Rapid Biodegradation of Methyl tert-Butyl Ether (MTBE) by Pure Bacterial Cultures

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ABSTRACT: *Two pure bacterial strains capable of rapid degrading methyl tert–butyl ether (MTBE) were isolated from an industrial wastewater treatment plant, identified and characterized. These strains are able to grow on MTBE as the sole carbon and energy sources and completely mineralize it to the biomass and carbon dioxide. The strains were identified as Bacillus cereus and Klebsiella terrigena. Both strains are able to grow in the presence of 48 gl^{-1} MTBE in water, which is almost the maximum concentration of MTBE in the water. They were able to completely degrade 10 gl^{-1} MTBE in less than a day. The specific degradation rate of MTBE at optimum conditions were 5.89 and 5.78 g(MTBE) $g(cells)^{-1}$ h^{-1} for B. cereus and K. terrigena, respectively. The biomass yield was 0.085 and 0.076 gg^{-1}, respectively. The cultivations were carried out successfully at 25, 30 and 37 °C, while they showed the best performance at 37 °C. Neither of the strains was able to grow and degrade MTBE anaerobically.*

KEY WORDS: *MTBE biodegradation, Bacillus cereus, Klebsiella terrigena, Pure bacterial strain, Mineralization, Anaerobic, Aerobic, Activated sludge.*

INTRODUCTION

Methyl tert–butyl ether (MTBE) has been used as an additive to gasoline since the late 1970s to replace tetraethyllead and other toxic chemicals and as an antiknoking agent to meet the vehicle emissions requirements of the 1990 Clean Air Act Amendments [1]. MTBE was introduced to the Iranian fuel market in large scale in 2001, where more than half a million tons are blended with gasoline annually. The widespread use of MTBE in gasoline has led to several accidental spills and its discharge into surface water, soils and groundwater. MTBE is highly soluble in water (ca 43,000-54,000 ppm, depending on the temperature) and has a low tendency to

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adsorb to soils, which results in rapid contamination of water [2]. The U.S. Environmental Protection Agency (EPA) lists MTBE as a possible carcinogen; however, toxicity limits are a subject of debate. MTBE can be detected by both taste and odor at concentrations as low as 35 μg l^{-1} [3].

There are few reports in which of pure or mixed cultured microorganisms were used to degrade MTBE. *Salanitro et al.* [4] were probably the first to report the bacterial degradation of MTBE. *Eweis et al.* [5] reported the enrichment of a second microbial consortium capable of degrading MTBE. *Mo et al.* [6] isolated three bacterial MTBE degrading strains and classified them as *Arthrobacter*, *Rhodococcus* and *Methylobacterium*. Among the other activities in this subject, we may mention the report by *Hanson et al.* [7] who isolated a pure culture (PM1), and *Hatzinger et al.* [8] who isolated a hydrogen-oxidizing bacterium *Hydrogenoplaga flava* for biodegradation of MTBE.

On the other hand, since some microorganisms may not be able to take up MTBE as the sole carbon and energy sources, some research were devoted to cometabolism of MTBE together with another carbon source. *Steffan et al.* [9] reported that some environmental isolates enriched with propane are capable of mineralizing MTBE. Mineralization of MTBE is probably carried out through conversion to tert-butyl alcohol (TBA), followed by methylhydroxylpropanol (MHP) and hydroy-iso-butyric acid (HIBA). It is then converted to propanol, acetone and hyroxyacetone and finally is mineralized to water and carbon dioxide [10]. There is also another report in which a strain of the fungus *Graphium* showed to be able of cometabolizing MTBE in the presence of n-butane [11].

Anaerobic biodegradation of MTBE has been observed in an aquifer [12], but not in several other sites [13,14]. However, a successful anaerobic degradation of MTBE was reported in the presence of Fe(III) and HS which are electron acceptors [15].

The goal of the current work was to look for a microorganism which is able to degrade MTBE quickly. Therefore, activated sludge from several municipal and industrial wastewaters were examined and two strains were isolated. The degradation of MTBE by the strains was tested and the medium composition and growth conditions were investigated.

