene terephthalate, unless the wastewater treatment is carried out. It's a well-known fact that chemicals have serious side effects on human health, so manufacturing companies should not dispose of such harmful chemicals without treatment. Biodegradation is an effective option for eco-friendly degradation of hydrocarbons. Hydrocarbon-degrading bacteria are everywhere in environment and can utilize these chemicals as sources of carbon and energy. In the present study, aerobic bacterial strains T1, T4, T5, and TK were isolated from activated sludge and crude oil deposits of a petrochemical company in Turkey. The strains were identified to be *Pseudomonas* sp., *Chryseobacterium* sp., *Burkholderia* sp., and *Arthrobacter* sp. according to morphological, physiological and biochemical characteristics. The strains were able to degrade about 100% of 100 mg/L terephthalic acid within, respectively, 8, 67, 52, 24 hour as sole carbon and energy source. Therefore, these isolates can be

effectively used for degradation of terephthalic acid contaminated sites. In addition to this, a Continuous Stirred Tank Reactor (CSTR) was used to test the biodegradation capabilities of the isolates in the activated sludge system. Throughout the biodegradation, bacterial existence and numbers were monitored using designed primer-probe sets in real-time polymerase chain reaction (PCR).

Keywords: biodegradation; Terephthalic acid; HPLC (High-Performance Liquid Chromatography); real-time PCR; CSTR (Continuous Stirred Tank Reactor).

INTRODUCTION

Terephthalic acid (TA) is one isomer of the three phthalic acids [1]. Phthalic acid isomers are used for manufacturing plastic bottles, fibers, dye, cellulose film coating, adhesives, polyvinyl chloride (PVC) resins and they are also used in cosmetics, insecticides, and propellants all over the world [2,3]. However, TA is the raw material of polyester fibers and polyethylene terephthalate (PET). PET is produced from TA and ethylene glycol. These chemicals are derived from a petroleum compound and several million tons of them are produced annually [4]. Phthalates are on the list of priority pollutants according to the USA Environmental Protection Agency [5,6]. Toxic chemicals are released to the atmosphere during the production of terephthalic acid. It's a well-known fact that chemicals have serious side effects on human health, so manufacturing companies should not dispose of such harmful chemicals without wastewater treatment. These are endocrine-disrupting chemicals and have been found to be harmful to human and animal health and have hepatotoxic, teratogenic and carcinogenic properties [7,8,9].

Recently, biological treatment technology has developed rapidly. Several studies have indicated that aerobic or anaerobic microorganisms play the major roles in the phthalates degradation in the environment [10,11,12,13,14,15]. Mechanical and chemical methods of removing hydrocarbons from contaminated sites are highly expensive. For this reason, biodegradation (biological method) is the preferred method in most wastewater treatment systems. Another advantage of biodegradation is converting the toxic material to less hazardous or nonhazardous compounds by microorganisms [16,17,18]. The biological treatment (biodegradation), particularly by the activated sludge process was generally used for degradation of chemicals like TA from petrochemical wastewater [19]. Bacterial cultures have recently been added to wastewater treatment as commercial products [20].

Many studies have reported that biodegradation of TA occurs in aerobic or anaerobic conditions. *Arthrobacter* sp. 0574, *Pseudomonas* sp., *Bacillus* sp., *Comamonas testosterone* and *Rhodococcus biphenylivorans* have studied on biodegradation of TA and effectiveness results have achieved [10,21,22,23,24,25,26,27]. Recent studies suggest that applications of molecular techniques using real-time PCR to environmental samples have proven to the practicable. Real-time PCR technique using a fluorogenic probe and specific primers allows for rapid detection and numbers of various environmental microorganisms [28,29,30].

The present research study was aimed to isolate TA degrading and with using bacterial isolates which have degradation abilities in Continuous Stirred Tank Reactor (CSTR). Also, *Chryseobacterium* sp. and *Burkholderia* sp. which are in CSTR were quantified using real-time PCR.

MATERIAL AND METHODS

Chemical

TA was purchased from Sigma Aldrich (185361) and used as hydrocarbon source. TA was dissolved in dimethyl sulfoxide (DMSO) as a stock solution which was 2000 mgL^{-1}. The stock solution was stored at 4 °C for further use.

