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*Full Length Research Paper*

# Biodegradation of 2,2,6,6-Tetramethyl-4-piperidone by three soil bacteria strains and their isolation and identification

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**In order to isolate and characterize 2,2,6,6-Tetramethyl-4-piperidone (TMPD)- degrading bacteria and to select the optimal degrading conditions, different bacterial types which can degrade TMPD were isolated from soil samples. Three strains (LF-1, LF-2 and LF-3) showed strong biodegradation ability by UV-visible spectrophotometer analysis. Morphological and biochemical analyses, as well as 16S rRNA gene sequence comparisons of three strains were further carried out. They were identified as *Klebsiella oxytoca*, *Staphylococcus pasteur* and *Bacillus flexus*, respectively. *K. oxytoca*, *S. pasteur* and *B. flexus* had degrading capabilities for TMPD in mineral salt medium of 80.9, 69.8 and 64.9%, respectively, during 24 days of incubation. Maximum degrading rates were obtained by an initial concentration of the TMPD of 200 mg/L, an incubation temperature of 30°C, and constant aeration (180 rev/min). In conclusion, *K. oxytoca*, *S. pasteur* and *B. flexus* show their potentials in TMPD detoxifying and in protecting the TMPD contaminated environment.**

**Key words:** 2,2,6,6-Tetramethyl-4-piperidone (TMPD), biodegradation, *Klebsiella oxytoca*, *Staphylococcus pasteur*, *Bacillus flexus.*

## INTRODUCTION

As a natural metabolite, 2,2,6,6-Tetramethyl-4-piperidone (TMPD) had been detected in a series of plants such as Egyptian goosefoot plant, *Syngonium podophyllum*, *Agelas oroides* (König and Wright, 1998), *Excoecaria venenata* (Lu et al., 2005) and *Cynanchum komanovi* (Zhao et al., 2002); *Armeniac mume* (Ren et al., 2004), and *Oxytropis glacialis*. *O. glacialis* exclusively grows in the Ali area of Tibet. It has been connected to a large number of animal poisonings in grassland areas (Li et al., 2007). The content of TMPD in *O. glacialis* amounts is 0.085% of dry weight. Synthesized TMPD is used as an additive especially for processing of light stabilizers (Jerzy et al., 2000), and is produced mainly in Germany and Italy. The annual production is about 20,000 t within the European Unit. TMPD is analytically used as a trapping probe to scavenge free oxygen radicals and for the detection of singulet oxygen in water and methanol mixtures. TMPD emission can be detected in wastewater at the production site; however, information on TMPT's risk to the environment is still inadequate (Kondo and Riesz, 1991). TMPD inhibits α-mannosidase in the blood, resulting in extensive accumulation of polysaccharides within cellular vacuolations. Clinical signs including central nervous system depression, roughened coat, halting walk, dullness, lusterless eyes, and weight loss. The $LD_{50}$ of TMPD is about 1 g/kg bodyweight by oral (Tan et al., 2002). TMPD was fed to goats constantly with 0.38 g/kg bodyweight per day. 40 days later, the goats started to stumble and fall down and could hardly stand up again. The serum α-mannosidase activity declined sharply. Histopathological changes at autopsy showed vacuolations in brain, liver and kidney (Li et al., 2007). Fish is the most sensitive organism, with a validated $LC_{50}$ (96 h) of 63 mg/L. The $EC_{50}$ (48 h) for daphniae (*Daphnia magna*) is 281.2 mg/L. The $EC_{50}$ (72 h) for the alga

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Desmodesmus subspicatus is 439.5 mg/L. TMPD is corrosive to the skin and eyes of rabbits (Advisory Committee on Existing Chemicals of Environmental Relevance, German Chemical Society, GDCh).

Although the toxicology of TMPD has been revealed, it remains a critical problem to protect animals from espousing to this poison due to limited food availability. The objectives of the present study were to find bacteria which can degrade TMPD and to further investigate the biodegradation process. Such bacteria could be used for environmental TMPD elimination and animal protection.

## MATERIALS AND METHODS

### Soil samples

Soil samples were collected 10 to 20 cm below the ground of the roots of *O.glacialis*. The collected samples were passed through a 2 mm sieve and stored at 4°C.

