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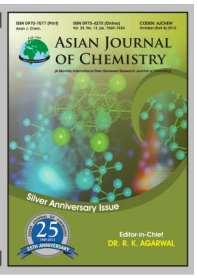

Characterization and Growth Optimization of a Novel Salt Resistant *Pseudomonas putida* Strain Zm Capable of Degrading Trichloroethylene by Cometabolism Under Aerobic Conditions

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A novel toluene-oxidizing strain capable of cometabolizing trichloroethylene was isolated and characterized. Combined with 16S rRNA sequence analysis, it was identified as *Pseudomonas* sp. It could grow with toluene as the sole carbon source. The analysis of the growth curve showed that Zm could reach the logarithmic phase within 8-10 h. Continuous sampling showed that toluene was exhausted within 1 d. Finally 29.1 % of trichloroethylene (50 mg/L) can be removed after incubation in mineral salt medium in the presence of 200 mg/L toluene for 3 d and 62.7 % for 7 d. The optimization of growth conditions was also conducted, the optimal growth conditions as the follows: carbon source toluene, at the concentration of 200-400 mg/L, medium pH 7, temperature 28 ºC and shaking speed 200 rpm. The tested strain Zm also showed substantial salt resistance including at 3.5 % NaCl concentration, indicating the potential application in bioremediation of coastal and marine trichloroethylene contaminants.

Key Words: Aerobic cometabolism, Biodegradation, *Pseudomonas putida*, Trichloroethylene, Salt tolerance.

INTRODUCTION

Trichloroethylene (TCE) is a halogenated volatile organic compound (VOC), which is believed to be carcinogenic and mutagenic[1]. It is considered a primary pollutant with 5 µg/L maximum contamination level allowed in drinking water.

Trichloroethylene has unique solvent properties which make it suitable for a wide range of industrial applications such as textile processing, refrigeration, lubricants and adhesives, along with the production of vinyl chloride, pharmaceuticals and insecticides[1,2]. Due to inappropriate disposal methods that are carried out, soil and groundwater contamination by trichloroethylene has become widespread. And this makes trichloroethylene become a recalcitrant groundwater pollutant[3].

The toxic effects associated with trichloroethylene have raised a serious public concern over the extensive contamination problems and knowledge of its effective remedial process will be highly valuable. Several methods are available for remediation of trichloroethylene pollution, including physical processes, chemical control methods and bioremediation. Of these processes, bioremediation technology is preferred over other technologies because it allows for complete mineralization of trichloroethylene to harmless chemical forms, including carbon dioxide, water and chlorine[4].

Up to date, although some researchers found direct degradation of trichloroethylene by some special organisms, such as endophyte of hybrid poplar[5], the co-metabolic degradation of trichloroethylene was recognized as the most efficient mechanism[6]. Besides methane-oxidizing bacteria[7,8], toluene oxidizers and phenol oxidizers have also received tremendous attention[9,10]. These bacteria produce oxygenases in response to phenol[11] or toluene, which initiate the oxidative degradation and mineralization of trichloroethylene. Among the toluene oxidizers, the *Pseudomonas putida* strains F1 and *Burkholderia cepacia* G4 was widely employed to study the co-metabolic degrading of trichloroethylene[6,12,13].

Toxicity and inaccessibility of trichloroethylene to trichloroethylene degraders and extreme environmental limitations will hinder the bioremediation. This is the main problems encountered so far. To overcome this problem screening of strains with more advanced technologies from a wider range of habitats is essential. For remediation of marine ecosystem, salt resistance to some degree might be also essential.

EXPERIMENTAL

NB medium (/L) contains 10 g peptone, 5 g beef extract and pH value was adjusted to 7.0. NA plates were prepared with NB medium with 15 g/L agar.

MSM (/L) contains 1.0 g KH_2PO_4 , 1.0 g K_2HPO_4,1.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$; 0.05 g $Fe_2(SO)_3$ and 0.05 g; $CaCO_3$ 0.02 g and pH value was adjusted to 7.0. Each of 120 mL serum bottles for fermentation contains 36 mL NB, MSM medium. All the media and materials are autoclaved at 121ºC for 20 min.

