

Full Length Research Paper

Kinetics biodegradation of triazophos by *Klebsiella oxytoca* TDB-1

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A triazophos (TAP)-degrading bacterial strain, *Klebsiella oxytoca* TDB-1, which could use TAP as sole carbon source, was isolated by enrichment culture technology. The growth characteristics of strain TDB-1 with pressure of TAP were tested, and the results show that optimum growth condition was at 30°C, pH 8 and 2% (v/v) inoculation. Strain TDB-1 was great promise in TAP biodegradation for extreme pH tolerance (pH 4 and 10). The kinetic of strain TDB-1 in degrading TAP followed first-order model under optimal growth conditions. Cell surface hydrophobicity (CSH) assays of strain TDB-1 reflected that the strain TDB-1 was of higher hydrophobicity while grown on higher concentration of TAP. The growth and TAP-biodegradation characteristics of strain TDB-1 revealed that it would be an excellent alternative degrader for bioremediation of TAP contaminants, especially for that with extreme pH, such as some industrial wastewaters.

Key words: Biodegradation, triazophos, *Klebsiella oxytoca* TDB-1, cell surface hydrophobicity.

INTRODUCTION

Organophosphorus pesticides (OPs) were poured into agricultural producing since the late 1970s, for the merits of high insecticidal activity and broad-spectrum (Kaloyanova and El-Batawi, 1991). The widespread use has caused severe environmental pollution since OPs applied in agricultural areas do not remain at their target sites, but often enter aquatic environments via soil percolation, air drift or surface run-off (Rovedatti et al., 2001; Anderson et al., 2003; Sawaya et al., 2000). The potential threat of OPs to the environment and to public health is proved by empirical analysis and experiment verification (Hayes et al., 1980). OPs are poisoning to peripheral and central nervous system, resulting in symptoms termed "organophosphorus induced delayed neuropathy" (Jokanović and Kosanović, 2010; Mearns et

al., 1994). Therefore, several species of OPs, such as parathion-methyl, monocrotophos, parathion, and so on were forbidden because of highly toxic to non-target organisms and mammal (Hreljac and Filipič, 2009).

Triazophos [o,o-diethyl-o-(1-phenyl-1,2,4-triazol-3-yl) thiophosphate] (TAP), one of alternatives to high-toxic OPs, has been widely used on main staple food crops (maize, rice) and vegetables since the late 1970s. It was reported that the mean half-life of triazophos in wheat plants (grain, stems, and leaves) was 5.22 days with a dissipation rate of 90% over 14 days, and the half-life in soil was 7.93 days with a dissipation rate of 90% over 21 days (Li et al., 2008). However, TAP-contamination of the environment could also not be ignored. TAP-contamination of the environment is accumulating and high level of TAP residue has been found in a wide variety of foods according to several US and European food monitoring schemes (Alexis et al., 2001). TAP is highly toxic to most fishes, except shellfishes (Lin and Yuan, 2005). In China, TAP has also been utilized as fungicide in intertidal aquaculture in farming shellfish such

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as *Sinonovacula constricta* and *Tegillarca granosa* (Lin and Yuan, 2005). The potential threat of coastal waters pollution by TAP is raising concern for the misuse of it in intertidal aquaculture, in recent years; fish kill accidents sporadically took place in coastal areas of Southern of China, mainly because of TAP contamination

Bioremediation of OPs is recognized as economical and reliable methods. In recent decades, the microbial degradation of OPs has been intensively studied, especially in high-toxic and high-residual OPs, such as parathion and methyl parathion. Several degradation bacteria, including strains in genus *Enterobacter* (Concepción et al., 2012), *Arthrobacter*, *Burkholderia*, *Sinorhizobium* and *Mycobacterium* (Seo et al., 2007), *Serratia* and *Pseudomonas* (Cycoń et al., 2009), and so on were isolated. Stimulating further studies demonstrated that all of them degrade OPs by hydrolase such as opd gene coding enzyme (Concepción et al., 2012; Steven et al., 2008). Nevertheless, some high-toxic and high-residual species of OPs, but not limited to parathion and methyl parathion, have been banned for use in agriculture in recent years. Many mid-toxic and low-residual Ops such as TAP, were widely utilized as alternative. Little studies have been published on the microbial degradation of TAP, although, Wang et al. (2005) isolated a TAP degrading bacterial strain, to their best knowledge, that was the first report of the microbiological degradation of TAP.

