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بررسی تجزیهٔ زیستی متیل ترشیاری بوتیل اتر با یک سویهٔ جدید *Azotobacter vinelandii* جداشده از خاک‌های آلوده شرکت پتروشیمی بندر امام خمینی (ایران)

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چکیده

مقدمه: متیل ترشیاری بوتیل اتر (MTBE) مکمل اکسیژنی محلول و کم تجزیه‌پذیری برای سوخت است که به سرعت خاک و آب را آلوده می‌کند. این مطالعه با هدف جداسازی و شناسایی باکتری‌های تجزیه‌کنندهٔ MTBE از خاک پتروشیمی بندر امام خمینی (ایران) و همچنین یافتن بهترین دما و pH برای تجزیهٔ MTBE انجام شد.

مواد و روش‌ها: نمونه‌ها از خاک آلوده خط تولید MTBE در شرکت پتروشیمی امام خمینی ماهشهر گرفته شد. غنی‌سازی و جداسازی باکتری‌های تجزیه‌کنندهٔ MTBE در محیط پایه نمکی (BSM) حاوی ۵۰ میلی‌گرم در لیتر MTBEو به دنبال آن، افزایش غلظت MTBE تا ۴۰۰ میلی‌گرم در لیتر طی ۵ مرحلهٔ غنی‌سازی انجام شد. فرایند تجزیهٔ MTBE توسط باکتری جداشده با روش GC ارزیابی شد. جدایهٔ تجزیه‌کنندهٔ MTBE با تکثیر و تعیین توالی ژن 16S rRNA شناسایی شد؛ درنهایت، پتانسیل باکتری برای تجزیهٔ MTBE در دماهای مختلف (۳۷-۲۵ درجه سانتی‌گراد) و مقادیر مختلف pH (۷/۵-۶/۵) تعیین شد.

نتایج: باکتری تجزیه‌کنندهٔ MTBE به‌عنوان *Azotobacter vinelandii* شناسایی شد. نتایج تجزیهٔ MTBE نشان دادند این باکتری یک سویهٔ قدرتمند با قابلیت تجزیهٔ ۹۱/۲۹ درصد MTBE در طول ۱۴ روز است. بیشترین تجزیهٔ MTBEتوسط باکتری در دمای ۳۰ درجه سانتی‌گراد و pH=۷ بدون تفاوت معنی‌دار به دست آمد.

بحث و نتیجه‌گیری: سویهٔ *Azotobacter vinelandii* جداشده در بررسی حاضر، توانایی زیادی برای تجزیهٔ MTBE طی ۱۴ روز انکوباسیون، بدون نیاز به افزودن سوبسترای هم‌تجزیه داشت و برای حذف این آلاینده از اکوسیستم‌های آلودهٔ خاکی پیشنهاد می‌شود.

واژه‌های کلیدی: متیل ترشیاری بوتیل اتر، خاک، تجزیهٔ زیستی، *Azotobacter vinelandii*

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The Analysis of Methyl Tertiary Butyl Ether Biodegradation by a Novel *Azotobacter Vinelandii* Strain Isolated from Contaminated Soils of Imam Khomeini Port Petrochemical Company (Iran)

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Abstract

Introduction: Methyl Tertiary Butyl Ether (MTBE) is a highly soluble and low degradable oxygenated supplement to fuel that causes rapid contamination of soil and water. The present study aimed to isolate and identify the potent MTBE degrading bacteria from the petrochemical soil of Imam Khomeini Port (Iran), as well as the finding of the best temperature and pH for MTBE degradation.

Materials and Methods: Samples were taken from contaminated soils of Mahsahar MTBE production site in Imam Khomeini Petrochemical Company. Enrichment and isolation of MTBE degrading bacteria were done in Basal Salt Medium (BSM) containing 50 mg/l MTBE followed by increasing the MTBE concentration up to 400 mg/l during 5 enrichment steps. The MTBE degradation process by the isolated bacterium was assessed by the GC method. The MTBE degrading isolate was identified by the amplification and sequencing of 16S rRNA gene. Finally, the MTBE degradation potential of the bacterium was detected in different temperatures (25-37 ºC) and pH values (6.5-7.5).

