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Biodegradation of MTBX by new bacterial isolate *B. sphaericus*

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Abstract

In the present study, a bacterial strain was isolated from biofilter which is able to utilize MTBX as a main source of carbon. With the help of biochemical test, this organism was identified as Bacillus sphaericus. This organism which was named B. sphaericus and capable to degrade MTBX quite effectively for the initial concentration 100, 200, 500 and 1000 mg L^{-1} in a liquid medium. The capability of B. sphaericus to degrade MTBX in 500 mg L^{-1} completely within the duration of 140 h. During the degradation of MTBX temperature was maintained from 15 to 45^0 C but the optimum range was 25 to 35^0 C. Ranges of pH value was kept 3 to 11 with an optimum range of 6 to 8 for the cultivation of isolates. The maximum degradation by B. sphaericus was found for n-butyl acetate with 99.99% for 500 mg L^{-1}. On the basis of results obtained in present study, B. sphaericus can be used effectively as an inoculum in a biofilter. for the treatment of MTBX.

Keywords: Bacillus sphaericus, MTBX, Growth rate, Biodegradation, Concentration.

Introduction

Air pollution is a global concern. Based on various sources of waste gas emissions different characteristics, type and concentration of air pollution can be identified and also the various types and concentrations of existing pollutants in the gas stream can be measure. Various air contaminants can be produced from livestock, from poultry buildings, on-site manure storage and treatment facilities, and are responsible for air pollution. Generally these pollutants can be classified into four groups i.e. fixed gases or gases, odors, particulate matter (PM), and volatile organic compounds (VOC)[1]. The different types of volatile organic compounds (VOCs) found in waste gases contribute to air pollution[2-4]. Ketones are used in aerosols, varnishes, window cleaners, paint thinners, and adhesives[5,6]. Methyl ethyl ketone (MEK), Toluene, n-butyl acetate and o-xylene are widely used industrial chemicals. Methyl ethyl ketone (MEK) is one of the ketone which is highly toxic and released in our atmosphere by various petrochemical, chemical, food processing, color printing, pulp and paper mills, paint and coating, electronic industries, etc. due to such high toxicity it leads to endanger the quality of air as well as public health[7]. The waste sector contributes up to 4% of VOC emissions[8]. In this type of waste sector, mechanical biological treatments (MBTs) have been become popular in the last twenty years, due to their importance in integrated systems for waste management[9,10] and also because of the their increasing awareness especially as a resource of energy[11].

There are various types of technologies which have been developed and used for the degradation of VOCs from gaseous streams and among all these technologies, biofiltration has considered as most efficient and cost-effective technique to control air pollution[12]. Biofilteration is based on microorganisms which are fixed to a packing material and a biofilm completely wetted along with the packed bed height[13-15] for degradation of the various contaminations present in the air[16]. In the Process of biofiltration, pollutants present in the air are removed biologically in a solid phase reactor[17]. For the removal the gaseous contaminants present in the air, contaminated wet-gas is supplied at the bottom of the biofilter so that the air contaminants diffuse into a wet and biologically active layer generally known as biofilm which is present on the surface of the filter. The biofilm act with aerobic bacteria for the degradation of the target pollutant(s) and produces CO_2, water, and microbial biomass[18]. According to recent study bacteria based biofilters are more efficient than fungi inoculated biofilters because of presence of a highly diversified population of microorganisms[19]. Biofilteration is the combination of several other techniques as absorption, adsorption and degradation[20-23]. The partition of gaseous contaminants between the gas and the liquid phases and also between the liquid and biotic phases in a biofilter system generally affects their transport and subsequent biodegradation[24].

It is very rare in literature[25] that VOCs especially releases from paint industry can be removed with the help of biological removal process in biofilter and biotrickling filters. Particularly, studies on the biodegradation of methyl ethyl ketone (MEK), toluene, *n*-butyl acetate and *o*-xylene (MTBX) in a corn based biofilter are scarce in the literature[26]. Only Lu et al (2001b)[27] had attempted to evaluate the performance of biotrickling filter by treating mixture of ethyl acetate (EA), toluene (T) and xylene (X) in a coal packed bed. Recently the gas phase MEK removal under various operating conditions has been performed using corn stack based biofilter inoculated with a mixed culture[28].

Deshusses et al. 1996[29] have studied for the degradation of MEK and MIBK with mixed culture and compost as packing materials. The degradation of MTBX (MEK, Toluene, n-Butyl acetate and o-Xylene) by using coal based biotrickling filter has been carried out by Mathur and Majumder 2008[30]. The performance of biofilter based on composite bead made up of PVA, peat, KNO_3 and GAC as filter material for the degradation of MEK, methyl isopropyl ketone (MIPK) and acetone can be studied by Chan and Peng 2008[31].

Due to its dangerous effect on the environment, it is necessary that MTBX should be removed completely. In the present research a bacterial strain, namely *B. sphaericus* has been used for the degradation of MTBX and it has been isolated from the biofilter unit and assigned a number 8103 after characterization. To investigate the performance of *B. sphaericus* for the removal of MTBX, present study has been carried out.

Materials and methods

Chemical and growth medium: First a basal salt medium was prepared by using deionized water (Milli-Q Millipore 18.2MΩ cm^{-1}resistivity) and then bacterial strain was allowed to grow in this medium. MTBX acts as the sole carbon source for the bacterial train. The BSM sterilization was done in three parts to avoid precipitation during autoclaving. These parts were denoted as solutions, A, B and C (Table-1). Solutions contained in these three parts were prepared and then filtered aseptically using (0.45µm filter, Sartorius, Goettingen, Germany) to insure that there should be no precipitation during storage.

Table-1: Composition of the Basal Salt Medium.