The cell surface hydrophobicity (CSH) is believed as one of the representation factors of degradation capacity of organisms, because the first step in the process of biodegradation is adhesion of the pollutants to cell surfaces of the organisms (Rosenberg et al., 1980). Bastiaens et al. (2000) reported that hydrophobicity is positively correlated to degradation efficiency of polycyclic aromatic hydrocarbon.

The objective of this study centered on the isolation, identification and degrading characteristics of TAP *in situ*. The study also involved the investigation of cell surface hydrophobicity property during the degrading progress.

MATERIALS AND METHODS

Chemicals and media

Sample of TAP (95.0% pure analytical grade) were kind gift from Dr Xingan Liu (Institute of Plant Protection of Chinese Academy of Agricultural Science). The standard samples of TAP (99.0% pure analytical grade) were provided by the Tianjing orient green technology Co., Ltd (Jiangshu province, China). TAP was dissolved in acetone as a stock solution (50 g/l). Medium for enrichment (EM) contained 10 g peptone, 1.0 g glucose, 1.0 g NaCl, and 1.0 g KH₂PO₄ in 1 L of deionized water. Medium for isolation (IM) contained 1.0 g NH₄NO₃, 0.5 g MgSO₄, 0.5 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.5 g NaCl, and 1.5g K₂HPO₄ in 1 L of deionized water.

In order to investigate whether isolates can use TAP as the sole carbon source, the TAP stock solution was added to IM medium at a final concentration of 50 mg/l. The solid medium contained 2% agar. The pH of all media was adjusted to 7.2 to 7.4 with 10 M NaOH or 2

M HCl. All other chemicals were of analytical grade and obtained from Shanghai Sango Biological Engineering Technology and Services Co., Ltd (Shanghai, China). The soil (100 g) was sampled from the top layer (0 to 10 cm) at rice field in Changsha, Hunan, China. This field has utilized OPs for pest prevention over 5 years.

Enrichment and isolation of TAP-degrading strain TDB-1

Soil samples (10.0 g) were added to EM medium containing 50 mg/l TAP and incubated at 30°C in a rotary shaker at 180 rpm. 10 ml of the culture broth was transferred daily to 90 ml of fresh EM medium, while its TAP concentration increased doubly. After the fourth enrichment transfer, each culture (10 ml) was centrifuged to get the pellets and was used to inoculate the corresponding plates of EM solid medium with TAP (100 mg/l) and incubated at 30°C until visible growth appeared. The biggest colony in plate was selected and named TDB-1. Strain TDB-1 was pre-cultured in EM medium and monitored by diluted plate method. The starting number of cells for the following tests was adjusted to approximate 2×10^9 cfu (colony forming unit)/ml.

Identification of TAP-degrading strain TDB-1

Strain TDB-1 was identified based on its morphological, physical and biochemical properties according to Bergey's Manual of Determinative Bacteriology (George et al., 1994). The genomic DNA was extracted as described previously (Seo et al., 2007) and 16S rDNA gene was amplified using polymerase chain reaction (PCR) with primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTGTACGACTT-3'). The PCR products were purified and sequenced by BGI Life Tech Co., Ltd. The homology was analyzed by BLAST in GenBank (<http://blast.ncbi.nlm.nih.gov/>). Phylogenetic relationships were analyzed by the evolutionary distance matrix calculated by the neighbor-joining method and a neighbor-joining tree was constructed using MEGA5.0 (Tamura et al., 2011).

Effect of different factors on the growth of strain TDB-1 under TAP pressure

Factors affecting growth of strain TDB-1 under TAP pressure such as TAP concentration (50 to 500 mg/l), pH (4 to 10), temperature (5 to 45°C) and initial inoculation (0.5 to 8% (v/v)) were investigated. To determine the effect of TAP concentration on strain TDB-1 growth, strain TDB-1 was grown in IM medium with different initial TAP concentration. The pH optimum experiments for strain TDB-1 growth were performed in different pH IM medium with TAP concentration of 100 mg/l. The temperature experiments for strain TDB-1 growth were performed when it was grown in IM medium with TAP concentration of 100 mg/l and cultured in different temperature. For evaluating inoculation on strain TDB-1 growth, the different initial inoculation was added in IM medium with TAP concentration of 100 mg/l. All of tests were in triplicate and incubated at 180 rpm. 2 ml of broth of each sample was measured spectrophotometrically at OD₆₀₀ at regular interval.

Determination of TAP degrading activity

The TAP degradation rate was determined by investigating the yield of the *p*-nitrophenol with the method mentioned previously (Yang et al., 2008). A standard curve of the relationship between the absorbance in 405 nm and the concentration of *p*-nitrophenol was established (the R^2 of the curve is 0.9916 that means the relationship is creditable, data not shown).

