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BIODEGRADATION OF MTBE BY A MICROORGANISM CONSORTIUM

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ABSTRACT

Methyl Tert-Butyl Ether (MTBE) is one of the ether oxygenates which its use has been increased within the last twenty years. This compound is produced from isobutylene and methanol reaction that is used as octane index enhancer and also increases dissolved oxygen in gasoline and decreases carbon monoxide emission in four phased motors because of better combustion of gasoline. High solubility in water (52 g/L), high vapor pressure (0.54 kg/cm^3), low absorption to organic carbon of soil and presence of MTBE in the list of potentially-carcinogens of U.S EPA has made its use of great concern. The culture media used in this study was Mineral Salt Medium (MSM). The study lasted for 236 days and in three different concentrations of MTBE of 200, 5 and 0.8 mg/L. A control sample was also used to compare the results. This research studied the isolation methods of microbial consortium in the MTBE polluted soils in Tehran and Abadan petroleum refinery besides MTBE degradation. The results showed the capability of bacteria in consuming MTBE as carbon source. Final microbial isolation was performed with several microbial passages as well as keeping consortium in a certain amount of MTBE as the carbon source.

Key words: MTBE, biodegradation, fuel additives, MSM

INTRODUCTION

The application of Methyl Tert-Butyl Ether (MTBE) has begun through the world since 1970. This compound is an octane enhancer agent as well as an oxygenate factor. Various researchers stated that the application this compound is very beneficial in reducing air pollution (U.S.EPA November 1993). As with other petroleum products, transportation, storage and processing of MTBE may result in serious environmental pollution due to its molecular structure. This compound, which differs with other petroleum products, can not be adsorbed easily by surface layer of soil, thus it will move through deep soil and will lead to serious groundwater pollution.U.S. EPA has listed MTBE as possibly carcinogens; as a consequence strict maximum permissible concentration (5µgl/L) has been set for it (U.S.EPA 2000).Although various physical, chemical and biological methods exist for MTBE removal from water and soil, an alternative method is to use microbial potential for MTBE elimination. By 1997, most researchers considered this compound as a refractory organic or in most cases as a non-degradable substance. Recently, many investigators have been focused on biological degradation of MTBE, resulting in considerable success in this regard (Fayolle *et al*., 2003). Early investigations suggested that MTBE biodegradation was not considerable in aerobic conditions, but more recent studies indicate that biodegradation is occurring in aerobic condition in a slower rate than that of methanogenic, sulfate reducing, iron reducing and nitrate reducing conditions (Alan *et al.,* 2001; Fayolle *et al.,* 2003; Mesdaghinia *et al.,* 2005). The results obtained from aerobic biodegradation of MTBE are of significant importance and it is believed that this process is based on two mechanisms. A group of microorganisms are capable of using MTBE

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carbon as energy source for growth. This group mostly has low growth rate in MTBE-bearing media and produce low biomass as well. The oxidation product of MTBE biodegradation is Tert Butyl Alcohol (TBA), which is not accumulative and readily undergoes degradation. As time proceeds, MTBE will act as a catalyst in the degradation process. Although monooxygenase has been reported as active enzyme, sufficient evidence does not exist. Enzymes can be early identification method for microbes, thus MTBE degradation is apparently known to be related to certain microbial species. Although all enzymes involved in MTBE degradation have not been known yet, perhaps due to the limited investigation on this subject, the results obtained from *Rubrivax* sp (PM1) and *Hydrogenophage flava* ENV735 showed that MTBE and TBA degradation are taken place by this enzyme. This is in agreement with the results obtained from MTBE metabolizing organisms or *Mycobacetrium austroafricanum* (Christy *et al.,* 2004).

Most of hydrocarbons act as substrate in this mechanism and contribute to accelerate the MTBE metabolism. Petroleum products such as branched, linear, aromatic and acyclic alkanes are included in the group of cometabolizer compounds. This idea seems to be very useful because in the past it was thought that MTBE degradation individually is much simpler than its degradation together with gasoline, which is a solvent for many hydrocarbons. However, at present it is believed that MTBE carbon in gasoline for biological degradation is not much dissimilar with that in individual MTBE.(Burbanou *etal*., 2003).