Component	Concentration (g L -1)	Solution
KH_2PO_4	0.91	A
MgSO4.7H2O	0.5	B
ZnSO4.7H2O	0.0004	C
CaCl2.2H2O	0.5	B
(NH4)2SO4	1.97	A
Na2HPO4.2H2O	2.39	A
KNO3	2.96	A
FeSO4.7H2O	0.02	C
MnSO4.7H2O	0.0008	C
Na2MoO4.2H2O	0.001	C
CoCl2.6H2O	0.0004	C

Isolation of strain: After the completing operating period of biofilter which was approximate six months to remove mixture of MTBX, samples were collected from compost based biofilter. Collected microbial samples from biofilters were then diluted using serial dilution technique. With the help of sterile steel forceps one gram of sample was taken from biofilter. After withdraw, this sample was mixed with 10mL of Millipore water and then shaken in a vortex shaker for the duration of 10 minutes for converting to desire concentration with the help of serial dilution technique. This diluted sample was then left undisturbed again for 10 min. 1 milliliter of this sample was then serially diluted up to 10^{-10} in sterile buffer (phosphate buffer, pH 7.0). The obtained sample was then aseptically spread on basal salts medium agar 2% (w/v) plates with MTBX as an energy and sole carbon. The poured agar plates were then incubated at the temperature range of 30±1 ◦C for 48 h and for the uniformity as well as differences in terms of growth of all developed colonies was carefully observed. Seven isolates were obtained after streaked on prepared BSM petri dishes. According to the obtained results four isolates monocultures were gram negative while three isolated monocultures were gram positive. Further detail of microscopic morphology, shape, and size as well as gram strain is represented in Table-2. From SEM micrographs it can be clearly observed that morphology, shape and size of three isolates are same while other four isolates are different. According to similarities three similar types of isolates were named as BG1, BG2, and BG3 while other four different isolated were represented by OG. These isolated strains were properly maintained by transferring periodically onto agar slant containing nutrient and can be stored at the temperature range of 4^0C for further study.

Identification of strain: Based on morphology, physiology and biochemical tests of isolates, obtained results from biofilters are listed in Table-3. MTCC, IMTECH, Chandigarh, India taxonomically identified this bacterial strain as *B. sphaericus* (Gram-positive) and also given a number for separate identification to it as MTCC-8103. Based on the similarities it can be visualize that isolates BG1, 2, and 3 belong to the same species as *genera Bacillus*. Isolate BG1 was dominant as compare to other isolates hence BG1 isolate was chosen for the further study of MTBX degradation. This bacterial strain was again confirmed with the help of 16S rDNA sequence analysis.

Genomic DNA isolation and sequencing of 16S rDNA gene: A standard bacterial procedure[26] was used to isolate Genomic DNA. 63f (5′-AGGCCTAACACATGCAAGTC-3′), 1387r (5′-GGGCGGAGTGTACAAGGC-3′)[27], primer which was suitable to use for PCR amplification of the 16S ribosomal DNA. The amplification of corresponding gene fragment was done from the genomic DNA. In this way the PCR products are generated with the help of Pfu enzyme (XT5 enzyme, Genei product).

The obtained products were ligated in pGEMT vector with the help of TA cloning method. When ligation process is completed the selection of successful clones was done with the help of

Blue- White screening. Colonies with desired insert and also with pGEMT vector were identified and positive clones were isolated. These isolated clones were sequenced in forward and backward direction from The Centre for Genomic Application (TCGA), New Delhi, India. ABI PRISM 300, Model DNA sequencer was used for sequencing purpose and analyzed with existing 16S rRNA sequence in either GenBank or EMBL or DDBJ. By using all these process taxonomical data were supported and BG1 was again identified as *Bacillus sphaericus*. After confirmation BG1 was employed for next batch study and biofilter purpose.

Acclimatization of culture and inoculum development: Cultivation of *B. sphaericus* was done in 500 mL flask which contains 100 mL of BSM along with MTBX as the sole carbon source. The acclimatization of cultures to MTBX was done by exposing the culture in a series of shake flasks (Figure-1). In 100 mL of Basal salt medium (BSM) which contains *B. sphaericus* from nutrient agar slants under sterile conditions in the presence of 10 mg L^{-1} of MTBX, the process of acclimatization can be startup. After the completion of inoculation at 30^0 C for 48 h, 5 mL of this obtained bacterial culture was again mixed with fresh Basal salt medium as inoculum again in presence of 10 mg L^{-1} of MTBX. After the time duration of 48 h, a third fresh BSM was also inoculated with 5 mL of the last culture for the confirmation that the isolation strain was already adapted to MTBX.

Table-2: Characterization of seven MTBX degrading strains isolated from an active compost based Biofilter.

Isolated bacteria	BG 1	BG 2	OG 1	BG 3	OG 2	OG 3	OG 4
Gram Straining of isolates	+	+	-	+	-	-	-
Microscopic Morphology							
Type of cell (Shape)	Rods	Rods	Short rod	Rods	Rods	Short rod	Rods
Color	Yellowish White	Yellowish White	White	Yellowish White	White	White	White
Size	0.5-0.6× 1.6-2.8 µm	0.5-0.6× 1.6-2.8 µm	0.5×1.6µm	0.5-0.6× 1.6-2.8 µm	0.5×1.6µm	0.5×1.6µm	0.5×1.6µm
Surface	Rough	Rough	Smooth	Rough	Smooth	Smooth	Smooth
Arrangement	Coherent Cluster	Coherent cluster	Groups	Coherent Cluster	Isolated	Isolated	Isolated
Density	Translucent	Translucent	Opaque	Translucent	Translucent	Opaque	Opaque
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Motility	+	+	-	+	-	-	-
Physiological Characteristics							
pH range							
3	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
6	++	++	++	++	++	++	++
7	+++	+++	+++	+++	+++	+++	+++
8	++	++	++	++	++	++	++
9	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+
RPM	125	125	125	125	125	125	125

Table-3: Biochemical tests of seven MTBX degrading strains isolated from an active biofilter.

