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Potential Ecotoxicological Implication of Methyl *tert*-butyl ether (MTBE) Spills in the Environment

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Abstract. Streptomyceticidal activity of Methyl *tert*-butyl ether (MTBE) elucidated for the first time. Adverse effect of MTBE, the gasoline additive, against 11 soil inhabitant *Streptomyces* spp. isolates was investigated. MTBE, an octane enhancer is added to gasoline to reduce atmospheric concentrations of carbon monoxide and ozone. It contaminates soil and groundwater by fuel leaks and spills. *Streptomyces* spp. are of the major contributors to the biological buffering of soils by exerting beneficial and antagonistic activity against wide range of bacteria and fungi. To evaluate anti-streptomycetidal activity of MTBE, it was tested against 11 soil isolates of Streptomyces isolates and also a plant-root bacterial pathogen, *Erwinia carotovora* and a plant-root fungal pathogen, *Fusarium solani*. MTBE did not reveal any growth inhibitory activity against *E. carotovora* and *F. solani*, but showed strong inhibitory effect against Streptomyces isolates. The Minimum Inhibitory Concentration (MIC) on Streptomyces isolates was 1/800 of the original MTBE. Fuel leaks and spills have the potential to suppress or eliminate the Streptomyces role in the soil causing alteration in the balance of soil micro flora. This change can promote the domination of micro-organisms with adverse biological or ecotoxicological effects.

Keywords: soil micro flora; soil contamination; MTBE

Introduction

Methyl *tert*-butyl ether (MTBE) is added to gasoline by many oil companies to enhance combustion efficiency of automobiles and reduce air pollution. It is the most commonly used oxygenate because of its low cost, high-octane level, and ease of blending with gasoline (Johnson et al., 2000). Due to its water solubility, high mobility and low biodegradability it leaches in soil subsurface at the speed of groundwater. Amending gasoline with MTBE has made a widespread contamination of groundwater, surface waters in coastal environments and at low levels in well water (Hoffert, 1998; Reuter et al., 1998; Brown et al., 2000; Johnson et al., 2000; Bennett, 2001; An et al., 2002). The carcinogenic effect of MTBE has been observed in animals and furthermore, its metabolites have shown mutagenicity effects in the Ames bacterial assay (Caprino and Togna, 1998; Williams-Hill et al., 1999). Although current public concern about MTBE contamination, its ban and phase out, or its substitution with ethanol is widely discussed in the USA media and is at the focus of environmental scientists, but its adverse effects on soil micro flora is not yet understood. To combat the problem of MTBE contamination, several workers reported laboratory methods of remediation, especially bioremediation (Salanitro

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et al., 1994; Hardison et al., 1997; Hanson et al., 1999; Fortin et al., 2001; Steffan et al., 2001). However, these methods are not practically established for widespread use in the natural environment.

At the present study, to investigate cytotoxic or growth inhibitory effects of MTBE against beneficial microorganisms of soil, 11 soil-inhabitant Streptomyces isolates were tested. Two ubiquitous soil pathogens of plant roots, *Erwinia carotovora* a bacterium, and *Fusarium solani* a fungus, were also included in the test to evaluate if there is any differential activity in MTBE. The growth-inhibitory activity of MTBE was measured by *In vitro* assay using Agar-well diffusion method. The aim of the study was to elucidate effects of MTBE on the growth of soil-beneficial Streptomyces in comparison with its effect on two of soil plant-pathogenic microorganisms.

Materials and methods

Preparation of microorganisms

Pure cultures of *Erwinia carotovora* (Jones, Bergey), *Fusarium solani* (Mart.) Sacc. and 11 *Streptomyces* spp. isolates (No. 17, 22, 32, 35, 44, 55, 65, 66, 73, 96, and 101) obtained from the Research Laboratory of Department of Plant Pathology, College of Agricultural Engineering, Bahonar University of Kerman, Iran. The Streptomyces isolates were isolated from agricultural soils, identified at Genus level and proved non-pathogenic but beneficial by the mentioned laboratory (personal communication).

Preparation of MTBE

The gasoline additive, MTBE was obtained from Oil Refinery of Abadan, Iran.