As the majority of MTBE enters the environment as part of gasoline, this range of co substrates raises the possibility that organisms involved in gasoline biodegradation could contribute to MTBE degradation in gasoline-impacted environments.

The initial reactions in the pathway of cometabolic MTBE oxidation have been examined, but incompletely recognized in both alkane utilizing fungi and propane oxidizing bacteria. Although both of these types of organisms generate tert-butyl alcohol (TBA) as an MTBE oxidation product, the fungal system also generates tert-butyl formate (TBF) (Strege *et al.,* 2002; Beguin *etal.,* 2003; Nario *etal.,* 2003).

MATERIALS AND METHODS

Different steps for experimental procedure of this research can be seen in figure 1.This research has been done in Iran in 2003-5.

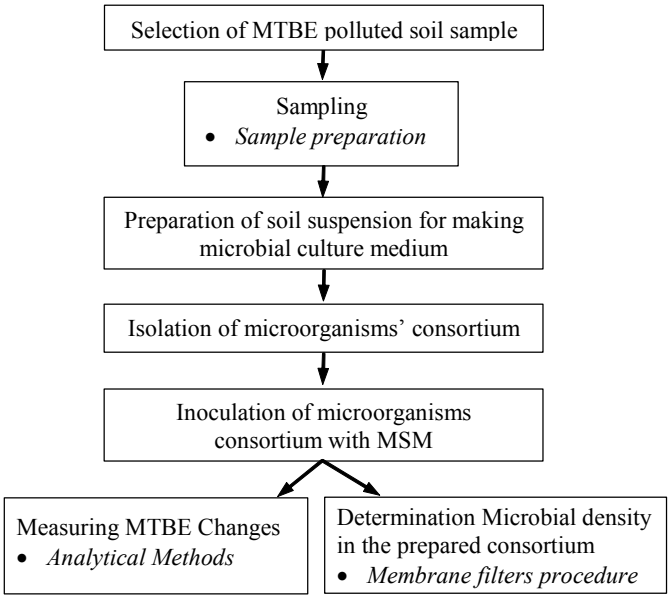

Fig.1: Schematic of different steps of the experimental study

Selection of MTBE polluted soil sample

This research was done on water and soil samples. Samples were taken from Tehran and Abadan petroleum refineries due to the transportation of produced MTBE from Imam Khomeini Port for storage and addition to gasoline. This also helped to have microbial consortium with long time contact and more suitable properties. Water drainage from MTBE and gasoline storage tanks was taken and carried to the laboratory. Another site that was selected for sampling was two gasoline stations in Tehran. One was located at the intersection of Valiasr and Mirdamad streets where for several years MTBE containing gasoline was distributed. The old gasoline storage tanks had high leakage during 2003 and polluted the environmental of this area. Samples taken at the end of 2005 from groundwater of Ameneh Nursery showed (68-150) µg/L MTBE.

These levels of MTBE were higher than the maximum threshold for taste and odor. For this reason, this gasoline station was closed by National Petroleum Company at the end of 2003 for rehabilitation of storage tanks; thus we could take samples from underground soil layers for our research.

The second gasoline station was located near Azadi Square and was selected because of high gasoline demand. Most soil samples were taken from surface soil layer in this station and sent to the laboratory where the samples were kept in special glass containers and at 4 ^{o}C (Table 1).

Table:1 Sites for sample collection

Number of samples	Sample characterization and microbial source
1	Adjacent Soil to MTBE storage tanks of Tehran petroleum refinery
2	Adjacent Soil to gasoline storage tanks of Tehran petroleum refinery
3	Adjacent Soil to MTBE storage tanks of Abadan petroleum refinery
4	Adjacent Soil to gasoline storage tanks of Abadan petroleum refinery
5	Drainage water of MTBE storage tanks of Tehran petroleum refinery
6	Drainage water of gasoline storage tanks of Tehran petroleum refinery
7	Drainage water of MTBE storage tanks Abadan petroleum refinery
8	Drainage water of gasoline storage tanks Abadan petroleum refinery
9	Adjacent Soil to gasoline storage tanks of Mirdamad Gas Station
10	Adjacent Soil to gasoline storage tanks of Azadi Gas Station

Sampling and sampling preparation

Soil samples were taken from the adjacent ground of MTBE and gasoline-bearing MTBE storage tanks. The soil samples were taken from the depth of 50-70 cm and kept and carried in screw-cap bottles to the laboratory. Soil samples were passed through a 2 mm sieve to be prepared for further microbiological examinations.

