微生物学通报
Microbiology China
tongbao@im.ac.cn

Feb. 20, 2016, 43(2): 279−284
http://journals.im.ac.cn/wswxtbcn
DOI: 10.13344/j.microbiol.china.150346

研究报告

Anaerobic biodegradation of 2,4-dinitrotoluene by *Rhodobacter sphaeroides*

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Abstract: [Objective] To investigate the biodegradation of 2,4-dinitrotoluene (2,4-DNT) under various environmental conditions by *Rhodobacter sphaeroides*. **[Methods]** 2,4-DNT was anaerobically biodegraded under 30 °C in illumination incubator using photosynthetic bacterium *Rhodobacter sphaeroides*. The concentration of 2,4-DNT in liquid medium were detected by HPLC. **[Results]** Optimum conditions for the removal of 2,4-DNT were initial concentration of 40 mg/L, initial pH 7.0 and inoculation quantity of 15%. In addition, 2,4-DNT could be absorbed by the cells in lag phase, then degraded as carbon source in exponential phase. The removal rate of 2,4-DNT achieved 98.8% at 72 h. Using HPLC, two different intermediate metabolites were also observed, however, these decreased gradually within 120 h. Furthermore, the removal kinetics of 2,4-DNT corresponded with the first-order rate model. **[Conclusion]** The removal rate of 2,4-DNT under different conditions indicated that *Rhodobacter sphaeroides* is efficient in biodegrading 2,4-DNT.

Keywords: Anaerobic biodegradation, 2,4-DNT, Kinetics, *Rhodobacter sphaeroides*

球形红细菌厌氧降解 2,4-二硝基甲苯

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摘 要:【目的】研究不同环境条件对 2,4-二硝基甲苯(2,4-DNT)生物降解的影响。【方法】采用光合细菌球形红细菌在温度为 30 °C 的光照培养箱中厌氧降解 2,4-DNT，并用高效液相色谱仪测定其浓度。【结果】去除 2,4-DNT 的最佳条件是初始浓度 40 mg/L、初始 pH 7.0 和接种量 15%。

Foundation item: Enterprise Program Foundation of Shanxi Province (No. 20080311027-1)
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Received: April 23, 2015; **Accepted:** June 16, 2015; **Published online (**www.cnki.net**):** July 03, 2015

另外，2,4-DNT 在菌体延滞期被细胞吸收,然后在指数期作为碳源被降解。2,4-DNT 的去除率在 72 h 达到 98.8%。从液相色谱图中观察到有 2 种中间代谢产物，但在 120 h 内产物被逐渐降解。2,4-DNT 的去除动力学符合一级速率模型。【结论】不同条件下 2,4-DNT 的去除率表明球形红细菌能有效降解 2,4-DNT。

关键词：厌氧降解, 2,4-DNT, 动力学, 球形红细菌

1 Introduction

2,4-Dinitrotoluene (2,4-DNT) is not only one of the most important explosives around active military firing ranges, but is also found as an intermediate compound in herbicides, dyes and synthetic foam industries[1]. The compound 2,4-DNT is ubiquitous and because of its high toxicity, carcinogenicity and mutagenicity to humans, 2,4-DNT has been classified by the United States Environmental Protection Agency (US EPA) as a priority pollutant[2-3].