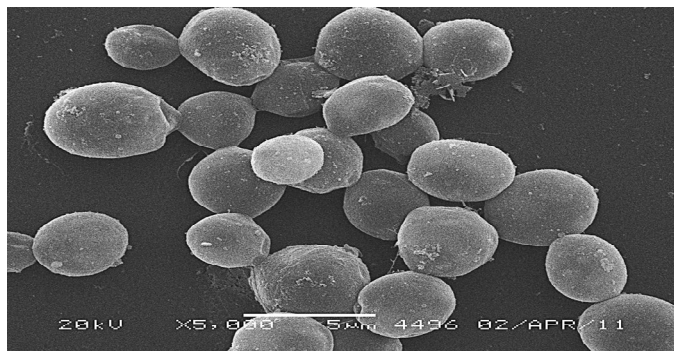


Figure 1. Scanning electron micrograph of strain TDB-1 ($\times 5,000$).

To study the degradation kinetics of TAP by strain TDB-1, first-order model (Equation 1) was used (Cycoń et al., 2009).

$$C_t/C_0 = e^{-kt} \quad (1)$$

Where, C_0 is the concentration of TAP at the initial time, C_t is the concentration of TAP at time t and the t is the degradation period in minutes. The degradation rate constant (k) was determined by measuring the C_0 and C_t .

The half-life ($t_{1/2}$) of TAP was determined using the algorithm as expressed in Equation (2).

$$t_{1/2} = (\ln 2)/k \quad (2)$$

Where, k is the rate constant (min^{-1}).

Measurement of the relative CSH

The relative CSH of strain TDB-1 in TAP biodegradation was tested by a modified microbial adherence to hydrocarbon (MATH) methods (Zhang et al., 2009; Canganella et al., 2011) with slight modifications. Briefly, 3 ml of broth was harvested by centrifugation (4,000 rpm, 10 min, 4°C), washed twice with 50 mmol/l phosphate buffer (22.2 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 7.26 g KH_2PO_4 per liter of deionized water, pH 7.0), and resuspended with the same buffer to give a final concentration of cells of approximately OD_{600} value at 0.6. Aliquots of the suspension was added to glass tubes and overlaid with 1 ml of *p*-xylene. The resultant aqueous/organic mixtures were mixed by vortexing for 2 min. After equilibration for 40 min, the contaminating xylene was removed by bubbling air (2 ml/s). The lower aqueous phase was then carefully removed and its absorbance at 600 nm was measured. The CSH value was calculated as the percentage of the decrease in absorbance of the aqueous phase before and after mixing with *p*-xylene, using the following equation: $\text{CSH}(\%) = 100 \times (A_i - A_f)/A_i$; where, A_i is the initial optical densities at 600 nm of the aqueous phase; A_f is the final optical densities at 600 nm of the aqueous phase.

RESULTS AND DISCUSSION

Isolation and identification of degradation strain TDB-1

Strain TDB-1 was sphere (Figure 1) and Gram negative

(G⁻) and it was positive for indole production, citrate utilization, reduction of nitrate, Voges-Proskauer test and methyl red test. The 16S rDNA sequence of strain TDB-1 was 1505 bp and was deposited in the GenBank (accession number: JQ068819), and has the highest similarity to that of the reference sequence of *Klebsiella oxytoca* (AB353046) and was in the same cluster by phylogenetic analysis (Figure 2). Based on these observations, the isolate was tentatively identified as *K. oxytoca*. Several bacterial strain for degrading OPs (including TAP) were isolated (Lin and Yuan, 2005; Zhang et al., 2002; Concepción et al., 2012), and as far as our knowledge, they did not include strain of *K. oxytoca*.

Effect of different factors on growth of strain TDB-1 under TAP pressure

The growth of bacteria was effected by different factors such as pH, temperature, initial pollutant concentration and initial inoculation, and biomass has a positive relation to biodegradation (Zhang et al., 2009). It is necessary to understand the effects of these factors to growth of strain TDB-1, to design efficient conditions to enhance TAP degradation.

As shown in Figure 3a, strain TDB-1 grow well from 25 to 35°C and the optimal temperature was 35°C. The comparative low and high temperature (< 15°C and > 45°C) almost completely inhibited the growth of strain TDB-1.

