Effect of humic acid on degradation of PTBP by strain Tas13

Hui Zhang[1*]

[1]Institute of Environmental Health and Ecological Security, School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, P. R. China

Abstract: p-tert-Butylphenol (PTBP) is a kind of alkylphenol endocrine disruptor. PTBP is not only toxic to aquatic organisms and animals because of its high persistence in the environment, but also has acute toxicity, chronic toxicity, endocrine interference and certain toxicity of reproductive development. It is also harmful to human health and has become an important environmental pollutant. Therefore, the pollution of PTBP and its removal have attracted wide attention. Humic acid affects its adsorption and biodegradation efficiency in water environment. In this study, the adsorption analysis of PTBP by humic acid and the mechanism of the effect of humic acid on the degradation of PTBP by Tas13 were investigated. The results showed that humic acid could adsorb PTBP and promote the degradation of PTBP by Tas13.Raman spectroscopy suggests that humic acid may be associated with relaxation of membrane proteins and lipid monolayers to promote subsequent intracellular metabolism. Zeta potential analysis showed that humic acid interacts with bacterial surface functional groups to generate and regulate the transmembrane transport of carboxyl, amino and hydroxyl groups. The addition of humic acid promotes the transport of PTBP from the outside to the inside of bacterial cells, thus effectively degrading it. In addition, by measuring the C23O activity of strain Tas13, humic acid stimulated the active C23O gene, which enhanced the expression of C23O gene mediated by strain Tas13.

1. Introduction

P-tert-Butylphenol (p-tert-butylphenol, PTBP), also known as 4-tert-Butylphenol (4-tert-butylphenol), is a kind of alkylphenol endocrine disruptors. With the mass production of enterprises and wide application in daily life, it has been frequently detected in the environment (0.003-64 μg/L) [1]. PTBP has become an important environmental pollutant due to its high persistence in the environment, acute toxicity, chronic toxicity, endocrine disruption and certain reproductive and developmental toxicity. It not only has toxic effects on aquatic organisms and animals, but also endangers human health [2].

Physical, chemical and biological remediation techniques are most commonly used to repair contaminated water bodies. But its cost is high, the amount of engineering is large, time consuming power. The residual chemical substances are easy to cause secondary pollution, and the addition of chemical substances has toxic effects on aquatic organisms. Compared with physical and chemical remediation, bioremediation technology which mainly uses plants, microorganisms and plant-microorganisms combination has the characteristics of energy saving, low cost and no pollution, so it has become a hot topic in the remediation technology of polluted water bodies [3]. Dracaena sanderiana was inoculated with two plant growth promoting (PGP) bacteria Bacillus thuringiensis and Pantoea dispersa in hydroponics to remove BPA from Dracaena Sanderiana. The combined microbial-plant system increased the removal rate of BPA by 92.32±1.23% [3]. The combined phytomicrobial remediation technology plays an enhanced role in the treatment of organic pollutants in the environment, improves the treatment efficiency and diversity of organic pollutants, and has great potential for the treatment of organic pollutants in the environment.

Natural organic matter (NOM) consists of a complex mixture of organic molecules produced by microbial and abiotic decay of plant and animal remains [4]. NOM is ubiquitous in nature, in all terrestrial and aquatic systems, and plays a role in many ecosystem processes. Concentrations range from a few mg/l in groundwater to several hundred mg/l of dissolved organic matter (DOC) in some surface waters [5]. Among them, humic acid accounts for about 50%-90% of natural organic matter, which is a common natural organic matter in the input water bodies of watershed [6]. Humic acid is composed of C, H, O, N, S and other elements, which is complex and has no fixed structure [7].