Inoculation of microorganisms in the drainage water of MTBE storage tanks

The prepared samples were passed through 0.45 mm membrane filters and then the filters were put in nutrient broth culture medium (100 mL) that was already made and sterilized. The culture media were kept at 35ºC for 72 h in incubator. Then the enriched media prepared for inoculation were poured into the MSM culture medium with MTBE.

Preparation of soil suspension for making microbial culture medium

10 g sieved soil sample was added to 100 mL distilled water and some drops of Tween 80. the solution was stirred for 15 minutes and then allowed to settle down. It was used to count and determine microbial density of soil samples.

Mineral salt medium

The mineral salt medium contained the following salts (g/L): $MgSO_4.7H_2O$ (0.25g), KNO_3 (0.5g), $CaCl_2.2H_2O$ (0.009g), KH_2PO_4 (0.5g), NaCl (1.0g) as well as trace elements solution $FeCl_2.4H_2O$ (1.5g), $CuCl_2.2H_2O$ (0.015g), $NiCl_2.6H_2O$(0.025g), $MnCl_2.4H_2O$ (0.1g), $CoCl_2.6H_2O$(0.12g), $ZnCl_2$ (0.07g), $NaMoO_4.2HO$ (0.025g), H_3BO_3 (0.06g), and EDTA. $4H_2O$ (5.2g). The final pH was 4.2.

Membrane filters procedure

Membrane filters procedure for total microbial consortium counting is a practical method is filtered which give reliable result. An appropriate volume of water sample was filtered through a 0.45 μm membrane filter to trap all existing bacteria. Then the membrane filter was placed in a solid culture media in order to let bacteria start growing and forming colonies. Each colony demonstrates a certain bacterial species. Results are reported as colonies per 100 mL, however, unit conversion was made in this study for reporting the results per one mL. Standard culture media is HPC Agar or R_2A Agar which in our study the latter was used due to presence of yeast extract with positive effect on microbial consortium growth.

For the selection of sample size A volume of 100 mL with different dilutions were used. Appropriate sample volumes should yield counts between 20 and 80 colonies per membrane from different bacterial species.Sterile filtration tools were used in each series of filtration in order to protect the samples filtration against any possible contamination. Whenever filtration interval between two samples was 30 min, the filtration was considered non-continuous. After this time interval, the same procedure was followed for new samples and all used membrane filter holders were resterilized. A membrane filter was placed by sterile forceps on the filter holder. The sample was filtered under vacuum condition. Funnel was rinsed with 20-30 mL sterile water. When rinsing and filtration came to an end, the vacuum was turned off. The membrane filter was removed by using sterile forceps and placed into the prepared R_2A agar culture media in such a way that no air was left between the filter and culture media.

All colonies with blue, non-shining purple color and without color were considered as heterotrophic bacteria. Culture media were kept in fridge 0.5 to 1 h before counting to stop colony growth for better colony determination.

For the calculation of microbial density Bacterial concentration in samples is reported as overall microbial consortium per 100 mL of water. There should be no more than 200 colonies and no less than 20 to 80 colonies. The following equation was used to calculate microbial consortium.

$$\text{Overall microbial consortium/100mL} = \frac{\text{Colonies counted} \times 100}{\text{Volume of filtered sample (mL)}}$$

Analytical Methods

One of the essential indicators showing biodegradation of MTBE by aerobic microorganisms is to measure MTBE concentration in various time intervals and compare them with initial concentration. The analysis was carried out according to the modified method of ChrompPack 1313.

