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ORIGINAL ARTICLE

Tailoring the nitrogen sources of bacterial culture to enhance methyl tert-butyl ether degradation

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Abstract Four methyl tert-butyl ether (MTBE)-degrading bacteria were isolated from a processing plant in Malaysia. Based on 16S rDNA sequences, the four isolates were identified as *Exiguobacterium profundum* P1M-2, *Bacillus megaterium* P1M-11, *Alishewanella* sp. P2A-12 and *Pseudomonas mendocina* P2M-8. Each of the isolates obtained optimum growth using a different source of nitrogen (0.1- 0.03 % yeast or peptone) and all four isolates were able to biodegrade 92.05-99.98 % of MTBE within seven days. Amongst the four isolates, the highest percentage of MTBE degradation was achieved using *B. megaterium* P1M-11. The highest growth on *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA) and 2-hydroxyisobutyric acid (2-HIBA) was also observed in *B. megaterium* P1M-11. This study suggests MTBE degradation by each of the bacteria can be enhanced by choosing the right nitrogen source. Furthermore, the ability of *B. megaterium* P1M-11 to grow on primary metabolites of MTBE and other structurally related ethers suggests the secretion of diverse degradative enzymes, making this isolate a good candidate to be applied in MTBE bioremediation strategies.

Keywords Degradation · *Exiguobacterium profundum* · *Bacillus megaterium* · *Alishewanella* sp. · *Pseudomonas mendocina* · MTBE · Nitrogen source

Introduction

Methyl tert-butyl ether (MTBE), synthesized from methanol and isobutylene, is a widely use gasoline additive to increase combustion efficiency of petrol, thus, leading to its widespread contamination of both groundwater and surface water (Squillace et al. 1997; Fischer et al. 2005). Numerous literatures on biodegradation of MTBE using microbes have been widely reported (Mesdagnhinia et al. 2005; Volpe et al. 2009; Hyman 2013) but with varying success due to multiple reasons such as low growth rate of MTBE degraders (Waul et al. 2008), domination of faster growing non-MTBE degrading heterotroph (Hatzinger et al. 2001), and partial degradation leading to generation of undesirable dead end metabolites (Finneran et al. 2001; Kolhatkar et al. 2002).

Low concentration of the nitrogen can inhibit the growth of microbes and, thus, impairs MTBE degradation (Leahy and Colwell 1990). Although addition of nitrogen (ammonium) has been shown to enhance MTBE degradation by indigenous bacteria (Volpe et al. 2009), contradictory results were reported when nitrate was used in a study on batch reactors (Waul et al. 2009a, b). This observation leads to a gap in the literature on why degradation of MTBE was enhanced only in certain instances when nitrogen sources were added.

Recent study by our group found out that different bacteria favoured a particular nitrogen source to achieve optimum growth, which directly affected their ability to dominate and eventually to accelerate degradation of hydrocarbons (Hamzah et al. 2013). Hence, the objective of this study was to identify locally isolated MTBE-degrading bacteria and to evaluate the effect of choosing the right nitrogen source to suit each isolate in order to achieve optimal growth and degradative capacity. In this study we discussed the significance of each isolate preferring a different nitrogen source to function at optimum capacity and the implication of using such knowledge to enhance MTBE degradation.

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Materials and methods

Isolation and identification of MTBE-degrading bacteria

Wastewater samples were collected from a pond at a MTBE processing plant in Malaysia. Enrichment and isolation of MTBE-degrading bacteria were performed in a mineral salt medium (MSM) supplemented with 1 % (v/v) MTBE as substrate. MSM medium (Zajic and Supplisson 1979) was fortified with 0.01 % trace elements (g/L); 0.1 g $MnSO_4 \cdot H_2O$, 0.025 g $CuCl_2$, 0.025 g $(NH_4)_2MoO_4$, $NaB_4O_7 \cdot 10H_2O$, 0.025 g $Co(NO_3)_2 \cdot 6H_2O$, 0.025 g $ZnCl_2$, and 0.01 g NH_4NO_3. For the initial bacteria isolation, the medium was further fortified with 0.1 % vitamin solution consisting of 2 mg biotin, 2 mg folic acid, 5 mg thiamine HCl, 5 mg vitamin B_{12}, 5 mg riboflavin, and 20 mg niacin in 100 mL of deionized water. The pH of MSM was adjusted to 7.0 and autoclaved at 121 °C for 15 min. After sterilization, the medium was left to cool to 30 °C before adding 1 % (v/v) MTBE (99.9 % purity, Merck Germany). A single isolate was obtained by the method of serial dilution of the enriched culture in MSM, plated on nutrient agar (Oxoid, UK), and incubated at 30±0.1 °C.

