

Experimental Study on Micro-biological Degradation of 1, 3, 5- TMB in Groundwater

Chu Yu, Dun-yu Lv*

Geology Environment Monitoring Institute of Zhejiang Province, Hangzhou, 310007

Abstract: 1, 3, 5-TMB (trimethylbenzene) has been considered as priority pollutant by several environmental agencies due to its high toxicity, carcinogenicity and mutagenic activity. Two bacteria with ability of degrading 1, 3, 5- TMB were isolated from crude oil contaminated soil. The optimal pH value and temperature for the growth of these bacteria were 7.0 and 30°C. 1, 3, 5- TMB was used as sole carbon and energy source by both strains. Strain A was identified as *Staphylococcus sciuri* and Strain C was *Microbacterium schleiferi*, both of which were facultative anaerobic bacteria. 1, 3, 5-TMB was degraded by strain C with efficiency of 41.2±1.8%. The bacteria offered new source for biodegradation of BTEX and bioremediation of oil-contaminated soil and groundwater.

Keywords: 1, 3, 5-TMB, Biodegradation, Facultative anaerobic strain

Introduction

1, 3, 5-TMB (trimethylbenzene) is one of primary constituents of petroleum and it is considered as priority pollutant due to its high toxicity, carcinogenicity and mutagenic activity. Contamination of groundwater with the benzene compounds is difficult to be remedied because they are relatively water-insoluble and can diffuse rapidly once introduced into aquifers (Harwood C S. et al., 1997; Chen J et al., 2007).

One of the most attractive means to remove 1, 3, 5-TMB is bioremediation. Despite of the toxicity and persistence, this kind of compounds can be degraded and utilized by many microorganisms as a sole source of carbon and energy (Fries M R. et al., 1994). Numerous studies have focused on the biodegradation of 1, 3, 5-TMB under various aerobic conditions.

The microbial metabolism patterns of 1, 3, 5-TMB was highly diverse (Barker JF et al., 1987; Burland SM et al., 1999; Edwards EA et al., 1992; Kasai Y et al., 2006). The research aimed to screen strains that are able to degrade 1, 3, 5-TMB under both aerobic and anaerobic conditions.

Materials and Methods

Culture Media and Growth Conditions

The experimental bacteria were grown in 100-mL shake flasks with 50-ml mineral salts (MS) liquid culture medium. The MS liquid medium contained 0.42 g NaNO₃, 0.27 g NH₄Cl, 7.9 g Na₂HPO₄·12H₂O, 1.5 g KH₂PO₄, 1.0 g MgSO₄·7H₂O, 1.2 mL trace elements solution and 1L distilled water, pH 7.0. Trace elements solution without carbon contained 2.14 g/L ZnCl₂, 2.5 g/L MnCl₂·4H₂O, 0.3 g/L CoCl₂·6H₂O, 0.2 g/L CuCl₂·2H₂O, 0.4 g/L NaMoO₄·2H₂O, 4.5 g/L CaCl₂·2H₂O, 2.9 g/L FeCl₃·6H₂O, 1.0 g/L H₃BO₃, and 0.1 g/L KI. For agar solid medium, 20 g/L agar was further added. After moist heat sterilization for 15 min, 1, 3, 5-TMB was added to the liquid medium as the sole carbon and energy source. All bacterial were cultured at 30°C± 2°C with 150 r/min shaking. When choosing the cultivation temperature and pH, the pH value of soil sample solutions and the ambient temperature of samples collected were taken into account.

The 1, 3, 5-TMB used was analytical grade. All other reagents were of the highest grade com-

*Corresponding author. Email: lvdunyu@hotmail.com

mercially available. Aqueous stock solutions for each of these compounds were prepared in shake flasks sealed with Teflon-coated rubber stoppers and aluminum crimps.

Bacterial Domestication, Screening and Isolation

The experimental bacteria were isolated from soil samples which were harvested from contaminated crude oil at an oilfield. The field moist soil samples were stored at 4 °C for further experiments.