Tests	BG 1	BG 2	OG 1	BG 3	OG 2	OG 3	OG 4
Gram Staining	-	-	-	-	-	-	-
Klingler Iron Agar Slant	Butt→ Red Slant→ Red	Butt→ Yellow Slant→ Red Bottom→ Black	Butt→ Red Slant→ Red Bottom→ Black	Butt→ Red Slant→ Red Bottom→ Black	Butt→ Red Slant→ Red	Butt→ Red Slant→ Red Bottom→ Black	Butt→ Red Slant→ Red
Catalase	+	+	-	+	+	+	-
Citrate	-	-	+	-	+	+	-
Indole	-	-	+	-	-	-	-
Voges-proskauer	-	-	-	-	-	-	-
Glucose	-	-	+	-	-	+	-
H_2S production	-	-	-	-	-	+	-
DNase	+	*	*	*	*	*	*
Methyl red	-	-	+	-	-	-	+
Sucrose	-	-	+	+	+	+	-
Lactose	-	-	+	-	+	+	-
Maltose	-	-	+	-	+	+	+
Oxidase	+	*	*	*	*	*	*
Ornithine decarboxylase	-	-	*	-	*	*	*
Urease	+	-	+	+	+	+	-
L-arabinose	+	*	*	*	*	*	*

Biodegradation of MTBX: The growth pattern of isolates can be understood by the deep study and various effects of different operating parameters such as temperature, pH value and also growth. Study of isolates was performed in basal salt medium (BSM) along with various concentration of MTBX. Table-4 show relations between parameters which are used to conduct every batch experiments, one parameter out of the other parameters was found changeable while other parameters were kept constant i.e. ph, temperature, MTBX concentration and reaction time.

Analytical method: To measure the growth of microorganisms, monitoring of the optical density (OD) at 600 nm can be done by using a spectrophotometer (Model Lamda 35, ParkinElmer, USA). A gas chromatograph named Netel India Limited (Model- MICHRO 9100) equipped with a capillary column type HP5 (30m × 0.249 mm × 0.25µm film thickness) along with a flame ionization detector was used to analyze the concentration of MTBX in gaseous form. The temperature of injector, oven and detector were kept at 210^0C, 60^0C and 230^0C respectively. The gases which were used as fuel and carrier gas are hydrogen and nitrogen gas respectively. The rate of flow for these gases were maintained at 20 mL min^{-1}. For the preparation of calibration curve known amount of the MTBX was injected into a sealed bottle which was equipped with a Teflon septum according to the standard procedure. The injected amount of MTBX, allowed evaporating in the air space inside the bottle at the temperature range of 30^0C. For the purpose of calibration air samples are drawn from the bottle by a 1 mL gas tight syringe and analyzed by using gas chromatography.

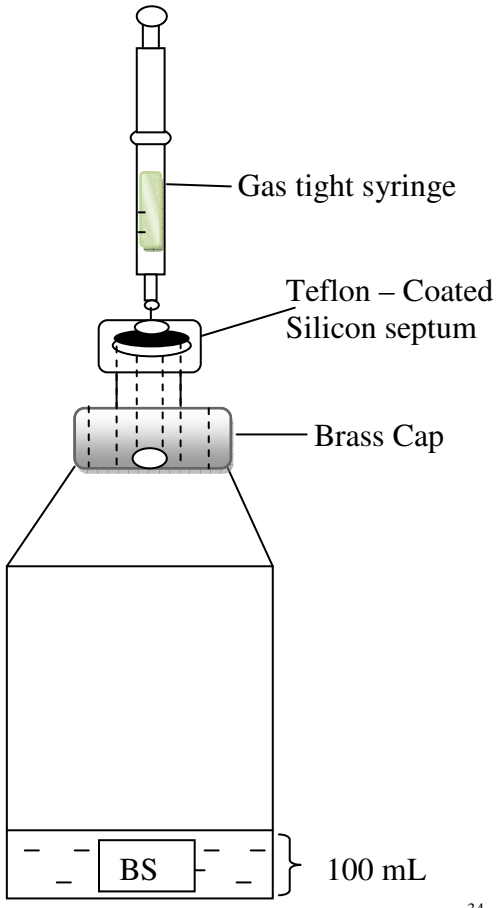


Figure-1: Schematic diagram for batch experiments[34]

Table-4: Set of batch experiments of MTBX used to test optimum degradation conditions.

Batch Test	Parameters	Temperature (^{0}C)	pH	Contaminant (mgL^{-1})	Reaction Test (h)
1.	pH	30	3,4,5,6,7,8, 9, 10, 11	50,100	72
2.	Temperature	15, 20, 25, 30, 35, 40, 45	7	50, 100	24
3.	Reaction Time	30	7	100	12, 24, 36, 48, 60, 72
4.	Contaminate Concentration	30	7	25, 50, 100, 200, 500	72

Results and discussion

Temperature effect on the biodegradation of MTBX by *B. sphaericus:* The removal percentage of MTBX by *B. sphaericus* for both low range (50 mg L^{-1}) and high range (100 mg L^{-1}) concentrations as a function of temperature at the fix value of pH (7.0) for the time duration of 24 h, is represented by Figure-2 (a) and (b). With the help of these figures the temperature effect on the degradation of MTBX was observed for both low and high concentrations. According to the obtained results it was found that the strain was a mesophilic bacterium. From the results it has been noticed that the removal percentage of MTBX by *B. sphaericus* gradually increases with increase in temperature. Figure-2 shows an increasing phase in terms of removal efficiency of MTBX with temperature range from 15^{0}C to 45^{0}C. This phase follow an exponential trend in the case of low as well as high concentrations. If temperature is increased beyond 35^{0}C it results remarkable decrease in the removal efficiency of MTBX in both cases of concentrations. At the temperature range of 25, 30 and 35^{0}C, degradation capability of *B. sphaericus* in terms of removal efficiency for n-butyl acetate was attained maximum compare to others as 97.84, 99.9 and

98.98 % respectively in case of low concentration while in the case of high concentration it was observed as 88.96, 96.8, and 87.98 % respectively.

