Biodegradation of long chain alkanes in halophilic conditions by *Alcanivorax* sp. strain Est-02 isolated from saline soil

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Received: 12 June 2018 / Accepted: 9 March 2019 / Published online: 15 March 2019
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Abstract

In this study, through a multistep enrichment and isolation procedure, a halophilic bacterial strain was isolated from unpolluted saline soil, which was able to effectively and preferentially degrade long chain alkanes (especially tetracosane and octacosane). The strain was identified by 16S rRNA gene sequence as an *Alcanivorax* sp. The growth of strain Est-02 was optimized at the presence of tetracosane in different NaCl concentrations, temperatures, and pH. The consumption of different heavy alkanes was also investigated. Optimal culture conditions of the strain were determined to be as follows: 10% NaCl, temperature 25–35 °C and pH 7. *Alcanivorax* sp. strain Est-02 was able to use a wide range of aliphatic substrates ranging from C₁₄ to C₂₈ with clear tendency to utilize heavy chain hydrocarbons of C₂₄ and C₂₈. During growth on a mixture of alkanes (C₁₄–C₂₈), the strain consumed 60% and 65% of tetracosane and octacosane, respectively, while only about 40% of the lower chain alkanes were degraded. This unique ability of the strain Est-02 in efficient and selective biodegradation of long chain hydrocarbons could be further exploited for remediation of wax and heavy oil contaminated soils or upgrading of heavy crude oils. Comparison of the sequence of alkane hydroxylase gene (*alkB*) of strain Est-02 with previously reported sequences for *Alcanivorax* spp. and other hydrocarbon degraders, showed a remarkable phylogenetic distance between the sequence *alkB* of Est-02 and other alkane-degrading bacteria.

Keywords Bioremediation · Hydrocarbon biodegradation · Tetracosane · Halophilic bacteria · *Alcanivorax*

Introduction

Crude oil is a complex mixture of different components. Most of the compounds in crude oil have been identified as hydrocarbons (organic matters mainly consisting of hydrogen and carbon atoms). Petroleum hydrocarbons can be subdivided into four fractions: The saturated (aliphatics), the

aromatics, asphaltenes and resins (Song et al. 2018; Cheng et al. 2014).

Alkanes are saturated hydrocarbons exist in different structural forms such as: linear (n-alkanes), cyclic (cyclo-alkanes) and branched (iso-alkanes). Due to the source of crude oil, alkanes can compose up to 20–50% of crude oil (Rojo 2009; El Mahdi et al. 2016). In addition, many organisms like plants, green algae, bacteria or animals can produce alkanes as a waste, structural element, defense mechanism, or as a chemoattractant (VanBeilen et al. 2003). Therefore, they exist in most of the natural environments and play an important role in nature. Although alkanes are apolar molecules and are very inert, they can be degraded by many microorganisms (aerobic and anaerobic) with specialized enzymatic systems and metabolic ability for utilization of n-alkanes (Rojo 2009; Wentzel et al. 2007). Numerous microorganisms such as phyla of eubacteria, yeasts, filamentous fungi, and algae have been reported to have ability to consume n-alkanes as their source of carbon and energy. Some specialized bacteria degrade hydrocarbons with special mechanisms; these microorganisms are able to

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-1670-3>) contains supplementary material, which is available to authorized users.

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use many petroleum originated compounds as sole carbon source (Margesin et al. 2003; Harayama et al. 2004). For instance, *Alcanivorax borkumensis* is a marine bacterium that is able to utilize a wide range of linear and branched alkanes (Schneiker et al. 2006; Yakimov et al. 2004). Previously, several n-alkane-degrading genera have been reported such as *Acinetobacter*, *Rhodococcus*, *Nocardia*, *Planococcus*, and *Pseudomonas* (Wang et al. 2006).