The identities of isolates were confirmed using PCR assay targeting the 16S rDNA gene. DNA of the isolates was extracted using the QIAamp DNA Mini Kit (QIAgen, USA) according to the manufacturer's instructions. The PCR reactions were performed in a Biometra® T-Gradient thermocycler (USA). Amplification was performed in a 50 μL reaction mixture using 16S rDNAs (universal F: 5'-AGAGTTTGATCCTGGCTCAG-3', universal R: 3'-GGTTACCTTGTTACGACTT-5') and 1 U Taq polymerase (Promega Master Mix, USA). The PCR product was purified using a QIAquick PCR Purification Kit (QIAgen, USA) and both 5' and 3' ends of 16S rDNA clones were sequenced with an ABI PRISM 377 sequencer and compared against the National Center for Biotechnology Information (NCBI) non-redundant protein database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997). Multiple sequence alignment was performed using ClustalW (Thompson et al. 1994) on the European Bioinformatics Institute (EBI) server <www.ebi.ac.uk/clustalw/index.html> to identify the isolated species. Phylogenetic inferences were made with SEQBOOT (for bootstrap analysis) and NEIGHBOR (for neighbour joining analysis) programs from MEGA4 version 4.0.2 (Tamura et al. 2007). Identified isolates were kept in glycerol stock at −80 °C.

Determination of the nitrogen source for optimum growth

The different nitrogen sources that can promote optimum growth of each isolate were determined as follows. In preparing the MSM, NH_4Cl was replaced with ammonium sulphate $[NH_4(SO_4)_2]$, peptone or yeast extract, providing similar amounts of nitrogen (26.21 %) in the media. Isolates were incubated in the modified MSM with 1 % (v/v) MTBE at pH 7, 30 °C, and shaken at 100 rpm for 144 h. At the end of incubation, the population of bacteria was enumerated using a plating technique to determine the best nitrogen source that supported the highest growth of each isolate. The experiment was repeated using the nitrogen source that supported the maximum growth of each isolate and the concentration was varied from 0.01, 0.03, 0.1 and 0.5 % (w/v). The population of bacteria again was enumerated using a plating method and expressed in terms of $\log_{10}$ colony forming units (CFU/mL) (Kok Kee et al. 2014).

Biodegradation of MTBE

The standard inoculum of each isolate was prepared according to Hazaimeh et al. (2014) in fresh nutrient broth, and the bacterial cells were harvested by centrifugation and resuspended in 0.85 % (w/v) sterile normal saline to give a cell concentration of 0.5 ($x10^7$ CFU/mL) at 550 nm wavelength using a spectrophotometer (UVmini-1240, Shimadzu, Japan). A total of 10 % (v/v) standard inoculum of each individual isolate was transferred into a 250 mL glass serum bottles equipped with a butyl rubber stopper, that contained 1 % (v/v) MTBE in 50 mL MSM. The cultures were incubated in an orbital shaker (Infors Multitron, Switzerland) at 100 rpm, and since all four isolates showed maximum growth at 30 °C (results not shown), the incubation was set at 30 °C for seven days in order to achieve maximum degradation. The negative control flask contained MSM with 1 % MTBE without inoculates. After seven days, the culture was analysed for bacteria growth using the pour plate method (CFU/mL) on nutrient agar (Kok Kee et al. 2014). The MTBE liquid samples were quantified by injecting the samples to a head-space connected to a gas chromatography-flame ionized detector (GC-FID) (Perkin Elmer Model 6000, US) with a capillary column (60 m×0.32 mm×0.25 μm; Supelco, Sigma-Aldrich Germany). Each vial was heated to 80 °C for 15 min. During this equilibrium period, the sample was mixed by mechanical vibration and each vial was pressurized with helium carrier gas to 10 psi. The GC-FID system was programmed as follows: ionization voltage: 70 eV; interface temperature, 320 °C; oven: 50 °C; injector: 200 °C; detector: 200 °C; and flow rate: 1.00 mL/min.

Growth of isolates on primary metabolites of MTBE

Bacteria isolates were inoculated in MSM supplemented with 1 % (v/v) of *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA) or 2-hydroxyisobutyric acid (2-HIBA) and incubated at 30 °C, 100 rpm for seven days. When no difference was observed in the turbidity compared to day-0, it was taken as no growth (−). Significant increase in turbidity was taken as good

growth (+) and luxuriant (++) when the turbidity was >4 fold compared to day-0.

