Biodegradation of Terephthalic Acid by *Rhodococcus biphenylivorans* Isolated from Soil

N. Suwanawat, P. Parakulsuksatid, N. Nitayapat, and W. Sanpamongkolchai

***Abstract*—Terephthalic acid (TA) is one of the main pollutants in wastewater from the polyester textile industry and extremely harmful to human health and the environment. Eighty-seven isolates were collected from soil around the wastewater basin of a polyester textile manufacturer in Thailand, based on their ability to grow in a minimal salt medium (MSM) containing 1 g/L TA as the sole carbon and energy source. Nineteen isolates were found to be TA-degrading bacteria. To obtain more efficient TA-degrading bacteria, the TA concentration in MSM was increased to 2 and 5 g/L. Isolate N2 showed TA-degrading ability higher than 90% and was identified as *Rhodococcus biphenylivorans* according to its morphological and biochemical properties and 16S rDNA sequence analysis. N2 degraded 99.6% of 10 g/L TA in 5 days. Optimal conditions for TA degradation were 30 °C, pH 7.0 and 200 rpm shaking speed. Results implied that *R. biphenylivorans* could be useful for future applications in bioremediation of TA in the environment.**

Index Terms*—Terephthalic acid, Polyester textile manufacturer, Biodegradation, *Rhodococcus biphenylivorans.

I. Introduction

In Thailand, polyester fiber is an important textile industry representing the highest annual man-made fiber production at 67% or 611,200 tons in 2015 [1]. The synthesis process from upstream to midstream discharges large volumes of wastewater and solid waste containing terephthalic acid and similar organic compounds as environmental pollutants [2].

Terephthalic acid or benzene-1,4-dicarboxylic acid ($C_6H_4(COOH)_2$, TA) is a raw material for the production of polyethylene terephthalate (PET) which is used in the plastics and polyester fiber industries [2]. TA is extremely harmful to human health and the environment because of its toxic properties which cause inhibition of microbial growth, bladder cancer and impair renal, liver and testicular functions [3], [4]. Therefore, widespread pollution resulting from TA discharge is closely monitored by the U.S. Environmental Protection Agency [5].

Because of its chemical characteristics, TA cannot be completely broken down and eliminated by natural processes [6]. Previous literature reported physicochemical pretreatments for degrading TA but these methods generate toxic intermediates and sludge as secondary pollutants and, in some cases, the cost of treatment is expensive [7]. Microbial degradation has recently become an attractive alternative method to remove TA with efficiency depending on the activity of the selected microorganisms. Several of these have been reported to degrade compounds such as *Pseudomonas aeruginosa* PP4 [8], *Rhodococcus pyridinivorans* and *R. rhodochrous* [9].

Here, high-capacity TA-degrading aerobic bacteria were isolated and their optimal cultivation conditions were investigated.

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II. Material and Methods

A. Reagents

Terephthalic acid (TA) with purity greater than 99% was purchased from Acros Organics (Geel, Belgium). All other chemicals used were of analytical grade.

B. Medium and Culture Conditions

The minimal salt medium (MSM) used in this study contained 1 g/L TA, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 0.02 g/L $FeSO_4 \cdot 7H_2O$ with pH 7.0. The seed medium contained 1 g/L TA, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $FeSO_4 \cdot 7H_2O$ and 0.2 g/L yeast extract, adjusted to pH 7.0. The screening medium contained 1 g/L TA, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $FeSO_4 \cdot 7H_2O$ and 18 g/L agar, adjusted to pH 7.0. All media were autoclaved at 121 ℃ for 15 min. For the liquid media, microbial cultivation was performed at 30 ℃ with shaking speed of 200 rpm.

C. Screening and Identification of Bacterial Strain

Soil samples were collected from the wastewater basin of a polyester textile manufacturer in Nakhon Pathom, Thailand. Ten grams of each soil sample were added to 100 mL of MSM containing 1 g/L TA and incubated for 5 days in an incubator shaker operating at 30 °C and 200 rpm. Then, TA-degrading bacteria were isolated on the screening medium following the serial dilution method and single colonies were obtained by streaking method.

An isolated strain with the highest TA-degrading ability was selected after cultivation on MSM containing 1, 2 and 5 g/L TA.

Morphological and biochemical characteristics of the selected strain were analyzed. DNA extraction and 16S rDNA sequence analysis were performed by Macrogen, Inc (Seoul, Korea). Polymerase chain reaction (PCR) was performed using the universal primer pairs of 785F (5'-GGATTAGATACCCTGGTA-3')/ 907R (5'-CCGTCA ATTCMTTTRACTTT-3'). Then, the 16S rDNA sequence alignment was conducted with MEGA 7.0 software by the

neighbor-joining method.