MTBE was quantified by a Varian 3800 Gas Chromatography fitted with a flame ionization detector (FID) and equipped with a capillary column (0.25 mm×30 m, ID coating cp-select 624 CB, DF = 1.4 μ). Headspace sampler was used (Model ComBIpal). The vials were incubated at 60°C with shaking (500 rpm) for 5 minutes. The injection temperature was 140 °C at a flow rate of 250 $\mu L / s$ and a spill ratio of 60. The pressure of the carrier gas was kept at 10 psi for 20 minutes. The column was maintained at 40 ºC and then increased to 180 °C with the rate of 20 °C/min. The capillary column temperature was kept 1 min at 180 °C. The detector temperature was 250 °C Helium was used as the carrier gas at a flow rate of 30 mL/min and the flow rate of air was 300 mL/min. The minimum limit of this method was 5 $\mu g / L$,used for Headspace and Direct Injection methods. Star 6 software of Varian was used to process the collected data.

RESULTS

Degradation of 200 mg/L MTBE by the isolated microbial consortium of this research as well as microbial growth and MTBE removal are presented in Figs. 2 to 5.

Fig. 2 shows MTBE concentration during 236 days. The best fitted curve for this process followed cubic equation. (Table 2) MTBE removal in the course of time (236 days) is presented in Fig. 2 which the best fitted curve followed cubic and quadratic equation (Table 2). Fig. 4 shows microbial density in consortium growth during 236 days and Fig. 5 presents the percent of microbial

growth due to MTBE consumption in 236 days. The best fitted curves for both Figs. 4 and 5 followed cubic equation. (Table 2)

Figs. 6 to 9 provides degradation of 5 mg/L MTBE in MSM culture media with a microbial loading of 1×10^6 bacteria per mL. Also, percent removal and biological growth rate due to MTBE biodegradation are presented in Figs. 6 to 9.