Formation of crude oil occurs in presence of high concentrations of salt, hence many crude oil-related environmental pollutants are spread in halophilic circumstances such as saline sea and salty soils. Therefore, halophilic microorganisms would be promising for application in different fields of oil industry (Martins and Peixoto 2012). Recently, some of the hydrocarbon-degrading halophilic and halotolerant bacteria have been reported such as *Marinobacter*, *Alcanivorax*, *Halomonas*, *Dietzia*, and *Bacillus* (McGenity 2010). The elimination of pollutants by microorganisms (biodegradation) can be affected by many limiting factors, which restricts the efficiency of biodegradation. Some of these limiting parameters are: concentration of pollutant, bioavailability of pollutants, factors affecting the growth of microorganisms including; salinity, temperature, pH, moisture, nutrients, and adequate oxygen supply (Wang et al. 2011). Despite these restriction factors, the biological processes are very noteworthy because of the ability to completely eliminate the pollutants, changing them to non-hazardous inorganic constituents such as CO_2 and H_2O , their compatibility with the environment, and low operational costs (King et al. 1992; Cole 1994).

Long chain alkanes and waxes have very low water solubility and bioavailability and thus have high persistence in different ecosystems. Recently, biodegradation of long chain alkanes and paraffins has attracted considerable attention for different applications including bioremediation, oil upgrading and well head clean up. Elumalai et al. studied the biodegradation of long chain n-alkanes of C_{32} and C_{40} by pure and mixed cultures of *Geobacillus* and *Bacillus* isolates and showed that degradation efficiencies were higher for C_{32} by mixed consortium, while the pure strains were better in degradation of C_{40} than mixed consortium (Elumalai et al. 2017). Park et al. studied the alkane biodegradation and alkane-degrading genes in *Acinetobacter oleivorans* DR1 which was able to utilize C_{12} – C_{30} alkanes as a sole carbon source but not short chain alkanes (C_6 , C_{10}). From the sequence analysis data they suggested that *alkB1* gene is responsible for long chain alkane utilization (C_{24} – C_{26}), and *alkB2* is important for medium chain alkane (C_{12} – C_{16}) metabolism (Park et al. 2017). Wang et al. investigated the potential of four salt tolerant *Bacillus* species for deposited paraffin removal. GC–MS analysis of paraffinic components before and after biodegradation showed that relative contents of n-alkanes between C_{15} – C_{23} and C_{27} – C_{30} evidently

decreased, indicating their good biodegradation properties (Wang et al. 2018).

The aim of this study was to isolate and characterize halophilic or halotolerant bacteria that are able to selectively degrade long chain n-alkanes for application in bioremediation of oil polluted soils and also upgrading of heavy oil fractions to lighter hydrocarbons.

Materials and methods

Chemicals and media

All chemicals used in this study were of analytical grade. Molecular biology reagents and kits were purchased from Cinnagen (Tehran, Iran). Hydrocarbons such as tetradecane (C_{14}), hexadecane (C_{16}), octadecane (C_{18}), eicosane (C_{20}), tetracosane (C_{24}), and octacosane (C_{28}) were purchased from Sigma-Aldrich. All other chemicals were from Merck (Darmstadt, Germany) unless otherwise stated.

Mineral medium with following composition was used for isolation and cultivation of alkane-degrading bacteria: KH_2PO_4 0.4 g, KCl 0.04 g, NH_4NO_3 0.5 g, MgSO_4 0.075 g, CaCl_2 0.02 g, NaCl 70 g, and 1 ml of trace element solution per liter of distilled water. The pH was adjusted to 6.8–7 and the medium was autoclaved at 121 °C for 20 min. Trace element solution contained $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ 1 mg, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 7 mg, $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ 18 mg, $\text{MnSO}_4 \cdot 5 \text{H}_2\text{O}$ 7 mg, BO_3H_4 1 mg, $\text{NiCl}_2 \cdot 6 \text{H}_2\text{O}$ 24 mg, $\text{CoSO}_4 \cdot 5 \text{H}_2\text{O}$ 18 mg, $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ 0.1 mg, and $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$ 0.1 mg in 1000 ml of distilled water. R2A agar medium containing: peptone (meat) 0.5 g, casamino acid 0.5 g, dextrose 0.5 g, yeast extract 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.05 g, K_2HPO_4 0.3 g, and agar 15 g per liter of distilled water was used as general purpose media in this study.