To date, many studies reported the remediation of DNTs in contaminated sites. While biodegradation is expected to be an economical and energy efficient approach in comparison to other remediation processes such as chemical or physical ones[4]. Previous studies shown that several strains have the ability of degradation of 2,4-DNT, such as *Pseudomonas*[5], *Alcaligenes*[6], *Burkholderia*[7], *Phanerochaete chrysosporium*[8], *Arthrobacter*[9]. Researchers also found that microbial consortia can degrade 2,4-DNT by interspecies metabolism[10], poultry litter leachate[11] or different plant species[12]. However, there are two problems in these studies. First, these strains degrade 2,4-DNT only under strict anaerobic conditions or strict aerobic conditions. Second, the degradation time is long. Therefore, these factors limit their further application. Fortunately, the phototrophic bacteria can overcome those drawbacks. Phototrophic bacterium *Rhodobacter sphaeroides*, a typical purple non-sulfur bacterium, are metabolically the most versatile among all the procaryotes, can anaerobically photoautotrophic and photoheterotrophic in the light or aerobically chemoheterotrophic in the dark, so they can use a broad range of organic compounds as carbon and energy sources[13]. So far, there were no reports in the literature about degradation of 2,4-DNT by *R. sphaeroides*. The objectives of this study was to investigate the biodegradation of 2,4-DNT by *R. sphaeroides*.

2 Materials and Methods

2.1 Microorganism and culture conditions

Rhodobacter sphaeroides was obtained from College of Life Science and Technology, Shanxi University, Taiyuan, China. *R. sphaeroides* was cultured in the medium containing (in 1 L) 2.5 g malic sodium, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 1 g yeast extract and 1.25 g $(NH_4)_2SO_4$, the resulting medium was adjusted to pH 7.0 and then autoclaved[14]. The stock solution (1 800 mg/L) of 2,4-DNT in methanol was injected respective volume into sterilized serum bottles and allowed to evaporate methanol in the laminar hood under the airflow, then added into the medium without other carbon source (2,4-DNT as the sole carbon source). Unless otherwise stated, experiments were carried out in 45 mL serum bottles with a working volume of 45 mL. For use as inocula, the cells suspension was adjusted an OD_{590} of 1.0 using sterile fresh medium. After inoculation (10%, *V*/*V*), the serum bottles were sealed with rubber stoppers (*R. sphaeroides* is not a strict anerobe) and cultured anaerobically at 30 °C under continuous illumination with incandescent lamps at a light intensity of about 2 500 lx. Each test was carried out in triplicates.

2.2 Biodegradation experiment

In consideration of abiotic 2,4-DNT degradation, control experiments were conducted with 40 mg/L of 2,4-DNT with *R. sphaeroides* or without *R. sphaeroides*. At the same time, media inoculated with dead cells were used as control by autoclaving at 1×10^5 Pa for 30 min. In addition, *R. sphaeroides* with distilled water containing 2,4-DNT (40 mg/L) was also prepared.

To further investigate the biodegradation capability of 2,4-DNT by *R. sphaeroides*, the effects of other operating parameters on the removal of 2,4-DNT were conducted in incubator under 30 °C. Various conditions can be described briefly as illumination and oxygen (anaerobic and illumination,

anaerobic and dark, microaerobic and illumination, aerobic and dark), initial concentration (20, 40, 60, 80 mg/L), initial pH value (5.0, 6.0, 7.0, 8.0, 9.0), and inoculation quantity (5%, 8%, 10%, 12%, 15%, 20%). For each batch experiment, one of the parameters was changed while the others kept constant. At regular intervals, 10 mL samples were collected from each bottle and centrifuged at 8 000×*g* for 20 min. The concentration of 2,4-DNT in the supernatant was determined by HPLC. The cells were washed and re-suspended with equivalent distilled water, then the biomass was monitored by OD_{590}. To obtain the intracellular 2,4-DNT fraction, cells was collected after the biomass was monitored, and subsequently kept in an ice bath during the cell disruption to prevent overheating. Cell disruption was performed at 650 W for 5 min (5 s: 10 s pulse on: off basis), then centrifuged at 15 000×*g* for 30 min[15].

In addition, the removal rate constant (k) and half-life removal time ($t_{1/2}$=0.693/k) were measured using a first-order kinetic equation, $C=C_0e^{-kt}$, where C is the 2,4-DNT concentration at time t (mg/L), C_0 is the initial concentration (mg/L), k is the removal rate constant (h^{-1}) and t is the time (h).

