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ORIGINAL ARTICLE

Biological degradation of toluene by indigenous bacteria *Acinetobacter junii* CH005 isolated from petroleum contaminated sites in India

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Abstract The bacterium *Acinetobacter junii* was isolated from petroleum-contaminated site in India and tested for its efficiency in degradation of toluene under aerobic condition. Within pH range 4–9, the optimum pH for toluene biodegradation was found to be 7.5. With increase in time, there was enhancement in degradation of toluene. Pure culture of *Acinetobacter junii* was able to degrade 69, 73 and 80% of 150, 100, and 50 ppm toluene, respectively, within 72 h at 37 °C. Simultaneous growth and degradation of toluene by the bacterium indicated the utilization of toluene as carbon source. After 72 h of treatment, toluene biodegradation was nearly stable. Scanning electron microscopic characterization of bacterial cells treated with toluene revealed the changes in surface morphology. Some of the cylindrical cells of bacterium got transformed into ovoid and spherical shape to escape the toluene toxicity. Degradation intermediates were identified by gas chromatography–mass spectroscopy. The major intermediate compounds identified after toluene degradation by bacteria were 1-isopropenyl-4-methyl-1,3-cyclohexadiene; 1,3-Cyclohexadiene; 2-methyl-5-(1-methylethyl); 4-methoxycarbonyl-4-butanolide; and vinyl (2E,4E)-2,4-hexadienoate, which are less-toxic in nature. The degradation of toluene into non-toxic intermediate compounds as well as the growth in the presence of toluene presents the suitability of *Acinetobacter junii* in biofiltration of toluene-containing petroleum waste.

Keywords *Acinetobacter junii* · Biodegaration · Mass spectroscopy · Petroleum waste · Phylogenetic relationship · Toluene

1 Introduction

The release of various recalcitrant organic pollutants in the environment from various anthropogenic sources has caused adverse effect on surface and ground water characteristics. Petroleum industries are known to generate large amounts of petrochemical waste which are harmful to the environmental health (Singh and Celin 2010; Singh et al. 2016, 2017). Petrochemical pollutants generated from wide range of industries have gained significant attention worldwide (Atlas 1985; Dilly et al. 2011). Benzene, toluene, ethylbenzene and xylene (collectively known as BTEX compounds) are being discharged without treatment in significant amount in the water bodies through the effluents from petrochemical industries such as refineries, paint, textile, paper and rubber (Rahul et al. 2013; Xin et al. 2013; Zhang et al. 2013; Huang et al. 2014; Rajamanickam et al. 2017). The exposure to these compounds and other hydrocarbons is extremely harmful for living organisms, including human beings (Chen et al. 2008; Zhang et al.

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2013). These compounds show bioaccumulation and biomagnification in food chains and causes serious health problems. Also, these compounds adversely affect the aquatic organisms (Floodgate 1984) by reducing their chlorophyll content, thus photosynthetic efficiency (Peng et al. 2015). Saturated aromatic hydrocarbons are released in huge amounts into the environment by both natural seepage of subsurface reservoirs and anthropogenic activities (Kim et al. 2014; Stasik et al. 2015; Vogt et al. 2016). These organic compounds have been categorized in the list of hazardous wastes due to their mutagenic/carcinogenic nature (Mastrangela et al. 1997; Singh et al. 2016; Zhang et al. 2018). Various studies have reported the genotoxic effects of these hydrocarbons in human beings as well as in plants (Gopinath et al. 2016).

Toluene is the most widely used industrial solvents for organic synthesis and instrumental cleaning. It is the major aromatic component in many petroleum products and is often found in ground water as a result of leakage in underground storage tanks and pipelines, improper waste disposal practices, inadvertent spills, leaching from landfills and some disastrous oil spill accidents (Robledo-Ortíz et al. 2011). Due to high water solubility (0.17–0.19%), higher volatility and high mobility, it is considered as toxic, carcinogenic and the universal environmental contaminant (Durmusoglu et al. 2010; Slominska et al. 2012). The toluene, occurring in all segments of environment, has adversely affected human and environmental health through various mode of interaction, i.e., soil, water and air (Mazzeo et al. 2010; Zhang et al. 2018). Therefore, degradation of toluene is an important environmental issue and application of eco-friendly indigenous practices for the environmental cleanup is the need of the hour. Different physical, chemical and biological treatment technologies have been proposed to treat the petrochemical contaminants at source as well as to remediate soil and water systems contaminated by them (Singh et al. 2016, 2017; Tian et al. 2016). Physical methods employ adsorption and coagulation as the commonly used treatment technique. The most common chemical process used for treatment of toluene containing effluents is oxidation (Abdelwahab et al. 2009); however, there are various problems including requirement of excessive quantity of chemicals (Guo and Al-Dahhan 2005), low reaction rate (Shu and Huang 1995) and production of higher amount of secondary sludge which requires further treatment.