### Media preparation

Two media, an enrichment medium (EM) and a mineral salt medium (MSM) were both used in enrichment culture and used to test the strains degrading abilities. EM contained (in grams per liter) 5 g yeast extract, 1 g peptone, 0.5 g NaCl, and 0.1 g $K_2HPO_4$ (pH 7.0), TMPD (99.5%, Langfang Flux Co., Ltd, China) dissolved in distilled water was added after autoclaving the medium adjusted to different concentrations. MSM contained (in grams per liter) 5.0 g $NH_4NO_3$, 1.5 g $MgSO_4$, 5.0 g $(NH_4)_2SO_4$, 5.0 g $KH_2PO_4$, 5.0 g NaCl, 1.5 g $K_2HPO_4$ (pH 7.0), and TMPD was added to the medium after autoclaving. The solid medium was prepared by adding 17 g agar per liter to the enrichment medium followed by autoclaving at 121°C for 20 min. All chemicals used in the study were of analytical grade.

### Isolation of TMPD-degrading bacteria

Enrichment culture technique was used for isolation of bacterial strains capable of utilizing TMPD as a sole carbon source. 10 g soil sample was suspended in 90 ml distilled water by shaking. After 30 min setting, 10 ml of the supernatant was inoculated into 90 ml of EM at 30°C, 180 rev/min for 24 h. 1 ml of this liquid culture was then added to the EM with different TMPD levels (50, 100, 150, 250 and 300 mg/L) and kept under the same incubation conditions for 4 days. After the last transfer, 1 ml of the liquid culture was serially diluted as a tenfold- dilution series into sterilized distilled water. Dilutions were spread onto MSM solid plates containing 150 mg/L TMPD and incubated at 30°C for 3 to 5 days. Colonies were picked from dilution plates based on distinct colony morphology and transferred onto fresh plates several times to ascertain culture purity (Siddique et al., 2003). Each strain was incubated in MSM medium containing TMPD (150 mg/L) and preliminary tested for its activity to TMPD degradation as the sole carbon source.

### UV-VIS analysis on TMPD degradation

The TMPD concentrations in all the liquid samples were determined by Ultraviolet-visible (UV-VIS) detector at 227 nm. The bacterial cultures were centrifuged at 7,500 g for 10 min at 25°C, and the supernatant was moved to a cuvette. The MSM worked as the blank control, absorbency of samples were measured as described by Tan et al. (2002).

### Inoculums preparation for degradation study

The inoculants for this experiment were bacteria growing on slant on MSM culture medium containing TMPD at 150 mg/L for 30 h. The cells were washed off with 5 ml MSM and then collected by centrifugation at 6,000 g for 10 min. Cells then were washed three times with 5 ml MSM and quantified by the dilution plate count technique. For all experiments, $10^6$ cells/ml were used for incubation.

### Screening on best TMPD degradation conditions

The degrading abilities of the isolated bacteria were incubated in MSM medium containing TMPD (150 mg/L) at 30°C, pH 7.0 and by shaking at 180 rev/min for 72 h unless otherwise stated. The TMPD concentration in MSM was analyzed by UV-VIS detector as previously described. MSM without TMPD was used as negative control. Effects of incubation time (0，6，12，18 and 24 days), initial TMPD concentration (100, 200, 300, 400 and 500 mg/L), temperature (4, 15, 25, 30 and 40°C) and pH (5, 6, 7, 8 and 9) on degradation were examined using three parallel treatments.

### Biochemical and phenotypic characteristics of bacteria

Cell morphology of the isolated strain was observed by transmission electron microscope (TEM: JEM-1230, JEOL Ltd., Japan) and scanning electron microscopy (JEOL JSM-6360LV, JEOL Ltd., Japan).

Conventional physiological characteristics of the isolated strains were determined according to the Manual of Identification for General Bacteriology (Garrity et al., 2004).