2.3 Analytical methods

2,4-DNT was analyzed using a Ultimate 3000 HPLC (USA) equipped with a UV-visible detector. The separation column used for HPLC was C_{18} reverse-phase column (250 mm × 4.6 mm inner diameter: 5 μm particle size) at 20 °C. The mobile phase was methanol/water (70:30, *V*/*V*), with a flow rate of 0.9 mL/min. The wavelength used for detection was 254 nm, and the injection volume was 20 μL.

3 Results

3.1 Degradation of 2,4-DNT in control experiments

As shown in Figure 1, 2,4-DNT was almost completely removed by *R. sphaeroides* within 72 h. However, under the condition of without *R. sphaeroides* or with dead cells or *R. sphaeroides* with distilled water, there was no significant change in the 2,4-DNT levels, and the concentration of 2,4-DNT was stable at about 39 mg/L over the whole incubations of 120 h. The results of the control experiments were in good agreement with other previous work, volatilization and adsorption were insignificant factors[16]. Fate of 2,4-DNT with *R. sphaeroides* was mainly due to biological process.

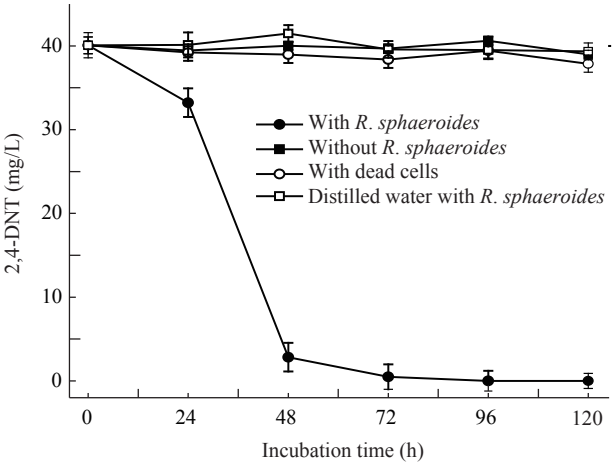

Figure 1 The removal of 40 mg/L 2,4-DNT by *R. sphaeroides* at pH 7.0 and 30 °C

图 1 在 pH 7.0、30 °C 时球形红细菌对 40 mg/L 2,4-DNT 的去除

Figure 2 shows that the strain inoculated from 0 h to approximately 48 h was in a lag phase, and the concentration of 2,4-DNT decreased sharply from 24 h to 48 h. The removal rate of 2,4-DNT achieved 92.9% at 48 h. In addition, the concentration of 2,4-DNT in the intracellular fraction incubated for 24 h and 48 h was 6.1 mg/L and 32.8 mg/L, respectively. Moreover, two different intermediates derived from 2,4-DNT were detected in the medium at 48 h, and reached the maximum at 72 h, then

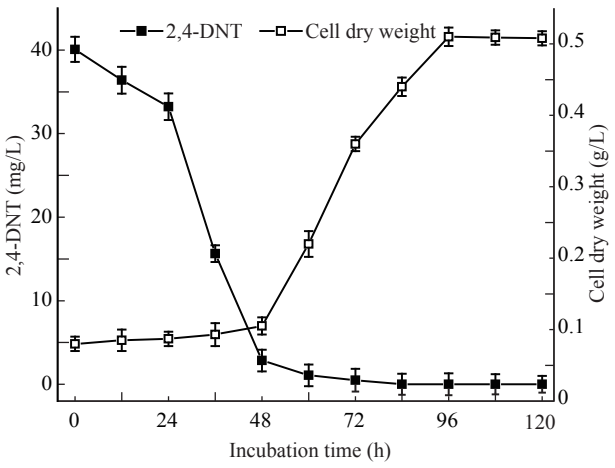

Figure 2 The curves of 40 mg/L 2,4-DNT removal and the *R. sphaeroides* growth

图 2 球形红细菌生长和去除 40 mg/L 2,4-DNT 的曲线

gradually disappeared within 120 h (Figure 3). The result indicates that 2,4-DNT is absorbed by the cells of the strain in lag phase, then degraded as carbon source in exponential phase.