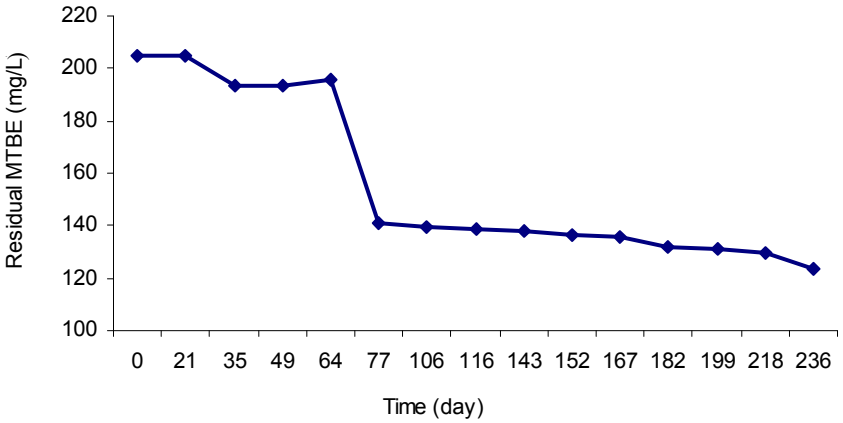

Fig. 2: Changes of MTBE during 236 days experiment

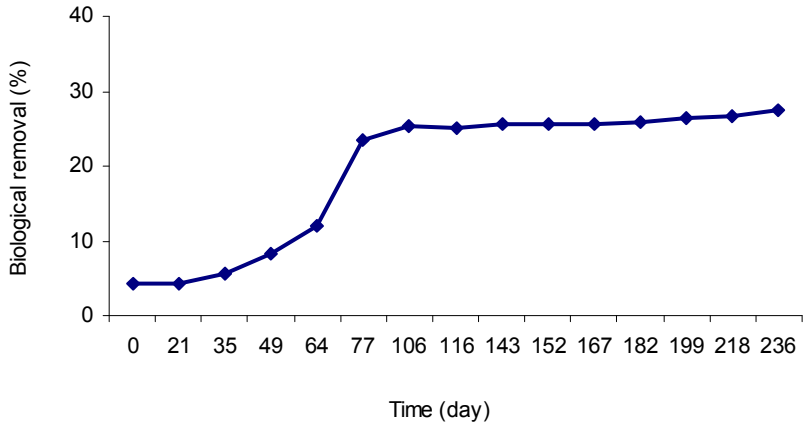

Fig. 3: Percent of biological removal of MTBE during 236 days experiment

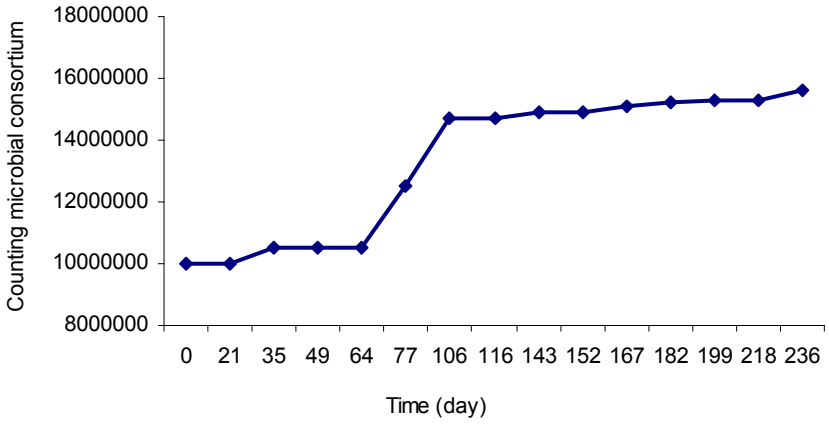

Fig. 4: Microbial density in consortium (cfu/mL) during 236 days of experiment

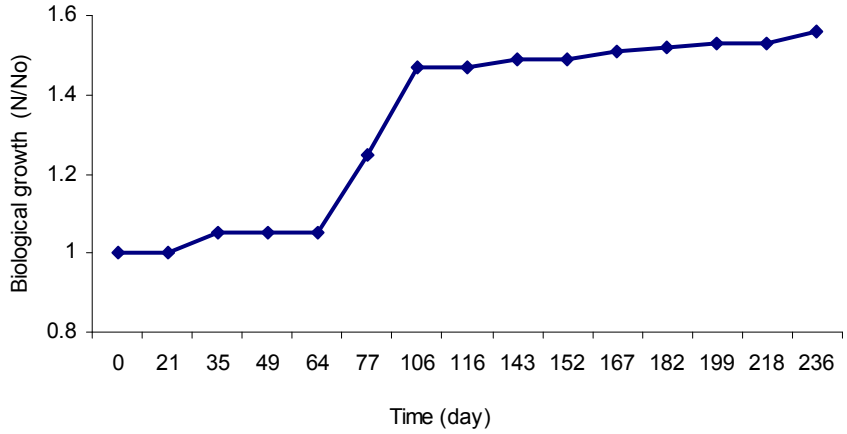

Fig. 5: The variation of (N/N0) during 236 days of experiment

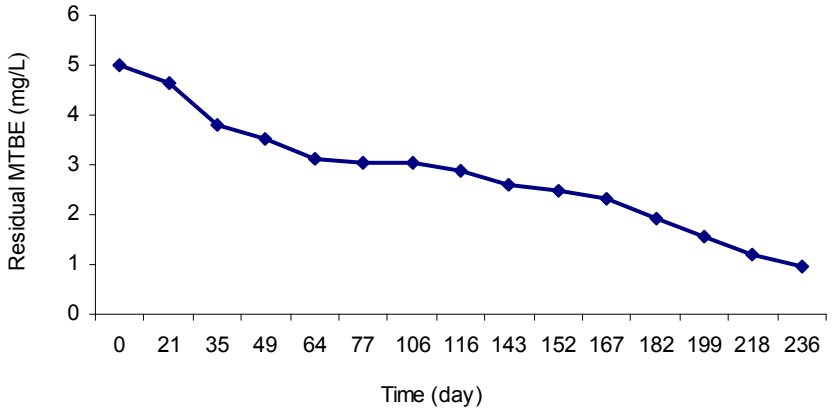

Fig. 6: Changes of MTBE during 236 days experiment

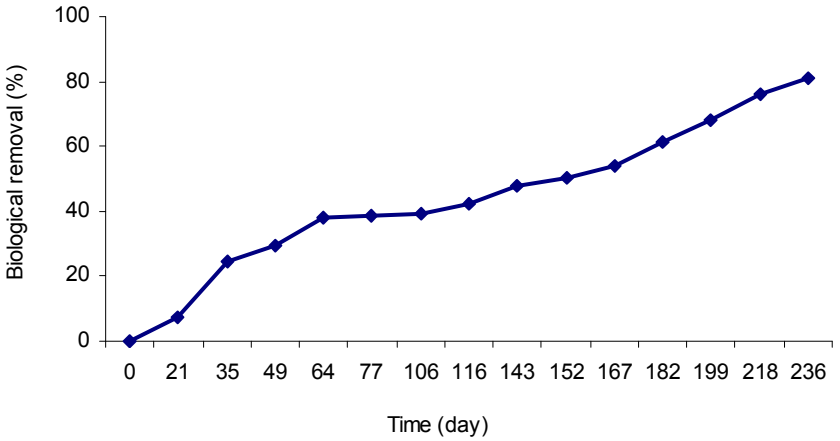

Fig. 7: Percent of biological removal of MTBE during 236 days experiment

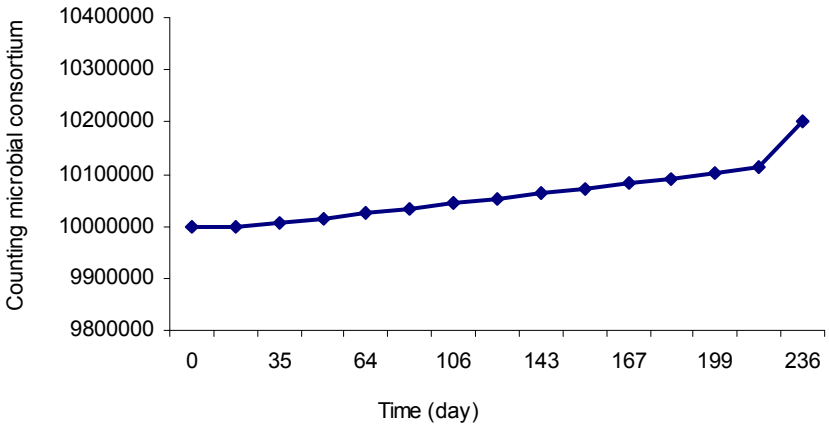