Results: The MTBE degrading bacterium was identified as *Azotobacter vinelandii*. The results of MTBE degradation showed that the bacterium is a potent MTBE degrading strain with the degradation ability of 91.29% during 21 days. The highest MTBE degradation by the bacterium was obtained at 30 ^{0}C and pH=7 with no significant differences.

Discussion and Conclusion: *Azotobacter vinelandii* strain isolated in the present study had the high ability to degrade high amounts of MTBE during 14 days of incubation without the need for the addition of co-catalytic substrate and is proposed for the removal of this pollutant from contaminated soil ecosystems.

Keywords: Methyl Tertiary Butyl Ether, Soil, Biodegradation, *Azotobacter Vinelandii*

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Introduction

Methyl Tertiary Butyl Ether (MTBE) is a low-cost compound that has been added to gasoline and diesel fuel all over the world since the 1970s, as a replacement for lead in order to increase the combustion and reduce the production of carbon monoxide (1, 2). MTBE is a polar, volatile, flammable, and colorless ether compound, which can easily be synthesized from methanol and isobutylene without any natural source. This liquid compound is highly soluble in water, alcohols, and other ethers at room temperature (3, 4).

MTBE has caused environmental problems, especially for groundwater (5), usually when leakages happened in gasoline underground storage tanks and to a lesser extent is an air pollutant when dispensed at a gasoline pump. Rain is a relatively quick washing-out agent for MTBE removal from the air, and MTBE is rapidly degraded in the air by the presence of light. MTBE is also rapidly biodegraded in the soils which are enriched with potent degrading microorganisms (6).

Because MTBE is a low taste and odor threshold substance, it is not easily recognized in drinking water by consumers. MTBE has a high solubility in water with at least 2 years' half-life in groundwater and low adsorption level to the soil which makes it an important pollutant in underground waters and surface soils. The standard concentration of MTBE in water is defined as 20-40 µg/l. This compound is also identified as a suspected carcinogen (4-8). MTBE removal from drinking water is done by physical and chemical methods including adsorption to substances such as activated carbon, aeration, and advanced oxidation including photo-oxidation by UV or chemical oxidation by Ozone or Perozone (4). These methods are expensive, especially when used in low depth water flows (2, 9). Biological remediation processes can be satisfactory alternatives to physical and chemical methods because of their cost-effectiveness and environmental friendship (1, 10).

MTBE is manufactured in Imam Khomeini Port Petrochemical Company along with other petrochemical products. MTBE pollution can cause soil and water pollution leading to adverse effects on human and animals' health. Therefore, this study aimed to evaluate MTBE degradation by the bacteria isolated from contaminated soils in Imam Khomeini Port (Iran) and investigate the degradation ability of the isolated bacteria in different temperatures and pH values.

Materials and Methods

Sampling: Samples were taken from the depth of 5 to 30 cm in MTBE contaminated soils around gasoline storage tanks (Ahwaz, Iran) of Mahsahar MTBE production plant (Imam Khomeini Petrochemical Company). The samples were transferred to the laboratory on ice in dark conditions and stored at 4 °C.

Isolation of Bacteria: MTBE (50 mg) as the carbon source was added to 1000 ml of basal salt medium (BSM) composed of 5.57 g Na_2HPO_4, 2.44 g KH_2PO_4, 2 g NH_4Cl, 0.2 g $MgCl_2.6H_2O$, 0.0004 g $MnCl_2.4H_2O$, 0.001 g $FeCl_3.6H_2O$, and 0.001 g $CaCl_2$ (pH=7) for enrichment and adaptation of bacteria (7, 11). Soil samples (5% v/v) were added to the medium and incubated at 30 ºC (on a shaker with 100 rpm agitation) for 10 days. Then, 5% of the enriched medium was added to BSM fresh media containing the serial carbon source concentrations (50, 100, 150, 200, and 400 mg/l) during 5 enrichment steps. BSM media containing the same concentrations of MTBE as above was considered as controls (11, 12). The bacteria were purified on BSM agar media containing the same concentrations of MTBE as above.