In addition, there are various biological methods which have been spotlighted as a promising and alternative technique for the degradation of organic pollutants (Lee et al. 2009; Mohan et al. 2006). Most of the studies performed on bioremediation of petrochemical wastes (Jones et al. 2007) have employed bacteria. Few studies have also focused on use of different types of algae (Ashwaniy and Perumalsamy 2017), fungi (Taha et al. 2017), biosurfactants, and consortium of microbes. However, studies based on in situ microbial isolation and identifying the degradation capacity by those strains are limited. Thus, the present study deals with the investigation of toluene degradation potential of *Acinetobacter junii* CH005 isolated from petroleum contaminated sites in India as well as identification of degradation intermediates by GC–MS.

2 Materials and methods

2.1 Chemicals

Toluene (purity, 99%) was used in the biodegradation process. In addition, concentrated sulfuric acid, potassium dichromate, starch indicator, ferrous ammonium sulfate, mercurous sulfate ($HgSO_4$), nutrient agar (NA), sodium hydroxide (NaOH), and ethanol were also used. All chemicals used in the experiment were of analytical grade and procured from Sigma-Aldrich, Merck, Himedia, and Thermo Fisher Scientific.

2.2 Sample collection and isolation, screening and identification of bacteria

Soil and effluent samples were collected in sterile polybags from the oil refinery situated in Mathura district, Uttar Pradesh, India. Soil solutions (1%) and effluents supplemented with 100 mg l^{-1} toluene in nutrient broth were incubated at 37 ± 2 °C for 4 weeks under both static and shaking conditions. The samples exhibiting visible bacterial growth were streaked on the fresh solid nutrient agar (NA) plates, having 100 mg l^{-1} toluene and again incubated at 37 ± 2 °C for a week. Different bacterial colonies were selected, picked up and were again streaked on solid nutrient agar plate for pure culture isolation of bacteria. The toluene degrading bacteria were identified on the basis of morphological details of colony, its color, Gram's staining and biochemical tests according to the Bergey's manual of determinative bacteriology. For molecular identification, DNA was isolated from the pure culture of bacterium and quality was evaluated on 1.2% agarose gel. A single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified from the isolated DNA segment by PCR using 8F and 1492R. A single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified and processed for the sequencing. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1488 bp 16S rDNA gene was generated from forward and

reverse sequence data using Aligner software. The 16S rDNA gene sequence was used to carry out BLAST (Basic Local Alignment Search Tool) of NCBI GenBank database. Based on maximum identity score, first 15 sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database, and the phylogenetic tree was constructed using MEGA (Version 6).

2.3 Batch degradation of toluene by pure bacterial culture

Batch studies were carried out to understand the biodegradation potential of toluene at different pH, ranging from 4 to 9 by using fixed concentration. Teflon liner screw cap flasks were used for the batch biodegradation studies. The flasks were kept on orbital shaker (150 rpm) at 37 °C. Batch experiments were performed in triplicate along with the control. For the sampling purpose, flasks were removed from the shaker and 2 ml of sample was collected from the sampling port with air tight syringe to estimate the cell density and toluene degradation.

2.4 Surface morphology characterization

The pure culture of *Acinetobacter junii* CH005 was observed under scanning electron microscope (SEM) for surface characterization and structure. Sample preparation techniques for the SEM analyses were as adopted by Paje et al. (1998). Sample fixations were performed in 2% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) for 1 h followed by washing with the buffer to eliminate any unbound chemicals. Thereafter, the specimens were dehydrated in a series of alcohol and acetone for 10 min. Further, drying was performed in a critical point drying chamber. Dried specimens were carefully mounted onto stubs and gold coated prior to observation under scanning electron microscope (ZEISS, model EVO-18 research; Germany).

