Screening for Terephthalic Acid (TA) Anaerobic Degrading Bacteria

Hong-xiang Zhu[1], Jiang-ping Qiu[2], Jin Wang[2]* and Xu-dong Li[2]*

[1]Department of Environmental Sciences, Insitute of Light Industry and Food Engineering, Guangxi University, Nanning, 530004, China.
[2]College of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China.

(Received: 21 August 2014; accepted: 14 October 2014)

An anaerobic bacterium was isolated and identified from an anaerobic expanded granular sludge bed reactor (EGSB) that was treating pure terephthalic acid (TA) wastewater. The bacterium could effectively degrade terephthalic acid. The screened anaerobic bacterium was determined to be *Bacillus cereus*. It could degrade 1000 mg/L TA within 72 h, and the maximum degradation rate was 98%. Its TA degrading enzymes were endoenzymes.

Key words: Anaerobic degradation, terephthalic acid (TA), screen, identification.

Terephthalic acid (TA) is the main material used to produce polyesters. During the production of the polyester textiles, about 70% of them are exposed to a "caustic treatment" that results in wastewater in which there is a high TA concentration. A variety of microbes can degrade TA by using it as the sole carbon source. The degradation of TA is performed mostly by gram negative microorganisms, such as *Pseudomonas* sp., and the majority of them are aerobic. Yu *et al*.[1] isolated a strain from the activated sludge of a wastewater treatment plant from a polyester fabric processing plant, and the strain could aerobically degrade TA at a 0.5% concentration and rate close to 100% within two weeks. Yang *et al*.[2] used an activated sludge process to deal with TA at 1100 mg/L, and the average degradation rate was 27.5 mg COD/g MLSS (mixed liquid suspended solids).h. Many studies have shown that TA and its derivatives can inhibit the proliferation of anaerobic microorganisms[3, 4]. Generally, TA does not inactivate the activated sludge, but it is difficult to decompose and the removal rate in an anaerobic treatment process is lower, around 40%. Under anoxic conditions, the TA removal rate at a hydraulic retention time (HRT) of 18 h was only about 7%[5]. Microbes can act as catalysts for biological wastewater treatment. Therefore, TA degrading microorganisms were screened, isolated, and their TA degrading characteristics were determined, to enable efficient biological treatment. This has important theoretical and practical significance, especially for the anaerobic treatment of PTA wastewater.

* To whom all correspondence should be addressed.
E-mail: wangjin100@sjtu.edu.cn (Jin Wang); lixudong@sjtu.edu.cn (Xu-dong Li).

MATERIALS AND METHODS

Medium and reagent

Murashige and Skoog medium (MS, pH 7) and TA inorganic salt culture medium were prepared per related papers[6-7]. Lysogeny broth (LB) medium (pH 7): 10 g peptone, 5 g beef extract, and 5 g NaCl, which was diluted to 1000mL with distilled water, had 1.8% agar added to prepare a solid medium (i.e. nutrient agar). Acetyl methyl methanol (V.P.) and methyl red (M.R.) medium: 5 g peptone, 5 g NaCl, and 5 g glucose, which was diluted to 1000mL with distilled water was prepared. The anaerobic incubator used, a YQX-II, was obtained from Shanghai Yuejin Medical Instruments of China.

Screen method of TA degrading bacteria

Sludge (1 g) was taken from an anaerobic expanded granular sludge bed (EGSB) reactor that had worked for one year, and put into pre-sterilized 250 ml Erlenmeyer flasks that contained 150 ml TA inorganic medium with 1000 mg/L of TA. Then to enrich the cultures, they were placed in the anaerobic incubator at 35°C until the medium became cloudy, and then the next batch of the static culture was started by taken 10 ml into the TA inorganic salt medium. Slowly, the TA concentration was increased to 3 g/L. Then 0.1 ml of liquid medium was drawn and moved to TA inorganic salt agar solid medium with a TA concentration of 3 g/L. This was performed repeatedly to screen for and purify the bacteria. Two anaerobic strains were isolated, and per their growth rate in the TA inorganic salt culture medium, one anaerobic strain was determined to be superior and used as the experimental strain, referred to as JD-2.