Statistical analysis

Data were reported as mean values±standard deviation of the number of replicates. Statistical analysis was performed using SPSS PASW Statistic 17 software (SPSS Inc., USA) for Windows Vista™ and Student's "*t*" test with levels of confidence at 95 % (α=0.05).

Results and discussions

Identification of isolated bacteria cultures and growth optimization using various nitrogen sources

From the wastewater of a MTBE-processing plant, the isolation procedures resulted in a total of 56 pure cultures growing in MSM with 1 % (v/v) MTBE as the carbon source. Of these, four isolates designated as P1M-2, P1M-11, P2A-12, and P2M-8 reached the optical density of 10^8 CFU/mL within 24 hours of incubation at 30 °C. Partial sequencing of the 16S rDNA gene of isolate P1M-2, P1M-11, P2A12 and P2M-8 showed 99, 98, 96, and 98 % similarity to *Exiguobacterium profundum* (AY818050.1), *Bacillus megaterium* (DQ105968.1), *Alishewanella* sp. (EU574916.2), and *Pseudomonas mendocina* (FJ840535.1), respectively (Fig. 1). *E. profundum* P1M-2 and *B. megaterium* P1M-11 were clustered under the Firmicutes phyla; whereas *Alishenawella* sp. P2A-12 and *P. mendocina* P2M-8 were found to be under Proteobacteria phyla.

All four isolates were found to grow on MSM media using MTBE as single carbon source supplemented with various nitrogen sources. Of these four, *E. profundum* P1M-2, *B. megaterium* P1M-11, and *Alishewanella* sp. P2A-12 were reported for the first time to grow using MTBE as a single carbon source. Figure 2 shows the effect of replacing ammonium chloride with yeast extract, peptone, and ammonium sulphate on the growth of *E. profundum* P1M-2,