Characterization of Zm: Pure culture was stocked into a 1.5 mL eppendorf with the 20 % glycerol at -20 ºC. A re-streak with pure culture stock was performed on NB plate at 37 ºC. After activation, Zm were submitted to plate culture and observation and physiological studies[14]. The 16S rRNA PCR and sequence analysis was carried out using modified protocols of reference[15]. The total DNA was extracted by DNA extraction kit from Hangzhou Sigmens Bioengineering Co. Ltd. (China). Two primers annealing at the 5' and 3' end of the 16 S rRNA gene were:

Forward primer (F): 5' AGA GTT TGA TCC TGG CTCAG 3'
Reverse primer (1492R): 5' TAC GGY TAC CTT GTT ACG ACT T 3'

The PCR was carried out accordingly. PCR products were sent to Shanghai Sagon Bioengineering Co. Ltd for sequencing. Afterwards, the partial rDNA sequences were analyzed using a BLAST search algorithm to estimate the degree of similarity to other rDNA sequences obtained from the NCBI/GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and the phylogenetic tree was also drawn using strains in the GenBank.

Acclamation to toluene: Zm pure culture was acclamated as the following procedures: it was enriched in the NB medium at 37 ºC for 24 h, then centrifuged to collect the cells, suspended in normal saline and inoculated in MSM plus 50 mg/L toluene and cultured at 28 ºC for 72 h as the first subculture, 4 mL inocula of the first subculture was inoculated in MSM plus 100 mg/L toluene and cultured at 28 ºC for 72 h as the second subculture and then inoculated in that plus 200 mg/L and culture at the same conditions as third subculture. Afterwards, subculture was conducted every week.

Growth curve determination: The 3rd subculture was inoculated in 150 mL serum bottles containing MSM plus 200 mg/L toluene and cultured on shaking table at 28 ºC. The culture broth was sampled every 4 h and the $OD_{600\ nm}$ of the broth was determined. The growth curve was drawn with Origin 8.0 software.

General procedure

Evaluation of bioremoval efficiency: Exact 4 mL of each acclamated pure culture was inoculated to bottles containing 36 mL MSM (according to the results of 1.2) plus 200 mg/L toluene and 50 mg/L trichloroethylene and cultured at 28 ºC and 150 rpm for 24, 48, 72, 96, 120 and 168 h to evaluate the removal efficiency and biodegradation rate of trichloroethylene. Controls were set without inoculation.

Optimization of cultivation conditions: (1) Carbon sources: Exact 4 mL of pure culture was inoculated to bottles containing 36 mL MSM plus 200 mg/L toluene, methanol, acetone and phenol and cultured at 28ºC and 200 rpm for 24 h. All the cultures was acquired by acclamation with corresponding carbon source individually as 1.3. The $OD_{600\ nm}$ of the broth was determined individually. (2) Concentration of toluene: Exact 4 mL acclamated pure culture was inoculated to bottles containing 36 mL MSM plus 200, 300, 400, 500 and 600 mg/L toluene and cultured at 28 ºC and 200 rpm for 24 h. The $OD_{600\ nm}$ of the broth was determined individually. (3) pH value: Exact 4 mL acclamated pure culture was inoculated to bottles containing 36 mL MSM plus 200 mg/toluene, with the pH value adjusted to 3, 5, 7, 9, 11, 12 and cultured at 28 ºC and 200 rpm for 24 h. The $OD_{600\ nm}$ of the broth was determined individually. (4) Salinity: Exact 4 mL acclamated pure culture was inoculated to bottles containing 36 mL MSM plus 200 mg/toluene, with the NaCl adjusted to 0.5, 3.5, 8, 10, 12.5 and 15 % and cultured at 28 ºC and 200 rpm for 24 h. The $OD_{600\ nm}$ of the broth was determined individually. (5) Temperature: Exact 4 mL acclamated pure culture was inoculated to bottles containing 36 mL MSM plus 200 mg/L toluene and 50 mg/L trichloroethylene and cultured at 15 ºC, 20 ºC, 25 ºC, 28 ºC, 30 ºC, 35 ºC, 45 ºC and 200 rpm for 24 h. (6) Dissolved oxygen concentration: The dissolved oxygen concentration was adjusted by shaking speed of shaker. Exact 4 mL acclamated pure culture was inoculated to bottles containing 36 mL MSM plus 200 mg/L toluene and 50 mg/L trichloroethylene and cultured at 28 ºC and 140, 160, 180, 200 and 220 rpm for 24 h.