2.5 Analytical investigation

HPLC investigations of toluene biodegradation were performed as per Rajamanickam et al. (2017). Inoculated samples were centrifuged for 10 min at 10,000 rpm to separate cell mass and the supernatant. Supernatant was extracted in an organic solvent (n-Hexane) for analysis through HPLC (Thermo Scientific Ultimate 3000) chromatograph equipped with UV detector at 254 nm. The column used was C18 bond packed 3 µm (25 × 4.6 mm) with mobile phase consisting of methanol and water (70: 30). The flow rate was set to 1 ml min^{-1}. The samples were also analyzed by GC–MS.

2.6 Detection of intermediate compounds produced after toluene biodegradation

Samples were extracted in n-hexane and analyzed through GC–MS in order to determine the metabolic intermediates formed during toluene biodegradation. GC–MS analysis of extraction was performed in GC–MS (QP–2010 plus system, Shimadzu). The column RTx-5 Sil MS having internal diameter of 30 m × 0.25 mm and 0.25 µm film thickness fitted in instrument was used for the intermediate compound analysis. The operational programmes of the column were as follows: oven temperature rise from 80 to 210 °C at 4 °C min^{-1} increase with hold time of 2 min and from 210 to 300 °C at 15 °C min^{-1} rise with stand time of 5 min. Final temperature was retained for 20 min. The injector temperature was kept at 270 °C. A total 0.2 µl volume of sample was injected into column. Pressure, total flow and column flow were 85.4 kPa, 76.8, 1.21 ml min^{-1}, respectively. The identification of compounds was performed by comparing their mass spectra with data available from NIST05 (National Institute of Standards and Technology, USA) and WILEY 8 libraries.

3 Results and discussion

3.1 Culture identification

The culture labeled as CH005 was identified as *Acinetobacter junii* (Genbank Accession Number: KT630871) based on nucleotide homology and phylogenetic analysis.

3.2 Phylogenetic relationship of the isolated bacteria

The evolutionary history was interpreted using the neighbor-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) was taken to show the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3 rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1447 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). The phylogenetic tree of isolate CH005 is presented in Fig. 1.

3.3 Effect of pH on toluene biodegradation

The pH is one of the most important factor controlling the growth and enzymatic activities in microorganisms. There was enhancement in the percent toluene degradation with increase in pH from 4.0 to 7.5. Beyond pH 7.5, reduction in percent degradation was observed which might be due to lowered enzymatic activity. In the present study, the bacterium demonstrated the best biodegradation performance at pH 7.5. Thus, optimum pH 7.5 was selected for further degradation studies. However, most of the microorganisms are reported to preferably grow at neutral or near neutral pH (Margesin and Schinner 2001). Effect of pH on toluene biodegradation is presented in Fig. 2.

3.4 Effect of toluene on surface morphology of *Acinetobacter junii* CH005

Surface morphology of untreated and toluene treated *Acinetobacter junii* was revealed by SEM (Fig. 3a, b). Prior to toluene treatment, all bacterial cells were cylindrical which can be clearly visualized in scanning electron microscope image. However, few cylindrical cells were transformed into ovoid and spherical structure after treatment as evidenced under SEM microphotographs, probably to escape themselves from toxicity. Such morphological changes have also been reported by Affandi et al. (2014). Bacterial systems are known to change themselves at biochemical and physiological level under stressed conditions such as presence of organic compounds including benzene, toluene, ethylbenzene and xylene (Michael et al. 2016). These protective mechanisms include changes in characteristics of cell membrane, alteration in cell shape, size, formation of vesicular structure on membrane, and proteins associated with stress tolerance, efflux pumps and energy pool maintenance.

3.5 Biodegradation studies of toluene by pure culture of *Acinetobacter junii*

Majority of the studies on toluene biodegradation have been performed with anaerobic microorganisms. In the present study, we have evaluated the toluene degradation ability under aerobic conditions. With an increase in time gap, concomitant increase in degradation by pure culture was observed. Pure culture of *Acinetobacter junii* was able to degrade 69, 73 and 80% of 150, 100, and 50 ppm toluene, respectively, within 72 h at 37 °C (Fig. 4). Toluene degradation with time shows that the degradation occurred due to the enzymatic activities resulting from bacterial growth. Nonlinear increase in percent degradation of toluene was determined up to 72 h. The percent degradation was observed to be reduced very much near to 72 h as compared to previous hours, showing saturation of enzymes involved in degradation. The increase in toluene concentration resulted into diminished percent biodegradation which may be explained by toxic impact of toluene on bacterial cells (Dorer et al. 2016).