Isolation of TA degrading strains from petrochemical wastewater

Activated sludge and wastewater samples were collected from aeration pool of wastewater treatment plants and crude oil deposits of a petrochemical company in Aliağa-Izmir, Turkey in different seasons and examined for isolation of TA degrading bacteria. The samples were stored at 4 °C until inoculation time. Bacterial cultures were selected in flasks filled with 50 mL Bushnell Haas (BH) medium (Sigma-Aldrich-B5051) which was autoclaved at 121 °C for 15 min [31]. As a sole carbon source, 100 mgL^{-1} TA was added to each flask. Then, approximately 2.5 g of activated sludge and 2.5 mL wastewater samples were put in the 50 mL flask and adjusted to pH 7.5-8. The flask was incubated at 30 °C on an incubator shaker (New

Brunswick Scientific Innova 4340) at 150 rpm for 7 days [24]. Then, 2.5 mL of the enriched culture was transferred to a new flask which contains fresh BH medium and incubated under the same conditions. This operation has been repeated four times to obtain enriched TA degrading bacteria.

At the end of the bacterial screening process, bacterial strains in the consortium were isolated by spreading the serially diluted ten-fold consortium onto BH agar plates added with 100 mgL^{-1} TA. Then, morphologically different bacterial colonies were selected and separately streaked on Plate Count Agar (PCA, Merck 105463) to obtain a pure culture of the bacterial isolates.

Identification of microorganisms

Purified strains were then identified by biochemical tests such as Gram staining, potassium hydroxide test (KOH), catalase and oxidase test. For the identification of isolated bacterial cultures, genomic DNA was extracted with Zymo-ZR Fungal/Bacterial DNA Kit. It was used in accordance with the manufacturer's instructions. The 16S rDNA genes were amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3) [24,32]. Polymerase chain reaction (PCR) is an amplification technique that includes three different steps (denaturation, annealing, and extension). Initial denaturation step: 1 cycle of 95 ºC for 120 s, denaturation step: 95 ºC for 20 s, annealing step: at 56 ºC for 40 s, extension step: 30 cycles of 72 ºC for 90 s; and final extension step: 72 ºC for 5 min.

HPLC analysis

High-performance liquid chromatography (HPLC) analysis was performed to screen the TA degradation ability of the isolates The bacterial strains were subjected to shake flask incubation (30℃, 150 rpm) with 100 mgL^{-1} TA in BH medium in 250 mL Erlenmeyer flasks. Inoculums were prepared in nutrient broth (0.5 McFarland). All of the flasks were incubated in the dark to prevent the possibility of photo-oxidation. 1 mL aliquots were transferred into 1.5 mL sterile microcentrifuge tubes at certain intervals and samples were centrifuged at 10000 rpm for 3 minutes. The supernatant (500 µL) was transferred to HPLC vial for TA analysis. HPLC analyses were carried out according to Wang and coauthors with slight modifications; the mobile phase was (80:20 methanol:water) at a flow rate of 0.4 mL/min. 1 µL sample was injected to the Agilent Zorbax Eclipse PAH Column at 25 °C column temperature and the detection wavelength was 240 nm. The analysis was carried out at ambient temperature. TA retention time was at 2.5 to 3 min [25].

Operating CSTR for TA degradation

We used artificial wastewater in terms of representing actual conditions in a continuous stirred tank reactor (CSTR). To test the microbial isolates with the best hydrocarbon degradation activities in a laboratory-scale reactor, an activated sludge sample was taken from pools in the aerobic biological treatment of the wastewater plant of the petrochemical industry.

The CSTR which used in operation was manufactured as previously explained [33]. Biological activated sludge was taken from the wastewater treatment unit from the petrochemical industry during a certain period was used as the initial sludge aging. From the moment sludge maturation takes place, it was seen that some parameters stable: pH was between 7.0-7.5; chemical oxygen demand (COD) was 1600 mgL^{-1} dissolved oxygen (DO) was 4 mgL^{-1}; Mixed liquor suspended solids (MLSS) was 2500 mgL^{-1}.