In the similar way, the removal efficiency by *B. sphaericus* in case of other elements was observed. The removal of MEK for low concentration was observed as 96.56, 97.34 and 94.98 % in the temperature range of 25, 30 and 35 ^{0}C, respectively while on the same temperature it was 74.32, 86.95 and 72.76% in case of higher concentration. Similarly removal of toluene was 97.77, 98, 96.19% at the temperature of 25, 30 and 35^{0}C, respectively in case of low concentration while in case of high concentration it was 75.34, 88.9 and 76.5 % on the same temperature range. The removal of toluene is higher than MEK but lower in comparison to n-butyl acetate. The slowest removal was observed for o-xylene which was 87.79, 88.20 and 83.21% in case of low concentration and 64.79, 69.40 and 64.21% in case of high concentration at the temperature range of 25, 30 and 35^{0}C, respectively.

From the batch study it was conclude that *B. sphaericus* can be grow properly in between the temperature range of 25 to 35^{0}C giving the maximum value of removal at 30^{0}C.

Effect of pH on the biodegradation of MTBX: pH value also play the major role in determining degradation efficiency of strains. The capability of *B. sphaericus* to degrade MTBX for both low and high concentration with respect to different pH value is represented in Figure-3a and b. At the different pH values degradability of MTBX was observed at the fix temperature of 30^{0}C for the time duration of 72 h. For the removal of MTBX, value of pH was kept from 3, 4, 5, 6, to 7. The observed graph shows that at this pH range the degradation capacity of *B. sphaericus* is fast in low concentration as compare to high concentration. The maximum removal of MTBX is found at the pH 0.7 specifically in between the range from 6.0 to 8.0. From the Figure-3a, it can be seen that the maximum degradation of MEK, toluene, n-butyl acetate and o-xylene at the pH 0.7 is 96.37, 98.16, 99.98 and 93.46% respectively for the low concentration while in case of high concentration this degradation was observed as 92.87, 95.34, 97.98 and 86.18 % respectively. When the removal percentage of MTBX is reached at the peak, the outlet concentration of MTBX found lowest at the pH range of 7.0 specifically between the optimum ranges from 6.0 to 8.0. Beyond this optimum rang the degradation of MTBX is comparatively low. The reason behind this low degradability might the inhibitory effect of super acidity of intracellular enzyme of *B. sphaericus.*

MTBX degradation by *B. sphaericus* in batch culture: The results obtained during the batch studies of biodegradation for MTBX in basal salt medium by *B. sphaericus* are shown in Figure-4a,b,c and d, respectively. The degradation of MEK by *B. sphaericus* is about 99.19, 99.98, 99.60 and 95.03% with respect to initial concentration 100, 200, 500 and 1000 mg L^{-1} in time duration of 36, 52, 98 and 135 h, respectively. In the similar way the degradation of toluene with *B. sphaericus* was observed as 99.98, 99.53, 99.61 and 95.68 % with an initial concentration 100, 200, 500 and 1000 mg L^{-1} in 40, 50, 110 and 135 h, respectively. Degradation for the n-butyl acetate was 99.50, 99.95, 99.99 and 95.99 % for the initial concentration of 100, 200, 500 and 1000 mg L^{-1} with respect to time 28, 42, 70 and 135 h respectively. For o-xylene the observed degradation was 99.19, 99.98, 97.94 and 94.18% with initial concentration of 100, 200, 500 and 1000 mg L^{-1} in 60, 85, 135 and 135 h, respectively.

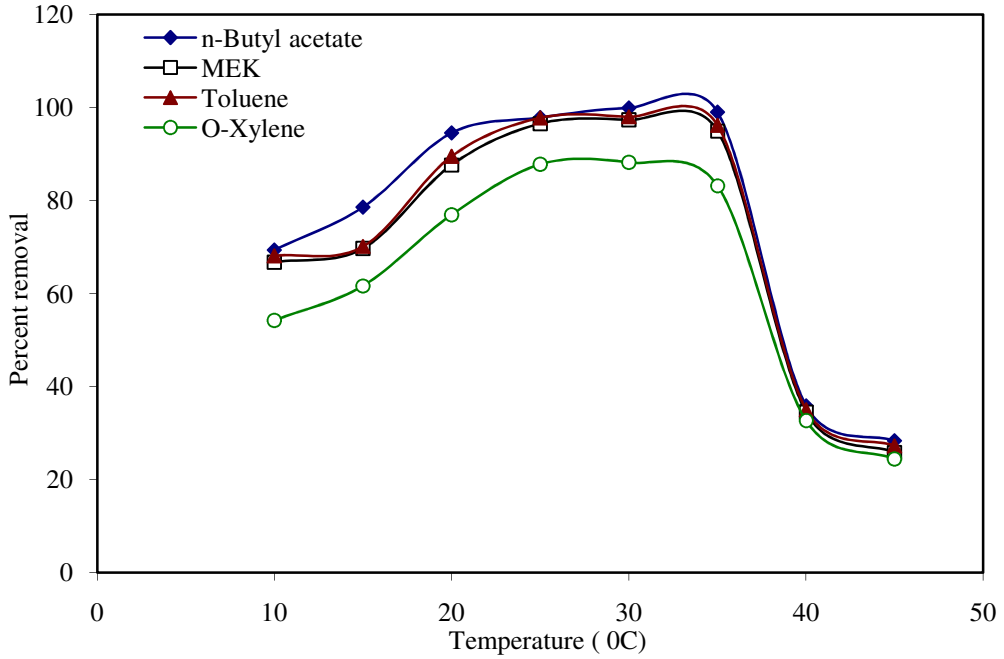
According to the obtained results it has been observed that the biodegradation time for MTBX was low for low substrate concentration as the degradation rate is high at same concentration. In terms of high concentration of MTBX rate of degradation is found low while degradation time is high. The reason behind these observations is that these experiments were completed in bottles of 500 mL capacity along with 100 mL working volume so it may be oxygen deficiency. On the basis of these results it can be seen that culture was unable for the degradation of high concentration of MTBX efficiently. Other than oxygen deficiency the fall in pH of the solution over a period of time also may be another reason of low degradation.