Growth of bacteria in the presence of aromatic compounds indicates its applicability for bioremediation of contaminated sites. Studies on biodegradation of toluene under both aerobic as well as anaerobic conditions have been reported worldwide. However, biodegradation studies under aerobic conditions are lesser as compared to the anaerobic ones.

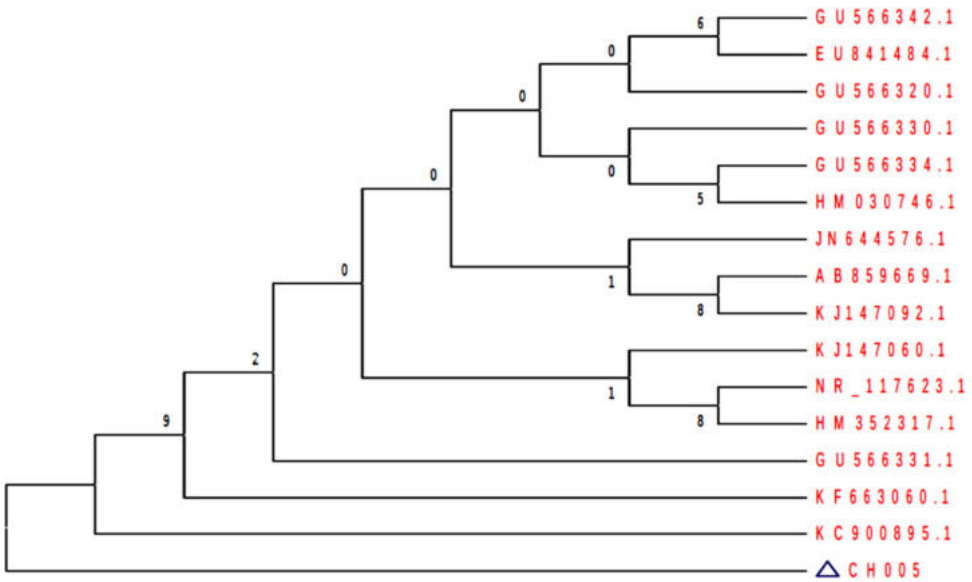

Fig. 1 Phylogenetic relationship of *Acinetobacter junii* isolate CH005 with other closely related taxa