CSTR has fed with artificial wastewater formulated as Glucose 1.5 g, Peptone 0.6 g, Urea 0.1 g, $Na_2HPO_4.12H_2O$ 0.0836 g, KH_2PO_4 0.0136 g, NaCl 0.03 g, KCl 0.014 g, $CaCl_2.2H_2O$ 0.01854 g, $MgSO_4.7H_2O$ 0.00262 g, $FeCl_3.6H_2O$ 0.0166 g per liter. Once the appropriate MLSS values were reached in the tank, the isolates were inoculated into flasks contained with 50 mL of Nutrient Broth were incubated at 30 °C in a rotary shaker for 48 hours Bacterial suspension (T1,T4,T5,TK) of concentration 10^8 cfu mL^{-1} was prepared and 500 mL of the prepared bacterial suspension (10% of the working volume) were added into the CSTR . The TA which previously prepared as the stock solution in DMSO was added to the tank at a final concentration of 50 mgL^{-1} and TA biodegradation rates were measured by HPLC in every 24 or 48 hours. The degradation process was continued for 11 days. All the operations described above were repeated for the control tank. However, microbial isolates were not added to the control tank.

Microbial Monitoring During Degradation

The microorganisms added to the tank were monitored by real-time PCR (LightCycler® 1.5, Roche Diagnostics, Germany) during TA biodegradation. For this, activated sludge samples were taken from the tank and total microbial DNA isolations were performed with the aforementioned DNA isolation kit in accordance with the manufacturer's instruction. Real-time PCR was performed using a TaqMan Probe system. LightCycler® TaqMan® Master Kit (Roche Diagnostics, Germany) was used in accordance with the manufacturer's instruction. PCR conditions were performed as an initial denaturation step at 95 °C for 10 min for 1 cycle, amplification steps follow as denaturation at 95 °C for 10 s for 1 cycle, annealing 46 °C for 30 s, extension at 72 °C for 1 s, for 45 cycles and cooling step at 40 °C for 30 s, for 1 cycle, respectively for *Burkholderia* sp., initial denaturation at 95 °C for 10 min for 1 cycle, amplification steps follow as denaturation at 95 °C for 10 s for 1 cycle, annealing at 55 °C for 30 s, extension at 72 °C for 1 s, for 45 cycles and cooling step at 40 °C for 30 s, for 1 cycle, respectively for *Chryseobacterium* sp. Primer-probe sets were designed for enumeration of the bacterial isolate in real-time PCR [34].

RESULTS

HPLC results of microorganisms

The HPLC analysis of the BH medium with TA showed a peak at 240 nm at 2.6 retention time. For the initial concentration of 100 mgL^{-1}, TA degradation was determined to be 100% for T1, T4, T5 and TK isolates at 8, 67, 52 and 24 hours, as shown in Figure 1. Although the most common bacteria in environment was T4, after 60 hours, there was still TA. Because we think that the genes that degradation of TA were expressed less than other bacteria. Sterile control flask, it was not observed biodegradation of TA.

