Original article

The enhancement of methyl tertiary butyl ether oxidation by *Gordonia* in the presence of nanosilver and nitrate

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Abstract

Introduction and objective: Several Gram-positive and Gram-negative bacteria isolated from methyl tertiary butyl ether (MTBE) reached soil. Among them one Gram-positive, oxidize positive, partial acid fast, which had strong nitrate reductase activities was identified as *Gordonia*. The effects of methanol, nitrate and nanosilver on MTBE removal were studied.

Materials and methods: *Gordonia* was isolated from MTBE enriched soil and identified by sequencing and biochemical test. The removal of MTBE was studied by *Gordonia* with COD Hach reagent. The production of formaldehyde and formate were determined by Hantzsch reagent and potassium permanganate, respectively. Also MTBE oxidation by nanosilver, nitrate and bacteria were compared.

Results: The results showed that the addition of 5 or 10ppm nanosilver inhibits the reaction, however 0.2ppm nanosilver increased MTBE oxidation by 15% and gives maximum 70% removal in 6h. The biodegradation of MTBE by this isolated strain only occurred when nitrate was added in media with or without Nanosilver. Also Methanol for induction or co-oxidation is necessary for MTBE oxidation by *Gordonia*.

Conclusion: *Gordonia* has a good capacity to remove MTBE only with nitrate respiration. Therefore nitrate as electron acceptor for respiration, methanol as co-oxidation and nanosilver as catalyses in special concentration help *Gordonia* for MTBE removal.

Keywords: Methyl tertiary butyl ether (MTBE); Nitrate reductase; *Gordonia*; Nanosilver

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Introduction

Among fuel oxygenates, methyl tertiary butyl ether (MTBE) is the most commonly used because of its high-octane level, low production cost, ease of blending with gasoline, ease of transfer and distribution [1,2]. The addition of MTBE to gasoline began on a relatively small scale in the late 1970's with its use as an octane enhancer to replace tetraethyl lead [3]. An average of 11% MTBE by volume is added to about 30% of the gasoline sold in the United States. However, currently the alcohol is used instead of MTBE [4].

Like most other gasoline components, MTBE is introduced into various environmental compartments during the production, distribution, use and storage of oxygenate-blended fuels. MTBE has been detected in urban air, soil, surface water, storm water and groundwater. In fact, MTBE has been shown to persist in aquifers, and MTBE plumes have been shown to migrate at rates comparable to groundwater velocities. The mobility of MTBE in the subsurface is due to high aqueous solubility, low water partition coefficient and chemical structure which are relatively resistant to microbial attack [3,5,6].

Scientific information on the assessment of the carcinogenicity of MTBE in humans comes from animal investigations. However, the potential carcinogenic effect of MTBE on humans remains a matter of debate [7]. Based on taste and odor concerns, the EPA's Office of Water has established a drinking water advisory level of 20-40µg/l as guidance for state and local authorities [8]. MTBE is poorly adsorbed, chemically and biologically stable and very soluble in water, making it very persistent in the environment. Therefore, effective technologies are in an urgent demand to remove MTBE from contaminated water. Conventional treatment of MTBE-contaminated groundwater is inefficient and unsatisfactory.

Removing of MTBE from air is difficult and requires a high air-to-water ratio (>200/1 l for 95% removal) because of its very low Henry's law constant [9]. MTBE removal, have been studied by several methods including physicochemical, chemical and biodegradation. Biological removal is very slow and hard also always co- metabolism with toluene and benzene is used for biological MTBE removal. Methylotroph bacteria also are responsible for MTBE degradation [6]. Alternative oxidation for MTBE is rely on of highly reactive hydroxyl radicals (OH) that result in oxidation and even complete mineralization of organic species. The chemical oxidations by UV/TiO2 process, UV/H2O2 process, O3/H2O2 and Fenton's Reagent have been used for the oxidation of MTBE in aqueous solution [9-11].