Enrichment, isolation, and identification of alkane-degrading bacteria

Ten water and soil samples used for bacterial enrichment were collected from oil-contaminated and non-contaminated saline water and soils in different parts of Iran (Tehran, Qom, Khark, Khangiran, Eshtehard, Orumieh, and Meighan). To enrich hydrocarbon-degrading bacteria, 1 g of soil and 1 ml of water samples were inoculated into 100 ml flasks with 30 ml mineral medium containing 7% NaCl (w/v). Filter sterilized 0.5% (w/v) tetracosane in n-hexane were added to medium as a sole carbon source. Flasks were incubated overnight prior to inoculation for elimination of n-hexane and were shaken at 120 rpm and 32 °C for 1 week. After that, 0.5 ml of first enrichment cultures were transferred to fresh media and incubated for 1 week. After four rounds

of enrichment, a sample of the culture was transferred to R2A agar media supplemented with 7% NaCl to isolate halophilic or halotolerant bacteria. The purified bacteria were re-inoculated to mineral medium containing 7% NaCl and tetracosane, to verify tetracosane degradation capacity of the isolates. After a week of incubation, the growth of the cultures was quantitatively investigated by measuring optical density of the whole cultures at 630 nm. Isolates with higher growth rates were selected for further studies.

To identify the selected strains, genomic DNA was extracted by modified Marmur et al. method (Marmur 1961), and the 16S rRNA gene was amplified and sequenced using universal primers 27F (5'-AGA GTT TGA TCM¹ TGG CTC AG-3') and 1492R (5'-TAC GGY² TAC CTT GTT ACG ACT T-3'). The 16S rRNA gene sequence was analyzed using NCBI BLAST online tool (Altschul et al. 1990). Sequence alignments and phylogenetic analyses were conducted using Bioedit, Clustal X, and Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura et al. 2007). Phylogenetic tree was constructed using Neighbor-joining method with 1000 bootstrap replicates.

Growth properties and tetracosane biodegradation

To investigate the growth characteristics, the strain Est-02 was inoculated to mineral medium containing 10% NaCl and 0.5% tetracosane. Flasks were incubated at 32 °C, and 120 rpm. The growth was assessed by measuring optical density at 630 nm every 24 h during 10-day test period.

To reduce the error caused by turbidity of colloidal hydrocarbons, the growth was also investigated by protein assay. Protein concentrations were determined by the Bradford method. The reaction mixture contained 20 µl of bacterial samples with 1 ml of Bradford reagent (Bradford 1976) (Coomassie Brilliant Blue G-250 100 mg l⁻¹, ethanol 95% 50 ml/l, phosphoric acid 85% 100 ml/l). After 10 min the optical density of the samples were measured at 595 nm.

To study the tetracosane degradation by *Alcanivorax* sp. Strain Est-02, every 24 h one flask was used to extract the hydrocarbon. Every time, an additional un-inoculated flask was used as control. The remained tetracosane of each flask was extracted three times by equal volume of n-hexane. Organic phase from the extract was dried over anhydrous sodium sulfate. Finally, after removal of solvent at room temperature, residual hydrocarbon in each flask was analyzed by gas chromatography (GC). The extracted samples were injected into Agilent gas chromatograph model 7890A equipped with FID detector with temperature 300 °C and an Agilent 19091J-413 column (30 m × 320 µm × 0.25 µm).

Nitrogen was used as a carrier gas with flow rate of 1 ml/min. Temperature program started at 100 °C by a rate of 25°C/min and finally increased to 290 °C and held in this temperature for 10 min.