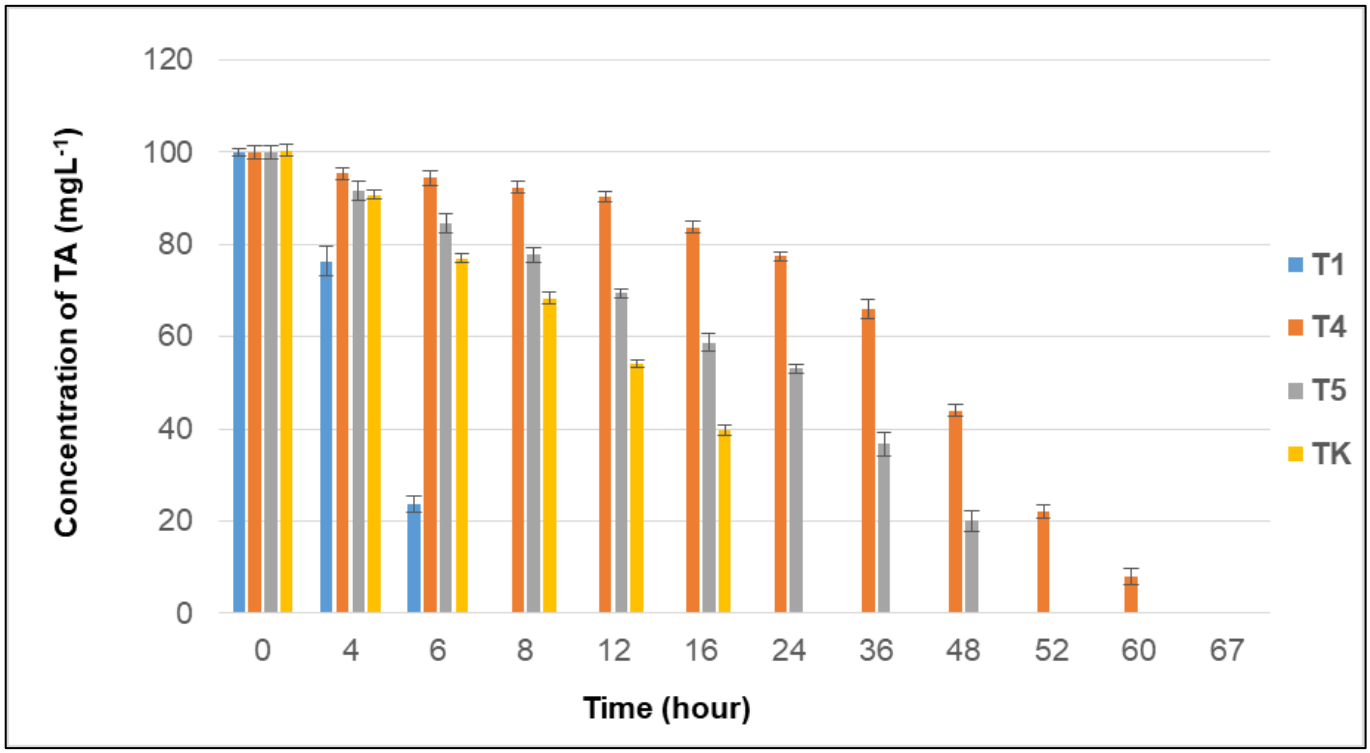

Figure 1. Biodegradation of TA by the isolated strains.

Isolation and identification of TA degrading microorganisms

TA degrading microorganisms were isolated from active sludge samples. 6 strains grew on TA containing BH medium. Among them, 4 strains were found to degrade TA. The biochemical and physiological characteristics of four strains are shown in Table 1. According to the results, Catalase and oxidase tests of all isolates were positively determined. T1, T4, T5 isolates were detected Gram-negative bacteria and TK was Gram-positive bacteria. However, the appearance of these isolates in the petri dish was determined to be different.

Table 1. Characteristics of four bacteria.

Identifying characteristic	T1	T4	T5	TK
Gram Stain	Gr -	Gr -	Gr -	Gr +
KOH Test	+	+	+	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Colonial color	light grey	orange	yellow	white

Subsequent 16S rRNA based phylogenetic analysis has demonstrated that the strains belonged to the genus as *Arthrobacter* sp., *Chryseobacterium* sp. *Burkholderia* sp. and *Pseudomonas* sp. The nucleotide sequences of 16S rRNA of four strains determined in this study have deposited in the GenBank database (NCBI) and the accession numbers have presented in Table 2.

Table 2. Affiliations of bacterial 16S rRNA gene sequences of T1, T4, T5 and TK.

Code of bacteria	Accession Number	Closest Species in GenBank	Similarity (%)
T1	JX480627	*Pseudomonas* sp.	100
T4	JX480628	*Chryseobacterium* sp.	99
T5	JX480629	*Burkholderia* sp.	100
TK	JX480630	*Arthrobacter* sp.	100

Evolutionary analyses were conducted in MEGA7 [35]. On the basis of the consensus sequences for the 16S rRNA gene, and a phylogenetic tree was constructed using sequences from the 4 strain isolates (Figure 2) The phylogenetic tree analysis showed that the 4 bacteria shared high 16S rDNA gene sequence similarities. Members of the genera *Chryseobacterium*, *Burkholderia*, *Pseudomonas, Arthrobacter* were supported by >99% bootstrap value and were well-established.