Growth of *B. sphaericus* in MTBX: To evaluate the biodegradation of MTBX along with growth of acclimatized microbes present in contaminants, batch studies were carried out in the presence of oxygen by using a mixed microbial culture which has acclimatized with single substrate. The growth profile of mixed microbial culture with respect to time is shown in Figure-5. It can be observed from the figure that the culture utilized MTBX as the sole carbon source and was effectively able to degrade them. The microbial growth was monitored regularly by measuring optical density (OD_{600}). As shown in the figure, a significance difference was observed in the growth of isolated bacterial strains. The strain BG1 is fastest, followed by BG2, BG3, OG1, OG2, OG3, and OG4. Although OG1, OG3, and OG4 isolates were grown on the same substrate but the lag phase was around 20 h around indicating lower growth rate. It is clearly shown that the growth of BG3 between 30 to 35 h was more as compare to BG1, so overall the growth of BG1 was higher as compare to all isolates.

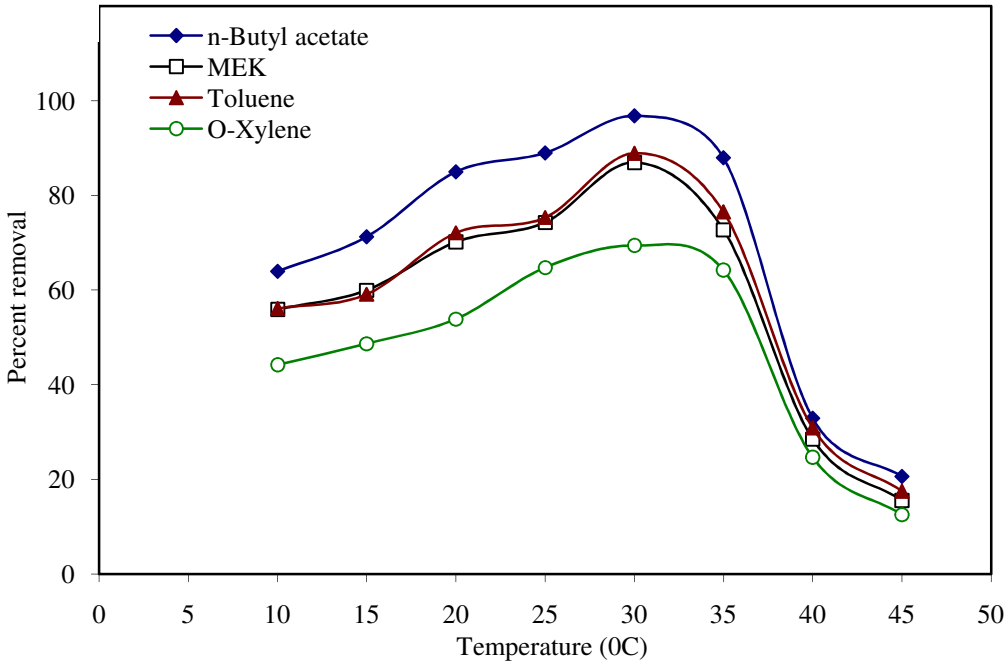
Conclusion

In this study the MTBX degrading bacteria was isolate from the biofilters and its biological characteristics were examined. According to obtained results the pure *B. sphaericus* strain quarantined and has a high capacity to degrade MTBX completely at concentration even lower than 200 mg L^{-1}. The maximum degradation was found in case of n-butyl acetate as 99.99% for the concentration 500 mgL^{-1}. But in the case of o-xylene the degradation was lowest as compare to MEK, toluene and n-butyl acetate. According to these results *B. sphaericus* has high capability to be use in biofilteration for degrading MTBX and create non-contaminated environments. According to this

study cultivation of *B. Sphaericus required* high range of concentrations of MTBX from the temperature 15-45^{0}C, while at the temperature higher than 40^{0}C it was less energetic. For the cultivation of all isolates, the pH range was kept from 3.0-11.0 with the optimum range of 6.0-8.0. Result analysis of this study showed that the operating conditions of biofilter were most suited for the degradation of n-butyl acetate followed by toluene, MEK and then o-xylene.