Nanoparticles are of great concern to the environment because of their small size and high catalytic properties, which provides better contact with microorganisms. The nanoparticles attached to the cell membrane and also penetrate inside the bacteria. Inhibition to nitrifying organisms correlated with the fraction of Ag nanoparticles less than 5nm in the suspension. It appeared that these size nanoparticles could be more toxic to bacteria. Cell membrane of bacteria contains proteins and the silver nanoparticles interact with these proteins in the cell wall and with the phosphorus containing compounds like DNA or ATP. The nanoparticles preferably attack the respiratory chain, and are toxic to nitrifying and enteric bacteria [12].

Nowadays tens of millions of chemical products are obtained through industrial processes involving silver-based catalysts, e.g. oxidation of ethylene to ethylene oxide,

1 methanol to formaldehyde, 2 ethylene glycol to glyoxal, 3, 4 and 1, 2-propylene glycol to methyl glyoxal. Recently, there has been great interest in the application of silver-based catalysts in the field of fine chemistry such as the partial oxidation of polyhydric alcohol [13]. The purposes of this research are for first time to investigate biodegradation of MTBE by *Gordonia* in addition to nanosilver.

Materials and methods

Chemicals and reagents

Toluene, methanol and MTBE were purchased in Iran that was made by Merck-Schuchardt Hohenbrunn.

Culture media and isolation of bacterial strain

Gordonia sp. strain was isolated from MTBE-contaminated soil by a standard culture enrichment technique using basal salt medium (BSM) supplemented with 791×10^3mg L^{-1} methanol and 29.6mg L^{-1} MTBE as the sole source of carbon. BSM contained 4g KH_2PO_4, 4g Na_2HPO_4, 2g NH_4Cl, 0.2g $MgCl_2$, 0.001g $CaCl_2$ and 0.001g $FeCl_3$ in 1000ml twice distilled water. The strain was stored in 50% (v/v) glycerol at -80°C. Each inoculum was grown to the late exponential phase at 30°C in BSM agar supplemented with methanol and MTBE as mentioned above [14].

Identification of isolated strain

Identification of the isolated strain was based on colony morphology, microscopic observation of the cell cycle, Gram stain, acid-fast stain, catalase test, oxidase test, oxygen requirement, motility, the ability to grow on different carbon sources, and in the presence of some inhibitors according to the standards for microbial identification in *Bergey's Manual of Systematic Bacteriology*. Sequences analysis of the 16S rRNA gene also was performed for identification of strain according to Bustos-Jaimes *et al.* by MacroGen Company (South Korea) [15].

Batch oxidation of MTBE by nanosilver

The oxidation capacities of nanosilver (Nanopac Persia Company, Iran) were studied in the batch experiments. To investigate the effect of nanosilver on degradation of MTBE, 0.2ppm nanosilver was added to MTBE and the absorption rate was measured at 150-300nm. To obtain reliable data, the initial concentrations of the aqueous phase of MTBE was 2ppm with different pH (4, 7, 9,) and the removal of MTBE was measured in 2h. The media with nanosilver (0.2, 5, 10ppm) incubated for 6h and the MTBE oxidation compared with the blank without addition of nanosilver. Each sample was measured three times.

MTBE removal assay

Removal of MTBE was assayed with UV spectrum at 200-600nm and the reaction with COD Hach reagent (4.913g $K_2Cr_2O_7$ was added to 500ml water with 167ml H_2SO_4 and 33.3g $HgSO_4$). This reagent was dissolved and diluted to 1000 ml. 1ml of the grown cells on MTBE was added to 1ml digestion solution (COD Hach reagent), the obtained blue green colour was measured by a turbidity measurement as (OD at 600nm) in a UV-visible spectrophotometer (Shimadzu UV-160, Japan) against blank. The reduction of blue green colour showed the removal of MTBE.

Determination of formaldehyde production

The concentration of produced formaldehyde by the studied strain was measured by Hantzsch method [16]. Equal volumes of Hantzsch reagent (2M ammonium acetate, 50mM acetic acid, 20mM acetyle acetone) were added to 2ml of centrifuge cell biomass grown on nutrient broth (Merck, Germany) and induced with

MTBE (4000ppm) for 2h. This mixture was incubated at 60°C for 10mins. The obtained yellow colour of 3,5 diacetyl 1,4 dihydrolotidin which was produced from reaction between formaldehyde and pentane 2,4 dion (acetyle acetone) in the present of ammonium acetate was centrifuged and measured at 412nm against blank.