Effects of different environmental conditions on growth of strain Est-02

The effects of different culture conditions on growth of the strain Est-02 was studied in a series of tests. The selected environmental parameters were different NaCl concentrations, temperatures, and pH. To determine the effects of these parameters a mineral medium containing 0.5% tetracosane was used. Every 24 h, the growth was examined by taking sample and measuring optical density at 630 nm during 10 days of incubation.

The effect of salinity was studied at different NaCl concentrations including: 0%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, and 25% (w/v). To investigate the effect of temperature on bacterial growth, inoculated flasks were incubated in different temperatures of 25 °C, 30 °C, 35 °C and 40 °C. The impact of pH on growth and tetracosane degradation by strain Est-02 was investigated within the ranges of 5–9 with interval of 1 pH unit.

Biodegradation of different n-alkanes

To determine of the ability of the strain Est-02 to utilize different n-alkanes, mineral medium containing 10% NaCl, containing different alkanes including tetradecane (C₁₄), hexadecane (C₁₆), octadecane (C₁₈), eicosane (C₂₀), tetracosane (C₂₄), and octacosane (C₂₈) was used. The flasks were inoculated with equal amount of the same stock culture and the growth was examined by taking sample and measuring optical density at 630 nm.

Utilization of the mixture of n-alkanes

To determine the order of alkanes biodegradation by strain Est-02, utilization of mixed alkanes was studied during a 2-week period. A fresh inoculum of the strain was inoculated to mineral base medium with 0.5% (w/v) of a hydrocarbon mix containing equal amounts of C₁₄, C₁₆, C₁₈, C₂₀, C₂₄, and C₂₈ alkanes and 10% NaCl. Cultures were incubated on a shaker at 120 rpm and samples were picked up on zero, 2, 5, 7, 9, 11, and 14 days for analysis. The remained hydrocarbon was extracted three times by equal volume of n-hexane and the organic fraction was dehydrated over anhydrous sodium sulfate. After removal of solvent at room temperature, the extracted hydrocarbon of each flask was analyzed by gas chromatography. All experiments performed in triplicates and standard errors calculated from the mean values and presented as error bars in the figures.

¹ M = A or C.

² Y = C or T.

Fig. 1 Phylogenetic tree of 16S rRNA gene sequence of *Alcanivorax* sp. strain Est-02 with related strains available in GenBank database. The tree was constructed by using Neighbor-joining method with

1000 Bootstrap replicates. *Bradyrhizobium rifense* was used as an Outgroup. Bootstrap values > 95% are indicated at the inter-nodes and GenBank accession numbers are shown in parenthesis

Molecular detection and phylogenetic analysis of alkane hydroxylase gene

To detect the existence of alkane hydroxylase enzyme in strain Est-02, a fragment of *alkB* gene was amplified in PCR reactions using highly degenerate primer pairs of MonF (5'-TCA AYA CMG S³N⁴C AYA AR⁵C-3') and MonR (5'-CCG TAR TGY TCN AYR TAR TT-3') designed based on conserved regions of several bacterial alkane hydroxylase genes (Wang et al. 2010). The alkane hydroxylase gene sequences were deposited into GenBank and sequence similarity analysis was performed by comparing its similarity to other GenBank records using online NCBI BLAST tool. Sequence alignments and phylogenetic analyses were performed as previously mentioned.

Results

Isolation and identification of tetracosane degrading bacteria

Among 10 soil and water samples, 5 enrichment cultures indicated suitable bacterial growth. After isolation of bacteria on R2A agar medium, almost 50 different colonies were obtained. Only 2 isolates (Est-02 and Est-03) were able to grow on tetracosane as sole carbon source. The 16S rRNA gene analysis of both Est-02 (GenBank accession No. MF116253) and Est-03 (Genbank accession No.

MK503770) strains illustrated 99.90% and 99.8% sequence similarity to the *Alcanivorax jadensis* (Fig. 1).