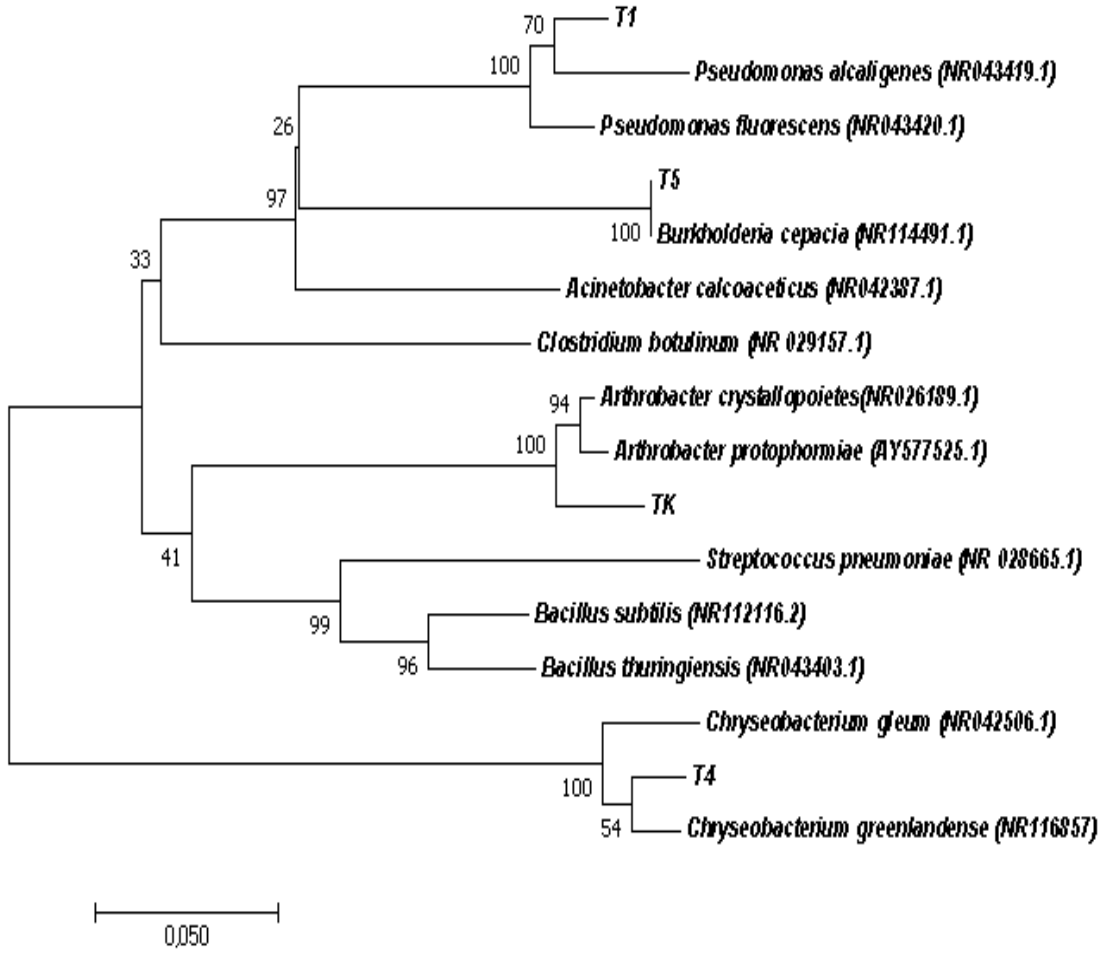

Figure 2. Dendrograms of sequence alignments of the 16S rRNA gene. T1:*Pseudomonas* sp. (JX480627), T4: *Chryseobacterium* sp. (JX480628), T5: *Burkholderia* sp.(JX480629), TK: *Arthrobacter* sp.(JX480630). The scale bar indicates 0.05 estimated changes per nucleotides and the numbers showed the bootstrap values representing percentage confidence of 1000 replicate analysis.