Identification of Superior Isolate according to MTBE Degradation: The most powerful MTBE degrading isolates were selected and characterized by morphological, biochemical, and molecular tests. Molecular identification was done based on the sequence of 16S rRNA gene. For this purpose, the DNA was extracted (GeneAll kit, South Korea) and PCR was done using universal primer pairs including FD1: 5´-AGAGTTTGATCATGGCTCAG-3´ and RP1: 5´-TACGGYTACCTTGTTACGACTT -3´ (13). The PCR steps included the steps as follows: incubation at 94 °C (5 min), 30 cycles consisting 94 °C (1 min), 55.8 °C (40 s), and 72 °C (150 s), and the final incubation (72 °C, 10 min). The size of PCR products was confirmed by 1% agarose gel electrophoresis and the resulting sequences were aligned in GenBank by using the NCBI BLAST and the phylogenetic trees were obtained by MEGA software (6).

Assessment of MTBE Degradation: The isolates were cultured in Luria Bertani (LB) medium and incubated at 30 °C for 18 h. Then, the cultures were centrifuged at 6000 rpm for 15 min in order to separate the bacterial cells. Afterward, the cells were added to a new LB medium and incubated to reach OD 600 nm=0.1. Then, 5% of the prepared suspension was inoculated into a BSM medium containing 200 mg/l MTBE as the sole carbon/energy source and incubated for 21 days at 30 °C on 150 rpm agitation. GC analysis was done on medium samples which were taken at 0, 3, 7, 14, and 21 days after incubation. The control non-inoculated culture medium was used for the comparison of the results. The experiments were done in 3 replicates (14, 15). GC analysis was performed in a Headspace Sampler Varian CP 380 with an injection rate of 250 µl/s and heating at 80 °C for 5 min on the rotation rate of 500 rpm. The capillary column was thermally programmed first at 40 °C for 5 min; then the temperature was increased stepwise up to 180 °C. The column temperature was then kept at this temperature for one min. A detector at the temperature of 300 °C with hydrogen flow of 30 ml/min with an inside flame ionization was used. Star Bar Software was used for processing the output information from the device. The obtained peak for the MTBE blank is shown in Figure 1. The calibration curve of the compound was plotted by using different concentrations of MTBE according to the area ratio under the MTBE curve to the region under the standard curve (Figure 2). The first-order kinetic model for the removal of MTBE was calculated by

$$\frac{\text{concentrati}\qquad\qquad\text{tion at day 0}}{\text{concentration at day 14}} \times 100$$

(16).

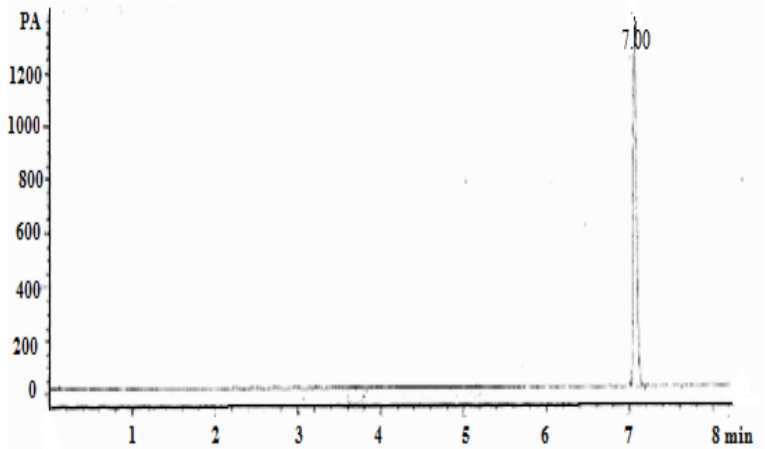

Fig. 1- MTBE Blank GC Analysis (the retention time was approximately 7 min.)