MATERIALS AND METHODS

Isolation, culturing and identification of the organisms

A sample of activated sludge were collected from an industrial wastewater treatment plant of Isfahan refinery (Iran). Volumes of 100 ml of this bacterial consortia were cultivated in 500-ml flasks at 25 °C for 72 h shaking at 150 rpm. The medium contained 10 gl^{-1} soy peptone, 5 gl^{-1} yeast extract and 48 gl^{-1} MTBE as the sole carbon and energy source. The biomass was then transferred to a similar medium and the cultivations were repeated four times. The prepared inocula were then enriched by plating on tryptic soy agar (TSA) (30 gl^{-1}), soy peptone (20 gl^{-1}), yeast extract (5 gl^{-1}) and MTBE (48 gl^{-1}). It should be noticed that MTBE was spread on the plates just before plating the microorganisms. The plates were then sealed and incubated at 25 °C for 48 h. The isolated colonies were then separated and kept in 4 °C for further investigation. Two different bacteria were isolated by this procedure.

Characterization of the two pure bacterial cultures degrading MTBE were carried out in cotton-sealed 250-ml flasks containing 150 ml liquid medium including 10 gl^{-1} MTBE as well as 0.5 gl^{-1} $NH_4H_2PO_4$, 1 gl^{-1} NH_4Cl, 0.5 gl^{-1} $MgSO_4$ and 0.5 gl^{-1} YE. The effect of $NH_4H_2PO_4$, NH_4Cl, $MgSO_4$, KCl and YE in the medium composition as well as the temperature of 25, 30 or 37 °C on the growth of the isolated bacteria were examined. All the experiments were duplicated, and an average standard deviation of 5.3% was obtained between the duplicated experiments.

Identification of the isolated bacteria were carried out by Iranian culture collection (PTCC) and the culture collection of university of Göteborg (CCUG).

CHEMICALS

Industrial MTBE (98%) was obtained from Isfahan refinery (Iran). TSA and yeast extract were obtained from Pronadisa, Hispadab u.s. (Spain). All others chemicals were obtained from Merck (Germany).

ANALYTICAL METHODS

The concentration of MTBE and TBA Tert Butyl Alcohol were determined using a gas chromatograph (Philips PU-4410, Cambridge, England) equipped with a flame ionization detector (FID) and a Carbowax OV1 capillary column. The samples were prepared using hexanol as internal standard and 10 μl was injected to the

GC. The initial, ramp and final temperature were 40 °C (for 13 min), 10 °C min^{-1} and 180 °C, respectively. The injector and detector temperature were adjusted at 200 and 250 °C, respectively.

Cell concentrations were determined from absorbance measurements at 610 nm after dilution of samples to obtain absorbance values less than 0.5, using a Shimadzu UV-VIS spectrophotometer (model 240). The measurements were calibrated to cell dry weight, which were determined from duplicated 10 ml samples. The samples were centrifuged, washed with distilled water and dried for 24 hours at 103 °C.

RESULTS

Isolation and characterization of the isolates

In order to isolate a strain capable of growing on methyl tert-butyl ether (MTBE), three different sources of activated sludge, obtained from the municipal and industrial wastewater treatment plants, were examined. The isolation process to obtain a MTBE degrading microorganism from the municipal activated sludge did not result in any specific strain, while 16 colonies were obtained by one week incubation of the microorganisms present in the activated sludge from Isfahan Refinery Co. (Isfahan, Iran) on the plates containing 48 gl^{-1} MTBE. The colonies were then separated, purified and tested in liquid culture. Although these strains were capable of growing in the presence of 48 gl^{-1} of MTBE, there were only two strains which were able to use MTBE as the sole carbon and energy source.

The first isolate consisted of rod shape gram positive bacteria, identified as *Bacillus cereus* and registered by culture collection of Göteborg university as CCUG 48624. The second isolate formed yellow colonies. The bacteria were rod shape gram-negative bacteria, identified as *Klebsiella terrigena*.

Cultivation of the isolated strains on MTBE

Cultivation of *B. cereus* and *K. terrigena* in liquid cultures containing 10 gl^{-1} MTBE as the sole carbon and energy source was investigated. The experiments were carried out in cotton-plugged shake flasks. The growth of these two strains on MTBE is presented in Fig. 1.