3.2 Effect of illumination and oxygen

Table 1 shows that *R. sphaeroides* can biodegradation 2,4-DNT in various conditions. The optimum condition was anaerobic and illumination, the removal rate of 2,4-DNT was reached 98.8% at 72 h. The results proved the property of *R. sphaeroides* that the strain can change the metabolism flexibly according to environmental conditions[17]. Hence, we chose anaerobic biodegradation of 2,4-DNT by *R. sphaeroides* in subsequent experiments.

3.3 Effect of initial concentration

As the initial concentration of 2,4-DNT increased from 20 mg/L to 80 mg/L, the removal rates at 72 h were 100%, 98.8%, 43.6% and 2.43%, respectively (Figure 4A). The higher levels may inhibit the growth and metabolic activity of microorganisms, thus further suppress the biodegradation of 2,4-DNT[18].

3.4 Effect of initial pH value

Figure 4B shows that the highest 2,4-DNT consumption occurred at initial pH 7.0, and more than 91.8% of 2,4-DNT was degraded when the initial pH of the culture medium ranged from 6.0 to 8.0 within 72 h. However, removal rate significantly declined at initial pH 5.0 or initial pH 9.0. The result agrees well with several reports that the optimal initial pH range for the growth of anaerobic microorganisms is between 6.0 and 8.0[19].

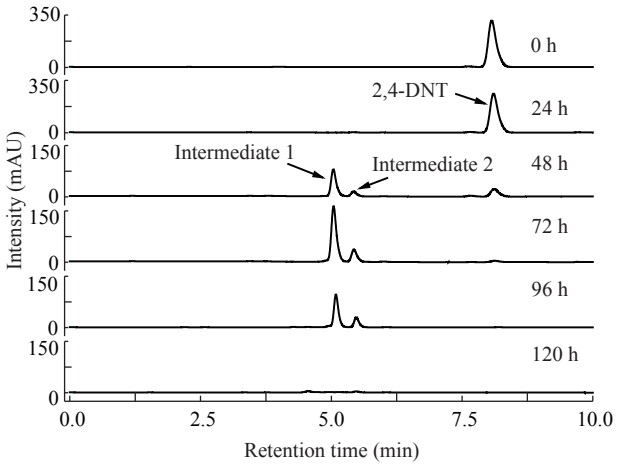

Figure 3 HPLC profiles for intermediate metabolites produced from 40 mg/L 2,4-DNT biodegradation by *R. sphaeroides* at different time

图 3 球形红细菌降解 40 mg/L 2,4-DNT 时出现中间产物的高效液相色谱图

Table 1 Effect of illumination and oxygen on *R. sphaeroides* growth and 2,4-DNT degradation

表 1 不同光照供氧对 *R. sphaeroides* 生长和 2,4-DNT 降解效果的影响

Illumination and oxygen	Dry cell weight (g/L)	Degradation efficiency (%)
Anaerobic and illumination	0.36	98.8
Anaerobic and dark	0.20	86.5
Microaerobic and illumination	0.29	90.6
Aerobic and dark	0.16	80.7

Note: Effect of illumination and oxygen on *R. sphaeroides* growth and the removal of 2,4-DNT were conducted at initial concentration of 40 mg/L, pH 7.0, inoculation quantity of 10% at 30 °C for 72 h.

注：在 30 °C、pH 7.0、接种量 10%时, 72 h 不同光照供氧对 *R. sphaeroides* 生长和 40 mg/L 2,4-DNT 去除的影响.

3.5 Effect of inoculation quantity

The trend of degradation rate of 2,4-DNT in Figure 4C revealed that the degradation time was shorten with the increase of inoculation quantity from 8% to 15%[20]. However, when inoculation quantity increased to 20%, there was no significant change for the degradation rate of 2,4-DNT. The result illustrates that the inoculation quantity of 15% is enough to meet the requirements of the degradation of 2,4-DNT. In addition, considering the economic factors, the optimum inoculation quantity is 15%.