Microbial monitoring was performed in the inoculated CSTR during 11 days period. Two isolates were monitored with real-time PCR. As seen in Figure 3, while *Chryseobacterium* sp. is the most abundant bacterium in the first 6 days in inoculated CSTR. The number of *Burkholderia* sp. has increased gradually during the degradation process. Although numbers of two bacteria are almost equal on the 7th day, numbers have decreased relatively from the 9th day. The first number of copy DNA of *Chryseobacterium* sp. was calculated as 4.0×10^6; at the end of 11 days, *Chryseobacterium* sp. was calculated as 5.4×10^5. Approximately 1 log of decrease has taken place in the number of *Chryseobacterium* sp., *Burkholderia* sp. was calculated as 8.1×10^4 at the beginning and 2.4×10^4 at the end of 11 days. It could be said that the number of *Burkholderia* sp. was not significantly changed when compared with the beginning.

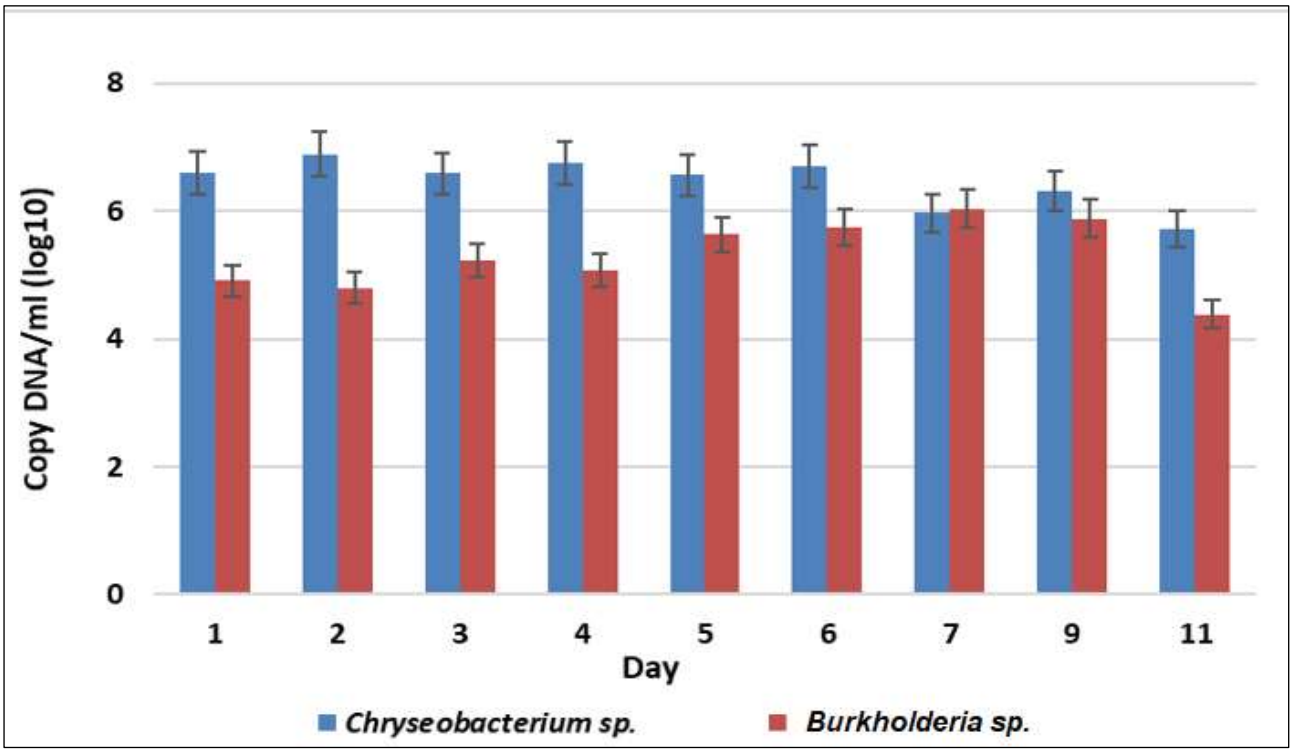

Figure 3. Copy DNA numbers of two selected bacteria during the biodegradation process. Each bar indicates that individual copy numbers of bacterial DNA at the log10 base (error bars calculated as a percentage).