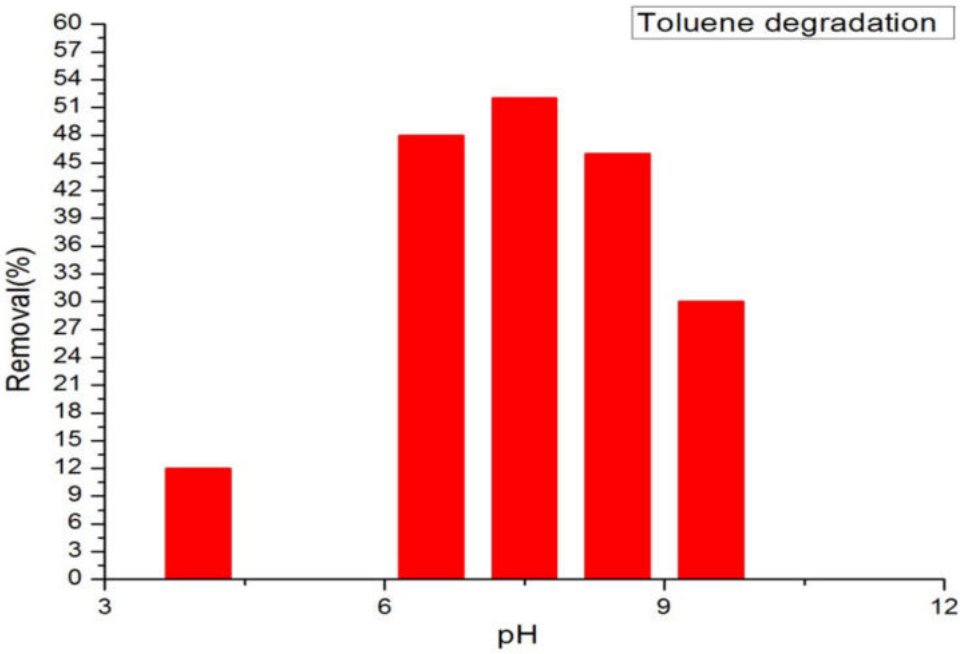

Fig. 2 Effect of pH on toluene biodegradation by bacteria

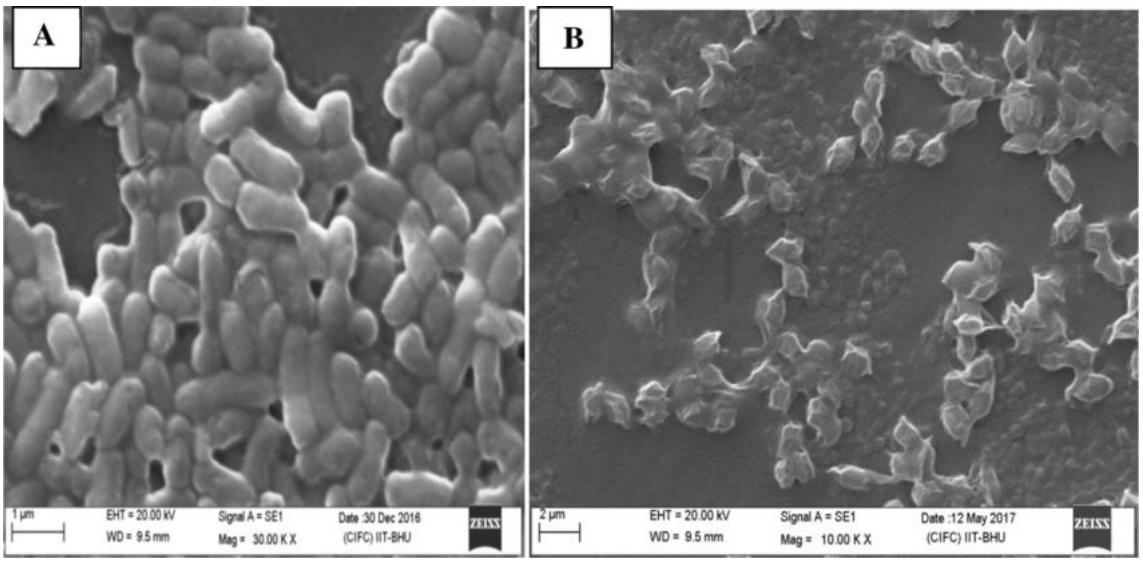

Fig. 3 SEM image of *Acinetobacter junii* isolate CH005 before (**a**) and after (**b**) treatment with toluene

3.6 General biodegradation pathway for petroleum hydrocarbons

BTEX compounds are mostly degraded by aerobic pathway. The main pathway for aerobic degradation of hydrocarbons by microorganisms is shown in Fig. 5. Aerobic conditions provide O_2 as a powerful oxidant to oxidize and cleave the ring of aromatic petrochemical compounds. Oxygen not only serves as the final electron acceptor but also as a co-substrate for some key catabolic processes (Fuchs et al. 2011; Díaz et al. 2013). Aerobic degradation of aromatic petrochemical waste generally starts with attack of dioxygenase enzymes on aromatic rings yielding cis-dihydrodiols (Cerniglia 1984). Subsequent dehydrogenation of cis-dihydrodiols to 1,2-dihydroxy compounds by a dehydrogenase is followed by metabolism of 1,2-dihydroxy compounds (Goyal and Zylstra 1997).