3.6 Biodegradation kinetics of 2,4-DNT

Table 2 shows that the removal of 2,4-DNT follows first-order kinetic reaction. With initial concentrations increase steadily from 20 to 80 mg/L, the removal rate constants decrease quickly from 0.206 9 to 0.000 4 h^{-1}, while the half-life increased from 3.4 to 1 732 h. The removal rate constants and half-life of inoculation quantity exhibited the opposite changing tendency to the initial concentrations (Table 3). The shift of inoculation quantity from 5% to 15% can significantly enhance the removal rate of 2,4-DNT by *R. sphaeroides*. Similarly, as Table 4 shows, with the initial pH values increased from 5.0 to 7.0 then to 9.0, the rate constants increased from 0.000 1 to 0.059 3 h^{-1}

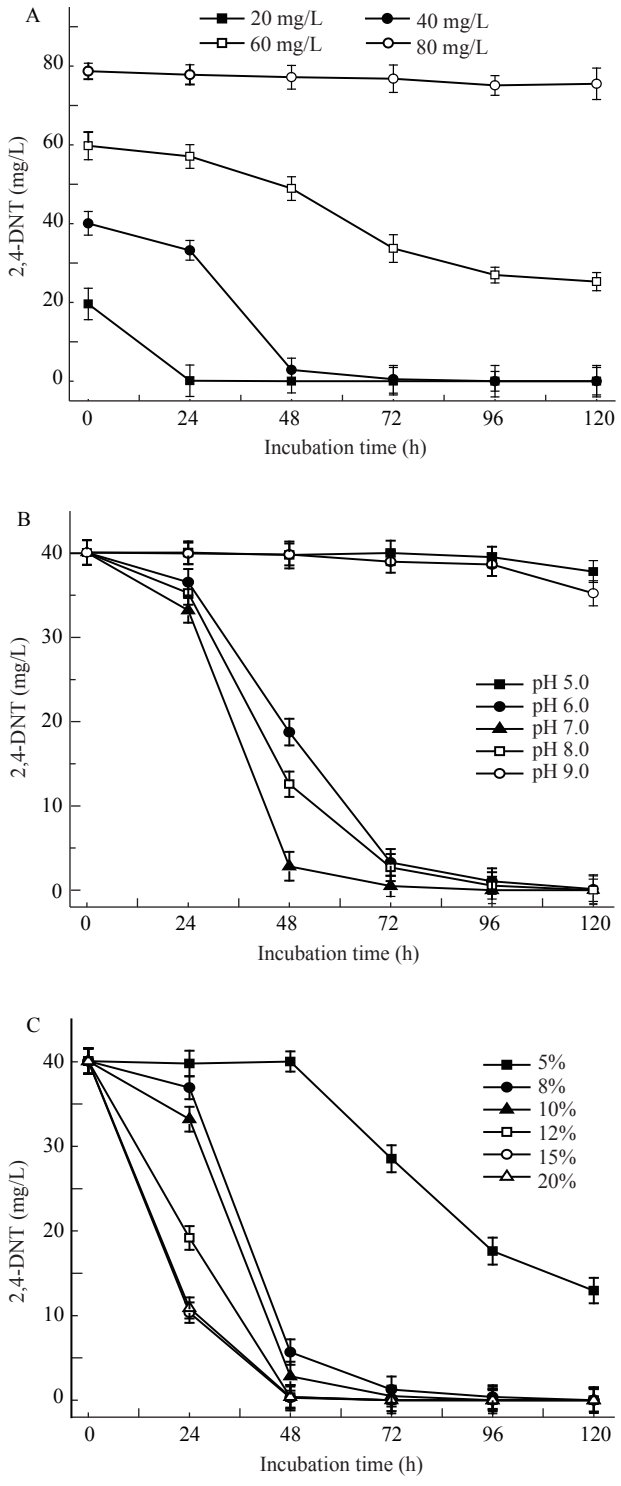

Figure 4 The effect of initial concentration of 2,4-DNT (A), initial pH (B), and inoculation quantity (C) on the removal of 2,4-DNT in batch culture