### 16S rRNA gene sequence determination and phylogenetic analysis of bacteria

Genomic DNA was isolated according to standard procedure as described by Singh et al. (2003). The 16S rRNA genes were amplified by PCR using Taq polymerase. The following pair of universal primers for bacteria was used, the forward primer BSF8/20:5'-AGAGT TTGAT CCTGG CTCAG-3' and the reverse primer BSR1541/20: 5'-AAGGA GGTGA TCCAG CCGCA-3' (Yang et al., 2005). PCR consisted of initial denaturation at 94°C for 2 min, followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min, with the last cycle followed by a 10 min extension at 72°C. The products were sequenced (Invitrogen Biotechnology Company Limited, Shanghai, China) and the obtained 16S rRNA gene sequence were compared with strains available in GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST sequence match routines. The sequences were aligned using multiple sequence alignment software CLUSTALW Version 1.81. Phylogenetic trees were then constructed by the neighbor-joining method using Mega 3.1 software based on the 16S rRNA gene sequences of 16 strains phylogenetically close to the isolated strain.

### Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). Significant differences between the groups were calculated by using One-way ANOVA and subsequent Student-Newman-Keuls test. Differences with $p<0.05$ were considered statistically significant.

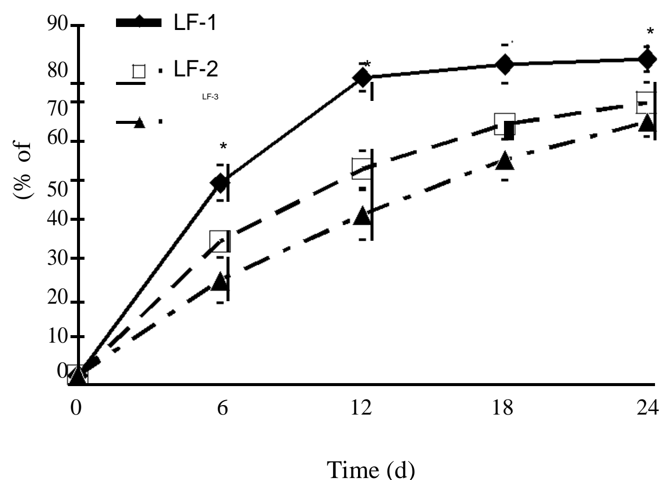


**Figure 1.** The degrading rates of TMPD by strains LF-1, LF-2 and LF-3-1 at different times. *: Significant difference between strains (p<0.05).

## RESULTS

### Isolation of the TMPD degrading strains

After enrichment culture procedure, 12 isolates were obtained that grew on MSM plates containing 100 and 150 mg/L TMPD as the sole carbon source. All isolates were tested for their degrading capabilities under 30°C, pH 7.0 by shaking at 180 rev/min for 72 h. After preliminary, TMPD degrading test (data not shown), 3 strain named LF-1, LF-2 and LF-3 that showed rapid and strong TMPD degrading ability were selected for further analysis.

### Screening on best TMPD degradation conditions

The degrading abilities of the isolated bacteria were further assayed in MSM. The TMPD concentration in MSM was analyzed every 6 days by UV-VIS detector. The time courses of TMPD degradation by strains LF-1, LF-2 and LF-3 were described in Figure 1. The TMPD concentration decreased with incubation time. After 24 days, the degrading rates of the strain LF-1, LF-2 and LF-3 were 80.9, 69.8 and 64.9% of the original concentrations, respectively. The degrading rate of strain LF-1 was significant higher than that of the strains LF-2 and LF-3 during the whole course (p<0.05).

Strains LF-1, LF-2 and LF-3 behaved similarly for degrading TMPD at different TMPD concentrations (Figure 2a), different temperatures (Figure 2b) and different pH values (from 5.0 to 9.0; Figure 2c). Maximum degrading rate of TMPD was observed when initial TMPD concentration was 200 mg/L. The degrading rates of strain LF-1, LF-2 and LF-3-1 were 28.1, 24.4 and 18.5%, respectively. The minimum degrading rate appeared in initial TMPD concentration 500 mg/L with no more than 2%. Biodegradation of TMPD was relatively higher at an incubation temperature ranged from 25 to 40°C, with a maximum at 30°C. The smallest degradation of TMPD was recorded at 4°C. Biodegradation of TMPD by these bacterial cultures was more pronounced at alkaline conditions but it was inhibited strongly at acidic pH. Maximum biodegradation of TMPD by bacterial strains was observed at an initial pH 8.0 and minimum at an initial pH of 5.0. All three selected bacterial strains showed high similarly in pH dependant biodegradation activity.