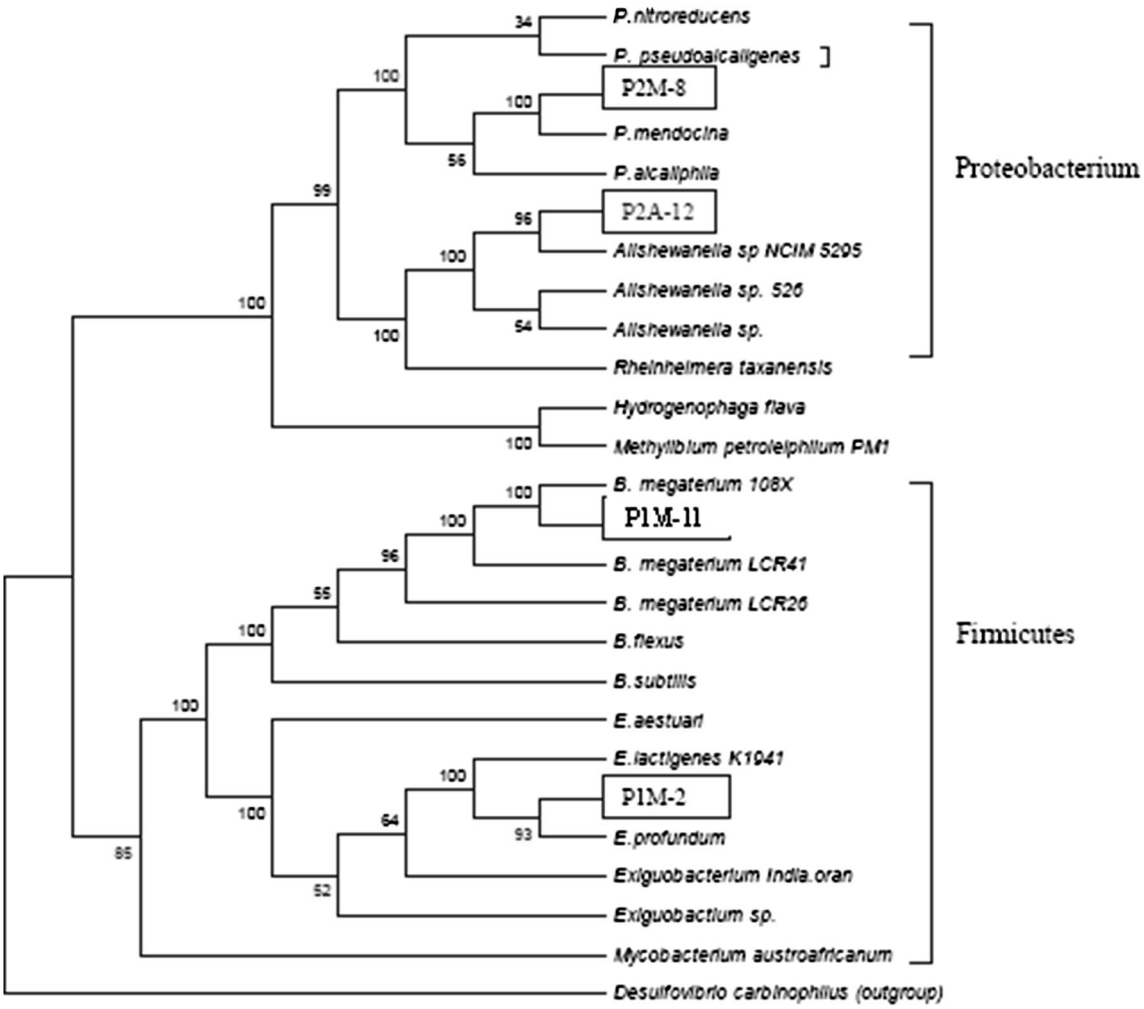

Fig. 1 Phylogenetic neighbours-joining tree of the 16S rDNA gene of P1M-2, P1M-11, P2A-12, and P2M-8. The sequence from *Desulfovibrio carbinoliphilus* was choosen as outgroup

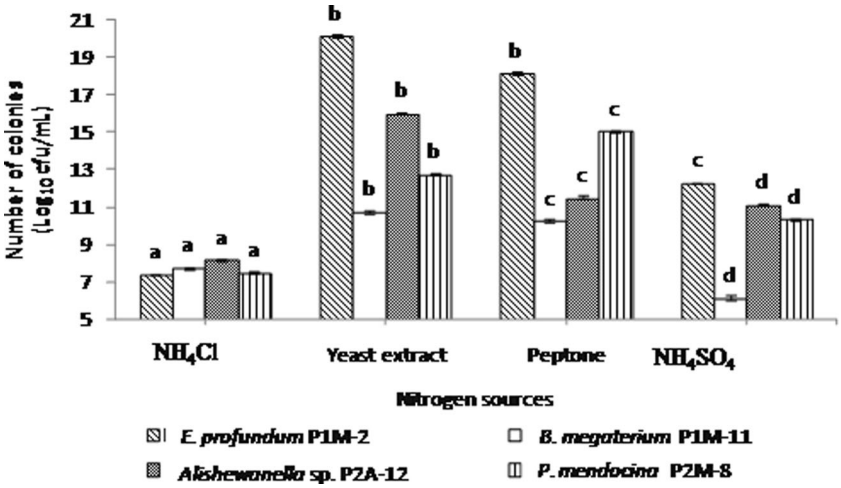

Fig. 2 Different nitrogen sources that supported bacterial growth of *E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8. Cultures were incubated at pH 7, 30 °C, and shaken at 100 rpm for 144 h. Different alphabets indicate significant differences ($p<0.05$) of growth ($\log_{10}$ CFU/mL) of each isolates when different nitrogen sources were used

B. megaterium P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8. In all cases, yeast extract, peptone, and ammonium sulphate significantly ($p<0.05$) increased the bacterial growth twofold to sixfold. However, growth of *B. megaterium* P1M-11 was observed to be twofold lower when ammonium sulphate was used. *E. profundum* P1M-2 (sixfold), *B. megaterium* P1M-11 (twofold), and *Alishewanella* sp. P2A-12 (threefold) were found to grow best using yeast extract and *P. mendocina* P2M-8 in peptone (twofold).

Using the best nitrogen source to suit each bacterial isolate, the nitrogen concentration was varied from 0.01, 0.03, 0.1 and 0.5 % (w/v). Figure 3 shows both *E. profundum* P1M-2 and *Alishenawella* sp. P2A-12 reached the maximum growth on 0.1 % (w/v) of yeast extract while *B. megaterium* P1M-11 reached the maximum growth on 0.03 % (w/v) of yeast extract. In the case of *P. mendocina* P2M-8, the optimal nitrogen source to support the maximum growth was obtained by using 0.1 % (w/v) of peptone.