Fig. 8: Microbial density in consortium (cfu/mL) during 236 days of experiment

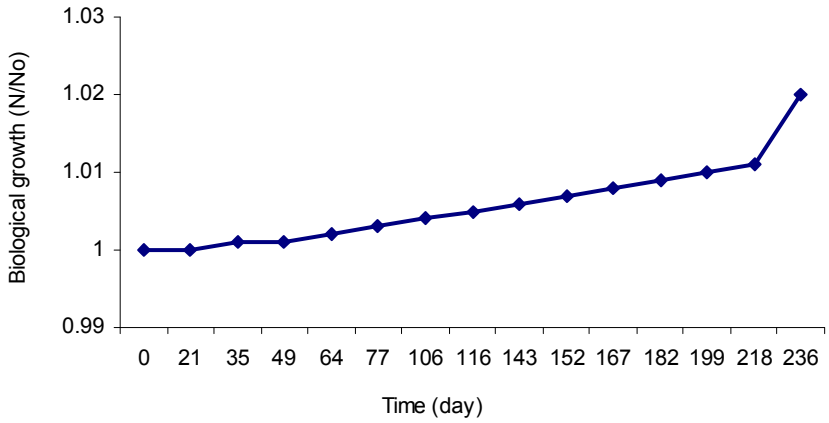

Fig. 9: The variation of (N/N0) during 236 days of experiment

The relation between the observed concentration of MTBE and time is presented in Fig. 5 which explains the best fitted curve equation followed the cubic equation (Table 2). Fig. 7 shows percentage of MTBE biological removal in 236 days and its reaction rate equation. The amount of microbial density in consortium growth within 236 (Fig. 8) followed the cubic equation (Table 2). Also, the trend of microbial growth due to MTBE consumption during 236 days and the computed equation are given in (Table 2).