### Characterization and identification of strains LF-1, LF-2 and LF-3

Strain LF-1 is Gram negative coccobacillus, straight rods, encapsulated, non-gemmas, motile, 0.5 to 9 µm in diameter and 1.0 to 2.0 µm in length (Figures 3a and b), and existed as single, double and acervate forms. The colony morphology of strain LF-1 on plain agar plate was round, smooth, moist, caelate, fringe- regular, gray and semitransparent.

Strain LF-2 is Gram positive coccus, non-encapsulated, non-gemmas, non motile, 0.8 to 1.0 µm in size (Figures 3c and d), and existed as single, double and acervate. The colony morphology of strain LF-2 on plain agar plate was round, smooth, moist, eminent, fringe- regular, gray and semitransparent. Strain LF-3 is Gram negative bacillus, rods, non-encapsulated, non motile, 1.0 to 1.2 µm in diameter and 1.5 to 5.0 µm in length (Figures 3e and f), gemmas were elliptic or columnar, and existed as single, double and acervate. The colony morphology of strain LF-3 on plain agar plate was shape of mycelia, non-smooth, dry, flat, fringe-irregular, gray and semitransparent.

After PCR amplification of 16S rRNA gene from strain LF-1, strain LF-2 and strain LF-3, single fragments of 1,477 bp (GenBank accession No. EF127829), 1,492 bp (GenBank accession No. EF127830) and 1,484 bp (GenBank accession No. EF127831) were obtained and completely sequenced. According to BLAST analysis, the resulting sequences were closely clustered to strain DR.Y14 (GenBank accession No. DQ226215), *Klebsiella* group with sequence identities of 99.52%, to strain AJ717376 *S. pasreuri.* SEQ (strain number not given, GenBank accession No.AJ717376) *Staphylococcus* group with sequence identities of 99.80% and to strain XJU2 (GenBank accession No.DQ837542); *Bacillus* group with sequence identities of 99.86%, respectively. Phylogenetic trees (Figure 4) again indicated that the isolated strains LF-1, LF-2 and LF-3 belong to the *Klebsiella* group, the *Staphylococcusi* group and the *Bacillus* group, respectively. Therefore, the isolates were designated as *K. oxytoca* strain LF-1, *Staphylococcus pasteuri* strain LF-2 and *B. flexus* strain LF-3, respectively.