These results suggested that growth of these isolates could be enhanced when suitable nitrogen sources, i.e., yeast extract and peptone were used to replace ammonium chloride. This demonstrated that growth of MTBE-degraders can be selectively promoted by pairing each of the isolate with a suitable source of nitrogen. Hamzah et al. (2013) also reported that by using an organic nitrogen source, i.e., yeast extract and peptone; the population of crude oil-degrading bacteria was increased significantly and ultimately the degradation of hydrocarbons was enhanced. Results from the same study further suggested that consortia constructed using different single isolates operated efficiently only when supplemented with a suitable source of nitrogen (Hamzah et al. 2013).

Biodegradation of MTBE and growth on primary metabolites

The degradation of 1 % (v/v) MTBE was analysed using GC-FID after seven days of incubation with the cultures. MTBE peaks in the chromatogram were significantly reduced to almost undetectable levels. Table 1 shows that all four isolates (*E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishenawella* sp. P2A-12, and *P. mendocina* P2M-8) were able to degrade more than 90 % MTBE after seven days of

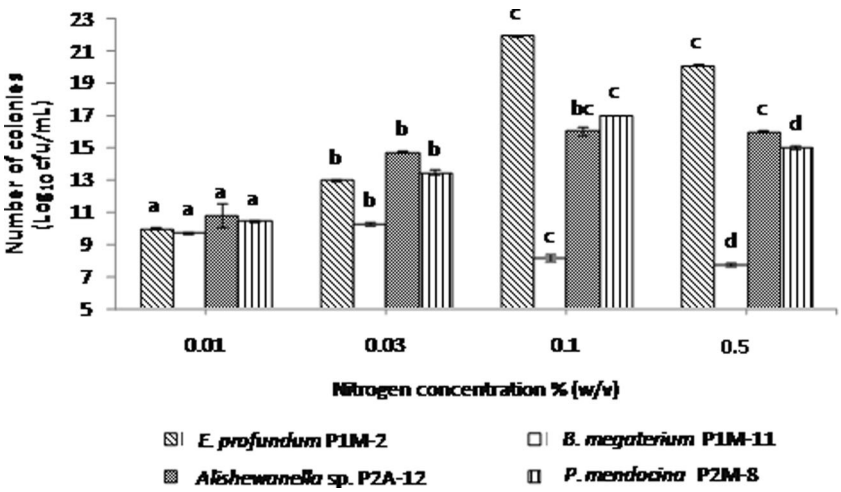

Fig. 3 Different concentrations of nitrogen sources that supported bacterial growth of *E. profundum* P1M-2 (yeast extract), *B. megaterium* P1M-11 (yeast extract), *Alishewanella* sp. P2A-12 (yeast extract), and *P. mendocina* P2M-8 (peptone). Cultures were incubated at pH 7, 30 °C, and shaken at 100 rpm for 144 h. Different alphabets indicate significant differences ($p<0.05$) of $\log_{10}$ CFU/mL of an isolate observed using suitable nitrogen sources at different concentrations

Table 1 MTBE degradation (%) by *E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8 in MSM supplemented with optimal concentration of the preferred nitrogen, and 1 % MTBE with starting pH 7 and shaken at 100 rpm for seven days at 30 °C

Isolate	Nitrogen source % (w/v)	Degradation (%)
Exiguobacterium profundum P1M-2	Yeast (0.1)	92.05±0.02
Bacillus megaterium P1M-11	Yeast (0.03)	99.98±0.01
Alishewanella sp. P2A-12	Yeast (0.1)	94.33±0.03
Pseudomonas mendocina P2M-8	Peptone (0.1)	93.07±0.05

incubation. *B. megaterium* P1M-11 was the most efficient MTBE-degrader since it was able to degrade almost 100 % MTBE within seven days of incubation.

In a separate MTBE-metabolites screening experiment, the growth of *E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8 in MSM supplemented with tert-butyl alcohol (TBA), tert-amyl alcohol (TAA), and 2-hydroxyisobutyric acid (2-HIBA) at 1 % (v/v) is presented in Table 2. Different isolates were observed to grow selectively on TBA, 2-HIBA and TAA. Only *B. megaterium* P1M-11 was found to grow on TAA. TAA is a pivotal and most frequently encountered intermediate metabolite of *tert*-amyl methyl ether (TAME), a fuel oxygenate structurally related to MTBE (Franziska et al. 2011).