Toluene can be degraded by many biodegradation pathways, some involving 3-methylcatechol as an intermediate. Similarly, many biodegradation pathways exist for degradation of ethylbenzene, some involving 3-ethylcatechol as an intermediate. However, degradation of xylene is a complex process because all xylenes get metabolized to mono-methylated catechols (*e.g.*, m-xylene

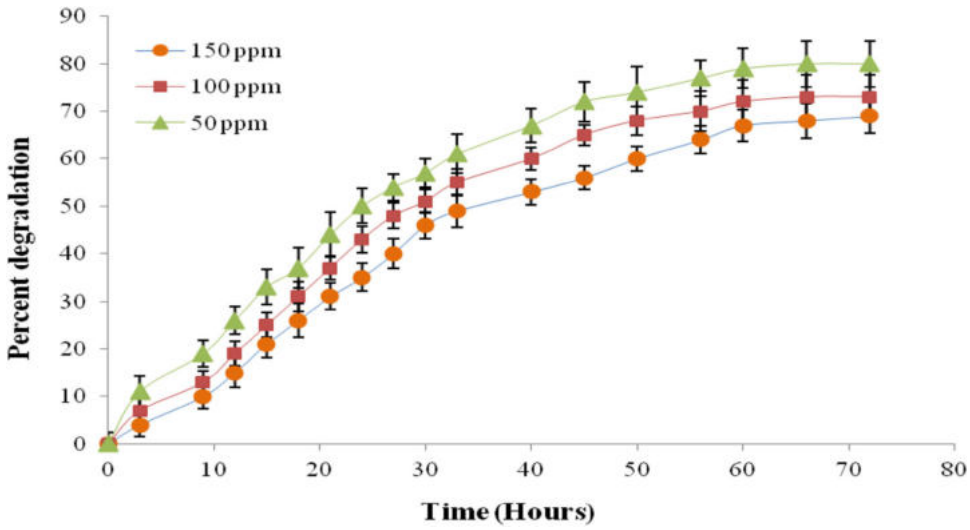

Fig. 4 Percent degradation of different concentrations of toluene by *Acinetobacter junii*