The excellent tolerance of strain TDB-1 to pH is indicated in Figure 3b. The growth of strain TDB-1 was scarcely inhibited from pH 5.0 to 9.0, and optimal pH was 7.0. Interestingly, even in extreme pH value of 4.0 and 10.0, only about 3 h delay of entering logarithmic phase was displayed. Although many bacterial strain are well tolerant to pH, but some strains of acidophile or alkaliphile, cannot commonly degrade pollutants (Canganella et al., 2011). This result implies that strain TDB-1 is extraordinary potential for TAP bioremediation in extreme pH environment.

The effects of different initial concentration of TAP on growth were measured and the results are shown in Figure 3c. The growth of strain TDB-1 was inhibited as much as the initial TAP concentration increased. Strain TDB-1 showed the highest growth activity under 100 mg/l of TAP concentration. This result was consistent with previous study (Cycoń et al., 2009; Wang et al., 2005), which reveal that the toxicity of OPs and its metabolites was depended on its concentration.

The results of the initial inoculation (Figure 3d) indicated that more rapid growth was displayed as initial inoculation increased. Previous study indicated that inoculation is an important factor that determines growth, and the efficiency of degradation of target pesticides was dependent on the biomass, as a higher inoculation could compensate for the initial population decline and so enhanced the degradation (Cycoń et al., 2009). Figure 3d also shows

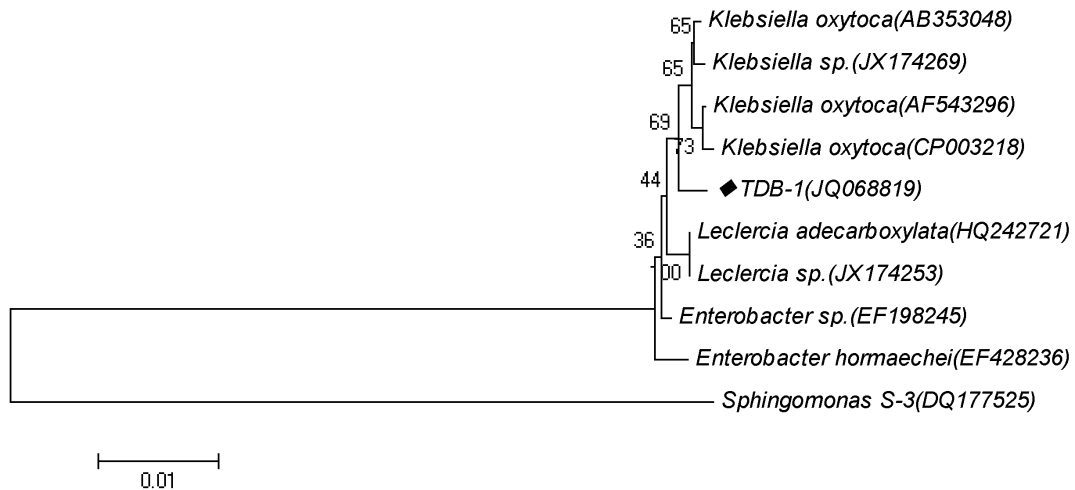


Figure 2. The phylogenetic tree of strain TDB-1 based on 16S rDNA gene sequence analysis, *Spingomonas S-3* was selected as the out group to root tree.