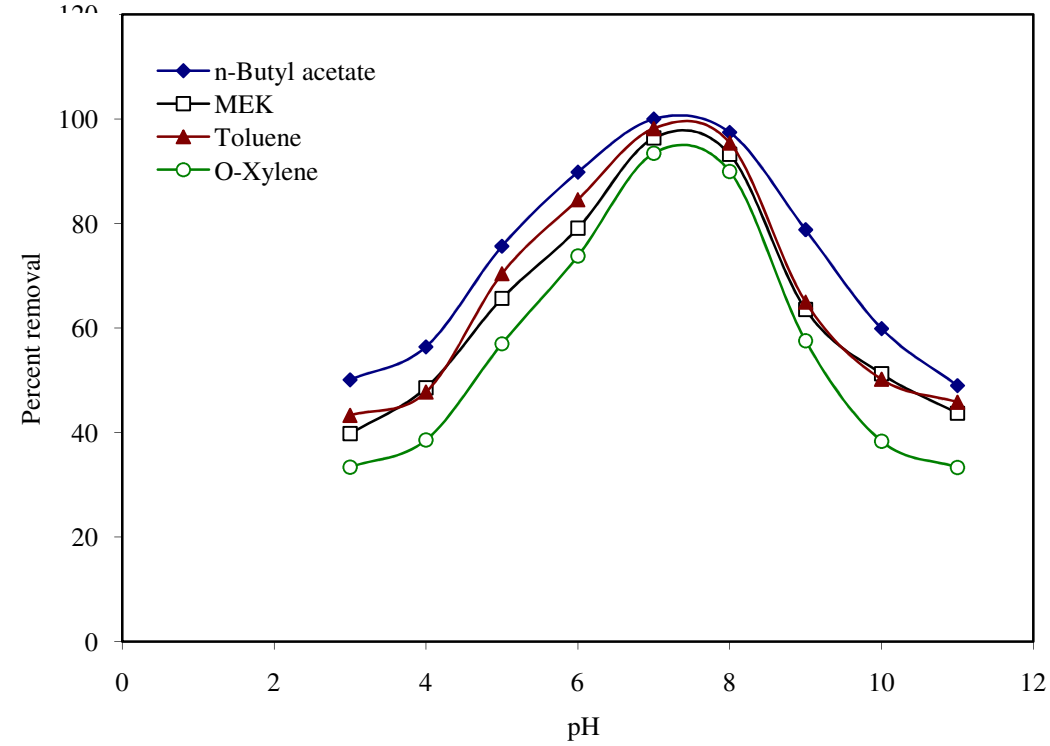


(a)

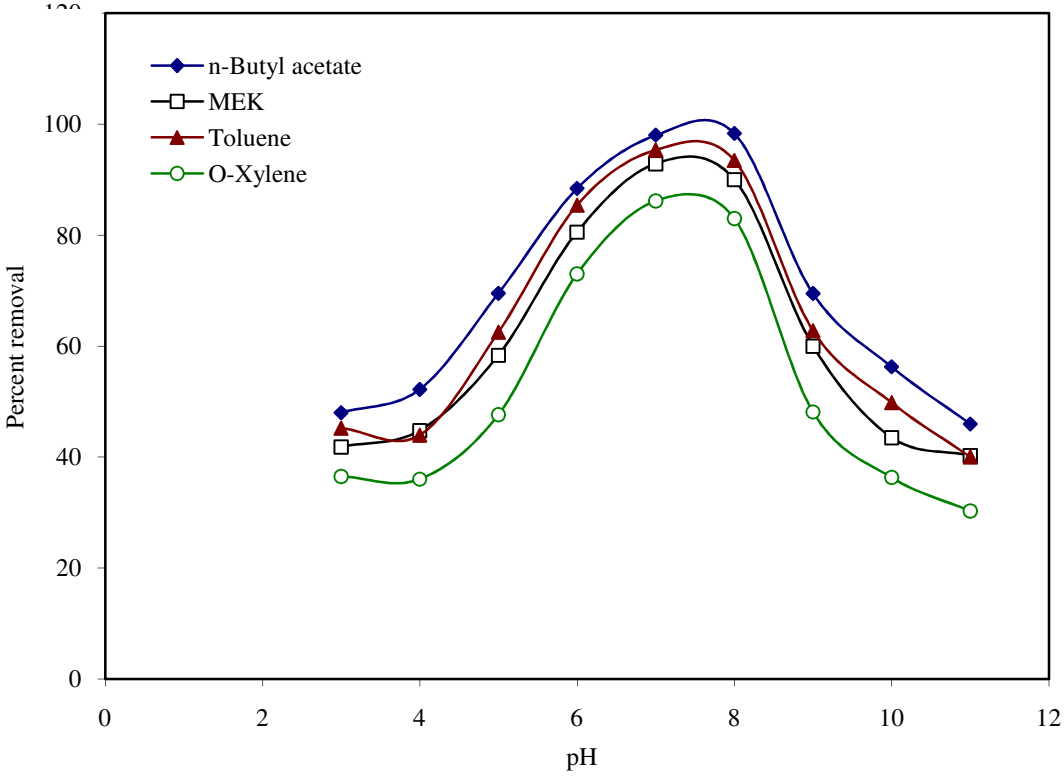


(b)

Figure-2: Effect of temperature on the biodegradation of MTBX by B. *sphaericus* of concentration of (a) 50 mg L^{-1} and (b) 100 mg L^{-1}.

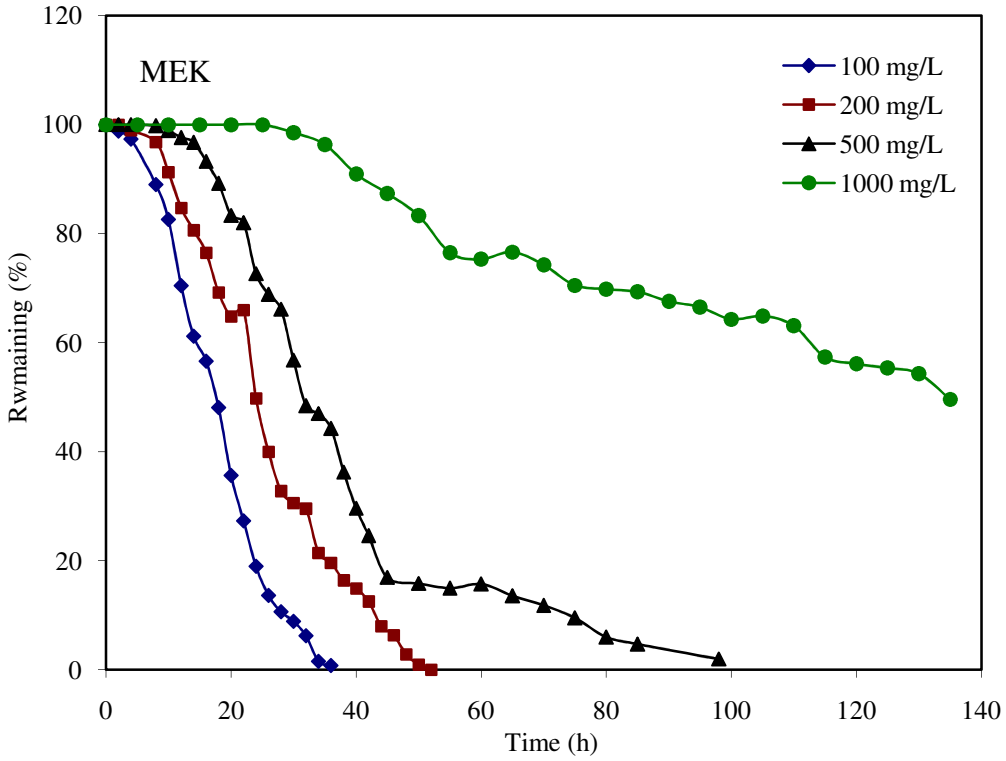


(a)