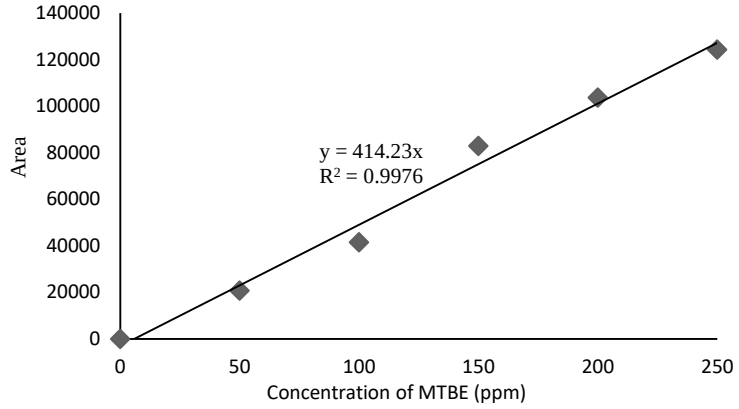

Fig. 2- Calibration Curve of MTBE obtained by GC Analysis

Detection of the Best Conditions for MTBE Degradation: The best temperature and pH values for MTBE degradation was detected by one factor at a time experiment designing. For this purpose, the bacterium was separately grown in BSM containing 200 mg/l MTBE at three growth temperatures of 25 °C, 30 °C, and 37 °C and three pH values of 6.5, 7, and 7.5 which were adjusted by 1 M NaOH and HCl (at the temperature of 30 °C). The inoculums contained 1.5×10^7 cells of the bacterium to 50 ml culture medium. The media then were incubated in an incubator shaker on 150 rpm agitation in different temperatures; then the amounts of residual MTBE in the culture media were detected after 21 h incubation (1, 7).

Results

The Isolated MTBE Degrading Bacteria: A total of 11 isolates were isolated from the contaminated soil samples, from which one isolate (PWB) was able to grow in BSM broth media containing different concentrations of MTBE. This isolate tolerated MTBE with a concentration of up to 800 mg/l.

Identification of the Selected Isolate: The selected isolate was a Gram-negative rod with positive catalase, oxidase, and gelatinase test results. Molecular identification based on sequencing of the 1500 bp band obtained from the amplification of 16S rRNA gene in agarose gel electrophoresis showed that the isolate was a strain of *Azotobacter vinelandii* (GenBank: OK035268). The phylogenetic tree is shown in Figure 3.

MTBE Degradation Potential of *Azotobacter vinelandii* Strain PWB: The first-order kinetic model for MTBE degradation potential by *Azotobacter vinelandii* strain PWB during 21 days of incubation in BSM broth containing 200 mg/l MTBE is shown in Figure 4. This strain decomposed 91.29% of MTBE on day 21. The result from the GC analysis of the inoculated BSM medium containing 200 mg/l MTBE after 21 days of growth of the strain at 30 °C on 150 rpm showed an intense decrease in MTBE amount (Figure 5).

Optimization of the Growth and MTBE Degradation by *Azotobacter Vinelandii* Strain PWB: The results from the investigation of MTBE biodegradation by the bacterium during 21 h incubation in BSM broth containing 200 mg/l MTBE in different temperatures and pH values are shown in Figures 6 and 7. The bacterium significantly removed the highest amounts of MTBE at the temperature of 30 ºC (91.30%), and pH = 7 (91.21%).