Evaporation of MTBE at identical condition to these experiments was examined by running similar experiment but at aseptic conditions (Fig. 2). The results show that

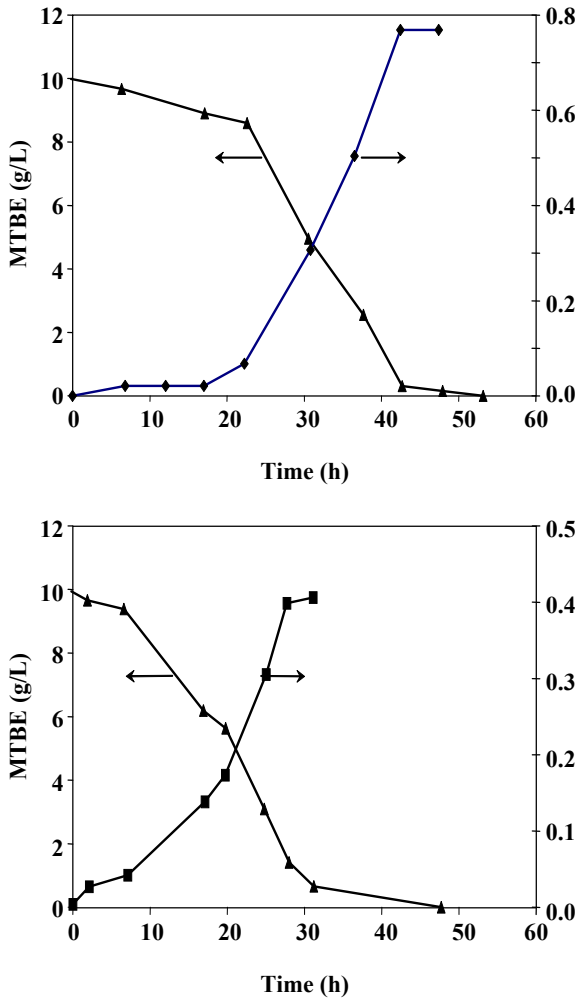

Fig.1: Profiles of biomass concentration (■) and MTBE degradation (▲) by (a) B. cereus and (b) K. terrigena in medium 1 including 10 gl^{-1} MTBE as the sole carbon source.

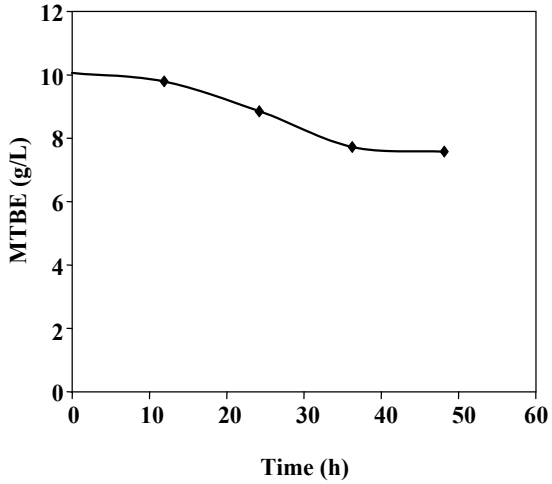

Fig.2: Profiles of evaporation of MTBE at identical conditions to experiments presented at Fig. 1.

only 11% and 21% of MTBE was evaporated after 24 and 36 h, respectively.

Both bacteria were able to completely degrade MTBE in less than 30 h. *B. cereus* was faster in taking up MTBE with a specific degradation rate of 4.56 $gg^{-1}h^{-1}$ compare to 3.52 $gg^{-1}h^{-1}$ for *K. terrigena*. Furthermore, *B. cereus* resulted in higher biomass yield of 0.0625 gg^{-1} compared to *K. terrigena* with a biomass yield of 0.0487 gg^{-1} at similar condition. Biomass and carbon dioxide were the only product of degradation of MTBE by either of the strains and no other metabolites was detected bygas chromatography. There was specific concern about the formation of tert-butyl alcohol (TBA) during degradation of MTBE, but TBA was never detected in any of the chromatograms throughout all the experiments.The effect of medium composition on the degradation rate of MTBE and growth of the bacteria were investigated. Neither *B. cereus* nor *K. terrigena* were able to grow in a medium containing only water and MTBE in at least 72 h.