Soil samples (5 g) were first washed with distilled water and centrifuged to remove most of the particles. Then the suspended samples (5 g) were collected in 150 mL shake flasks, with sterilized MS medium (50 mL) and a headspace of about 100-mL with gas/liquid ratio of 2:1, and supplemented with 0.472 g/L sodium succinate, 12.5 µL 1, 3, 5-TMB. An aliquot of 1 mL bacterial suspension was sub-cultured to fresh medium every two to four days, with the concentration of 1, 3, 5-TMB increasing from 12.5 µL to 50 µL and that of sodium succinate decreasing from 0.472 g/L to 0 g/L. After four cycles, some bacteria were domesticated to use 1, 3, 5-TMB as only C-source. From this bacterial suspension, a serial dilution method was used for bacterial isolation. The gas-liquid was controlled at a ratio of 2:1, because facultative anaerobes could be selected by limiting microbial oxygen consumption.

The isolated bacterial strains which were observed higher ability to grow on agar medium were selected for further studies. Colonies formed on the plates were purified by agar plate dilution method. Strains were routinely maintained on agar MS medium slants at 4 °C in the fridge and were transferred into fresh MS liquid medium once a month.

Bacterial Identification

The identification of strains of two bacteria with 1, 3, 5-TMB biodegradation ability was initially preceded on the basis of colony morphology, phenotype, physio-biochemical traits. Cell morphology and cell motility were examined by phase-contrast light microscopy. Gram-staining, catalase testing and other classical tests were

performed according to the eighth edition of Bergey's Manual of Determinative Bacteriology. Anaerobic growth was investigated by incubation in the presence and absence of oxygen using the Anaero cult system (Merck). Further analysis of 16S rDNA sequence was used for molecular identification.

Purified bacterial strains were grown in MS medium, and genomic DNAs were extracted using a genomic DNA purification kit (Promega) in accordance with the manufacturer's instructions. Amplification reaction was performed on Master cyclerepgradient S PCR system (Eppendorf AG, Germany) using primers P1 (5'-AGAGTTT GATCCTGGCTCAG-3') and P2 (5'-GGTACC TTGTACGACTT-3'). Each PCR mixture (50 µL) was composed of 20 mM Tris-HCl (pH 8.0), 0.5 µL MP1 and P2, deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 0.1 µg template DNA, and 2 U of Taq DNA polymerase. The amplification program consisted of an initial denaturation at 94 °C for 5 min; 38 cycles of 94 °C for 30 s, 61-65 °C for 30 s, and 72 °C for 60 s and finally, an additional extension step at 72 °C for 10 min. Amplification products were subjected to gel electrophoresis in 1% agarose, followed by ethidium bromide staining. DNA patterns were visually analyzed after photography by EC3 600 Imaging System (UVP, LLC, US). PCR amplification products purification and sequencing with the Applied Bio systems 373A Automated DNA Sequencer was done by Shanghai Invitrogen Biotechnology Co., Ltd. and sequences were aligned using BLAST+2.2.22 program available in NCBI website.

Optimum Grow Conditions

The optimum pH was determined, with pH values adjusted to 4, 5, 7, 9 with HCl (1 M) or NaHCO₃ (1 M), in 200 µL bacteria cultures growing in 50 mL MS medium in the presence of 50 µL (about 879 mg/L) 1, 3, 5-TMB. The optimum temperature was determined at different incubation temperature conditions (4, 15, 30, and 40 °C) assessed in triplicate incubation series. Cell growth was monitored once every 24 hours by measuring the optical density (OD) of culture at

600 nm with 722N visible spectrophotometer (Shanghai Jinpeng Analysis Instrument Co., Ltd, China).

Analytical methods

The concentration of 1, 3, 5-TMB was measured by GC analysis of the headspace (Alvarez Pedro J. J., Vogel Timothy M, 1991; Lovely, D. R, 1997). Controls containing no microorganism were also assayed to compensate the abiotic loss. The compound consumed was determined by the comparison of gas phase 1, 3, 5-TMB concentration in a sample flask with that in a control flask. All samples, irrespective of the incubation temperature of the cultures, were equilibrated at 60 °C for 30 mins before GC analysis. After 500 µL of headspace was withdrawn by a 5 mL Hamilton gas-tight syringe, it was injected into a gas chromatograph (HP 5890N) equipped with an HP-5capillary column and a flame-ionization detector (Chakraborty, R., Coates J. D, 2004). Chromatography was accomplished over 8 mins using an oven temperature of 40 °C for 1 min followed by a 6 °C/min rise up to 80 °C and held for 4 mins at this temperature. The injector and detector temperatures were set to 220 and 250 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 2 mL/min. 1, 3, 5-TMB was separated with retention time of 10.3 mins, respectively. Detection limits ranged from 8.67 to 13.85 µg/L and recovery rates from 85.3 to 102.6%.