Amongst the four isolates, *B. megaterium* P1M-11 degraded the highest percentages of MTBE (Table 1) despite having the lowest bacterial count (Fig. 3) on yeast extract at 0.03 % (w/v). The reason can be attributed to *B. megaterium* P1M-11's ability to grow rapidly on TBA and 2-HIBA (Table 2). Both are the primary metabolites of MTBE. This suggests that *B. megaterium* P1M-11 can express monooxygenase, which is known to be involved in the initial oxidation of MTBE to TBA and subsequently to 2-HIBA (Ferreira et al. 2006). Furthermore, *B. megaterium* P1M-11 was able to grow on TAA, a central metabolite in the degradation pathway of *tert*-amyl methyl ether (TAME), a related fuel oxygenate with higher structure complexity than MTBE. This suggests that *B. megaterium* P1M-11 may have evolved more than one catabolic pathway to degrade MTBE and other structurally related compounds. This observation is further supported by reports of *Bacillus simplex* and *B. drentensis* isolated from hydrocarbon-contaminated soil degrading MTBE, ETBE and TAME (Purswani et al. 2008). Activation of more than one possible pathway by *B. megaterium* P1M-11 can lessen the metabolic bottlenecks caused by *tert*-butyl alcohol (TBA), *tert*-amyl alcohol (TAA), and 2-hydroxyisobutyrate (2-HIBA), thus, accelerating MTBE degradation and structurally related ethers.

Table 2 Growth of *E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8 on TBA, 2-HIBA, and TAA

Isolate	Metabolites		
	TBA	2-HIBA	TAA
Exiguobacterium profundum P1M-2	++	+	-
Bacillus megaterium P1M-11	++	++	+
Alishewanella sp. P2A-12	++	+	-
Pseudomonas mendocina P2M-8	++	++	-

Semiquantitative level of growth of different isolates tested on different MTBE metabolites: ++ (luxuriant); + (good), − (none)

B. megaterium P1M-11 favouring yeast extract might also provide another key answer to the isolate efficiency in degrading MTBE. Volpe et al. (2009) reported that, although the addition of nitrogen accelerated biodegradation of MTBE, the choice of nitrogen can also adversely affect the degradation process. In their study, nitrogen source in the form of nitrate can inhibit MTBE degradation due to the accumulation of nitrite from nitrification, but not ammonium. Furthermore, another study found that when nitrogen source was replaced with yeast extract, secretion of nitrite by *Arthrobacter* sp. (strain 9006) was suppressed but not the growth (Witzel and Overbeck 1979). This suggests that *B. megaterium* P1M-11 was favouring yeast extract as a nitrogen source to enhance growth, which subsequently accelerated MTBE degradation possibly due to the absence of undesirable nitrite being generated either by nitrification or secretion. Kim et al. (2005) also reported on other *Bacillus* spp., i.e., *B. cereus, B. subtilis*, and *B. licheniformis* were able to perform aerobic nitrification of ammonia to N_2 without forming the undesirable nitrite-mediated nitrous oxide. This clearly suggests that not only the presence of nitrogen is essential, but also the form of nitrogen played a fundamental role in promoting MTBE degradation. This aspect of a different form of nitrogen source and its effect on MTBE degradation and on the degraders should be further explored.

It has long been thought that degradation of MTBE is solely species-dependent. However, this study suggests that, in order to achieve optimal degradation, choosing the right source of nitrogen is vital to boost the potential of each degrader. This information can be use to enhance degradation in MTBE-contaminated areas by using the right source of nitrogen to maximise effectively the targeted bacterial strain

rather than introducing foreign strains to the area, which can upset the balance of natural flora. Moreover, by knowing the nitrogen source preference of each isolate, we can construct effective consortia without promoting a particular species and putting other members at a disadvantage in terms of growth. The results in Table 2 suggest that *E. profundum* P1M-2 and *Alishenawella* sp. P2A-12 both showing optimised growth on 0.1 % (w/v) yeast extract that can be developed into a consortium, thus, reducing the negative effect of interspecies competition. The distinct advantage of using a constructed consortium is that more enzymes can be harnessed to facilitate and enhance the MTBE degradation.

Conclusion

This study has identified four isolates, namely, *E. profundum* P1M-2, *B. megaterium* P1M-11, *Alishewanella* sp. P2A-12, and *P. mendocina* P2M-8 from a local wastewater MTBE processing plant. Results suggest degradation of MTBE can be enhanced by increasing the population of MTBE-degraders by choosing the right nitrogen source and concentration to suit the respective isolates. *B. megaterium* P1M-11 showed the highest MTBE degradation, possibly due to existence of more than one metabolic pathway being activated. *B. megaterium* P1M-11's preference for yeast extract as a nitrogen source prevented the formation of nitrite than can adversely affect MTBE degradation.

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