At present, studies at home and abroad have shown that humic acid can (1) promote photosynthesis in plants, (2) enhance root vitality (promote root length), (3) promote respiration (4) stimulate the activity of various enzymes in crops [8]. The higher the concentration of humus (HS) (1,2,4mg/L), the slower the degradation rate of IM1 strain was. Moreover, studies have shown that the type of NOM (i.e. AOM or HS) and its concentration may have different effects on MC-LR biodegradation [9]. Other studies showed that the initial degradation rate of acephate decreased after adding humic acid. The associative dissociation mechanism of acephate and humic acid slows down the initial rate and aggregates are formed through h

*Corresponding Author, zh10040903@163.com

and van der Waals bonds. The formation of aggregates leads to the reduction of acephate toxicity [10,11]. Previous studies have investigated the effect of HA on the degradation of pyrene by Mycobacterium NJS-1, and the presence of HA significantly accelerates the degradation of pyrene by inducing mycobacterium [12]. This work will provide information for more accurate assessment of duckweed Tas13 degradation in natural aquatic environments.

2. Experimental Design

2.1 Materials

The duckweed (*S. polyrhiza*) sample is the original sterile duckweed in the laboratory. It was collected from a pond on the campus of Jiangsu University after surface disinfection with NaClO. it was subcultured in Hunter nutrient solution as a follow-up experimental material. The cultivation condition was 28±1℃, illumination was 8000 lux, light: dark=16 h: 8 h.

The strain Tas13 (*Sphingobium phenoxybenzoativorans)*, which efficiently degrades PTBP, is an original strain in the laboratory and was selected from the activated sludge in the secondary wastewater treatment tank of a wastewater treatment plant in Zhenjiang City, Jiangsu Province. After being confirmed as pure bacteria, they were cultured in BSM medium.

2.2 Laboratory related reagents

(1) Hunter medium: 20 mg KNO_3，35.7 mg Ca（NO_3）$_2$ • $4H_2O$，50 mg MgSO4 • $7H_2O$, 40 mg KH_2PO_4，5.71 mg H_2BO_3，2.03 mg $MnSO_4$ • $4H_2O$，6.585 mg $ZnSO_4$ • $7H_2O$，2.52 mg Na_2MoO_4 • $2H_2O$，0.394 mg $CuSO_4$ • $5H_2O$，0.162 mg $CoCL_2$ • $6H_2O$，56.7 mg Na_2-EDTA，2.49 mg $FeSO_4$ •$7H_2O$ per liter of distilled water.(2) Inorganic salt based (BSM) culture medium
(3) To prepare HA, weigh 1 g of humic acid and dissolve it in 1 L of ultra-pure water. adjust the pH to 7 with HCl. Filter with a 0.22 um sterile water system filter membrane, and store it in a refrigerator at 4 ℃ away from light.

2.3 Adsorption and desorption characteristics of PTBP by humic acid

The final concentration of HA in Hunter solution was 5 mg/L, 10 mg/L, 20 mg/L, 100 mg/L and the volume percentage of methanol was maintained at &lt on a super clean table; 0.22% (v/v) and 5mg/L PTBP were mixed. The conical bottle was wrapped with tin foil and placed in a shaking bed (30℃, 200 rpm/min) for fixed-point sampling at 0h, 3h, 6h, 12h, 24h, 30h, 36h and 48h. HPLC was used to detect the content of PTBP in the solution. The adsorption equilibrium was achieved when the concentration of PTBP in the solution remained constant. After the adsorption equilibrium was reached, 50mL $CaCl_2$ without HA was added into each bottle and placed in a shakable bed for 0h, 3h, 6h, 12h, 24h, 30h, 36h and 48h for fixed point sampling to determine the total amount of PTBP desorption in the solution. Absorption equilibrium is achieved when the concentration of PTBP in solution remains unchanged

$$Q_e=\frac{V(C_0-C_e)}{W} \quad (1) \qquad X=\frac{(C_0-C_e)}{C_0}\times 100\% \quad (2)$$

Including Qe- adsorption, X-ray absorption rate, C_0 - PTBP initial concentration, C_e - PTBP equilibrium concentration, V-solution volume, W-absorbent quality.