The isolated strains were compared based on growth rate and tetracosane biodegradation in mineral medium and strain Est-02 was selected for further studies because of its faster growth and better tetracosane degradation capabilities. The strain Est-02 was isolated from the enrichment culture of salty non-contaminated Eshtehard soil. The Est-02 produced mucoid and round shaped colonies on R2A agar medium and appeared as rod shape and gram negative cells in microscopy. The selected strain was deposited into the open collection of the bank of microorganisms of Iranian Biological Resource Center (IBRC) under the reference number of IBRC-M 10412.

Growth and tetracosane biodegradation by strain Est-02

During a 10-day test period, the growth rate and biodegradation ability of the strain Est-02 were monitored in mineral base medium containing 0.5% tetracosane and 10% NaCl. As depicted in the growth curve in Fig. 2, after a long lag phase of 1 day, the logarithmic growth was started and the maximum growth of the bacterium was recorded by the 5th day. Moreover, the n-alkane degradation by strain Est-02 was determined by gas chromatography. The results of GC illustrated that alkane degradation was also started with 1-day delay, an effective biodegradation of tetracosane was observed from days 2 to 5 simultaneous with the bacterial growth. Eventually, the maximum amount of tetracosane degradation of 52.8% was observed on the 10th day of the experiment.

³ S = C or G.

⁴ N = A, C, G or T.

⁵ R = A or G.

Fig. 2 Time course of growth and tetracosane degradation by *Alcanivorax* sp. strain Est-02 during growth on mineral salt medium containing 500 mg l⁻¹ of tetracosane

Effects of environmental conditions on growth of strain Est-02

The salinity tolerance range of *Alcanivorax* sp. strain Est-02 was investigated on R2A agar medium with different NaCl concentrations. It was determined that the strain could grow on R2A agar in salinities ranging from 1 to 15% of NaCl and no growth was observed in the absence of NaCl. The study of the Est-02 growth in mineral base medium containing 0.5% tetracosane as sole carbon source showed that the strain Est-02 could grow and degrade tetracosane in salinities ranging from 2.5 to 12.5% and its optimal salinity is at 10% NaCl (Figure S1, provided as supplementary material). According to these results, *Alcanivorax* sp. strain Est-02 is a moderate halophilic bacterium.

As indicated in Fig. S2 (Supplementary Material), strain Est-02 was able to growth in mineral base medium with 0.5% tetracosane and 10% NaCl over a temperature range of 25–35 °C and could not grow at 40 °C. Therefore, strain Est-02 is a mesophilic bacterium.

The effects of different pH on growth of strain Est-02 was investigated during 1 week. The optimum pH for growth of the strain on tetracosane was found to be neutral pH (Figure S3, given as supplementary material).

Biodegradation of different n-alkanes

To determine the ability of strain Est-02 to utilize different aliphatic hydrocarbons, its growth in mineral salt medium in presence of some middle and long chain n-alkanes was examined. As demonstrated in Fig. 3, strain Est-02 was able to utilize n-alkanes with chain lengths from C₁₄ to C₂₈ as sole carbon source but it exhibits a clear tendency towards

longer chain alkanes, namely, tetracosane and octacosane in comparison to the other hydrocarbons.

Utilization of the mixture of n-alkanes

The biodegradation of mixture of alkanes ranging from C₁₄ to C₂₈ by strain Est-02 during a fortnight period was investigated in mineral salt medium through gas chromatography analysis. As depicted in Fig. 4, strain Est-02 was able to degrade n-alkanes (C₁₄–C₂₈) and showed a clear affinity to consume long chain n-alkanes such as tetracosane and octacosane (C₂₄ and C₂₈) in comparison to middle chain n-alkanes. While 65% and 60% of octadecane and

Fig. 3 Comparison of growth of *Alcanivorax* sp. strain Est-02 in mineral base medium using different alkane hydrocarbons

Fig. 4 Biodegradation of a mixture of alkanes (C_{14} – C_{28}) in mineral salt medium by *Alcanivorax* sp. strain Est-02

Fig. 5 The phylogenetic tree based on alkane hydroxylase gene (*alkB*) sequences of *Alcanivorax* sp. strain Est-02 and some related sequences from GenBank. The tree was constructed by using Neigh-

bor-joining method with 1000 Bootstrap replicates. GenBank accession numbers are included in parenthesis

tetracosane were degraded after 2 weeks, only about 40% of other alkanes were consumed.