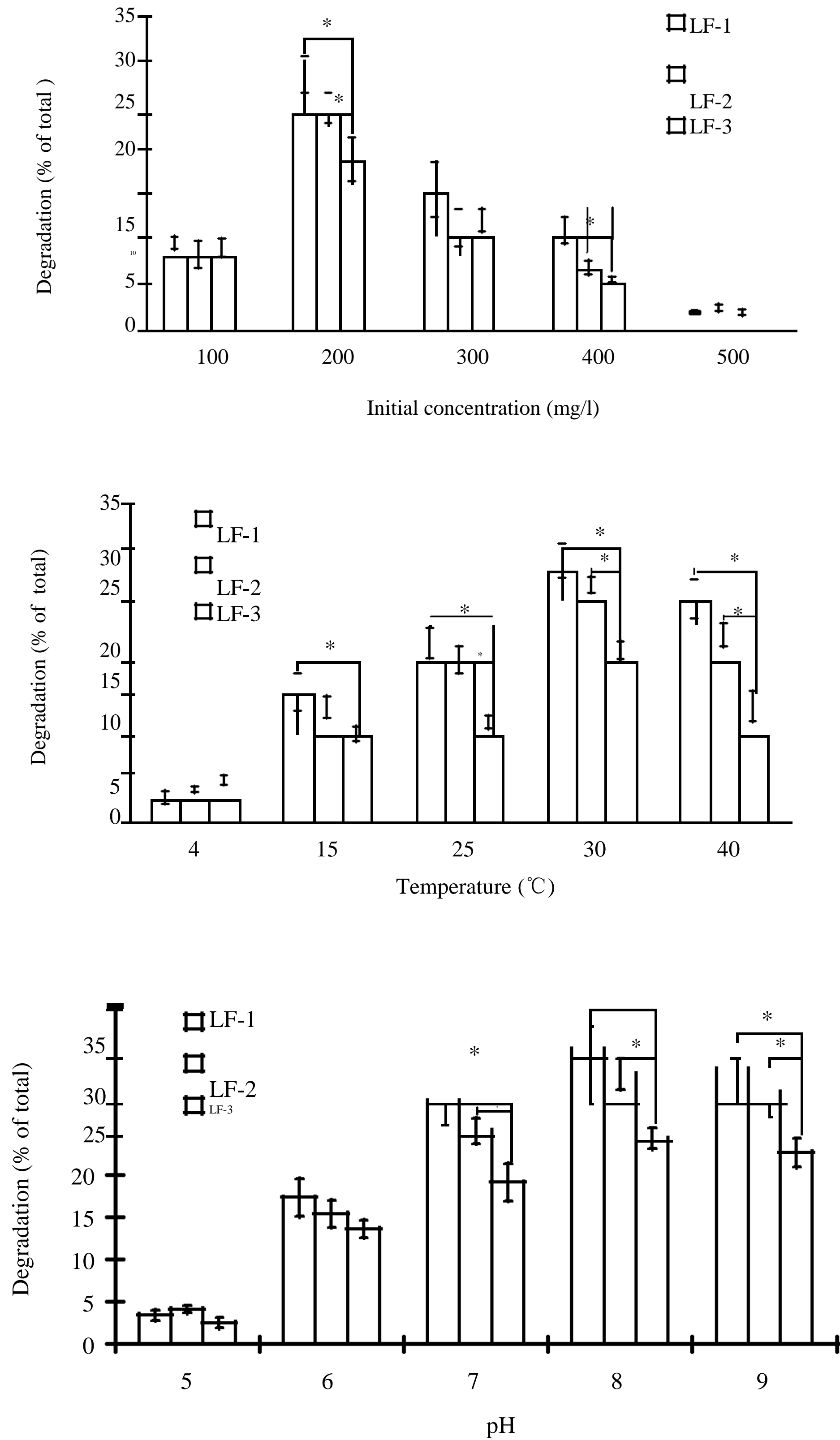


**Figure 2.** Effect of initial TMPD concentrations (a) temperatures (b) and pH values (c) on the TMPD degrading rate by strains LF-1, LF-2 and LF-3. *: Significant difference between strains ($p<0.05$).