D. Biodegradation Experiments

The selected strain was cultured on MSM medium containing various TA concentrations (1, 2, 5 and 10 g/L of TA) by inoculating 2% seed culture with a concentration of 2.0×10^6 CFU/mL and incubated at 30 °C and 200 rpm for 5 days.

E. Optimization of Physical Factors for TA Degradation

An orthogonal $L_9(3^3)$ test design was adopted, including three factors (temperature: A, pH: B, and shaking speed: C) with three levels (**Table I**). The selected strain was cultured with MSM containing 1 g/L TA by inoculating 2% seed culture with a concentration of 2.0×10^6 CFU/mL for 48 h.

TABLE I: FACTORS AND LEVELS FOR THE ORTHOGONAL DESIGN

Factor	Level		
	1	2	3
Temperature (°C)	30	37	40
pH value	7	8	9
Shaking speed (rpm)	50	100	200

F. Analytical Methods

Measurement of terephthalic acid, based on the characteristic of its aromatic ring structure which can absorb UV light at 240 nm, was performed using a UV-Vis Spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific) [10]. Concentration of TA was calculated based on a standard calibration graph.

Cell concentration was determined by measuring the optical density at 600 nm.

III. RESULTS AND DISCUSSION

A. Bacteria Isolation and Identification

A total of 87 isolates from soil nearby a wastewater basin of a polyester textile manufacturer located in Nakhon Pathom, Thailand were obtained in MSM containing TA (1 g/L) as the sole carbon source. Nineteen isolates showed the ability to degrade 1 g/L TA. When TA concentration was increased to 2 and 5 g/L, only isolate N2 could grow well with TA degrading ability higher than 90%. Morphological characteristics of the strain were determined as aerobic, Gram positive, rod-shaped bacilli, non-spore-forming and non-motile. N2 colonies were smooth, pale orange-pink, convex, sticky and opaque with regular edges on the screening medium.

Biochemical characteristics of isolate N2 tested positive for catalase activity but negative for urease activity and indole production (**Table II**). Isolate N2 was able to utilize fructose, sorbitol, mannitol and arabitol as the sole sources of carbon for energy and growth. This strain also showed esculin hydrolysis activity but could not utilize citrate and malonate as a sole carbon source.

Isolate N2 was closely related to *Rhodococcus biphenylivorans* (16S rRNA gene sequence identities ranging at 99% analyzed by Macrogen, Inc). For neighbor-joining analysis, isolate N2 shared highest similarities with the group of *R. biphenylivorans* TG9, *R. gordoniae* W4937 and *R. rhodochrous* DSM43247T (supported by 83% bootstrapping) (**Fig. 1**).

R. biphenylivorans is a novel strain identified for the degradation of TA. Previously, *R. biphenylivorans* strain $TG9^T$ was reported as a Gram-positive aerobic bacterium which was isolated from a polychlorinated biphenyl (PCB)-contaminated site in China [11]. Although this bacterium has not been reported for biodegradation of PCB or other xenobiotics on conventional bacteriological media, the strain was still alive and retained metabolic activity such as naphthalene degradation, bisphenol degradation and polycyclic aromatic hydrocarbon degradation [12]. *R. biphenylivorans* has ring-cleavage pathways for bacterial catabolism of aromatic compounds that support TA degradation.

TABLE II: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATE N2

Characteristic	Result
Colonial morphology	Pale orange-pink appearance with rounded, convex, sticky, opaque and regular edges
Gram stain	Positive
Cell shape	Rod-shaped bacilli
Spore	Non-spore-forming
Mobility	Non-motile
Catalase activity	Positive
Urease activity	Negative
Indole production	Negative
Utilization as sole carbon source and acid production	
Lactose	Negative
Xylose	Negative
Maltose	Negative
Fructose	Positive
Dextrose	Negative
Galactose	Negative
Raffinose	Negative
Trehalose	Negative
Melibiose	Negative
Sucrose	Negative
L-Arabinose	Negative
Mannose	Negative
Inulin	Negative
Sodium gluconate	Negative
Glycerol	Negative
Salicin	Negative
Dulcitol	Negative
Inositol	Negative
Sorbitol	Positive
Mannitol	Positive
Adonitol	Negative
Arabitol	Positive
Erythritol	Negative
∝-Methyl-D-glucoside	Negative
Rhamnose	Negative
Cellobiose	Negative
Melezitose	Negative
∝-Methyl-D-mannoside	Negative
Xylitol	Negative
ß-galactosidase activity	Negative
Esculin hydrolysis	Positive
D-Arabinose	Negative
Citrate utilization	Negative
Malonate utilization	Negative
Sorbose	Negative

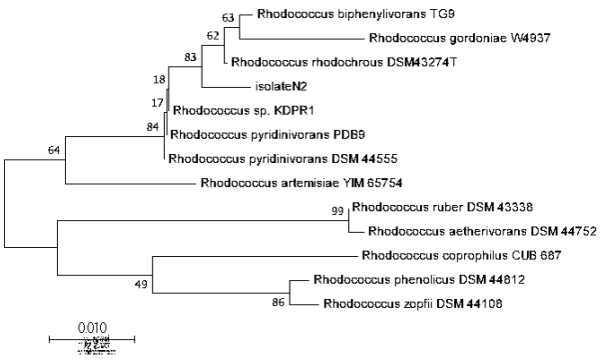

Fig. 1. A neighbor-joining analysis tree of the isolated strain (isolateN2) 16S rDNA.