The initial concentration of TA is 50 mgL^{-1} in inoculated and non-inoculated CSTR. 50 mgL^{-1} 56.4% of TA degraded in the inoculated CSTR in first 24 hours by activated sludge which inoculated with degradative microorganisms, while 32% of 50 mg L^{-1} of TA degraded in the non-inoculated CSTR. 85.8% of TA degraded in inoculated CSTR after 11 days, but 42.1% of TA degraded in non-inoculated CSTR after 11 days. The efficiency of inoculated CSTR approximately 50% more effective than non-inoculated CSTR, and in the light of these data, it is clear that inoculated CSTR has better performance (Figure 4).

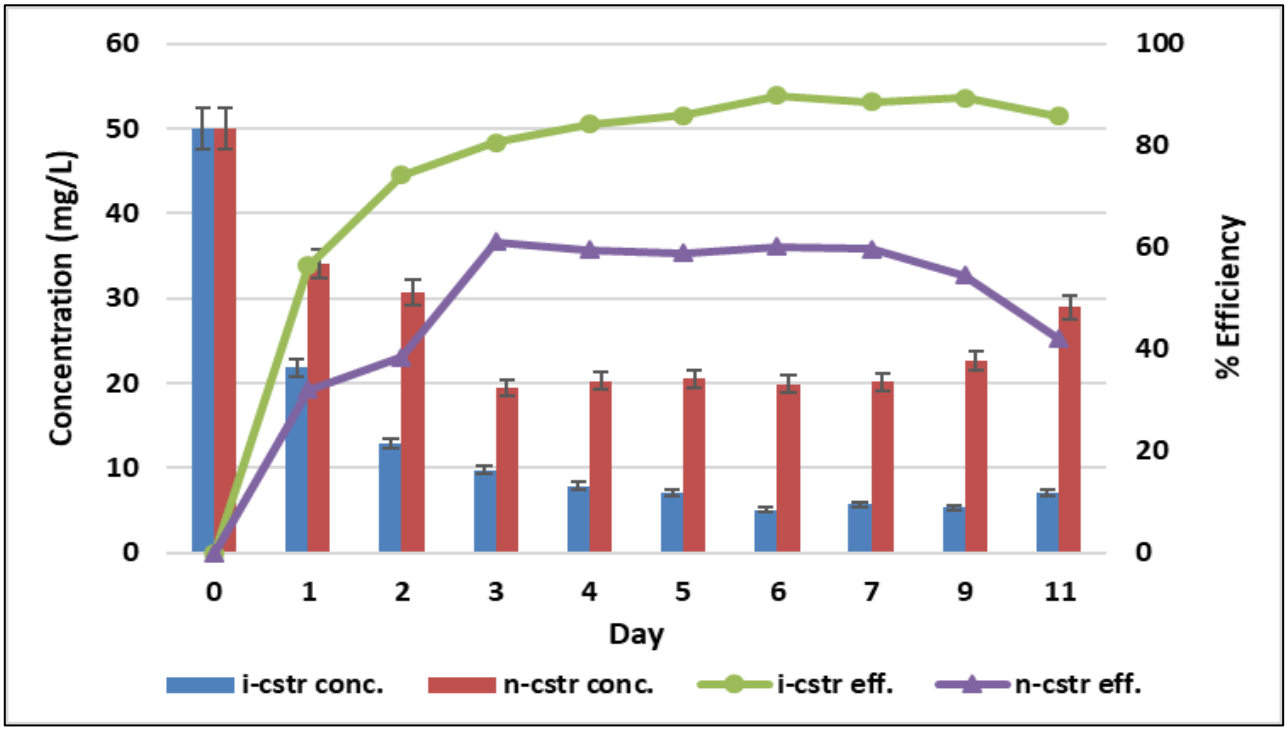

Figure 4. The graph shows the concentrations of TA determined in the effluent during the degradation and the removal efficiencies. (i-cstr: inoculated CSTR with degradative isolates, n-cstr: non-inoculated CSTR as control).