Bacterial identification methods

According to the Bacteria Identification Manual[8], the following identification test were performed: oxidase test positive, methyl red (M.R.) experiment, acetyl methyl methanol (V.P.) test, Gram stain, gelatin liquefaction test, pyocyanin test, and 42°C growth test.

Sequence analysis

The DNA of the strain was extracted according to the CTAB method[9], and used as the 16S rDNA template in PCR (Polymerase chain reaction) amplification. The bacterial universal primer[10-11] was adopted to amplify the 16S rDNA. The sequence of the reversed primer BSR1541/20 was 5'-AAGGAGGTGATCCA GCCGCA-3' and the forward primer BSF8/20 was 5'-AGAGTTTG ATCCTGGCTCAG-3'. Through the Blastn program, a homologous comparison was made between the obtained nucleic acid 16S rDNA sequence and GenBank data (http://www.ncbi.nlm.nih.gov/blast)[12-13].

RESULTS AND DISCUSSION

Physiological and biochemical identification of bacteria

The strain JD-2 was determined to be a Gram-positive bacteria. It had a short rod or oval shape when observed via electron microscope (Fig. 1), an end flagella, and cell size of 0.3-0.5 µm × 1-1.5 µm. It was non-capsule, had ovate spores, was motile, a facultative anaerobic, degraded glucose to produce acid with no gas, and could perform the gelatin liquefaction. On a solid medium, the colony was milky white, opaque, convex, and the colony surface appeared to be like frosted glass (Fig. 2).

Based on the physiological and biochemical characteristics of strain JD-2, it was identified as *Bacillus cereus*. To verify the identification, the automatic identification card method of bioMerieux VITEK-2 (France) was adopted, as shown in Table 1. Again the identification showed *B. cereus*.

Molecular biology identification

After extracting DNA from the strain, the full-length 16S rDNA sequence was amplified using bacterial universal primers, which gave a DNA fragment of about 1.5 Kb. Part of the 16S rDNA was sequenced, and the sequences were submitted to the GenBank nucleotide database registration, and given the access number EF489293 (738bp). Once the sequences were input into GenBank, the Blastn program was used to perform a homology comparison. The results showed that the 16S rDNA sequence of JD-2 had 99% homology with *B. cereus*. Therefore, both the biochemical and molecular identifications showed that the anaerobic TA degrading bacterium identified in this work was *B. cereus*.

Degradation characteristics on TA

The TA medium (1000 mg/L) (150 ml) was put into 250ml triangle bottles, seeded with 0.5 ml fresh seed solution (JD-2 strain), and anaerobically

Table 1. Results of VITEK test

NEG-	GLU+	XYL-	AGA-	TRE+	PLA-	SOR-	NAG+
SUC+	INO-	MAN-	INU+	AMY+	KCN+	MEN+	OLD-
TZR-	GAL-	BAF+	RIB+	NAA+	ARB+	PAS-	NAE-
TAG+	ARA-	SAL-	MLT+	ESC+	7NC-	THRM+	

Table 2. TA degradation efficiency of the JD-2 strain.

Duration, h	Strain JD-2, %
0	0
12	9
24	20
36	34
48	64
60	76
72	98
Max (%)	98

statically cultivated at 35°C. The TA determination method was the First Order Derivative UV Spectrophotometric method [14], and the results are given in table 2.

From Table 2, it can be seen that the maximum degradation efficiency of TA by JD-2 strain was 98% after 72h. The initial pH was 7, and the pH value under the anaerobic conditions decreased initially and then increased gradually.