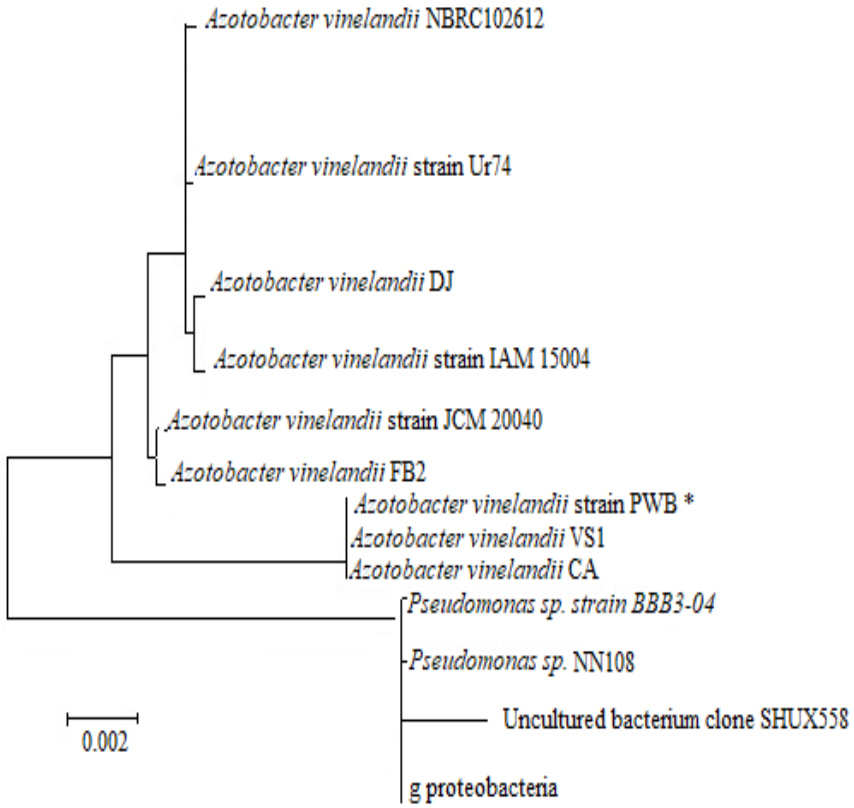

Fig. 3- The Phylogenetic Tree (Neighbor-joining) of the isolate PWB (*)

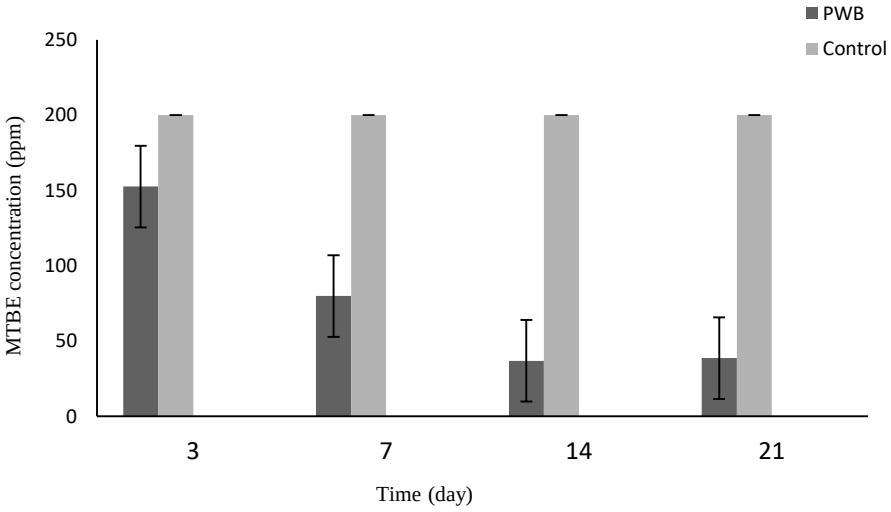

Fig. 4- The Rate of MTBE Removal in BSM Broth Medium Containing 200 mg/l MTBE by *Azotobacter Vinelandii* Strain PWB in Different Incubation Times

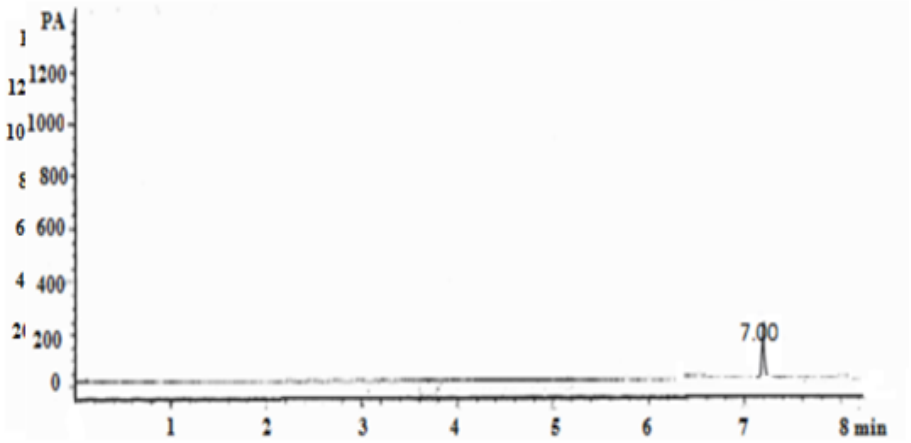

Fig. 5- Analysis by GC shows an approximate 91% decrease in MTBE content of the medium by *Azotobacter vinelandii* PWB