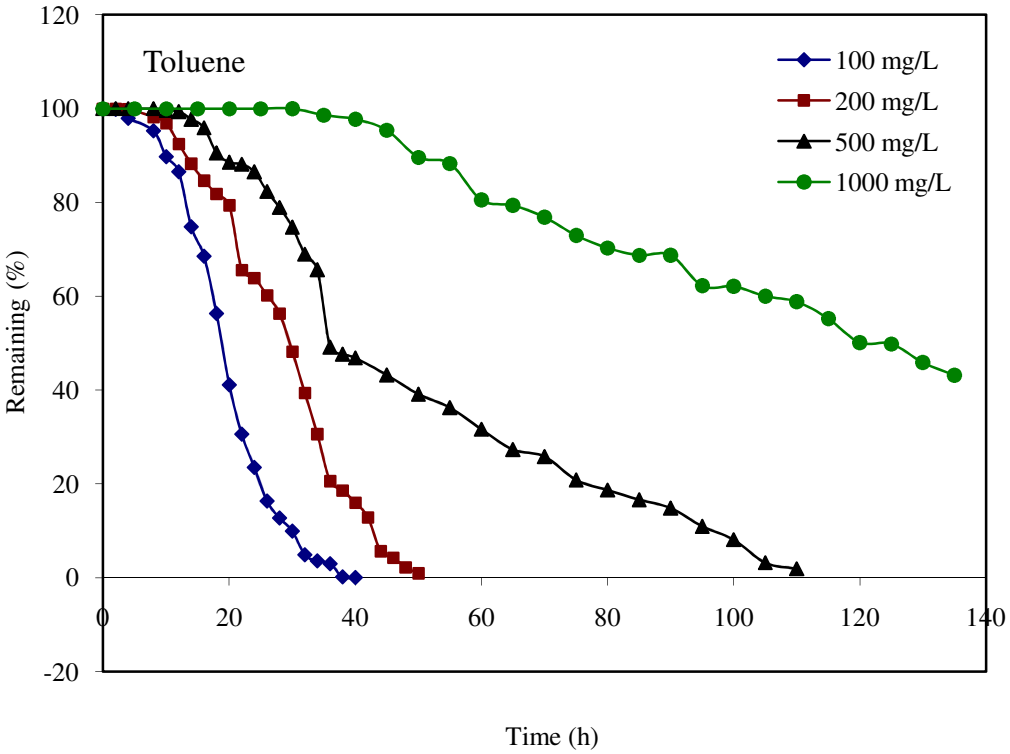


(b)

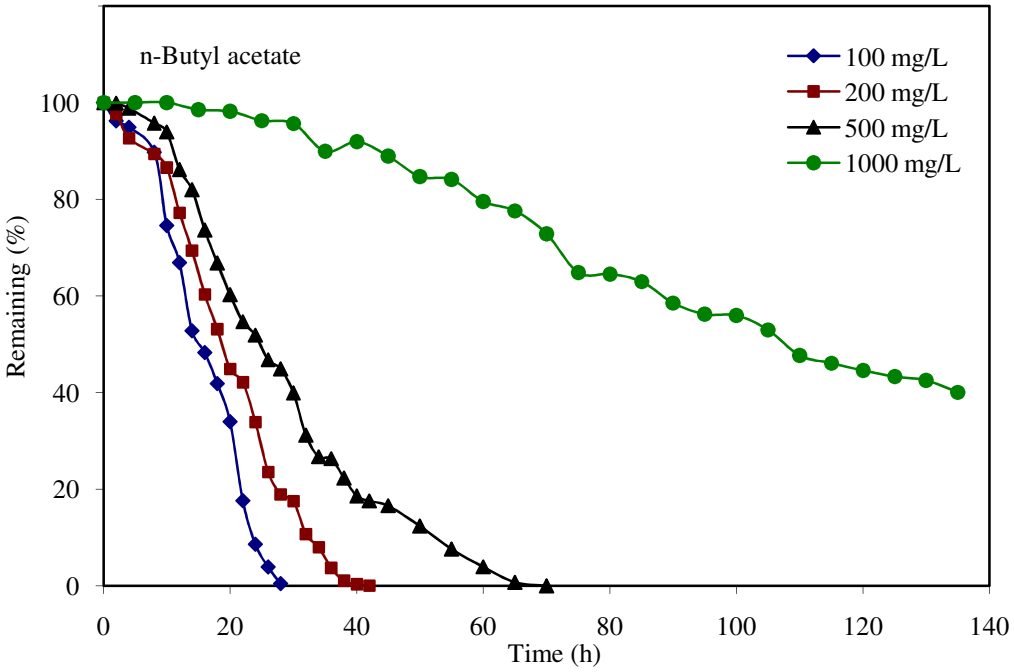
Figure-3: Effect of pH on the biodegradation of MTBX by B.*sphaericus* of concentration of (a) 50 mg L^{-1} and (b) 100 mg L^{-1}.