The total desorption amount and desorption rate are calculated by the following formula:

$$S=C_j\times\frac{V}{W} \quad (3) \qquad E=\frac{S}{Q_e}\times 100\% \quad (4)$$

Which S-desorption amount, E-desorption rate, C_j-desorption experiment PTBP concentration in the upper clear liquid.

2.4 Effect of humic acid on degradation of PTBP by strain Tas13

In order to verify the effect of humic acid on the growth of strain Tas13 and the degradation ability of strain Tas13 to PTBP in the presence of humic acid, we set up the following six treatment groups.Tas13, HA+Tas13, HA+Tas13+PTBP, Tas13+PTBP, HA+PTBP, PTBP. The specific experimental operation is as follows:

Inoculate PTBP degrading strains into BSM-PTBP (50 mg/L) liquid medium, culture them in a shaking table (30 ℃, 200 rpm/min) until the logarithmic growth stage, and centrifuge (10000 × g, 4 ℃ ,10 min), collect the bacteria. We wash them twice with PBS buffer, resuspend the bacteria, and prepare the bacterial suspension. The prepared bacterial suspension was inserted into BSM medium with or without the addition of 100mg/L HA at an initial concentration of OD_{600}=0.1, and then incubated in a shaking table (30 ℃, 200 rpm/min) with the addition of PTBP as a blank control. Each treatment group had three replicates. The prepared bacterial suspension was inserted into a BSM-PTBP (130 mg/L) liquid medium with or without 100 mg/L HA at an initial concentration of OD_{600}=0.1, and then incubated in a shaking table (30 ℃, 200 rpm/min). HA and PTBP were added as blank controls. Take regular samples, detect the residual concentration of PTBP by HPLC, and detect the bacterial concentration (OD_{600}) by spectrophotometer. Zeta potential measurement: Collect the above Tas13 cells for biodegradation experiments, and immediately measure them at room temperature using zetaphoremeter4.20 using a laser Doppler velocimeter. Raman spectroscopy.

2.5 Enzyme Activity Determination of the Degrading Gene C23O of Strain Tas13

The presence of humic acid can promote the growth of strain Tas13. To verify whether humic acid can regulate the enzyme activity of the main degradation gene C23O of Tas13, the specific experimental operations are as follows:Collect Tas13 bacterial suspension according to the method described in 1.3, suspend the bacterial weight in BSM medium, adjust the initial concentration of

OD_{600}=0.08 to the BSM-PTBP (200 mg/L) medium with/without HA (100 mg/L), culture in shaking table (30 ℃, 200 rpm/min), sample and measure the enzyme activity for 6h.

C23O determination of strain Tas13: centrifugation of bacterial solution (10000 × g. 4 ℃, 10 min), collect the bacteria, and then wash them with PBS (0.01M pH=7.5) buffer solution twice. Suspend the bacterial weight in 3 mL of buffer solution.[30]. The C230 enzyme activity determination mixture contains 0.01M PBS (2.8mL pH7.5), 0.01M catechol (0.1mL), and 0.1mL of enzyme solution. It is fully mixed and uniform. The reaction mixture is continuously reacted at 30 ℃ for 30 minutes. At the 0^{th} and 30^{th} minutes after mixing, the absorbance is recorded at 375nm using an ultraviolet spectrophotometer. The enzyme activity is calculated according to the following equation.

Enzyme activity (μmol / min)=$\varepsilon\frac{L}{V}\frac{\triangle OD}{30}$

L=path length (cm), V=reaction volume (mL), ε = Molar extinction coefficient. In the case of C23O, ε = 14700 $mM^{-1}cm^{-1}$ (molar extinction coefficient of 2-hydroxymyxofuroic acid semialdehyde)

3. Results and Discussion

3.1 Adsorption and desorption characteristics of PTBP by humic acid

The results show that humic acid isothermal adsorption of PTBP at 30℃. The total adsorption amount and adsorption rate of PTBP increased with the increase of humic acid concentration. The highest adsorption rate is 48.81%. The total amount and desorption rate of humic acid to PTBP also increased with the increase of total adsorption amount. In conclusion, the initial concentration of humic acid affects the adsorption level of PTBP, and humic acid has a strong adsorption effect on PTBP. The possible reason is that there are a large number of hydroxyl (-OH) and carboxylic (-COOH) active functional groups in humic acid.