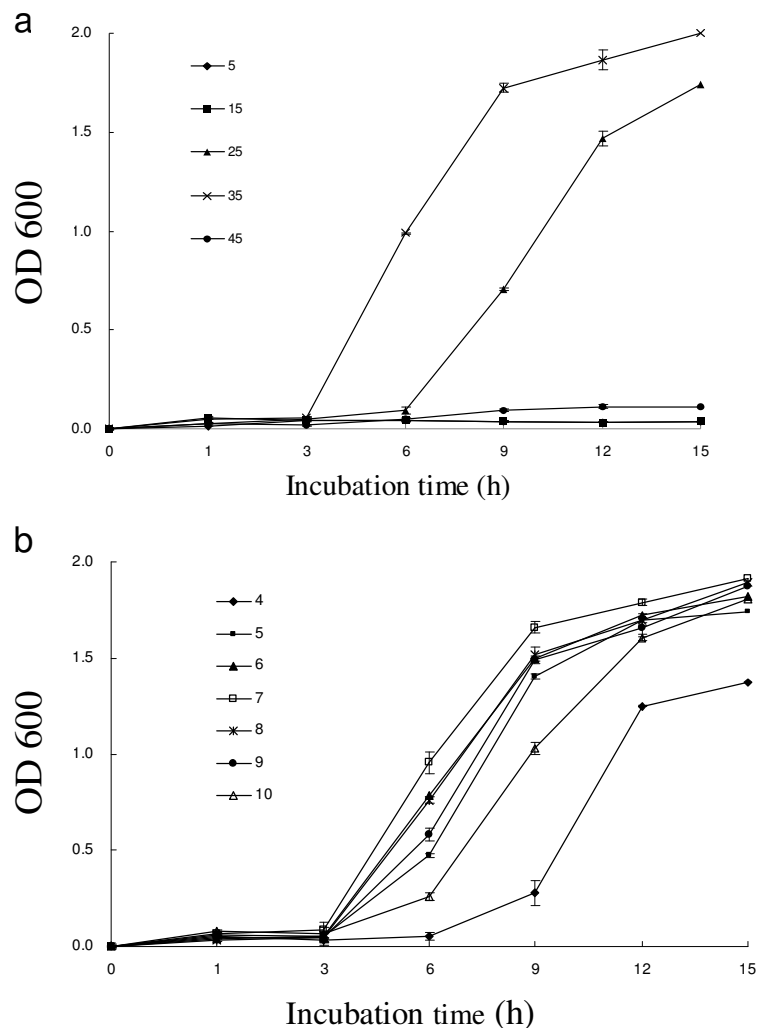
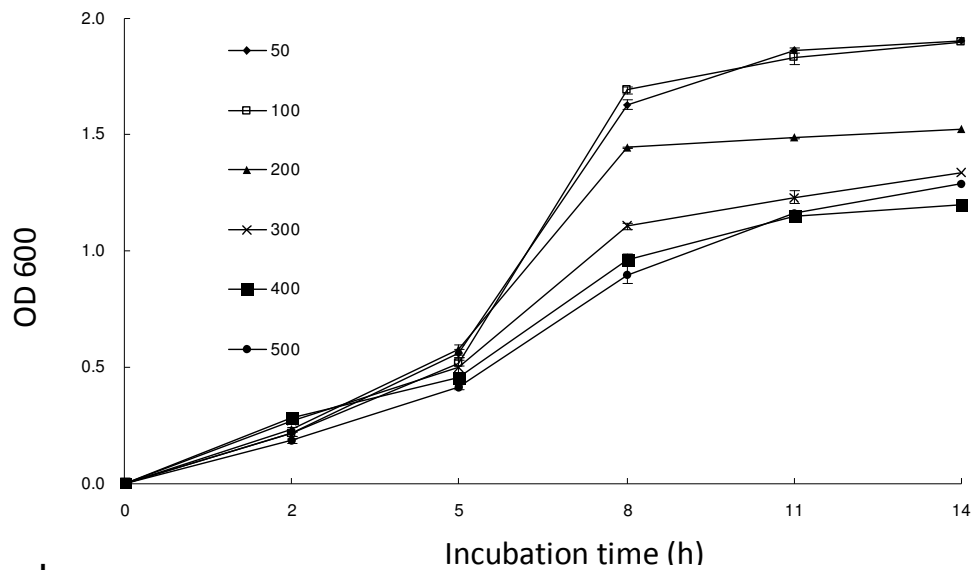


Figure 3. Effect of different factors on growth of strain TDB-1. (a) Temperature (°C); (b) pH;

Figure 3. Contd

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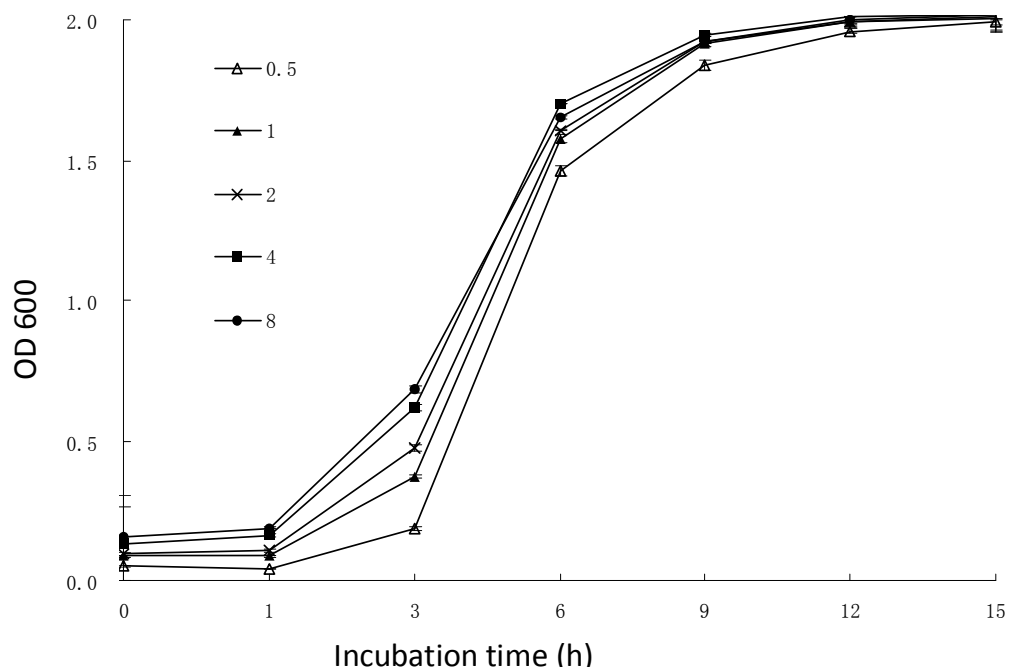