Culturing and assay method

The bacterium, *E. carotovora*, was cultured on Mueller-Hinton-Agar medium (MHA). For assays, suspension of approximately 1.5×10^8 cells ml^{-1} in sterile normal saline were prepared as described by Baron et al. (1990), and about 1.5 ml of it was uniformly seeded on MHA in 9×1.2 cm glass Petri dishes, left aside for 15 min and excess of suspension was then drained and discarded properly. Wells of 6 mm in diameter and about 2 cm apart were punctured in the culture media using sterile cork borers and MTBE administered to fullness in the corresponding wells. *F. solani*, the plant root-pathogenic fungus, was cultured on Potato Dextrose Agar medium (PDA). For assays, suspension of fugal spores was uniformly seeded on PDA medium using sterile cotton swabs and assayed as mentioned. *Streptomyces* spp. isolates were cultured on Casein Glycerin Agar medium (CGA). For assays, suspension of spores was uniformly seeded on CGA using sterile cotton swabs and assayed as above. Culture plates, were incubated at 29 °C for 24 h for *E. carotovora* and 3–5 days for *F. solani* and Streptomyces isolates. All samples were tested in

Table 1. Growth inhibitory effect of MTBE serial dilutions against 11 *Streptomyces* spp. isolates, *E. carotovora* and *F. solani*, tested in Agar-well diffusion method and indicated as diameter of growth-inhibition zones in mm

	MTBE Dilutions				
Microorganisms	1:100	1:200	1:400	1:800	1:1600
Streptomyces sp. isolate No. 17	27	25	20	14	–
Streptomyces isolate No. 22	28	22	16	14	–
Streptomyces sp. isolate No. 32	48	40	29	23	–
Streptomyces sp. isolate No. 35	30	27	20	16	–
Streptomyces sp. isolate No. 44	27	25	20	14	–
Streptomyces isolate No. 55	41	35	22	17	–
Streptomyces sp. isolate No. 65	48	40	29	23	–
Streptomyces sp. isolate No. 66	30	27	20	16	–
Streptomyces sp. isolate No. 73	24	20	16	14	–
Streptomyces sp. isolate No. 96	24	20	16	14	–
Streptomyces sp. isolate No. 101	23	19	15	12	–
Erwinia carotovora (Jones, Bergey)	–	–	–	–	–
Fusarium solani (Mart.) Sacc.	–	–	–	–	–

– = Zero growth inhibitory effect.

triplicate. Growth inhibitory effect was determined by measuring diameter of growth-inhibition zones in mm. Solvent controls (DM solvent) were included, although no antimicrobial activity noted in the solvent employed for the test.

Minimum inhibitory concentration (MIC) of MTBE

Dilution series of 1:100, 1:200, 1:400, 1:800 and 1:1600 of MTBE were prepared in DM solvent and assayed against Streptomyces isolates, *E. carotovora* and *F. solani* as mentioned.

Elucidation of mechanism of action of MTBE

To deduce whether mechanism of action of MTBE is Streptomyceticidal or Streptomycetistatic, using transfer needles, smears from MTBE inhibitory zones of all sensitive isolates were streaked to new plates of CGA medium aseptically. As controls, similar transfers were made from non-inhibitory arias of the corresponding isolates. All tests were performed in triplicates. Plates were incubated at 29 °C for 3–5 days and then evaluated for the presence or lack of Streptomyces growth.

Results

Inhibitory action of MTBE

E. carotovora and *F. solani* showed no growth inhibition but strong inhibitory effect of MTBE was noticed against *Streptomyces* spp. isolates as indicated in Table 1. Figure 1 shows inhibition zones of serial dilutions (1:100, 1:200, 1:400, 1:800 and 1:1600) of MTBE on *Streptomyces* sp. isolate No. 32 (a) and isolate No. 17 (b), however, similar growth inhibitions noticed for other isolates too. This data indicates that Minimum inhibitory concentration (MIC) of MTBE is approximately 1/800 of the original MTBE against all used Streptomyces isolates.

Mechanism of action of MTBE

No growth was recovered in transfers from inhibition zones. However, normal growth observed in the cultures from non-inhibitory arias of the corresponding isolates. This indicates that mechanism of action of MTBE is streptomyceticidal in all isolates of this study.