Analysis of degradation products

Fresh seed culture (0.5 ml) was inoculated

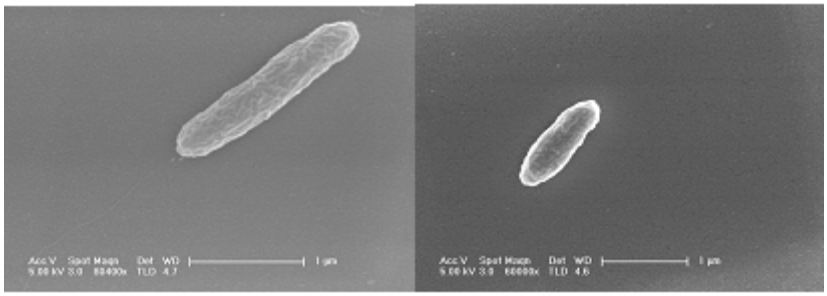

Fig. 1. Scanning electron microscope micrograph of JD-2

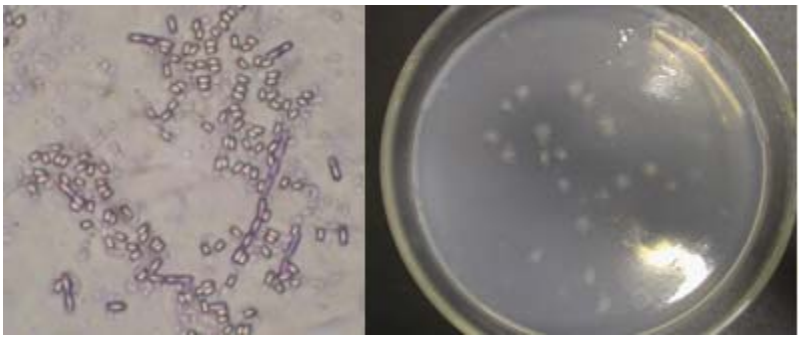

Fig. 2. Micrograph (1000×magnification) of JD-2 and bacterial colony on solid media

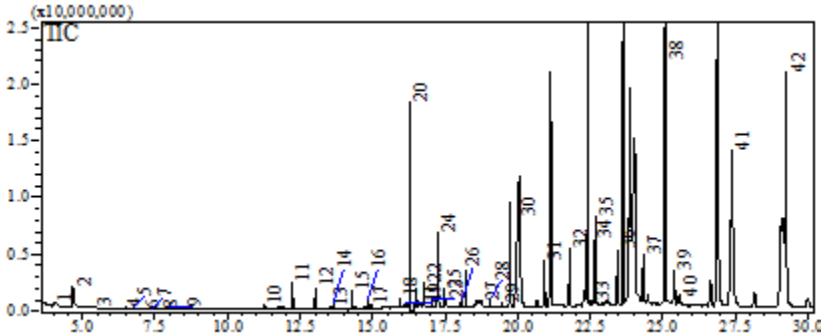

Fig. 3. GC-MS of anaerobic degradation of TA by JD-2

into nine sets of 150 ml Erlenmeyer flasks containing sterilized PTA medium in the enrichment culture, at 35°C and under anaerobic conditions for 72 h. Every 8 h one flask was removed and centrifugation at 3000 r/min for 20 min was performed before the supernatant was concentrated to 25 ml at 50°C, and then the pH was adjusted to 2 by adding H_2SO_4, before filtering twice using filter paper.

All the collected liquids were individually extracted with 200 ml of ether three times. The three extractions were combined and passed through a glass funnel filtration with 30-40g anhydrous Na_2SO_4. They were then poured into a 500 ml cylinder and purged to about 3 ml with nitrogen. Then they were analyzed using GC/MS-QP2010NC gas chromatography/mass spectrometer, which was made in the USA by Angilent Instrument Company. Qualitative analyses were performed using the NOVA-4 computer data processing system.

Comparative analysis of TA degradation products

Samples were taken every 8 h and dosed with acid. In the first 24 h, all samples still had obvious white precipitate in the solution after dosing with acid, which showed that most of TA samples were not transformed. While at 48h, when the taken samples were dosed with acid, only a small amount of white precipitate (about 1/10 of the former) was present, which suggested most of the TA in the samples was transformed into other substances by JD-2. After 72 h, when dosed with acid, there were no white precipitates in the samples, which means all the TA in the culture was converted to other chemicals.

Fig. 3 shows the GC/MS chromatogram of the anaerobic degradation products, and this shows that both degradation and metabolite products were complicated, with there being 42 intermediate degradation products. The main compounds were alkanes, acid, benzene, alcohol, and small molecule esters.