Figure 3. Effect of different factors on growth of strain TDB-1. (c) initial TAP concentration (mg/l) and (d) Inoculation (v/v).

that there was no obvious difference in biomass (OD₆₀₀) in inoculation over 2% (v/v), indicating that higher inoculation limited growth, probably because of shortage

of nutrient and mutual competition of the organisms themselves (Chen et al., 2011). The optimal inoculation was 2% (v/v).

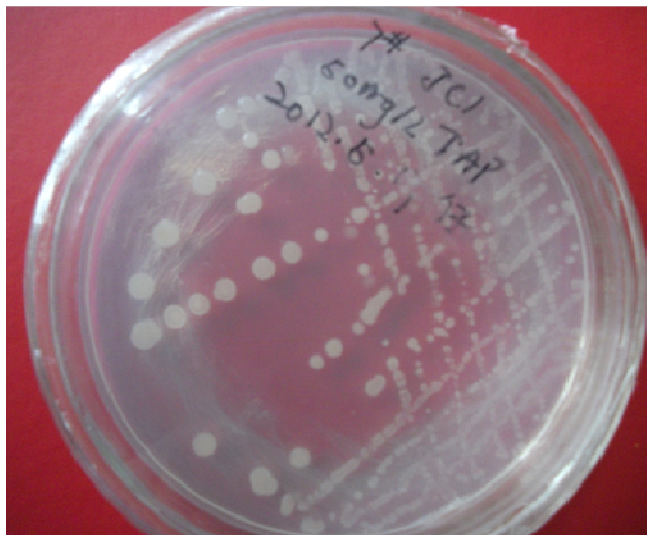


Figure 4. Strain TDB-1 growth in plate using 100mg/l of TAP as sole carbon.

Table 1. Kinetic parameters of TAP degradation by strain TDB-1

Treatment (min)	K (min ⁻¹)	t _{1/2} (min)	R ²
CK	0.00141	491.5937	0.9415
Strain TDB-1	0.06757	10.2582	0.9212

Determination of TAP degrading activity

Strain TDB-1 could utilize TAP as sole carbon, because of its growth on IM medium (remove carbon source); using 50 mg/l of TAP as sole carbon (Figure 4). This result shows that TAP of strain TDB-1, has detoxifying capacity and further study of gene(s) of detoxification enzyme(s) to show the detoxification mechanism of TAP should be done.

Strain TDB-1 would be capable of utilizing TAP as sole carbon (data not shown), which shows its potential in TAP bioremediation. The validation of the first-order kinetics was tested and is shown in Table 1. The TAP degradation process by strains TDB-1 was characterized and k and t_{1/2} was 0.06757 and 10.2582 min, respectively. A non-inoculated control showed that the k and t_{1/2} was 0.00141 and 491.5937 h (Table 1). This result shows that strain TDB-1 could efficiently degrade TAP.