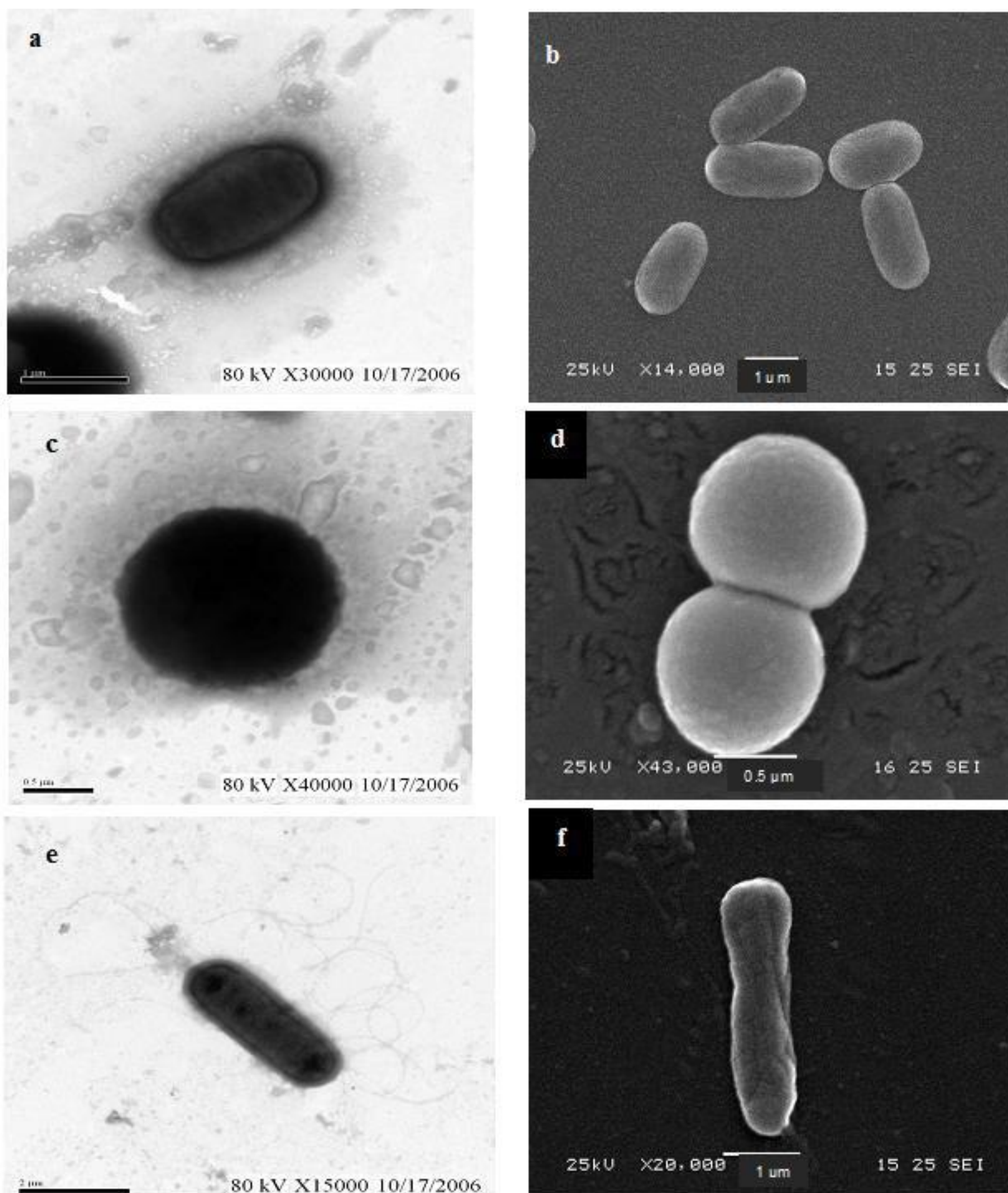


**Figure 3.** Bacteria morphological observation by transmission electron microscope (a, c, e) and scanning electron microscope (b, d, f) of strains LF-1 (a, b), LF-2 (c, d) and LF-3 (e, f).

## DISCUSSION

The German Chemical Society (GDCh)-Advisory Committee (2004) reported that the available degradation investigations showed TMPD as not readily biodegradable after 28 days (11%, based on the DOC, or 1%, based on the substance). However, no experimentally data are available on its potential biodegradability. The present study describes the enrichment, isolation and identification of microbial strains of degrading chemical substance. In this study, 12 TMPD-degrading isolates were obtained from soil using a traditional enrichment