In order to examine the medium complexity, four different media were prepared according to Table 1. The bacteria were able to grow in all the media, but the growth rate and the rate of MTBE degradation were different. The time courses of MTBE degradation by the two strains in different media are shown in Fig. 3. The results show that the basic minerals containing ammonium, magnesium, phosphate, chloride and sulfate in addition to MTBE (medium 2, Table 1) is enough to stimulate the growth of both of the strains and mineralize MTBE.

However, *B. cereus* and *K. terrigena* need at least 40 and 50 h to completely degrade 10 gl^{-1} MTBE, respectively (Fig. 3). Addition of potassium ions in the form of KCl was enough to decrease the time of degradation to 30 h by *K. terrigena*, while the addition of yeast extract did not further stimulate the degradation rate (Fig.3b). However, the degradation rate of MTBE by *B. cereus* was affected by both KCl and yeast extract. Addition of KCl to the medium had some positive effect on *B. cereus* to decrease the total time of degradation of 10 gl^{-1} MTBE by about 10 h, while the addition of yeast extract decreased the total degradation time of the MTBE to less than 24 h (Fig. 3a). It probably shows that there are some other chemicals in yeast extract which could stimulate the growth and degradation rate of *B. cereus*, while no further chemicals than those present in medium 3 (Table 1) are necessary for the growth of *K. terrigena* on MTBE.

Table 1: Different medium composition used to cultivate the isolated strains, B. cereus and K. terrigena.

Medium number	NH_4Cl (1 gl^{-1})	$MgSO_4$ (0.5 gl^{-1})	$NH_4H_2PO_4$ (0.5 gl^{-1})	Yeast extract (0.5 gl^{-1})	KCl (0.5 gl^{-1})
1	✓	✓	✓	✓	-
2	✓	✓	✓	-	-
3	✓	✓	✓	-	✓
4	✓	✓	✓	✓	✓

Both of the strains were cultivated in the best medium (Medium 4 in Table 1) at different temperature of 25, 30 and 37 °C, in order to investigate on the effect of temperature. The most important results of these experiments are summarized in Fig. 4. Both *B. cereus* and *K. terrigena* are able to grow at three different temperatures, but the best growth and MTBE degradation occurred at 37 °C for both strains (Fig. 4).

The specific growth rate of *B. cereus* at 25, 30 and 37 °C was 0.273± 0.011, 0.352±0.04 and 0.588±0.021 $gg^{-1}h^{-1}$, $g^{-1}h^{-1}$ at 25, 30 and 37 °C, respectively (Table 2).

The growth rate of *K. terrigena* was not as fast as *B. cereus*, i.e. *K. terrigena* grew at 25, 30 and 37 °C on 10 gl^{-1} MTBE by the specific growth rate of 0.142±0.028, 0.265±0.045 and 0.509±0.012 $gg^{-1}h^{-1}$, respectively. The degradation rate of MTBE by this strain in three different temperatures were 3.055±0.01, 3.77±0.1 and 5.78±0.012 $gg^{-1}h^{-1}$, respectively. The biomass yield of both strains in different medium compositions and temperature are summarized in Table 2

Anaerobic degradation of 10 gl^{-1} MTBE in the best medium composition (medium 4, Table 1) and temperature (37 °C) by *B. cereus* and *K. terrigena* was examined in shake flasks equipped with a loop-trap which allowed no entrance of oxygen into the flasks [16]. The cultivations lasted 192 h, but no growth and no degradation of MTBE occurred by neither of the strains, which means that both of the strains are strictly aerobic, while MTBE is used as the sole carbon and energy source.

DISCUSSION

The identified strains in the current work with such high capacity of MTBE degradation are probably suitable strains for industrial applications, where MTBE's are present in surface water or wastewater. MTBE belongs to a family of ethers, which are relatively unreactive

Table 2: The specific degradation rate of MTBE and biomass yields of B. cereus and K. terrigena at different temperature and medium composition.