Molecular detection and phylogenetic analysis of alkane hydroxylase gene

After extracting the whole genomic DNA, polymerase chain reaction with degenerated primers was performed to amplify a fragment of *alkB* gene (alkane hydroxylases). Sequencing of the fragment with almost 400–500 bp length indicated that strain Est-02 has an alkane hydroxylase gene(s) responsible for the first step of alkane hydrocarbons degradation. The phylogenetic relationship based on alkane hydroxylase gene sequence of the Est-02 and some related sequences from GenBank was constructed and shown in Fig. 5.

Discussion

Ten oil-polluted and non-polluted saline soil and water samples were used to isolate long chain alkane-degrading halophilic bacteria using a multistep enrichment process. Despite the generally accepted idea that hydrocarbon-degrading microorganisms are more conceivable to isolate from oil-polluted sites, the selected strains of the current study were isolated from non-contaminated salty soil. There are some similar results reported by Nicholson and Fathepure and also Tapilatu et al. indicating that microorganisms capable of degrading oil components such as aromatic hydrocarbons were isolated from saline and non-pollutant environments (Nicholson and Fathepure 2004; Tapilatu et al. 2010).

Several previous studies showed that bacteria of the genus *Alcanivorax* appropriately degrade petroleum hydrocarbons in marine environments. Species of *Alcanivorax* are found in non-polluted sea water in few low amounts and constant numbers. Their existence might be due to the production of alkanes by plants, algae and other marine microorganisms. *Alcanivorax* strains have a critical role in biodegradation of oil contaminations after oil spills in marine environments (Rojo 2009).

Bacteria which belong to this genus, consume not only a wide range of petroleum constituents as energy and carbon source, but also many of them can produce biosurfactants that increase the availability of hydrocarbons to the living microorganisms. These unusual metabolic and physiologic abilities of *Alcanivorax* genus make them suitable candidates for bioremediation of oil polluted environments (Liu et al. 2010).

Most of the strains belonging to *Alcanivorax* are abundantly found in oil-polluted and non-polluted marine environments and are considered to be marine bacteria (Rojo 2009). In most of the previous studies *Alcanivorax* spp. are found in marine environments, while *Alcanivorax* sp. strain Est-02 was isolated from a saline soil. Dastgheib and coworkers also reported the same observation in their study and isolated *Alcanivorax dieselolei* from contaminated saline soil (Dastgheib et al. 2011). These findings are inconsistent with the commonly accepted idea that *Alcanivorax* species are absolute marine hydrocarbon-degrading bacteria.

The time course of tetracosane degradation by strain Est-02 was studied in mineral salt media containing 10% of NaCl. As presented in Fig. 2, the growth and biodegradation of the strain during 10 days of the study showed a lag phase of 1 day, after which the maximum growth rate of bacterium and an effective biodegradation of tetracosane were observed and continued up to the 5th day. The highest rate of tetracosane biodegradation was 52.8% on the 10th day of the experiment. In comparison to a similar study by Dastgheib et al. who demonstrated that during a 20-day experiment, *Alcanivorax dieselolei* Qtet3 strain showed a 5-day lag phase and the highest biodegradation rate was observed during days 5–10 of the study (Dastgheib et al. 2011). Liu et al. reported that the growth rate of *Alcanivorax* sp. using octadecane as sole carbon source was very slow and the log phase was started after 60 h of inoculation and only 16.07% of octadecane was degraded on second day of the experiment and afterwards the degradation rate was gradually and slowly increased (Liu et al. 2010). The comparison of our results with findings of other researchers reveals a great potential of the strain Est-02 for effective and fast biodegradation of long chain alkanes.