Detection method: Residue trichloroethylene and toluene contents were measured by gas chromatograph (GC, Agilent, 6890N, Agilent Technologies Co., Ltd, China) equipped with a flame ionization detector (FID) and a capillary column (HP-5; 30 m × 0.53 μm I.D. with a stationary-phase film thickness of 0.88 μm). Samples were withdrawn in accordance to the sampling scheme using sterilized syringes. About 1 mL of the liquid sample was then injected into the amber vial (WHEATON) and secured with PTFE/silicone liner (WHEATON) using crimper. One microliter of liquid samples was injected by the autosampler injector (7638 Series, Agilent Technologies Co., Ltd., China) equipped with a tapered microsyringe (5181-1267, Hamilton Company, USA).The tapered microsyringe in the autosampler was preinjected with solvent (ethanol) twice before the injection of the sample into the GC and two post-injections were done after the injection. Nitrogen was used as carrier gas at a flow rate of 20 mL/min. The flow rates of hydrogen gas and synthetic air were 40 and 400 mL/min, respectively. The inlet and detector temperatures were 260 and 280 ºC, respectively. The column temperature was programmed as initial temperature, 40 ºC (hold for 2 min), then incrementally increased at 12 ºC/min to 300 ºC, then hold for 10 min.

RESULTS AND DISCUSSION

Characterization of strain Zm: Zm could form round colonies *ca.* 3 mm in diameter on beef-peptone agar plate and blood agar plate, but cannot form clear haemolytic circles after cultivation for 2 d on the blood agar plates. Zm was gram-negative and rod-shaped bacteria. The sequencing analysis of Zm using the 16 S rRNA gene nucleotide sequences data showed that this strain had the highest homology (over 99 %) with *Pseudomonas putida* or *Pseudomonas plecoglossicida* (Fig. 1).

Combined with other results of morphological, physiological and biochemical tests (Table-1), Zm was identified as *Pseudomonas putida*. This strain could grow well in 10 % NaCl supplemented NB, showing good salt resistance.

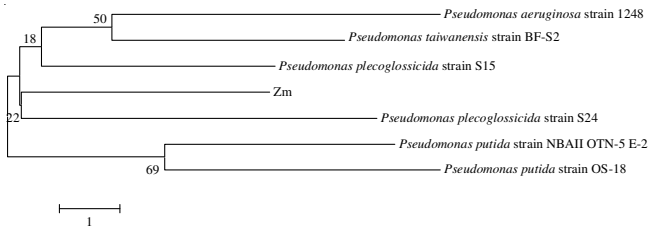

Fig. 1. Neighbor-joining trees based on the sequences of 16 S rRNA gene. [Numbers on the tree represent bootstrap value (1000 replications)]

TABLE-1
OTHER MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL IDENTIFICATIONS OF Zm

Tested items	Results
Endospore	–ve
Flagella	+ve, mobile
Pigment producing in NB	–ve
Oxydase	+ve
Contact enzyme	+ve
Aerobic/anaerobic	Aerobic
Citrate utilization test	+ve
sodium malonate utilization	+ve
Tartrate utilization	+ve
Nitrate reduction test	+ve
Glucose fermentation	+ve, acid produced
Lactose fermentation	+ve, acid produced
Lactic acid fermentation	+ve, acid produced
Sorbitol fermentation	+ve, acid produced
Dulcitol fermentation	+ve, acid produced
Arabinose fermentation	–ve
Esterase	–ve
Urease	+ve
V.P.	–ve
Methyl red test	–ve
Glutin liquefaction	+ve
NB + 0.5 % salt	+ve
NB + 2 % salt	+ve
NB + 4 % salt	+ve
NB + 6 % salt	+ve
NB + 8 % salt	+ve
NB + 10 % salt	+ve

Growth curve: The growth curve in MSM plus toluene was determined by continuous samplings. It could be concluded from Fig. 2 that based on optical density at 600 nm, the Zm showed similar growth curve with *Escherichia coli*. It could reach the log phase at about 8h and death phase at about 20 h.