Biodegradation of 1, 3, 5-TMB

After strains pre-incubated for 36 hours, bacterial suspension was diluted to the same OD

value with sterile water for batch experiments. The experiments were conducted using 100 mL shake flasks, sealed with Teflon-lined caps and aluminum crimps, containing 50 µL bacterial cultures, 50 mL medium and 10 µL 1, 3, 5-TMB individually, there was no additional carbon source added. The compounds were injected as pure stock with a 10 µL syringe. The initial concentration of 1, 3, 5-TMB was approximately 175.7 mg/L. All the experiments were conducted at 30°C, pH 7.0 in a shaking incubator at 200 r/min.

Three replicates were used for each batch. Sterile controls, prepared by autoclaving for 15 minutes at 120°C, were set to discern volatilization and adsorption losses of 1, 3, 5-TMB. The cultures were routinely examined by CPH-500E phase contrast microscope (Shanghai Changfang Optical instrument Co., Ltd, China) to ensure the absence of bacterial contamination (Jindrova E, Chocova M, Demnerova K, Brenner V, 2002). Biodegradation was assessed by comparing the disappearance of 1, 3, 5-TMB in samples and controls over time. The biodegradation of 1, 3, 5-TMB was measured after 72 hours.

Results and Discussion

Identification of the Degradation Bacteria

The indigenous bacteria which were capable of utilizing 1, 3, 5-TMB as the sole source of carbon and energy were isolated from polluted soil. Strain A and C were selected for further studies because it showed better growth on agar plate with 1, 3, 5-TMB. Their physico-biochemical characters were shown in Table 1.

Table 1 Differential Morphological and Physico-biochemical Characteristics of Strain A and C (+, Positive; -, negative)

Characteristic	Strain A	Strain C
Cell Shape	Round	Rod-shaped
Motility	Immobility	Immobility
Pigment	No Pigment	Yellowish
Growth at 4 °C	+	+
Growth at 41 °C	+	+
Growth at 60 °C	-	-
Gram Staining	+	+

Table 1 (continued)

Characteristic	Strain A	Strain C
Glucose	+	+
Fructose	+	+
Sucrose	+	+
Maltose	-	-
Gelatin Liquefaction	+	-
Nitrate Reduction	+	+
Oxidase	-	+
Catalase	+	+
Aerobism	Facultative Anaerobic	Facultative Anaerobic

Colonies of strain A were circular, slightly protruding, jagged, opaque, glossy and creamy white with a maximum colony diameter of 2 mm after 72 hours (photograph not shown). Growth occurred at 4 °C and 41 °C. Strain A was found to be facultative anaerobic, gram-positive, catalase-positive, oxidase-negative and colorless. The cells were shown to be round when visualized by phase contrast microscopy. Nonmotile cells generally occurred in groups. This morphologic appearance fitted the original description of *Staphylococcus* spp in the eighth edition of Bergey's Manual of Determinative Bacteriology.

Colonies of strain C were circular, slightly sunken, smooth, semi opaque, glossy and yellow pigmented with a maximum colony diameter of 1.5 mm after 72 hours. Growth occurred at 4 °C and 41 °C. Strain C was found to be facultative anaerobic, gram-positive, catalase-positive, oxidase-positive, and yellow-pigmented. The cells were shown to be rod-shaped when visualized. This morphologic appearance fitted the original description of *Micro-bacterium* in the eighth edition of Bergey's Manual of Determinative Bacteriology.

Environmental Factors on the Growth of Strains

The temperature influence on the metabolic activity differed between strain A and C, as seen in Fig1. At 4 °C the growth of both strains was considerably slow. Comparing the temperature effect results, strain A adjusted itself less quickly than strain C. After 48 hours log phase, strain A stepped into exponential growth phase and OD 600

nm increased rapidly. Both strains grew at temperature 15-40 °C with 1, 3, 5-TMB as carbon and energy source. The temperature at 30 °C was most favorable for the growth of both strains.