To better determine the potentials of strain Est-02 for biodegradation in various environmental conditions, the effect of different environmental factors on growth and

hydrocarbon degradation by the strain was investigated. The results of NaCl optimization indicated that the maximum growth rate of strain Est-02 was at 10% NaCl. However, no substantial degradation of tetracosane by strain Est-02 was observed at 15% NaCl, demonstrating characteristics of a moderate halophilic bacterium. Yakimov et al. observed that the optimal growth of *Alcanivorax borkumensis* strain Sk2 was at 3–10% NaCl (Yakimov et al. 1998). Moreover, Liu and colleagues reported that optimal growth of halophilic *Alcanivorax* sp. strain 2B5 using octadecane as carbon source was 2–9% NaCl (Liu et al. 2010).

Temperature strongly affects the physical properties of petroleum hydrocarbons, diversity of microbial population and the rate of hydrocarbon metabolism by microorganisms and consequently affects the biodegradation of petroleum hydrocarbons (Leahy and Colwell 1990). Therefore, the effect of various temperatures (25–40 °C) on tetracosane biodegradation by strain Est-02 was studied. Whilst Yakimov et al. (1998) reported that the optimal temperature for growth of *Alcanivorax* genus is between 20 and 30 °C; our results demonstrated that the highest growth and biodegradation rates of strain Est-02 were at 25–35 °C. The strain Est-02 showed no growth at temperatures above 40 °C which is similar to the characteristics of mesophilic bacteria. Another report by Dastgheib et al. also claimed that the optimal temperature of an *Alcanivorax* isolate was about 35 °C (Dastgheib et al. 2011). Similarly, Liu et al. isolated mesophilic *Alcanivorax* strain which used octadecane as sole carbon source and its optimum temperature was shown to be at 30–37 °C (Liu et al. 2010). Moreover, Golyshin et al. showed that the optimal temperature for growth of *Alcanivorax* spp. is between 25 and 30 °C (Golyshin et al. 2007).

According to our results, the optimal pH for growth of *Alcanivorax* sp. strain Est-02 cells was at pH 7. Generally, the optimal pH of the genus *Alcanivorax* was reported at neutral pH. Kwon et al. reported the isolation of a new species of *Alcanivorax* from sediments collected from Korea and the optimum pH for growth of the strain was reported to be 7–8 (Kwon et al. 2015). Liu et al. also isolated alkane-degrading *Alcanivorax* sp. from the sea mud in China with the optimal growth pH and octadecane degradation at pH 6–7 (Liu et al. 2010).

The ability of the strain Est-02 to consume alkanes with different chain lengths was investigated in basic mineral salt medium. The strain Est-02 was capable of using a wide range of aliphatic substrates from C₁₄ to C₂₈ with very different efficiencies. According to Fig. 3, the maximum degradation rate of the strain was recorded during growth on tetracosane as its carbon source and it was not surprising because tetracosane was the only alkane added to medium as the sole carbon source in the enrichment and isolation steps and the strain was acclimated to tetracosane degradation. The octacosane was the second substrate that was efficiently

consumed by strain Est-02 indicating its clear tendency to utilize long chain hydrocarbons. The affinity of the strain Est-02 to consume heavy chain alkanes was also proved during growth on mixed alkanes. As shown in Fig. 4 during growth on a mixture of alkanes ranging from C_{14} to C_{28} , the strain consumed heavy chain alkanes of tetracosane and octacosane with better efficiencies. The strain was degraded about 60% and 65% of tetracosane and octacosane, respectively during 2 weeks of incubation and only about 40% of lower chain alkanes were consumed at the same time. These findings are different from the results of similar investigation by Mohanty and Mukherji who studied the n-alkanes biodegradation by *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. Their observations showed that the highest degradation rate of the bacteria was in the range of low and medium chain alkanes of C_9 – C_{18} (Mohanty and Mukherji 2008). The ability of the strain Est-02 in efficient and selective biodegradation of long chain hydrocarbons makes it a potential strong candidate for remediation of heavy oil contaminated soils. Moreover, this distinctive feature of Est-02 may be very attractive for bio-upgrading of heavy crude oils by breaking down of heavy chain alkanes and waxes into shorter ones.