Table2: The best fitted equations for curves in (Fig. 2 to Fig. 13)

FIGURE No.	R^2	EQUATION
2	0.885	y = 0.0259x3 - 0.0964x2 - 10.613x + 223.93
3	0.9135	y = -0.006x3 - 0.0591x2 + 4.1386x - 3
4	0.9199	y = -5566x3 + 100440x2 + 121116x + 9E+06
5	0.9199	y = -0.0006x3 + 0.01x2 + 0.0121x + 0.9385
6	0.9884	y = -0.0032x3 + 0.0805x2 - 0.8154x + 5.7553
7	0.9883	y = 0.0642x3 - 1.6096x2 + 16.299x - 15.072
8	0.9439	y = 120.32x3 - 2180.7x2 + 18980x + 1E+07
9	0.9430	y = 1E-05x3 - 0.0002x2 + 0.0017x + 0.9976
10	0.9758	y = 0.0003x3 - 0.0047x2 - 0.0605x + 0.8976
11	0.9875	y = -0.0339x3 + 0.5096x2 + 6.7664x + 0.1905
12	0.9840	y = -59.085x3 + 1148.6x2 - 4109.5x + 1E+07
13	0.8957	y = -6E-06x3 + 0.0001x2 - 0.0003x + 1.0002

Figs. 10 to 13 present biodegradation of 0.87 mg/L MTBE and the microbial growth resulted from MTBE consumption with a microbial loading of 1×10^6 per mL in MSM culture media. MTBE concentrations during 236 days, together with the best fitted curve, are also presented in Fig. 10. After 190 days, substrate concentration reached to zero. The biological removal percentage of MTBE with a concentration of 0.87 mg/L and the best fitted curve are given in Fig. 11. The increased number of bacteria related to 0.87 mg/L MTBE and its best fitted curve, which is of third degree, are given in Fig. 12. The percentage of microbial growth due to the existing substrate consumption and its best fitted curve are given in Fig. 13.