TA degrading enzyme

A loop was used to inoculate a slope with a culture of JD-2 and this was incubated at 35°C for 24 h. This was then used to inoculate 100 ml of the LB culture media in a 250 ml Erlenmeyer flask, which was shaken for 18 h until the logarithmic growth phase was reached. Then 10 ml of the culture was removed, and sterilized using a microporous membrane filter. Then 7 ml was dosed into 100 ml sterile TA (1 g/L) medium and shaken at 35°C. The TA concentration was measured via regular sampling. The remaining 3 ml of the bacteria containing medium was dosed to another 100 ml of sterile TA culture medium as a control. The results showed that the sterilized culture medium had no degradation activity against TA, while the degradation ability appeared in the presence of bacteria. This suggested that the degradation enzymes for TA from the JD-2 bacteria could only exist inside the cell, they were endoenzymes.

CONCLUSION

An anaerobic TA degrading bacterium called JD-2 was isolated from an EGSB reactor. Biochemical and molecular methods identified the anaerobic bacterium JD-2 as being *B. cereus*. The TA degradation rate of JD-2 was 98% for 1000 mg/L TA in 72 h. Its enzyme was located intracellularlly.

ACKNOWLEDGEMENTS

This work was financially supported by the Natural Science Foundation of China (Project no 51108261), and the Start Program for Young Talent of Shanghai Jiao Tong University (Project no 12X100040059).

The authors acknowledge Wei LI from Instrumental Analysis Center, Shanghai Jiao Tong University for their assistance in SEM analyses.

REFERENCES

1. Yu J.L.,Tengtian Z.M. Cultivation and screening of terephthalic acid degradation by microorganisms. *Journal of Tianjin Polytechnic University*, 2004; **23**(2):48-50.
2. Yang Q.Y., Chen J.H., Sun X.J., *et al*. Study on aerobic biodegradability of terephthalic acid in polyester fabric alkali peeling process wastewater. *Techniques and Equipment for Environmental Pollution Control*, 2006; **7**(7):38-42.
3. Fajardo J.P., Gupot H., Macarie, *et al*. Inhibition of anaerobic digestion by terephthalic acid and its aromatic by products. *Water Science & Technology*, 1977; **36**(6-7): 83-90.
4. Chidambara Raj C. B, Ramkumar N. Biodegradation of Acetic, Benzoic, Isophthalic, Toluic and Terephthalic Acids Using a Mixed Culture: Effluents of PTA Production. Process

Saf. Environ.: Transactions of the Institution of *Chemical Engineers*[C]. Part B, 1997; **75**(4): 245-256.

5. Zhao, H.B., 1994. Pilot study on terephthalic acid (PTA) wastewater treatment using an A/O biofilm process. *Chinese Journal of Environmental Science*, 1994; **15**(6): 47-50, 60.
6. Zhong W.H. Microbiological Degradation of 2,4-Dichlorophenol and cloning of relative genes. Doctoral dissertation of Zhejiang University, Hangzhou, 2001.
7. Chen Y.X. Biodegradation of aromatic compounds and construction of genetically engineered bacterium for aromatic compounds biodegradation. Doctoral dissertation of Zhejiang University, Hangzhou, 2003.
8. Dong X.Z., Cai M.Y. Common bacterial systems Identification Handbook (in Chinese). Academic Press, first edition, 2001; pp. 364-398. Beijing.
9. Sambrook J., Fritsch E. F., Maniatis T. Molecular cloning: a laboratory manual.(20d ed),New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA, 1989
10. Han R.Y., Min H., Che M.C., *et al*. Isolation, identification and phylogenetic analysis of a thermophilic cellulolytic anaerobic bacterium. *Acta Microbiologica Sinica*, 2002; **42**(2): 138-144.
11. Edwards U., Rogall T., Blocker H., *et al*. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research,* 1989; **17**(19): 7843-7853.
12. Altschul S.F., Gish W., Miller W., *et al*. Basic local alignment search tool [J]. *Journal of Molecular Biology,* 1990; **215**(3): 403-410.
13. Zhang J., Madden T. L. Power BLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. *Genome Research,* 1997; 7(6): 649-656.
14. Wang X.P. Determination of terephthalic acid in dyeing wastewater and characterization for vater quality and biochemical effect. *Science & technology in Chemical Industry*, 2000; **8**(1): 47-49.