Liu et al. investigated the capability of *Alcanivorax* sp. to degrade petroleum hydrocarbons (C_{13} – C_{30}) and they reported that after 15 days of inoculation, almost all of them were equally biodegraded (Liu et al. 2010). Dastgheib et al. also studied the growth of *Alcanivorax dieselolei* Qtet3 using different hydrocarbons (C_{10} – C_{34}) and reported that the maximum growth rate of strain was during growth on C_{14} – C_{16} alkanes (Dastgheib et al. 2011). Wu et al. isolated *Alcanivorax* sp. that has the ability to degrade n-alkanes from C_8 to C_{26} (Wu et al. 2009). Santisi and colleagues showed that isolated *Alcanivorax* strain was able to grow by consuming a wide range of hydrocarbons (C_{12} – C_{30}) with no special preference (Santisi et al. 2015).

Many aerobic and anaerobic microorganisms have effective strategies to consume alkanes as carbon and energy sources that include specific enzymatic systems and metabolic pathways. Aerobic alkane degraders use oxygen molecule to activate alkanes by monooxygenase enzymes (Minerdi et al. 2012). In bacteria, several key enzymes are identified through alkane oxidation processes that involve alkane hydroxylases such as cytochrome P450, *alkB*, dioxygenases or monooxygenases. PCR amplification of *alkB* gene using special primers revealed that the strain Est-02 possessed a gene coding for alkane degradation (*alkB*). The comparison of the sequence of the Est-02 *alkB* gene with some Genbank records of the *alkB* for *Alcanivorax* and other hydrocarbon degraders showed a remarkable phylogenetic distance between *alkB* gene of strain Est-02 and other previously reported bacteria. This unique and different *alkB* sequence and hence function of the alkane hydroxylase, may

be a good explanation for the exclusive ability of the strain Est-02 for selective degradation of long chain alkanes. Some bacterial genera such as *Rhodococcus*, *Pseudomonas* and *Alcanivorax* carry several *alkB* genes. It seems that each gene is responsible for oxidation of different alkane molecules in each strain. Many studies showed that diversity of *alkB* genes in natural environments or in hydrocarbon-degrading bacteria isolated from aquatic ecosystems could be successfully used to evaluate the hydrocarbon degradation abilities of the native bacteria (Paisse et al. 2011).

Conclusions

In current study, several different oil contaminated and uncontaminated soil and water samples were used for isolation of long chain alkane-degrading halophilic bacteria. After a multistep enrichment and isolation procedure an efficient alkane-degrading *Alcanivorax* strain was isolated from uncontaminated soil sample. Isolation of *Alcanivorax* species from soil samples could eventually change the common notion of the role of *Alcanivorax* spp. as absolute hydrocarbon degraders of the aquatic ecosystems. Determining the biodegradation capability of *Alcanivorax* sp. strain Est-02 in different environmental conditions using different alkanes revealed efficient and selective potential of the strain for degradation of long chain hydrocarbons (especially C_{24} and C_{28}). This special and unique ability of the strain Est-02 in efficient and selective biodegradation of long chain hydrocarbons could be further exploited for remediation of wax and heavy oil contaminated soils. This distinctive characteristic of the Est-02 can also be very promising for upgrading heavy crude oils.

Acknowledgements This work was supported by a general graduate student fund from Tehran University. The authors are grateful to Dr. Hojatollah Kazemi and Ms Sima Ghadernia for their assistance in gas chromatography analysis. We also would like to thank Ms Ghanimi fard for reading and editing the English text.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest regarding the publication of this paper.

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