B. TA Biodegradation Experiments

The effect of TA concentration (1, 2, 5 and 10 g/L) on the degrading ability of *R. biphenylivorans* is shown in **Fig. 2**. At concentrations of up to 5 g/L, TA was completely degraded within 60 h while 120 h was required to completely degrade 10 g/L TA. An acclimation of 24 h was observed at the 10 g/L TA concentration.

Increasing the TA concentration decreased the degradation extent, indicating reduced TA-degrading ability of the strain. A high initial TA concentration inhibited cell growth and the ability of the remaining cells to degrade TA [6], [13]. For example, a TA concentration greater than 1 g/L inhibited the growth and degrading ability of *Pseudomonas* sp. [6]. The ability to degrade TA by *Arthrobacter* sp. 0574 was inhibited when TA concentration was increased above 10 g/L [13]. Interestingly, cell growth and TA-degrading ability of *R. biphenylivorans* did not show any inhibitory effect at high concentration of TA such as 10 g/L, indicating that this strain had a strong ability to degrade TA compared to other reported strains.

C. Optimization of Physical Factors for TA Degradation

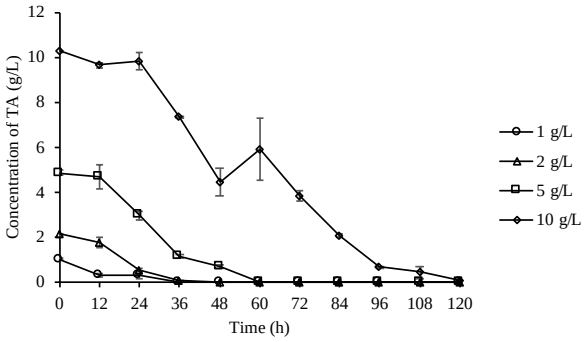

Fig. 2. Time course of TA degradation by *R. biphenylivorans* on various initial TA concentrations

To select the optimal TA degradation conditions, three physical factors (temperature, pH and shaking speed) were determined using an orthogonal $L_9(3^3)$ test design. A range analysis was performed to determine the effect of the factors. A high range value (R) implies that the factor has a strong effect on the results [14]. The orthogonal test results indicated the ranking order of the three factors that affected TA degrading ability as shaking speed > temperature > pH as shown in **Table III**. To confirm which factors had the strongest effect on TA degradation, an analysis of variance (ANOVA) was carried out and results are shown in **Table IV**. Findings also indicated that the effect of shaking speed was significantly different ($P < 0.05$). Therefore, shaking speed was the most important factor regarding the rate of TA degradation after cultivation for 48 hours in a medium containing 0.1 g/L of TA. For TA degradation, the optimal condition was 30 °C, pH 7 and shaking speed of 200 rpm. This was considered the maximum mean value of temperature, pH and shaking speed from single-factor statistical data and pairwise comparison data (data not shown).

TABLE III: RESULTS OF ORTHOGONAL TEST L9(33) OF THE STRAIN ON RATE OF TA DEGRADATION (%) FOR 48 HOURS

Treatments	Factors			Rate of TA degradation (%)
	Temperature (℃), A	pH, B	Shaking speed (rpm), C	
1	40	9	50	10.0
2	30	8	200	99.3
3	40	7	200	99.4
4	30	9	100	99.4
5	37	9	200	99.6
6	40	8	100	98.4
7	37	8	50	28.0
8	37	7	100	99.4
9	30	7	50	39.0
K_1	79.2	79.3	25.8	
K_2	75.7	75.2	99.1	
K_3	69.4	69.8	99.4	
R	9.87	9.50	73.7	
Ranking	C > A > B			

K_i = The average values of degradation rate for level i = 1, 2, 3
$R = K_i max - K_i min$; larger R value indicating more significant effects

TABLE III: VARIANCE ANALYSIS OF ORTHOGONAL EXPERIMENT OF THE STRAIN ON RATE OF TA DEGRADATION (%) FOR 48 HOURS

Variables	Sum of Squares	df	Mean Square	F- value	Sig.
Temperature, A	149.762	2	1847.661	1.119	0.472
pH, B	136.402	2	50295.538	1.019	0.495
Shaking speed, C	10799.802	2	74.881	80.670	0.012
Error	133.876	2	66.938		