图 4 初始浓度(A)、初始 pH (B)和接种量(C)对 2,4-DNT 去除的影响

Table 2 Kinetic equations and kinetic parameters of the removal of 2,4-DNT under different initial concentration by *R. sphaeroides*

表 2 不同初始浓度下球形红细菌去除 2,4-DNT 的动力学方程及动力学参数

Initial concentration (mg/L)	k (h^{-1})	$T_{1/2}$ (h)	R^2	$C=C_0e^{-kt}$
20	0.206 9	3.4	0.964 0	$C=19.59e^{-0.206\,9t}$
40	0.059 3	11.7	0.994 1	$C=40.08e^{-0.059\,3t}$
60	0.007 3	94.9	0.929 7	$C=59.76e^{-0.007\,3t}$
80	0.000 4	1 732	0.921 8	$C=78.71e^{-0.000\,4t}$

Table 3 kinetic parameters of the removal of 2,4-DNT under different inoculation quantity by *R. sphaeroides*

表 3 不同接种量下球形红细菌去除 2,4-DNT 的动力学参数

Inoculation quantity (%)	k (h^{-1})	$T_{1/2}$ (h)	R^2
5	0.008 3	83.5	0.887 4
8	0.045 9	15.1	0.929 4
10	0.059 3	11.7	0.994 1
12	0.084 2	8.2	0.837 5
15	0.093 4	7.4	0.923 9
20	0.087 6	7.9	0.928 9

Table 4 kinetic parameters of the removal of 2,4-DNT under different initial pH by *R. sphaeroides*

表 4 不同 pH 下球形红细菌去除 2,4-DNT 的动力学参数

Initial pH value	k (h^{-1})	$T_{1/2}$ (h)	R^2
5.0	0.000 1	6 930	0.931 4
6.0	0.044 3	15.6	0.963 5
7.0	0.059 3	11.7	0.994 1
8.0	0.038 6	17.9	0.889 4
9.0	0.000 4	1 732	0.999 7

and finally dropped to 0.000 4 h^{-1}. Nevertheless, there is an opposite trend for the half-life, which drop steeply from 6 930 h to 11.7 h as the initial pH value jumped from 5.0 to 7.0, and then went up to 1 732 h with further rose initial pH to 9.0[21]. Thus, the maximum removal rates of 2,4-DNT and optimum specific bacterial growth are obtained near initial pH 7.0 and inoculation quantity of 15%, which agreed well with our experimental results described above.

4 Conclusion

This is the first study to report the biodegradation

of 2,4-DNT using *R. sphaeroides*. The strain was able to grow in medium with 2,4-DNT as sole carbon source. Notably, 2,4-DNT was removed through biological processes. The optimum reaction conditions were initial concentration of 40 mg/L, initial pH 7.0, and inoculation quantity of 15%. In addition, 2,4-DNT could be absorbed by the cells in lag phase, then degraded as carbon source in exponential phase, the removal rate of 2,4-DNT achieved 98.8% at 72 h. Moreover, two different intermediate metabolites detected as a result of biodegradation of 2,4-DNT. Further studies are needed to identify the metabolites by GC-MS, then reveal the anaerobic 2,4-DNT degrading pathway under photoheterotrophic growth conditions by *R. sphaeroides*.

Acknowledgements

We thank Dr. Vincent from National Research Centre for Environmental Toxicology (Entox), the University of Queensland Australia for revising the manuscript. Also, we thank Professor Jiao Weizhou and Feng Zhirong from Research Center of Shanxi Province for High Gravity Chemical Engineering and Technology, North University of China for technical support in carrying out the experiments.

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