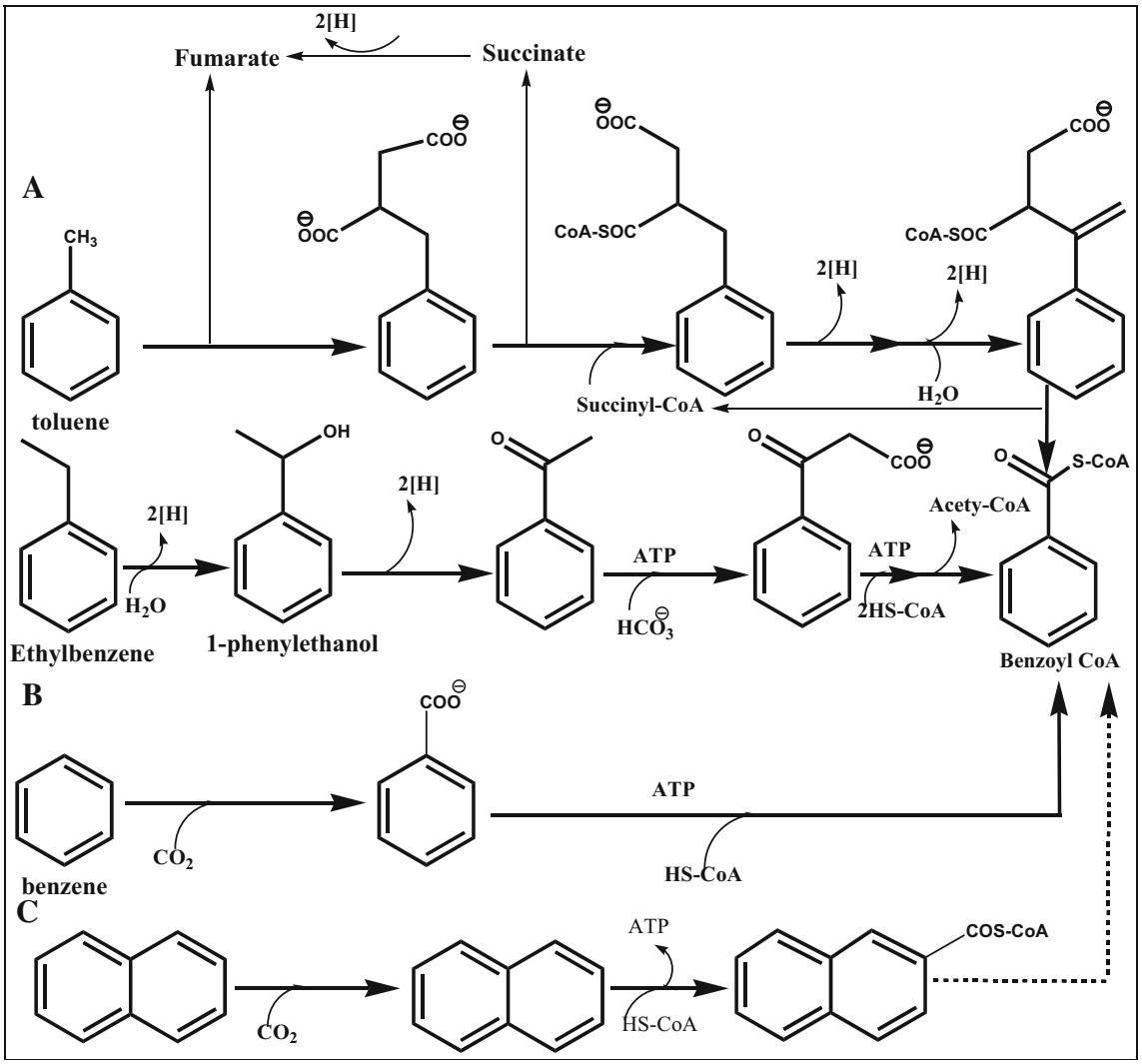

Fig. 5 General aerobic biodegradation pathway of benzene, toluene and ethylbenzene (*adapted from* Fuchs et al. 2011)