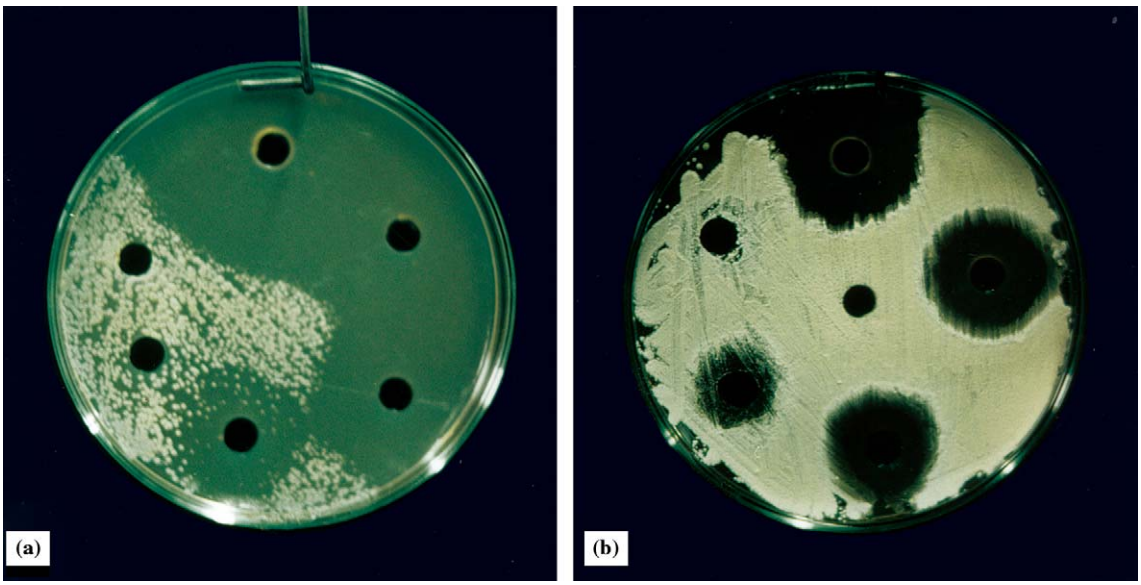

Figure 1. Inhibitory effect of MTBE on *Streptomyces* spp. isolates No. 32 (a) and No. 17 (b). Clockwise from top, dilution series of MTBE in wells represent (a) 1:100, 1:200, 1:400, 1:800, 1:1600 and solvent control; (b) 1:100, 1:200, 1:400, 1:800, 1:1600 and solvent control (center). Similar growth inhibitions noticed in other isolates of Streptomyces as indicated in Table 1.

Discussion

Streptomyces are prokaryotes which contribute to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production. Besides, Streptomyces have been much studied as potential producers of antibiotics and exert antagonistic activity against wide range of harmful bacteria and fungi in soil (Sykes and Skinner, 1973; Okami and Hotta, 1988). Fading the Streptomyces role can alter the balance of soil micro flora and dominate microorganisms with hazardous biological or ecotoxicological effects. *E. carotovora* and *F. solani* are major plant-root pathogens which under normal conditions are partially suppressed by antagonistic activity of soil *Streptomyces* spp. (Demain, 1998; Getha and Vikineswary, 2002). One major way in spread of MTBE in agricultural soils is by spills or leakage of gasoline in the vicinity of reservoirs or gasoline pumps constructed in the fields during in and out refills. From there, by many ways as irrigation, runoffs after precipitations, field animals, soil levelers, and contaminated mud on field machinery-tires, MTBE spreads around the field. As a result, concentration of MTBE increases in soil upon time which may cause suppression of the beneficial Streptomyces. Consequently, the increase in soil contamination by MTBE can lead to reduction of soil fertility, eruption of harmful microorganisms causing detrimental changes in soil health and fertility. For better elucidation of its adverse effects and spectrum of bioactivity, further inhibitory and cytotoxic activities of MTBE should be investigated against wider range of soil-inhabitant organisms. Results of the present study highlights that MTBE spills could have a potential ecotoxicological impact in the environment.

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