MEK
100 mg/L
200 mg/L
500 mg/L
1000 mg/L
Rwmaining (%)
Time (h)
0
20
40
60
80
100
120
140

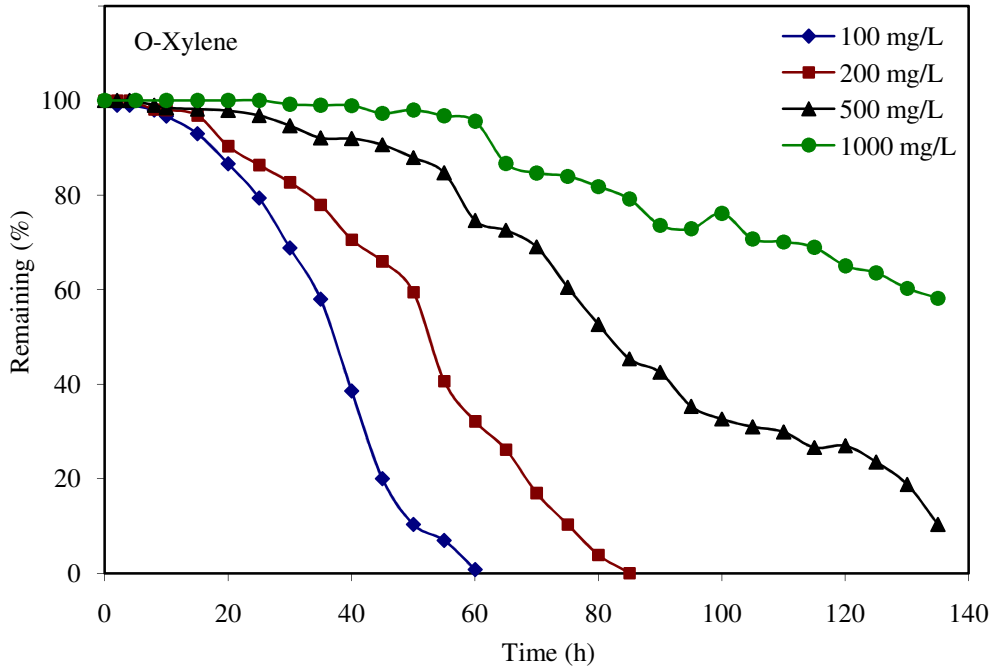
(a)


Toluene
100 mg/L
200 mg/L
500 mg/L
1000 mg/L
Remaining (%)
Time (h)
-20
0
20
40
60
80
100
120
140

(b)



(c)



(d)

Figure-4: Effect of substrate concentration on the biodegrading of (a) MEK (b) Toluene (c) n-butyl acetate and (d) o-xylene.

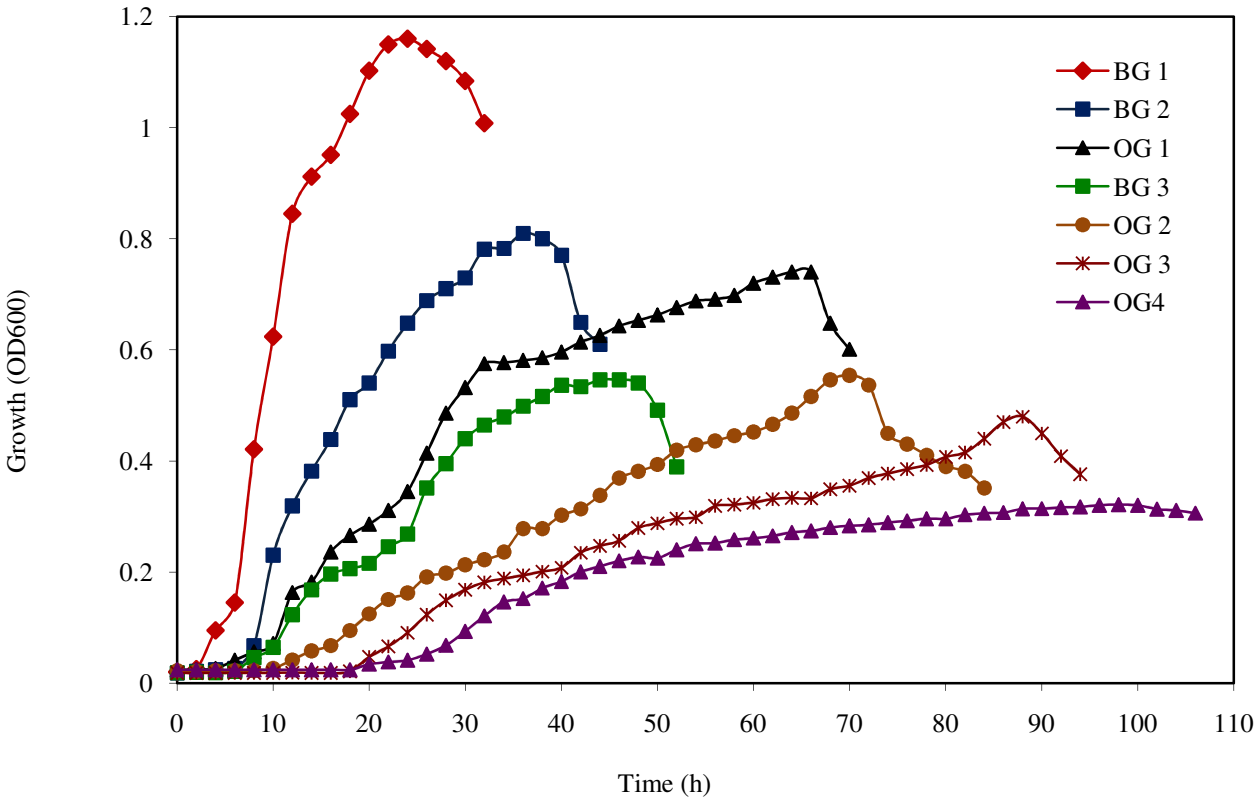


Figure-5: Growth of isolated strains from compost based biofilter on the mixture of MTBX.

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