Formate determination by potassium permanganate

Production of formate was tested with significant reduction of purple colour of potassium permanganate (1mM) [17].

The effect of nitrate in MTBE removal

Gordonia was grown in nutrient agar (Merck, Germany) with and without methanol for induction. The cell colonies transferred to MTBE media with the addition of nitrate (1g/l) in aerobic and anaerobic condition. The MTBE degradation was studied in nitrate respiration by isolated *Gordonia*.

The effect of methanol and toluene on MTBE oxidation

The isolated strain was prepared in media with methanol or without it. Also in batch experiment, methanol (10ml/l) was added to MTBE to study the effect of methanol for degradation. Toluene (1ml/l) was added to MTBE to study co-metabolism degradation of MTBE with another carbon sources.

Production of CO_2 from MTBE

Filter paper Whatman number one (2×1cm) was cut soaked with 0.1ml KOH 40% and dried. The weight of this paper was measured and inserted into small minifuge tube in sealed cap universal tube with MTBE (0.02ml/ml) and nanosilver (20ppm). The MTBE oxidation by CO_2 was measured by CO_2 reaction with KOH. This experiment was done in triplicate.

Results

The soil was contaminated with MTBE for three months and enriched bacteria were isolated on MTBE noble agar. The MTBE removal was tested by eight different strains with nitrate respiration and aerobic condition which are shown in table 1. As it is shown, strain B reduced MTBE by 64% with nitrate respiration and the removal was negative in the absent of nitrate. This strain reduced nitrate strongly. However strain C reduced MTBE by 43% in aerobic respiration and addition of nitrate to this strain has negative effect on MTBE degradation (Table 2).

Table 1: MTBE removal by cell biomass (OD=0.7) isolated from enriched soil with MTBE grown on nutrient broth aerobically

Strain	MTBE removal without nitrate	MTBE removal in nitrate	$OD_{500\ nm}$ reduced nitrate
A	0.24%	19%	0.15
B	0.0%	64%	0.32
C	43%	0.0%	0.09
D	36%	37%	0.15
E	8%	28%	0.1

Table 2: MTBE removal by cell biomass (OD =0.7) isolated from enriched soil with MTBE grown on nutrient broth anaerobically

Strain	MTBE removal in nitrate	OD $_{500\ nm}$ reduced nitrate
A	27%	0.14
B	10%	0.06
C	24%	0.1
Combination	16%	1.3

It is worth noting that strain B is highly positive nitrate reduction while C is negative nitrate reduction strain. The strains were grown on methanol and toluene agar to see the effects of these compounds on biodegradation of MTBE and the results are shown in table 3, which shows that the pure culture on methanol and toluene is not useful for MTBE degradation by isolated strains. However strain B has high effect on the reduction of MTBE and the identification of this strain has done by preliminary test (Table 4). This strain was identified as *Gordonia*, therefore further test was carried out on this isolate.

The addition of methanol to media for co-oxidation increased degradation of MTBE from 41% to 58% by biomass obtained from *Gordonia* grown on nutrient broth. Whereas this degradation was 10% by biomass obtained from *Gordonia* grown on MTBE + methanol + toluene. This suggests that co-metabolism of MTBE with methanol occurs while MTBE co-oxidation with toluene by *Gordonia* negative. The production of formate and formaldehyde from oxidation of MTBE for this strain was negative.