T (°C)	Medium number	Specific degradation rate (g g^{-1} h^{-1})		Biomass yield (g g^{-1})	
		B. cereus	*K. terrigena*	*B. cereus*	*K. terrigena*
25	1	4.56	3.52	0.0625	0.0487
25	2	2.80	2.80	0.0598	0.0401
25	3	4.41	3.80	0.0503	0.0549
25	4	5.47	3.05	0.0808	0.0525
30	4	5.68	3.77	0.0827	0.0556
37	4	5.89	5.78	0.0847	0.0763

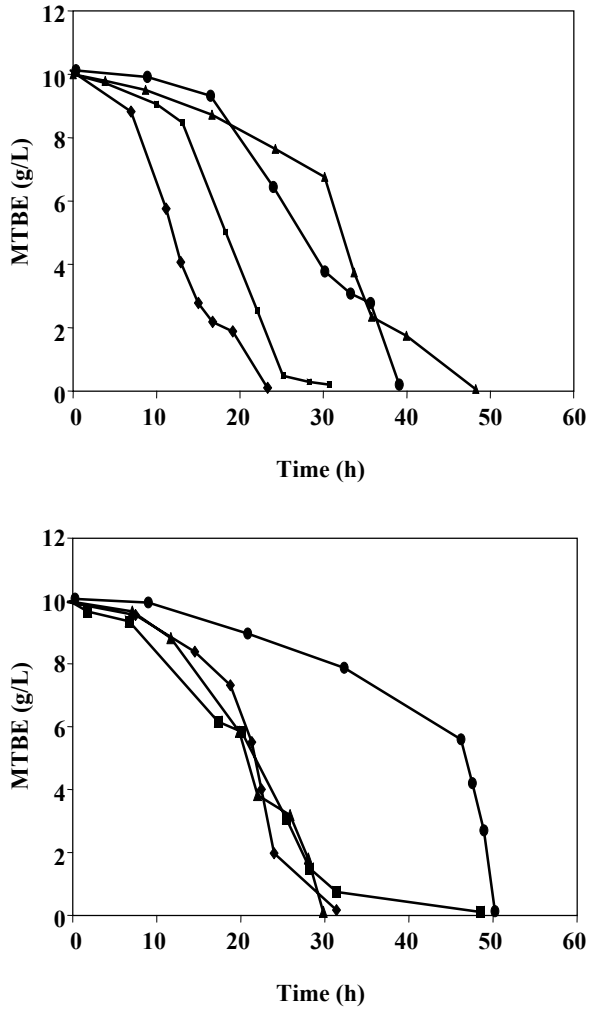

Fig. 3: The effect of medium composition on degradation of MTBE by (a) B. cereus and (b) K. terrigena at 25 °C. The media are medium 1 (▪) medium 2 (▲), medium 3 (●) and medium 4 (◆) of Table 1.

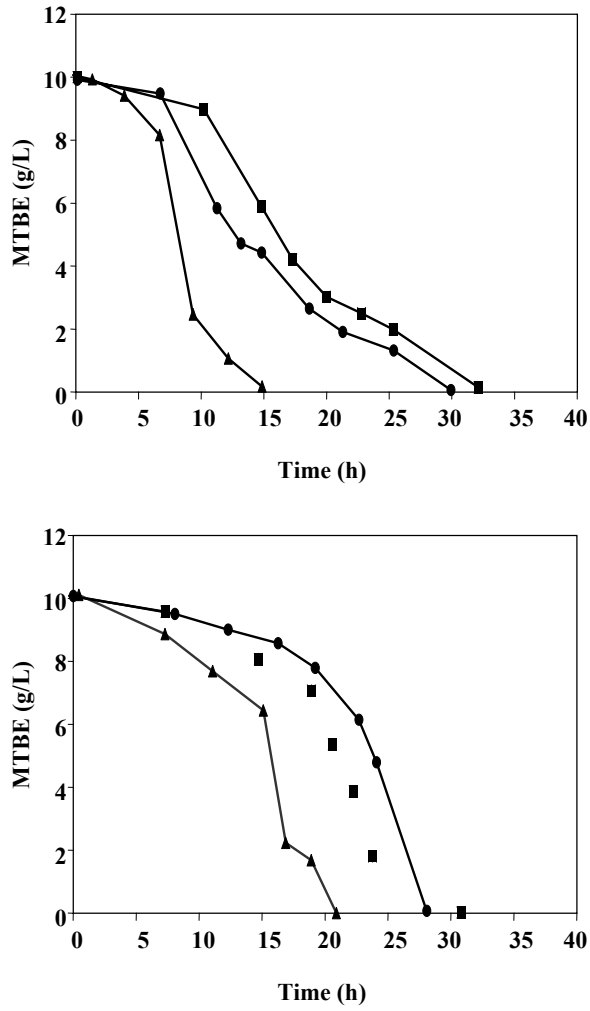

Fig. 4: The effect of cultivation temperature on degradation of gl^{-1} MTBE by (a) B. cereus and(b) K. terrigena in medium 4 (Table 1). The symbols represent 25 °C (▪), 30 °C (●) and 37 °C (▲), respectively.