Measurement of the relative CSH

The CSH of strain TDB-1 during growth on different TAP concentration was tested. Figure 5 shows the time-variation of CSH in 0, 100, 200 mg/l TAP, respectively. Without supplying TAP, the MATH increased

to 28.2%, but then decreased quickly and remained around 5%. When 100 mg/l TAP was added, the MATH increased to a maximum of about 27.2%. And then, a slight increase occurred after a short decrease. After that, the MATH remained constant at around 20%. However, with concomitant increase in the TAP concentration from 100 to 200 mg/l, the maximum hydrophobicity rose to 50.9%, while a decrease occurred after reaching the maximum hydrophobicity. The decrease was just for a short time, and the MATH increased to 34.9% and kept around 30%. On the whole, the higher the concentration of TAP was, the bigger the MATH of strain TDB-1 was. It coincides with that of *Rhodococcus* sp. CN6 during growth on *p*-nitrophenol (Zhang et al., 2009). The higher CSH implied more adherence ability of the pollutants to the cell surface of the biodegrader, so the high CSH of strain TDB-1 can enhance its biodegradation. The CSH is dependent on the constituents of the cell outer membrane, and also is influenced by environment factors (Roseberg et al., 1980). So, further studies of strain TDB-1 would enhance its potential application in TAP bioremediation.

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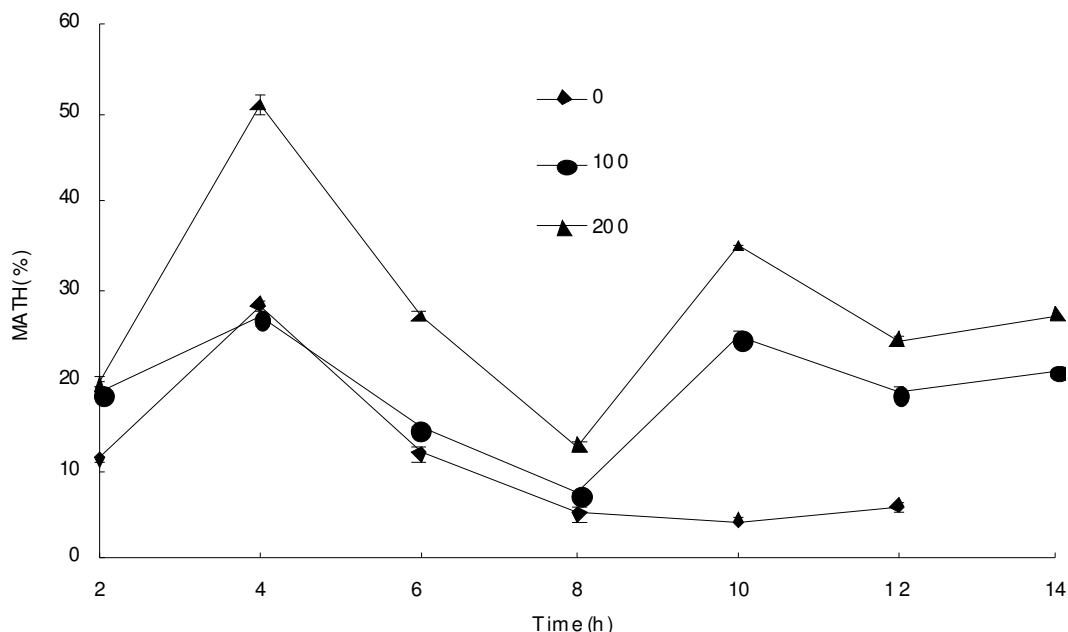


Figure 5. Hydrophobicity (as MATH%) of strain TDB-1 in degrading different TAP concentration (mg/l).