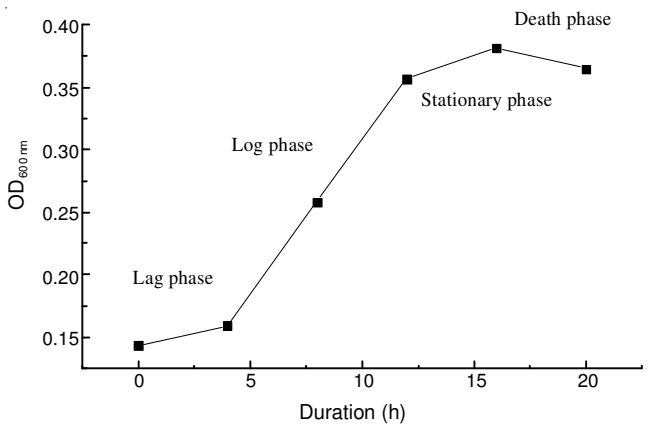

Fig. 2. Growth curve in MSM plus toluene

Then in the following experiments, all the acclamated pure cultures were inoculated at 8 h.

Degrading characteristics study: Table-2 showed that toluene as the substrate was consumed very quickly and was used up within 24 h. The residual trichloroethylene decreased slowly. About 29.1 % of trichloroethylene (50 mg/L) can be removed after incubation in mineral salt medium in the presence of 200 mg/L toluene for 3 d and 62.7 % for 7d, indicating that although the toluene was exhausted, the dioxygenase produced by Zm were still working for degradation of trichloroethylene.

Optimization of growth conditions

Effect of medium conditions on growth of Zm: Fig. 3 shows different medium conditions, *i.e.*, carbon sources, toluene concentrations, initial pH value of MM and NaCl concentration, on growth Zm measured by optical density value at 600 nm. It can be concluded from Fig. 3a that toluene was the best sole carbon source, significantly differing from the other three organic matters. Phenol could also work as the sole carbon source, but the growth of Zm was much slower than using toluene. Methanol and acetone were not suitable carbon source for Zm.

Toluene concentration also influenced the growth of Zm, but not so obviously as carbon source did. Judging from Fig. 3b, with the rising of toluene concentration, the optical density values also rose, reaching the climax at 400 mg/L. Then with the rising of toluene concentration, the growth went down sharply. It seemed that 200-400 mg/L toluene were all appropriate concentrations for Zm growth, with no significant differences. To shorten the acclamation period, 200 mg/L were preferred.

It can be seen from Fig. 3c that pH value of MM also influenced the growth of Zm. Neutral conditions of MM (pH = 7) were the best conditions for Zm.

Fig. 3d illustrates the changing trend of OD values at 600 nm of Zm when subjected to different NaCl concentrations. With the rising of NaCl concentration from 0.5 to 3.5 % (the average salinity of the ocean), the optical density value didn't change remarkably, dropping from 0.42 to 0.35, indicating that Zm could grow well in seawater prepared medium and thus good potential of employment in bioremediation of toluene and trichloroethylene in marine ecosystem. Even at higher concentrations (> 8 %), Zm could hardly grow.

Effect of environmental conditions on growth of Zm: Environmental factors especially temperature and dissolved oxygen concentration are also considered important for the growth of environmental bacteria. As shown in Fig. 4A, with the continuous increasing of temperature, optical density value rose to climax at 28 ºC and then dropped but there were no significant differences among 25, 28, 30 and 35 ºC, showing that Zm could grow rigorously at the temperature range at 25-30 ºC. But at 45 ºC, the optical density value dropped significantly to below 0.1, indicating Zm could not grow at normal speed at high temperature at 45 ºC or above. But dissolved oxygen concentration (shaking speed) didn't influence the growth significantly, although the $OD_{600\ nm}$ kept increasing with increment of shaking speed (Fig. 4B). Then 200 rpm should be a suitable speed for cultivation of Zm.