Table 3: MTBE removal by cell biomass (OD=0.7) isolated from enriched soil with MTBE grown on methanol or toluene agar in aerobic condition

Strain	MTBE removal in nitrate (biomass obtained in methanol agar)	MTBE removal (biomass obtained in Toluene agar)	OD $_{500\ nm}$ reduced nitrate
A	18	0	0.1
B	17	0	0.07
C	27	10	0.1
D	0	0	0.08
E	0	0	0.09

Table 4: Preliminary identification of isolated *Gordonia*

Test	Reaction
Gram stain	+
Shape	Coryne form
Spore	–
Catalase	+
Oxidase	+
Acid fast stain	Partially +
OF test	O/F
Growth aerobically	+
Growth anaerobicaly	+
Motility	–
Acid from glucose	+

The effect of nanosilver on biodegradation was studied and the results are shown in figure 1. As it is shown, the addition of 0.2ppm nanosilver reduced the pick of MTBE but increased the absorption at 178nm and the addition of 5ppm nanosilver to MTBE gives adsorption on 270nm. Table 5 shows some intermediate compound with their λ max. As it is shown, the alcohol and ketone have absorption at 178 and 270nm respectively. Therefore the addition of nanosilver oxidizes MTBE to alcohol or acetone and we can use it to oxidize the MTBE to OH or CO radical. Addition of

0.2ppm nanosilver to media with MTBE helps *Gordonia* to reduce MTBE up to 68% in few hours (Table 6). It is obvious that the MTBE oxidation by nanosilver occurs in the light environment and the production of hydrogen peroxide is possible too.

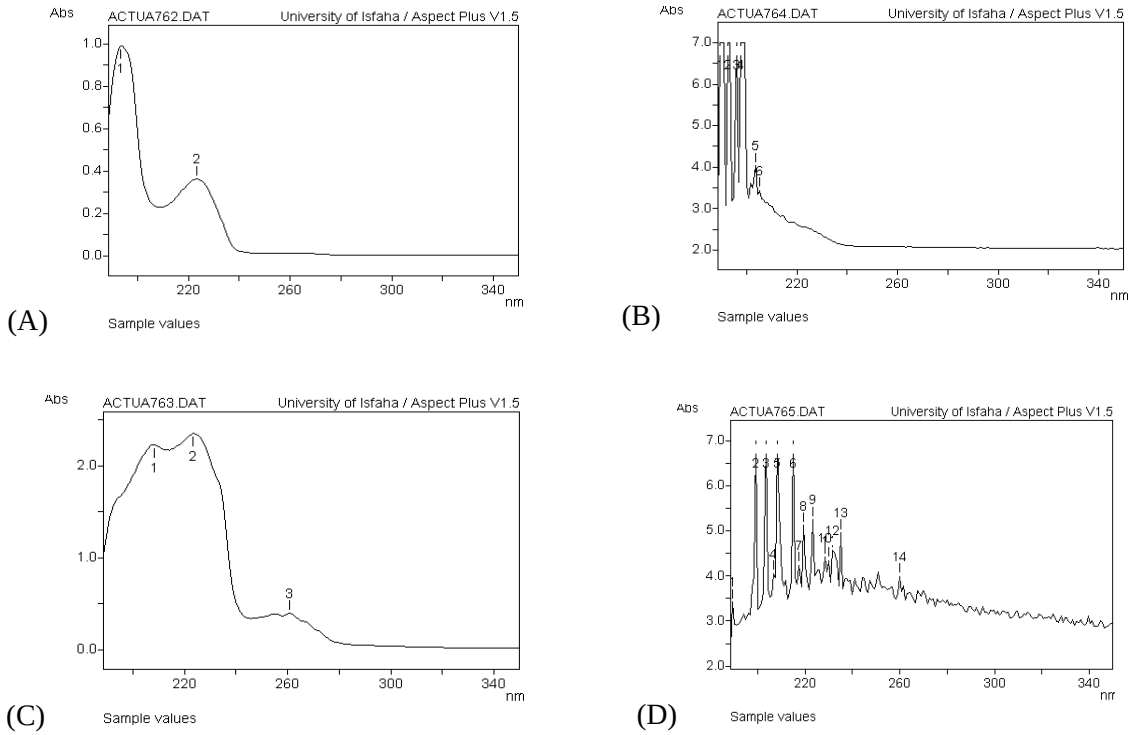


Fig. 1: UV absorption of MTBE treated with 0.2ppm and 5ppm nanosilver in present of light during 30 minutes (A=0.2 ppm nanosilver, B= 0.2ppm nanosilver + MTBE, C=5ppm nanosilver, D=5ppm nanosilver + MTBE)