compounds and the cleavage of an ether linkage normally requires vigorous conditions involving concentrated acids and high temperature. Ethers tend to be quite resistant to biodegradation by microorganisms [17]. There are few reports in the literature on biodegradability of alkyl ethers in soil, groundwater and activated sludge environment.

There are a few publications reporting biological degradation of MTBE under aerobic conditions. *White et al.* [18] reported a degradation rate for MTBE of 0.034 $gg^{-1}h^{-1}$ by an aerobic mixed culture. *Hatzinger et al.* [8] achieved a better result of 0.45 $gg^{-1}h^{-1}$ for degradation rate of MTBE by *Hydrogenophaga flava* Methylo-bacterium mesophilicum, Arthrobacter and Rhodococcus were not able to take up more than 29% of 0.2 gl^{-1} of MTBE in two weeks [19]. *Steffan et al.* [9] reported two microorganisms similar or closely related to Nocardia sp., which were able to utilize MTBE together with propane. They reported a specific degradation rate by the microorganisms at 9.2 nmol min^{-1} $mgprotein^{-1}$, which is equal to about 0.1 $gg^{-1}h^{-1}$. *Deeb et al*. [20] reported a specific degradation rate of MTBE of 0.05 $gg^{-1}h^{-1}$ by *Rubrivivax gelatinosus*. *Graphium* sp. specific degradation rate of MTBE as 0.00017 $gg^{-1}h^{-1}$ [21]. Other data was presented by *Garnier et al.* [22] where Pseudomonas aeruginosa degraded MTBE in cometabolism with pentane by of about 0.04 $gg^{-1}h^{-1}$.

Comparison of these reports with our strains which were able to degrade MTBE by specific degradation rate of more than 5.7 $gg^{-1}h^{-1}$ (Table 2) shows excellent performance of both *B. cereus* and *K. terrigena* for biological degradation of MTBE and *terrigena* for biological degradation of MTBE.

None of the strains *B. cereus* and *K. terrigena* was able to grow anaerobically on MTBE and therefore, they will not be suitable for anaerobic degradation of MTBE in, for example, groundwater. This fact can be discussed using redox balance. If we consider the degree of reduction of CO_2, H_2O and NH_3 as zero, the degree of reduction of MTBE is calculated as 6. Therefore, if we assume the degree of reduction for the biomass as 4.2 [23], and consider the total conversion of MTBE to the biomass and CO_2, it would not be possible to produce them by anaerobic degradation of MTBE. It means the degree of reduction will not be in balance if we convert MTBE to the biomass and CO_2 anaerobically. This problem can be solved theoretically either by addition of a chemical with a low degree of reduction potential (electron acceptor) such as oxygen (with a degree of reduction of -4) or production of another product than CO_2 and biomass with a high degree of reduction such as methane (degree of reduction of +8). Consequently, for anaerobic degradation of MTBE in e.g. groundwater, one should probably look in the groups of methane-producing bacteria or provide an additional electron acceptor component. One of the few successful reports on anaerobic biodegradation of MTBE was by *Finneran & Lovley* [14]. They reported no degradation of MTBE in aquifer sediments without addition of Fe (III) and H_2S, which act as electron acceptor.

It can be concluded that the two identified strains of *B. cereus* and *K. terrigena* show the highest specific degradation rate of MTBE compare to the previously reported microorganisms. Since they are able to completely mineralize MTBE to the biomass and CO_2, they will probably be good candidates in cleaning the surface water or wastewater polluted with MTBE.

CONCLUSIONS

It can be concluded that the two identified strains of *B. cereus* and *K. terrigena* show the highest specific degradation rate of MTBE compare to the previously reported microorganisms. Since they are able to completely mineralize MTBE to the biomass and CO_2, they will probably be good candidates in cleaning the surface water or wastewater polluted with MTBE.

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