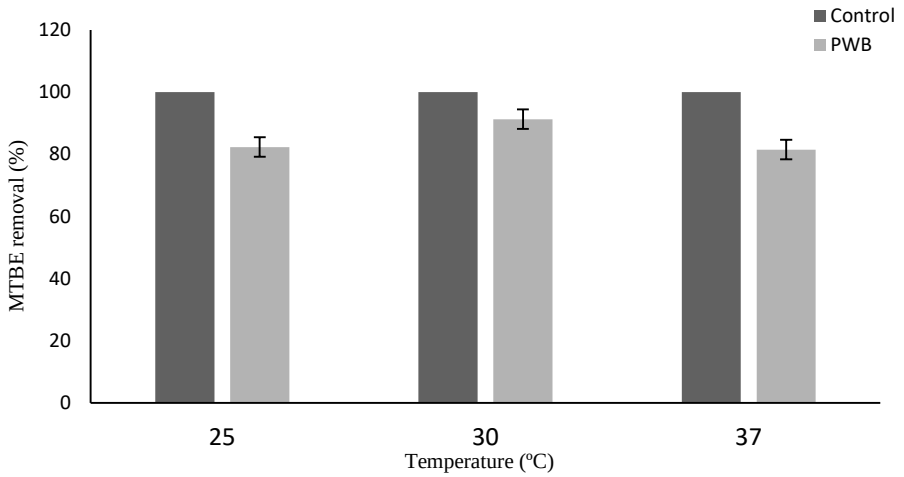

Fig. 6- The rate of MTBE removal in the BSM broth medium containing 200 mg/l MTBE by *Azotobacter vinelandii* strain PWB in different temperatures during 21 h incubation time. The highest degradation efficacy was achieved at 30 ºC.

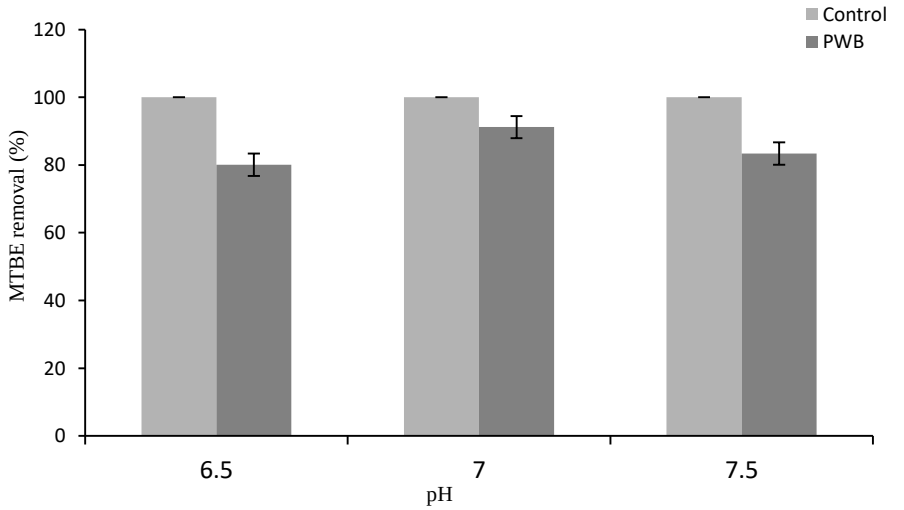

Fig. 7- The rate of MTBE removal in the BSM broth medium containing 200 mg/l MTBE by *Azotobacter vinelandii* strain PWB in different pH values during 21 h incubation time. The highest degradation efficacy was achieved at pH = 7.