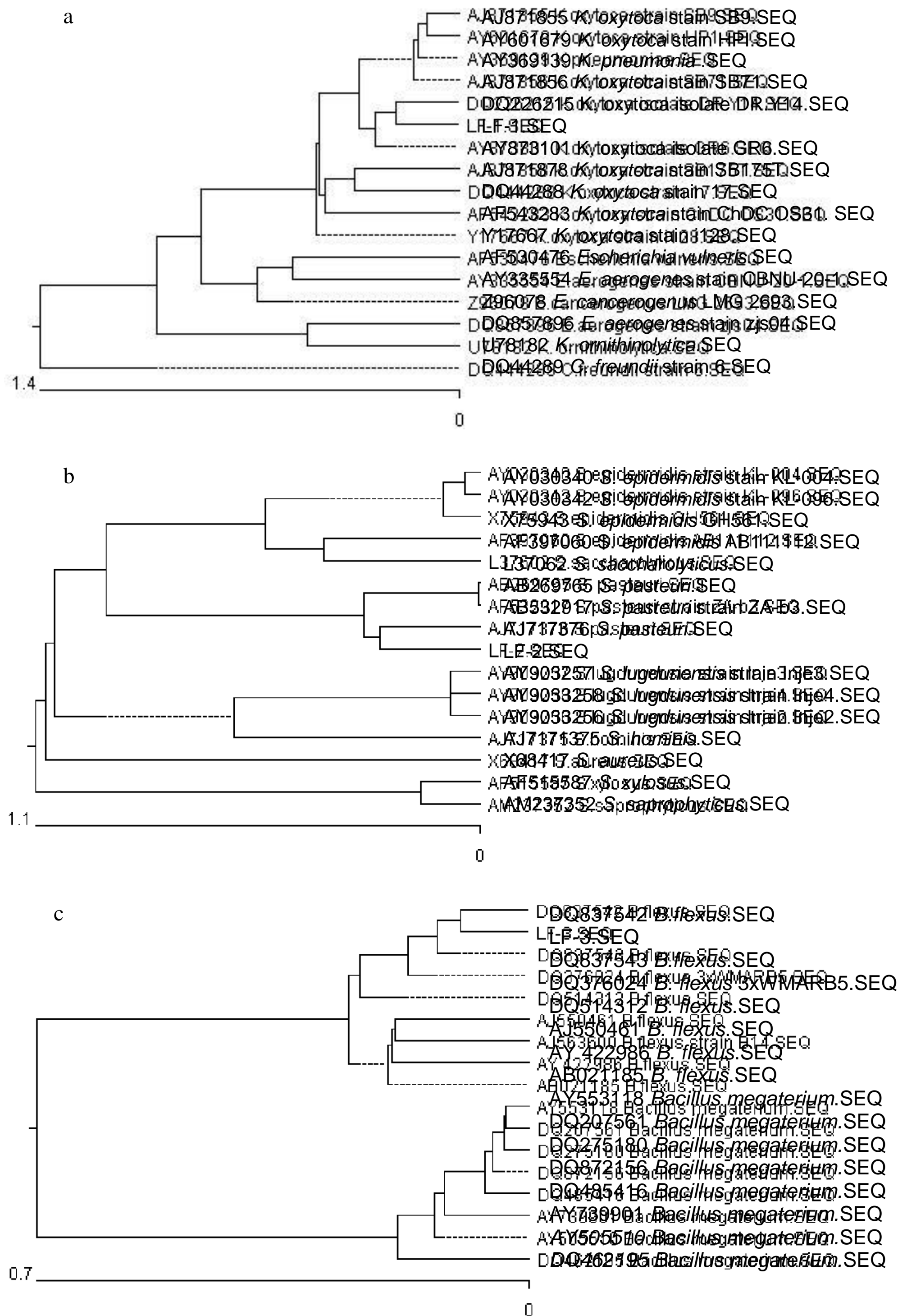


**Figure 4.** Phylogenetic trees based on 16S rDNA sequences of strains LF-1 (a), LF-2 (b) and LF-3 (c).

procedure (Siddique et al., 2003), and 3 of them showed the best degrading ability. Enrichment was achieved by providing TMPD as sole carbon source.

Various studies have reported that batch tests need to be designed with appropriate substrate concentrations for analyzing the bacterial biodegradation abilities (Bielefeldt and Stensel, 1999). It is highly likely that the incubation conditions could directly influent the bacterial growth. Our study showed that high TMPD concentration (that is, 500 mg/L) could inhibit the degradation ability (less than 2%),

which might be due to inhibitory effect of high TMPD concentration. This phenomenon has been also reported by various researchers during batch degradation studies for toxic organics (Rhee et al., 1997; Kumar et al., 2005; Arutchelvan et al., 2006).

In general, neutral to slightly alkaline conditions are considered more favorable for bacterial growth than acidic conditions (Hussain et al., 2007). It is very likely that the initial alkaline pH 8.0 of incubation could better support TMPD degradation. The biodegradation under shaking condition was preferable over static conditions (Hussain et al., 2007). That might due to better bioavailability of chemicals to the microbes coupled with physicochemical degradation.

According to our study, biodegradation of TMPD by the 3 efficient strains *K. oxytoca*, *S. pasteur* and *B. flexus* was found to be maximal at an initial concentration of TMPD of 200 mg/L incubation temperature of 30°C and under shaking conditions (180 rev $min^{-1}$). To our knowledge, this is the first relevant systemic study on TMPD biodegradation condition.

In the present study, we showed that the *K. oxytoca*, *S. pasteur*, *B. flexus* are effective in TMPD degradation. However, the TMPD pathway under these bacteria remains unknown. Further characterization on the TMPD degrading products is required, as they could be more toxic than their parent compounds, and this is currently studied in our laboratory by GC-MS method together with the identification of proteins which are related to the degradation pathway. From the point of environmental protection and economic value, the present observation of using bacteria for TMPD degradation may be exploited further in biotechnology for the effective detoxification of *O. glacialis* and for protecting the TMPD contaminated environment. According to our understanding, future study on identification and isolation of the enzymes involved in TMPD degradation is necessary.

## ACKNOWLEDGEMENT

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