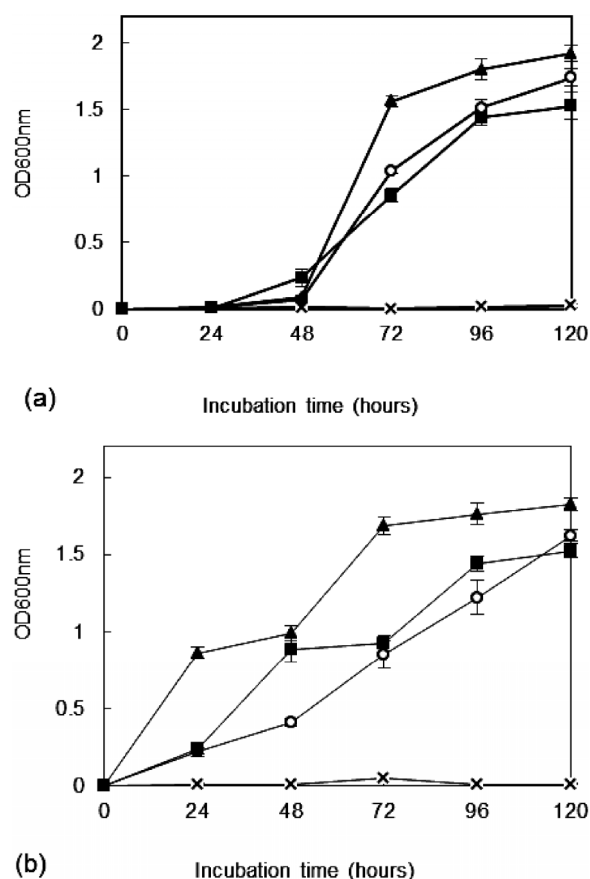
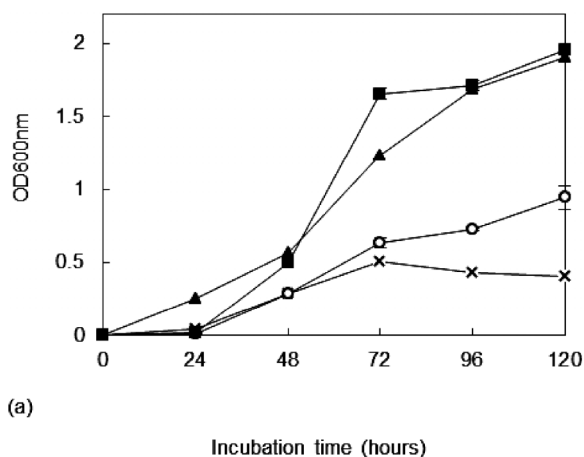


Fig1. Effects of Temperature on Bacterial Growth During a 120 hour Incubation Period

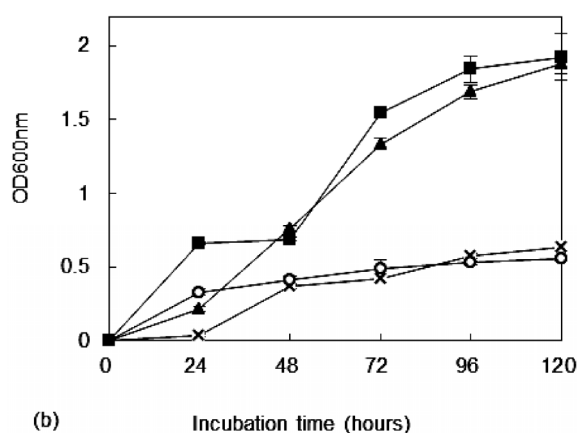
(a), Strain A; (b), strain C; x, 4°C; o, 15°C; ▲, 30°C; ■, 40°C. Error bars represent standard deviation of results (may be covered by symbols)

The influence of pH on bacterial growth was shown in Fig.2. The growth rate increased with the

increase of pH between 4.0 and 9.0. Both strains grew well at pH at 7.0 and 9.0; however, the bacteria could adapt more quickly and grow steadier at pH 7.0.