Discussion and Conclusion

Biodegradation of MTBE in soils has received little attention so far. The extensive contamination of water resources and soil because of wide production, distribution, and use of MTBE has been concerned as an important environmental problem. The main source of MTBE entrance to the environment is the leakage from underground storage tanks. This compound is highly persistent and low biodegradable; therefore, efforts should be done to identify more bacteria capable of MTBE biodegradation as biological treatment of polluted waters and soils (4, 11, 12, 17, 18). Because of cost-effectiveness and adaptability to different environmental conditions, biodegradation is the more attractive strategy for MTBE degradation (11, 19) than different other methods such as physicochemical remediation (ozone utilization, activated carbon, and vaporization extraction) (4, 11, 20, 21).

Although ether oxygenate contaminants are usually degraded in soils by a diverse range of methylotrophic bacteria (22), several other microorganisms have been isolated from uncontaminated and gasoline, or MTBE-contaminated soils with the ability to grow on MTBE and other ether oxygenate compounds under aerobic conditions by using them as the sole carbon and energy sources (23-26). Although actinobacteria (large members of the Nocardiaceae) have been identified as the major predominant MTBE degrading bacteria, the bacteria in the beta and gamma Proteobacteria have also been detected (22). In the present study, MTBE and gasoline contaminated soils were used to isolate MTBE degrading bacteria in order to obtain an isolate that may be compatible with soil conditions for further usage in the degradation of MTBE from contaminated soils. MTBE degrading bacteria also have been isolated from soils or wastewaters in previous studies (9, 11, 12, 15, 27). The enrichment in the present study was performed in 5 steps by increasing the trend in MTBE concentration from 50 to 400 mg/l in order to select resistant bacteria to higher MTBE concentrations and to maintain slow-growing bacteria (12). In the end, a novel strain of *Azotobacter* was isolated with a high MTBE degradation ability. Okeke and Frankenberger Jr (2003) isolated 12 bacterial strains during two enrichment steps in a BSM broth medium containing 200 ppm of MTBE. Among the isolates, the *Sterptomyces* sp. and *Sphingomonas* sp. from MTBE contaminated soils were able to degrade 29.6% and 27.8% of MTBE during 28 days' incubation, respectively (2).

Zhang et al. (2007) isolated a *Chryseobactrium* sp. from MTBE-contaminated soils by increasing MTBE concentration in a BSM broth medium, from 50 to 200 ppm. The bacterium was able to remove 52.6% of MTBE during 10 days of incubation (11). Kariminik et al. (2013) isolated 12 MTBE degrading strains with the maximum biodegradation rate of 93.2%, 60%, and 97.97% which was resulted from the cultivation of *Micrococcus luteus*, *Bacillus subtilis*, and *Bacillus megaterium*, respectively, at the concentration of 500 ppm MTBE during 14 days of incubation (28). In the present study, *Azotobacter vinelandii* strain PWB showed a maximum MTBE degrading potential (91.30%) in BSM broth containing 200 mg/l MTBE during 21 days of incubation at 30 ºC and pH = 7.

The results of the above studies are shown that using various bacterial strains in different concentrations of MTBE, different incubation times and incubation conditions influence MTBE biodegradation results. On the other hand, stepwise increasing the concentration of carbon substrates during bacterial enrichment may influence the adaptation and degradation potential of the

strains (4). *Azotobacter vinelandii* strain PWB, in the present study, showed the highest degradability at 30 °C and pH = 7 which has been reported in some previous studies as the optimum temperature and pH for the highest rates of MTBE degradation (3, 11, 29). The *Azotobacter vinelandii* strain PWB also was able to degrade MTBE at the other studied temperatures, and pH values with no significant differences, which is important for the biological treatment of contaminated soils and waters in conditions such as groundwater which has low temperatures. Also, *Azotobacter vinelandii* which is a member of Azotobacteriaceae is an effective agent in soil fertility by nitrogen fixation, has genetic pliability and natural competence has made it a favorite for different research projects (30). This bacterium also can be used in co-metabolism processes which is an important mechanism for biodegradation of other oxygenated compounds in gasoline-contaminated soils (31).

Bioremediation is proposed as the most promising technique to reduce MTBE contamination in the environment. The novel strain of *Azotobacter vinelandii* was able to remove high amounts of MTBE during 15-21 days of incubation without the addition of a co-catalytic substrate. This bacterium may be used for cost-effective removal of MTBE from the environment, especially soil which is the main environmental source of the bacterium.

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