TABLE-2
BIOREMOVAL EFFICIENCY OF TOLUENE AND TRICHLOROETHYLENE BY Zm

	Toluene bioremoval efficiency (%)	TCE concentration (mg/L)	TCE removal efficiency (%)		
			Total	Biotic	Abiotic
1dck	100	26.5	47.0	0.0	47.0
1d	100	23.0	54.0	13.3	40.8
3dck	100	25.7	48.5	0.0	48.5
3d	100	18.2	63.5	29.1	34.4
5dck	100	26.8	46.4	0.0	46.4
5d	100	17.0	66.0	36.5	29.4
7dck	100	25.0	50.1	0.0	50.1
7d	100	9.3	81.4	62.7	18.7

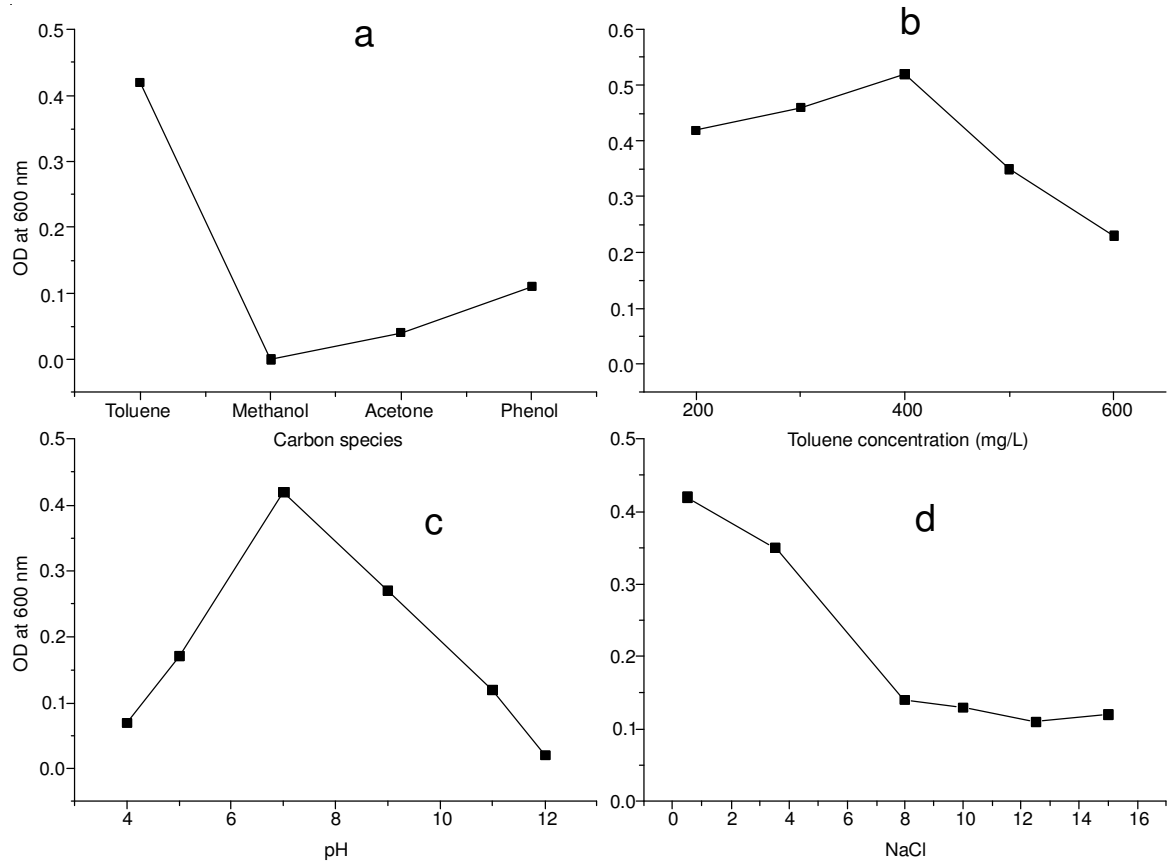

Fig. 3. Effect of medium conditions on growth of Zm. (a) Carbon sources, (b) Toluene concentration, (c) pH, (d) NaCl concentration. All the data were average values of triplicates. To make the figures more concise, the error bars were not given