DISCUSSION

Biodegradation, one of the popular topics, basically involves the conversion of complex polluants to simpler ones by using microorganisms. This biological method proves to be less expensive than other methods used for cleaning up hazardous waste. Numerous studies have been reported that on the biodegradation of wastewater treatment. Biodegradation of chemicals such as phthalates, polyaromatic hydrocarbons (PAH), benzene, toluene, phenol was studied by many researchers [24,36,37,38,39]. In this study, the removal of TA via bacteria and bioaugmented sludge is effective. We understand that, different bacteria can degrade TA in different times.

Four isolates from petrochemical wastewater were obtained in BH medium containing TA (100 mgL^{-1}) as the sole carbon source. T1 isolate showed the ability to degrade 100 mgL^{-1} TA in 8 hours. Morphological characteristics of the 3 isolates were found to be a Gram-negative and 1 isolate was Gram-positive on the screening medium. These bacteria were identified as *Arthrobacter* sp., *Chryseobacterium* sp. *Burkholderia* sp. and Pseudomonas sp. (T1) have the best result for biodegradation of TA. This study petrochemical wastewaters in Turkey, to obtain 4 different bacteria differ in terms of degrading TA. Also, *Chryseobacterium* sp. has not been reported to be isolated in TA biodegradation studies so far. T1 isolate (degrade of TA in 8 hours) revealed its difference from other studies.

To date, many phthalates degrading bacterial strains such as; *Flavobacterium* sp., *Pseudomonas aeruginosa* PP4, *Comamonas acidovorans* Fy-1, *Burkholderia cepacia* DB01 were isolated and characterized in a few studies [23,40,41,42]. *Pseudomonas* and *Burkholderia*, which were used in our study, were used in many previous studies for phthalate degradation. Numerous studies have shown that TA is intrinsically biodegradable under aerobic conditions. To remove TA from wastewater, the biodegradation method is very efficient and rapid. *Rhodococcus* and *Pseudomonas* play a fundamental role in biodegradation [43,44]. Biodegradation of phthalates may be carried out syntrophically by the microbial consortium [45]. The other study reported that degradation of phthalate was accelerated by consortia which are *Corynebacterium* sp. O18 and *Sphingomonas* sp. DK4 [46].

Pseudomonas sp. and *Arthrobacte*r sp. were not detected in real-time PCR, because of selected primers did not work in real-time PCR, but they were determined by conventional PCR. We have commented that primer specificities of these bacteria are less adequate in fluorescent technology than in conventional ones. We observed that isolate T1 had degraded TA in 8 hours in the flask assays before CSTR. However, the elapsed time in the degradation process is thought to extend due to different parameters such as physicochemical interactions and reaching of TA to targeted microorganisms within the activated flocs in CSTR. The fluctuation of the TA in non-inoculated CSTR directly depends on the microbial situations in the population. This situation is a significant sign of microbial dynamics in the population. Also, it could be considered as a difference in microbial lifespans. Although some bacterial groups utilize TA as carbon and energy source more quickly and reach the plateau phase in the logarithmic curve, other ones can behave in the opposite ways such as low doubling in time and need more time reaching the plateau phase.

Further research on biodegradation pathways, microorganisms' responsibility and limiting factors for degradation of hazardous chemicals are necessary before applying microbes in the field, which helps to achieve success in biodegradation of dangerous chemicals. Results from this study suggest that inoculation and acclimation of degradative microorganisms into activated sludge positively affect the degradation process via increasing the degradation capacity and more concentration. Acclimation and bioaugmentation processes could be the critical factors for the degradation of some xenobiotics and chemicals, which will be more effective in this way.

Funding: This work was funded by the research project SANTEZ-00639 STZ 2010-2 of the Republic of Turkey, Ministry of Science, Industry of Technology and Ege University, Scientific Research Project (10-FEN-060).
Acknowledgments: This work was funded by the research project SANTEZ-00639 STZ 2010-2 of the Republic of Turkey, Ministry of Science, Industry of Technology and Ege University, Scientific Research Project (10-FEN-060).
Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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