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REFERENCES

- Alexis, JH, Li C, Ian, CS (2001). Thermal stability of organophosphorus pesticide triazophos and its relevance in the assessment of risk to the consumer of triazophos residues in food. *J. Agric. Food Chem.* 49: 103-106
- Anderson BS, Hunt JW, Phillips BM, Nicely PA, Vlaming de V, Connor V, Richard N, Tjeerdema RS (2003). Integrated assessment of the impacts of agricultural drainwater in the Salinas River (California, USA). *Environ. Pollut.* 124: 523-532.
- Bastiaens L, Springael D, Wattiau P, Harms H, Wachter R, Verachtert H, Diels L (2000). Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. *Appl. Environ. Microbiol.* 66: 1834-1843.
- Canganella F, Wiegel J (2011). Extremophiles: from abyssal to terrestrial ecosystems and possibly beyond. *Naturwissenschaften* 98: 253-279
- Chen SH, Hu MY, Liu JJ, Zhong GH, Yang L, Rizwan-ul-Haq M, Han HT (2011). Biodegradation of beta-cypermethrin and 3-phenoxybenzoic acid by a novel *Ochrobactrum lupini* DG-S-01. *J. Hazard. Mater.* 187: 433-440.
- Concepción CF, Edgar DG, Alejandra VR, Raunel TV, Rafael DM, Enrique SS, Ma-Luisa CG, Fernando RQ, Laura OH (2012). Isolation of the opdE gene that encodes for a new hydrolase of *Enterobacter* sp. capable of degrading organophosphorus pesticides. *Biodegradation*, 23:387-397
- Cycoń M, Wójcik M, Piotrowska-Seget Z (2009). Biodegradation of the organophosphorus insecticide diazinon by *Serratia* sp. and *Pseudomonas* sp. and their use in bioremediation of contaminated soil. *Chemosphere*, 76: 494-501.
- George, MG, Julia, AB, Timothy GL (1994). *Bergey's Manual of Determinative Bacteriology*, New York: Springer, pp. 473-476.
- Hayes, AL, Wise, RA, Weir, FW (1980). Assessment of occupational exposure to organophosphates in pest control operators. *Am. Ind. Hyg. J.* 41: 568-575.
- Hreljac I, Filipič M (2009). Organophosphorus pesticides enhance the genotoxicity of benzo(a) pyrene by modulating its metabolism. *Mutat. Res.* 671: 84-92.
- Jokanović M, Kosanović M (2010). Neurotoxic effects in patients poisoned with organophosphorus pesticides. *Environ. Toxicol. Pharmacol.* 29: 195-201.
- Kaloyanova FP, El-Batawi MA (1991). Organophosphorus compounds Human Toxicity of Pesticides. CRC Press, Boca Raton, FL. pp. 3-33.
- Li W, Qiu SP, Wu YJ (2008). Triazophos residues and dissipation rates in wheat crops and soil. *Ecotoxicol. Environ. Saf.* 69:312-316
- Lin KD, Yuan DX (2005). Degradation kinetics and products of triazophos in intertidal sediment. *J. Environ. Sci.* 17: 933-936.
- Mearns J, Dunn J, Lees-Haley PR (1994). Psychological effects of organophosphate pesticides: a review call for research by psychologists. *J. Clin. Psychol.* 50: 286-293.
- Rosenberg M, Gutnick D, Rosenberg E (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS. Microbiol. Lett.* 9: 29-33.
- Rovedatti MG, Castañé PM, Topalián ML, Salibián A (2001). Monitoring of organochlorine and organophosphorus pesticides in the water of the Reconquista. *River. Wat. Res.* 35: 3457-3461.
- Sawaya WN, Al-Awadhi, FA, Saeed T, Al-Omair A, Husain A, Ahmad N, Al-Omirah H, Al-Zenki S, Khalafawi S, Al-Otaibi J, Al-Amiri H (2000). Dietary intake of organophosphate pesticides in Kuwait. *Food. Chem.* 69: 331-338.
- Seo JS, Keum YS, Harada RM, Li QX (2007). Isolation and characterization of bacteria capable of degrading polycyclic aromatic hydrocarbons (PAHs) and organophosphorus pesticides from PAH-contaminated soil in Hilo, Hawaii. *J. Agric. Food. Chem.* 55: 5383-5389.
- Steven BB, Tara DS, Chip G, John O, Colin S, Michael D (2008). OpdA, a bacterial organophosphorus hydrolase, prevents lethality in rats after poisoning with highly toxic organophosphorus pesticides. *Toxicology* 247: 88-92.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28: 2731-2739.

- Wang LH, Zhang L, Chen HL, Tian QG, Zhu GN (2005). Isolation of a triazophos-degrading strain, *Klebsiella* sp. E6 effectively utilizing triazophos as sole nitrogen source. FEMS. Microbiol. Lett. 253: 259-265
- Yang C, Zhao Q, Liu Z, Li QY, Qiao CL, Mulchandani A, Chen W (2008). Cell Surface Display of Functional Macromolecule Fusions on *Escherichia coli* for Development of an Autofluorescent Whole-Cell Biocatalyst. Environ. Sci. Technol. 42: 6105-6110
- Zhang JS, Sun ZT, Li YY, Peng X, Li W, Yan YC (2009). Biodegradation of *p*-nitrophenol by *Rhodococcus* sp. CN6 with high cell surface hydrophobicity. J. Hazard. Mater. 163: 723-728.
- Zhang SB, Zhang DY, Liu Y, Luo XW, Cheng FX, Luo YH (2009). Isolation, identification and degrading gene cloning of a pyrethroids-degrading bacterium. Acta. Microbiologica. Sinica. 49: 1520-1526.
- Zhang ZL, Hong HS, Wang XH, Lin JQ, Chen WQ, Xu L (2002). Determination and load of organophosphorus and organochlorine pesticides at water from Jiulong River Estuary, China. Mar. Pollut. Bull. 45: 397-402.