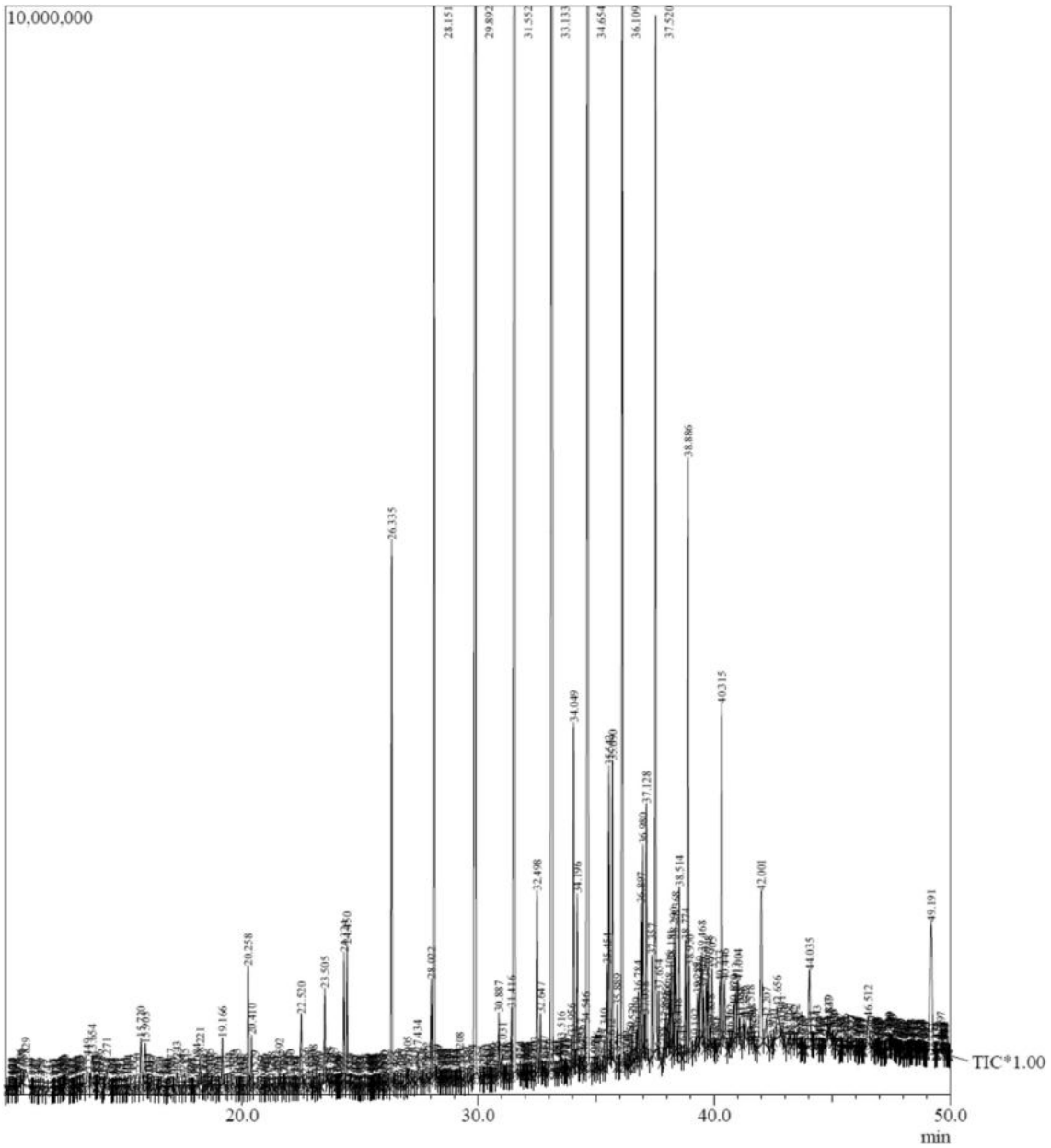

Fig. 6 GC−MS chromatogram of toluene degradation product after treatment with *Acinetobacter junii*

into 3-methylcatechol). The aromatic ring of all these substituted catechols is later cleaved under the action of dioxygenase enzymes.

3.7 Determination of intermediates formed after toluene degradation by *Acinetobacter junii*

Formation of intermediate compounds such as 1-isopropenyl-4-methyl-1,3-cyclohexadiene, 1,3-cyclohexadiene, 2-methyl-5-(1-methylethyl), 4-methoxycarbonyl-4-butanolide (Dobslaw and Engesser 2015), vinyl (2E,4E)-2,4-hexadienoate (Cho et al. 2000) was observed after toluene degradation by *Acinetobacter junii* isolate CH005. GC–MS chromatogram of biodegradation products are shown in Fig. 6.

Structures of intermediate compounds formed after toluene biodegradation are presented in Fig. 7.

4 Conclusion

Petrochemical waste management is one of the most serious concerns faced by environmentalists today. Bioremediation practice has gained huge success under laboratory

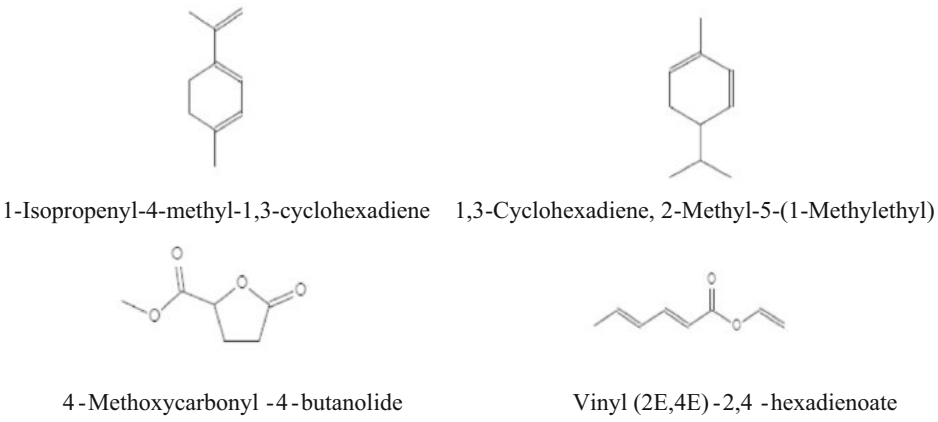

Fig. 7 Intermediate compounds formed after toluene biodegradation by *Acientobacter junii*

condition for toluene degradation. In the present study, we have reported the toluene degradation by pure bacterial culture. The biodegradation of toluene by isolate *Acinetobacter junii* CH005, isolated from petroleum contaminated water and soil showed that percent degradation of toluene, was best achieved at pH 7.5 and 37 °C temperature. Bacterial species under investigation demonstrated varying degree of toluene degradation ability. Moreover, it is important to facilitate and promote the degradation activities of these indigenous microorganisms by bioaugmentation and biostimulation. Since, the biodegradation of aromatic compounds is affected by numbers of physical, chemical, and biological factors, therefore, studies on other factors must also be taken in account in order to maximize the degradation processes.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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