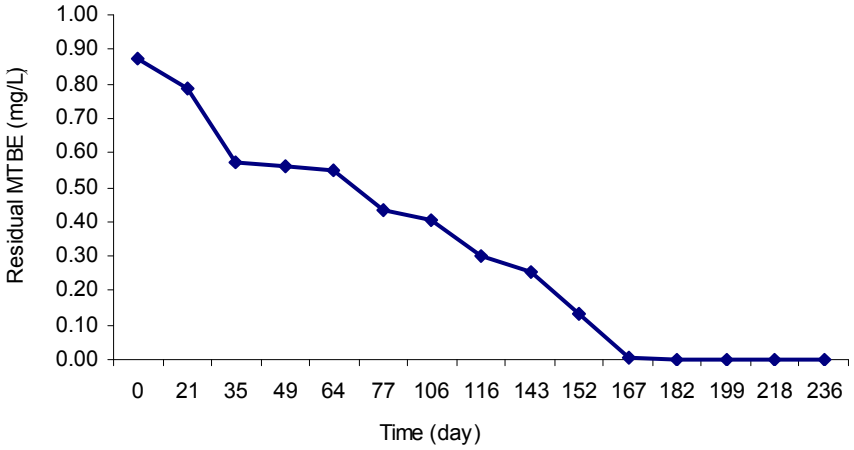

Fig. 10: Changes of MTBE during 236 days experiment

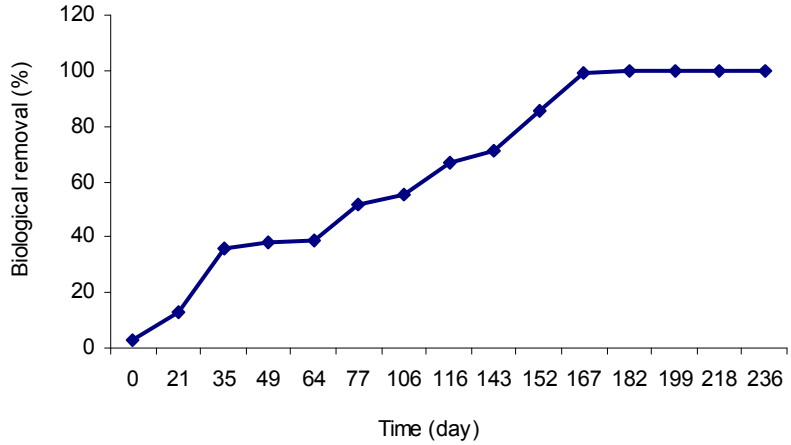

Fig. 11: Percent of biological removal of MTBE during 236 days experiment

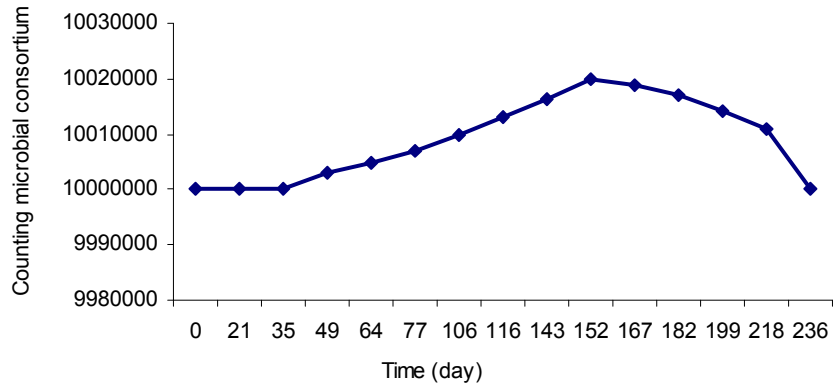

Fig. 12: Microbial density in consortium (cfu/mL) during 236 days of experiment

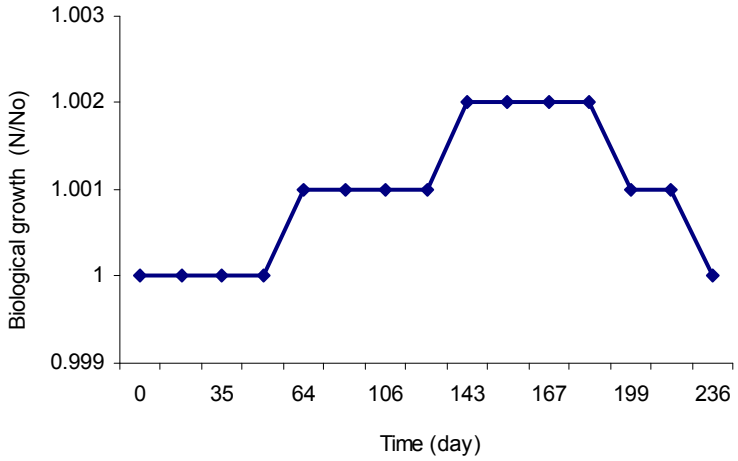

Fig. 13: The variation of (N/N_0) during 236 days of experiment

DISCUSSION

To date, few pure or mixed culture of bacteria have been shown to grow on MTBE as a carbon source. The culture that have been isolated grow relatively slowly on the compound and have low cell yields (Hirstova *etal.,*2003).These growth characteristics could result from slow initial biological oxidation of the MTBE, poor energy yield, and poor utilization o the compound and its metabolites in the biosynthetic process, specific nutritional requirement of the organism, or a combination of these factors. As the time proceeded, metabolites concentrations were increased, while concentrations of MTBE was decreased indicting the evidence of MTBE degradation by microbial consortium. Since the sole carbon source was MTBE, microbial growth in MSM culture media was an indication for biodegradation of MTBE. Meanwhile, it is apparent that MTBE can not be adsorbed to active and non-active microbial cells due to its chemical properties. The results showed that ether compounds can be decomposed by certain microorganisms or acclimatized microbial consortium, however, earlier studied pointed out that these compounds are not biodegradable or are highly resistant to mineralization due to the presence of ether bond (Erika *etal.,* 2004).

Although earlier researchers indicated very slow production of biomass due to the biodegradation of petroleum compounds and hydrocarbons, especially MTBE, this study contradicts those researcher's findings and the reason is the production of high energy resulted from MTBE biodegradation. Biological growth was confirmed by the number of microbial growth compared with the number presented in the initial time (N/N_o). It can be stated that N/N_o ratio is a good indication showing MTBE carbon utilization by the isolated microbial consortium. With regard to the provision of specific substrate for MTBE without providing hydrocarbons used under the mechanisms as co-metabolizing, the predominant process can be contributed to MTBE metabolizing microbial consortium.

Finally, the main finding at this research showed that the degradation of MTBE is feasible and the carbon resulting from MTBE is applied as a source of energy and microorganism productivity.

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