Table 5: The UV adsorption for possible MTBE restructure compounds

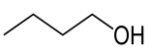
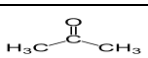
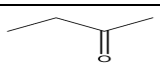
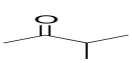
Name	Structure	Lambda max
Butanol		178 nm
Acetone		272 nm
Butanone (or methyl ethyl ketone)		273 nm
Methyl isopropyl ketone		280 nm

Table 6: The effect of nanosilver on MTBE removal by *Gordonia*

Conditions	MTBE removal (in light)
MTBE removal in methanol	58%
MTBE removal with 0.2 ppm nanosilver	45%
MTBE removal with nitrate	53%
MTBE removal in presence of methanol + 0.2 ppm nanosilver + nitrate	68%

Discussion

Many investigations have shown that the cellular yield of microorganisms utilizing MTBE as the sole organic carbon source can be expected to be very low [3,6]. The

same results were obtained in the previous study in which three isolated bacterial strains have been observed to grow at a relatively very slow rate and did not have any significant growth on MTBE (unpublished data). Here we found that *Gordonia* is the best isolate to degrade MTBE. Although the oxidation state of the silver nanoparticles may influence their (biological and toxicological) activity, little attention has been paid to the oxidation state of the silver nanoparticles of silver which exert an antibacterial effect, most likely due to the combination of nanocarrier material (i.e., silver nanoparticle).

Liu *et al.* [18] showed the removal of gaseous methyl mercaptan for odor control by $AgNo_3$ films prepared by a simple dip-coating method. They show CH_3-SH-Ag is formed on surface of $AgNo_3$. Today removal of metals, (e.g. cadmium, copper, lead, mercury, nickel, zinc) nutrient (e.g., phosphate, ammonia, nitrate and nitrit) viruses, bacteria, parasites and antibiotics are shown by nanoparticles, nanomembranes and nano powders. Zinc oxide nanopartichles have been used to remove arsenic from water, even though bulk zinc oxide cannot absorb arsenic [19].

Biosorption of metals, protein and fat by nanoparticles in waste water have been studied before [19]. Also reduction of the dye and metal has been reported by Zhong *et al.* [20] and Slocik *et al.* [21]. They have shown that catalytic activites of silver nanoparticles are able to remove azo dies.

Removal of MTBE from water using Fenton's reagent have been studied by Ray *et al.* [22]. Although they have shown that complete mineralization of MTBE by Fenton's reagent was not achieved, but greater than 99% destruction of MTBE was seen. Their results were accomplished at a Fe^{++}, H_2O_2 ratio of 1:1 in one hour of contact time. They found that the major by-products were tertiary butyl alcohol, tertiary butyl formate and acetone with trace of 2-methyl-1-poropen. The production of CO_2 was not observed by them. However, in the our study the small amount of CO_2 (0.35mM) was monitored by adsorption on KOH in sealed cap vial when 2mM MTBE was treated with 20ppm nanosilver.

There is not any report about the oxidation of MTBE by nanosilver and this is the first time that MTBE removal by nanosilver in the presence of light is reported. The production of H_2O_2 by nanosilver in light can be the real reason of MTBE oxidation, just like Fenton reaction (Ag is a cation like Fe in Fenton's reagent). Degradation of MTBE by H_2O_2 has been reported also by Zang *et al.* [23] and Acero *et al.* [24]. This concentration of nanosilver is not toxic to *Gordonia* and is helpful for biodegradation.

Conclusion

Gordonia has a good capacity to remove MTBE only with nitrate respiration. Therefore nitrate as electron acceptor for respiration, methanol as co-oxidation and nanosilver as catalyses in special concentration help *Gordonia* for MTBE removal. UV radiation, hydrogen peroxide and ozone are expensive, toxic, and also have hazardous properties, therefore low concentration of nanosilver which is cost effective, is low cost, continuous and nontoxic could be on interesting option for MTBE oxidation.

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