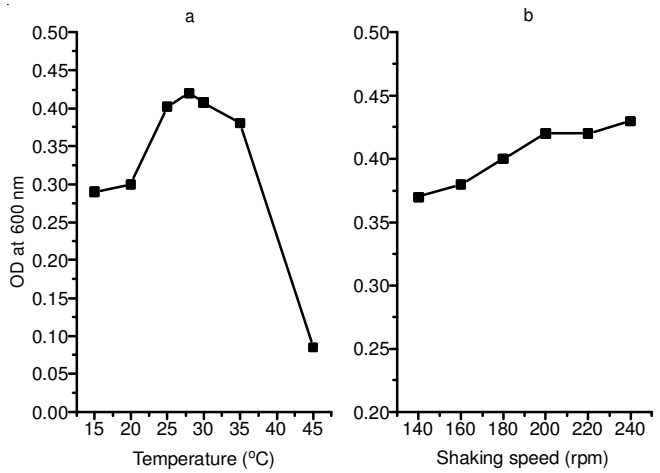

Fig. 4. Effects of environmental conditions on growth of Zm-1. (All the data were average values of triplicates. To make the figures more concise, the error bars were not given)

Conclusion

A novel toluene-oxidizing strain Zm capable of cometabolizing trichloroethylene was isolated and identified. Combined with 16S rRNA sequence analysis, it was identified as *Pseudomonas putida*. The analysis of the growth curve showed that Zm could reach the logarithmic phase within 8-10 h. It could remove trichloroethylene efficiently using toluene as the sole carbon source. The optimal growth conditions as the follows: carbon source toluene, at the concentration of 200-400 mg/L, medium pH 7.0, temperature 28 ºC and shaking speed 200 rpm. The tested strain Zm also showed substantial salt resistance including at 3.5 % NaCl concentration.

Reliability of the experimental system: For toluene and trichloroethylene are both volatile, some researchers developed special instruments, including Erlenmeyer flasks with specifically prepared glass stoppers for cultivation of trichloroethylene co-metabolic degraders[16]. In this study, only common

silicon glass serum bottles were used. After injection of toluene and trichloroethylene, the serum bottles were quickly sealed with bottle sealed with Teflon-faced butyl rubber stoppers and aluminum crimps and inverted for cultivation to prevent possible abiotic losses of toluene and trichloroethylene by volatility and absorption of glass. The residual trichloroethylene of the blanks kept constant (unreported in this study), the closed system was reliable.

Bioremoval efficiency: *Pseudomonas putida* is a frequently used species in environmental bioremediation[4,17]. Our *P. putida* strain Zm could grown in mineral media with toluene as the sole carbon source and 29.1 % of trichloroethylene (50 mg/L) can be removed after incubation in mineral salt medium in the presence of 200 mg/L toluene for 3 d and 62.7 % for 7 d, which indicating that this strain could remove trichloroethylene in water body efficiently in a short time. The trichloroethylene concentration used in our study was higher than most other literatures, for instance, 5 mg/L[18], 0.2-20 mg/L[10], thus it seemed that the removal efficiency of trichloroethylene by Zm was comparatively low. Next the optimization of degrading conditions (nutrimental and environmental) is essential for further improvement in trichloroethylene removal efficiency.

Importance of salt resistance of trichloroethylene degraders: Trichloroethylene contaminations sometimes take place in saline environments, including coastal wetland[19] and saline sediments, seawater and saline wastewater. Under these conditions, salt resistance of trichloroethylene degrading microorganisms shows its importance in *in situ* trichloroethylene bioremediation. However, salt resistant trichloroethylene degraders are rarely reported up to date. In present study, Zm could grow vigorously at 3.5 % NaCl concentration as well as degrading trichloroethylene very efficiently. It should be of great potential to be employed in toluene and trichloroethylene remediation in the saline environments as above and deserves further study on the mechanisms of salt resistance with toluene as growth substrate or/and trichloroethylene.

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