R^2 = .988 (Adjusted R^2 = 0.952)

IV. CONCLUSION

We initially identified a *Rhodococcus biphenylivorans* as an attractive bacterial strain for use in microbial biodegradation of TA in TA concentrations up to 10 g/L. The effect of physical factors (temperature, pH and shaking speed) on TA degradation were investigated and the optimal conditions for degradation were found to be temperature 30 °C, pH 7.0 and shaking speed 200 rpm for 48 hours. To further develop and utilize this bacterium, the metabolic pathways or its mechanism, kinetic parameter for its growth and degradation, toxicity and others need to be studied.

REFERENCES

[1] Thailand textile institute (THTI), *Thai Textile Statistic 2015/2016*, Thailand Textile Institute, Bangkok: THTI, 2016, ch. 2, pp. 11-18.
[2] A. Ordaz-Cortés, F. Thalasso, E. Salgado-Manjarrez, and C. Garibay-Orijel, "Treatment of wastewater containing high concentrations of terephthalic acid by *Comamonas* sp. and

Rhodococcus sp.: kinetic and stoichiometric characterization," *Water Environ. J.* vol. 28, pp. 393-400, September 2014.
[3] G. Dai, L. Cui, L. Song, N. Gong, J. Chen, and R. Zhao, "Terephthalic acid occupational exposure and its effect on organ functions in fiber workers," *Environ. Toxicol. Pharm.*, vol. 20, pp. 209-214, July 2005.
[4] Z. Zhang, L. Ma, X. X. Zhang, W. Li, Y. Zhang, and B. Wu, "Genomic expression profiles in liver of mice exposed to purified terephthalic acid manufacturing wastewater," *J. Hazard Mater*., vol. 181, pp. 1121-1126, September 2010.
[5] US Environmental Protection Agency (US EPA). (March 2018). Substance Registry Services (SRS): Terephthalic acid. [Online]. Available: https://iaspub.epa.gov/sor_internet/registry/substreg/searchandretrieve/advancedsearch/externalSearch.do?p_type=CASNO&p_value=100-21-0#
[6] Z. J. Wang, L. H. Teng, and J. F. Zhang, "Study on optimal biodegradation of terephthalic acid by an isolated *Psuedomonas* sp.," *Afr. J. Biotechnol.*, vol. 10, no. 16, pp. 3143-3148, April 2011.
[7] M. O. Daramola, E. F. Aransiola, and A. G. Adeogun, "Comparative study of thermophilic and mesophilic anaerobic treatment of purified terephthalic acid (PTA) wastewater," *Nat. Sci*., vol. 3, pp. 371-378, January 2011.
[8] C. Vamsee-Krishna, Y. Mohan, and P. S. Phale, "Biodegradation of phthalate isomers by *Pseudomonas aeruginosa* PP4, *Pseudomonas* sp. PPD and Acinetobacter lwoffii ISP4," *Appl. Microbiol. Biotechnol*., vol. 72, pp. 1263–1269, October 2006.
[9] S. Sookkasem, "Isolation and Screening of Terephthalic acid (TA)-degrading microorganisms from textile industry," M.S. thesis, Dept. Biotech., Kasetsart Univ., Bangkok, Thailand, 2013.
[10] T. Kimura and Y. Ito, "Two bacterial mixed culture systems suitable for degrading terephthalate in waste water," *J. Biosci. Bioen*, vol. 91, no. 4, pp. 416-418, January 2001.
[11] X. Su, Y. Liu, M. Z. Hashmi, J. Hu, L. Ding, M. Wu, and C. Shen, "*Rhodococcus biphenylivorans* sp. nov., a polychlorinated biphenyl-degrading bacterium," *Antonie Leeuwenhoek*, vol. 107, pp. 55-63, January 2015.
[12] X. Su, F. Sun, Y. Wang, M. Z. Hashmi, L. Guo, L. Ding, and C. Shen, "Identification, characterization and molecular analysis of the viable but nonculturable *Rhodococcus biphenylivorans*," *Scientific Reports*, 2015.
[13] Y. M. Zhang, Y. Q. Sun, Z. J. Wang, and J. Zhang, "Degradation of terephthalic acid by a newly isolated strain of *Arthrobacter* sp. 0574," *S. Afr. J. Anim. Sci.*, vol. 109, pp. 1-4, January 2013.
[14] J. Zhou, R. An, H. Zhang, and Y. Liu, "Orthogonal design of pharmaceutical experiment based on SPSS," in Proc. *ICICA 2012*, Chengde, China, September 14-16, 2012, pp. 552-560, 2012.

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