(a)



(b)

Fig2. Effects of pH on Bacterial Growth during a 120 hour Incubation Period

(a), Strain A; (b), strain C; ×, pH=4; ○, pH=5; ▲, pH=7; ■, pH=9. Error bars represent standard deviation of results (may be covered by symbols)

Degradation of 1, 3, 5-TMB

The two isolated strains were examined for the ability to degrade 1, 3, 5-TMB. Loss of 1, 3, 5-TMB in control was approximately $7.2 \pm 0.6\%$. The results demonstrated that there were great differences between the biodegradability of two strains. Almost $3.1 \pm 0.3\%$ of 1, 3, 5-TMB was degraded by *Staphylococcus sciuri* strain A, while strain C was capable for degrading 1, 3, 5-TMB with degrees of $41.2 \pm 1.8\%$.

Conclusions

Two indigenous bacteria for degrading of 1, 3, 5-TMB were isolated. Strains A and C, belonging to *Staphylococcus sciuri* and *Micro bacterium schleiferi*, respectively, are of particular interest because of their facultative anaerobic characteristics. The optimal pH and temperature was 7.0 and 30°C respectively. Mechanism of degradation and whether the bacteria could adapt to a wider range of temperature and pH need further study.

Acknowledgments

This work was supported by The National Key Technology R&D Program "Technology research and demonstration to assess, prevent and control geological environment risk during urban development" (No. 2012BAJ11B04) and Basic Scientific Research Program "Transportation behavior and mechanism of emerging contaminants in vadose clay layer" (No. SK201307).

References

- Harwood C S., Gibson J. Shedding Light on Anaerobic Benzene Ring Degradation: A Process Unique to Prokaryotes [J]. *Journal of Bacteriology*, 1997, 179: 301-309
- Chen J, Li X, Li J, Zhao Q, Xu C, Shu W. Degradation of Environmental Endocrine Disruptor Di-2-Ethylhexyl Phthalate by a Newly Discovered Bacterium, *Microbacterium* Sp. Strain Cqo110Y[J]. *Applied microbial and Cell Physiology*, 2007, 74: 676-682
- Fries M R., Zhou J, Chee-Sanford J, Tiedje J M. Isolation, Characterization, and Distribution of Denitrifying Toluene Degradors from A Variety of Habitats[J]. *Applied and Environmental Microbiology*, 1994, 8: 2802-2810
- Barker JF, Patrick GC, Major D. Natural attenuation of Aromatic Hydrocarbons in A Shallow Sand Aquifer[J]. *Ground Water Monitor Rev*, 1987, 7: 64-71
- Burland SM, Edwards EA. Anaerobic Benzene Biodegradation Linked to Nitrate Reduction[J]. *Appl Environ Microb*, 1999, 65(2): 529

- Edwards EA, Wills LE, Reinhard M, Grbic-Galic D. Anaerobic Degradation of Toluene and Xylene by Aquifer Microorganisms Under Sulfate-Reducing Conditions [J]. Appl Environ Microb, 1992, 58(3):794
- Kasai Y, Takahata Y, Manefield M, Watanabe K. Rna-Based Stable Isotope Probing and Isolation of Anaerobic Benzene-Degrading Bacteria From Gasoline-Contaminated Groundwater [J]. Appl Environ Microbiol, 2006, 72 (5):3586-3592
- Alvarez Pedro J. J., Vogel Timothy M. Substrate Interactions of Benzene, Toluene, and Para-Xylene during Microbial-Degradation by Pure Cultures and Mixed Culture Aquifer Slurries [J]. Applied and Environmental Microbiology, 1991, 10:2981-2985
- Lovely, D. R. Potential for Anaerobic Bioremediation of BTEX in Petroleum-contaminated Aquifers. Journal of Industrial Microbiology & Biotechnology, 1997, 18:75-81
- Chakra borty, R., Coates J. D. Anaerobic Degradation of Mono Aromatic Hydrocarbons [J]. Applied Microbiology and Biotechnology, 2004, 64:437-446
- Jindrova E, Chocova M, Demnerova K, Brenner V. Bacterial Aerobic Degradation of Benzene, Toluene, Ethyl benzene and Xylene[J]. Folia Microbial (Praha), 2002,47:83-93