Table 1 Different concentrations of humic acid isothermal adsorption of PTBP（30℃）

HA concentration（mg/L）	Initial concentration of PTBP（mg/L）	Equilibrium concentration of PTBP（mg/L）	Total adsorption amount of PTBP（mg/g)	PTBP adsorption rate(%)	Total desorption（mg/g)	desorption rate(%)
5	5	3.96	6.15	20.75	2.94	47.81
5	5	3.50	10.57	29.96	0.37	3.51
5	5	4.02	5.75	19.59	1.41	24.52
10	5	3.74	9.23	25.16	1.77	19.21
10	5	3.93	4.12	21.40	0.21	5.00
10	5	2.94	17.50	41.18	5.95	33.98
20	5	3.34	14.07	33.22	3.64	25.84
20	5	3.80	2.83	23.91	2.88	101.74
20	5	3.76	10.27	24.77	8.70	84.68
100	5	3.80	10.59	24.04	3.83	36.17
100	5	2.99	16.96	40.16	5.25	30.97
100	5	2.56	21.74	48.81	4.31	19.81

3.2 Effect of humic acid on degradation of PTBP by strain Tas13

3.2.1 Strain growth status

The effect of humic acid on the growth of the strain is shown in Figure 3. The growth rate of the Tas13 strain in the treatment group is humic acid+PTBP+Tas13, PTBP+Tas13, HA+Tas13, and Tas13 in order from high to low. The growth rates of strain Tas13 at 24 h were 176.29%, 147.67%, 71.70%, and 71.32%, respectively. The growth rate of Tas13 strain in the treatment group with only humic acid added was higher than that in the treatment group with only Tas13. Strain Tas13 is a screened PTBP degrading bacterium that can grow using PTBP as the sole carbon source and energy source. In the treatment group with the addition of humic acid and PTBP at the same time, the growth rate of Tas13 was higher than that of the treatment group with PTBP as the sole carbon source, and the growth rate of Tas13 significantly increased by 28.62%, which means that humic acid can provide the nitrogen source required for microbial life activities, and the carbon source significantly promoted the growth and reproduction of Tas13.

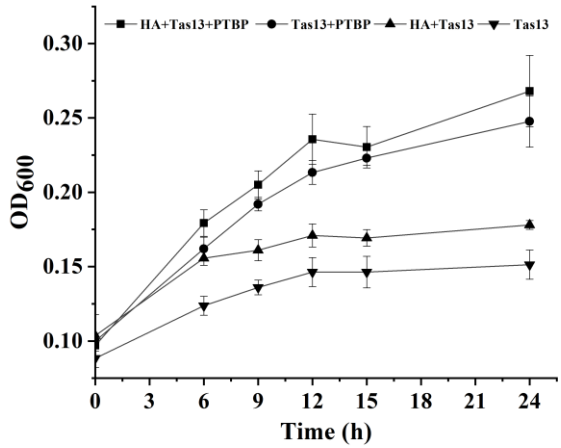

Fig 3. Growth curve of Tas13 strain in the presence of humic acid. The error bars showed the standard error (n=3).

3.2.2 PTBP residual concentration

The effect of humic acid on the degradation of PTBP by Tas13 is shown in Figure 4. The addition of humic acid significantly promotes the degradation of PTBP. During the entire experimental period, the treatment group strain Tas13 with both humic acid and PTBP can degrade 130 mg/L of PTBP within 12 hours, and the treatment group Tas13 with PTBP can degrade 130 mg/L of PTBP within 15 hours. In addition, it can be seen from this experiment that the adsorption amount of humic acid on PTBP also depends on the initial amount of PTBP.

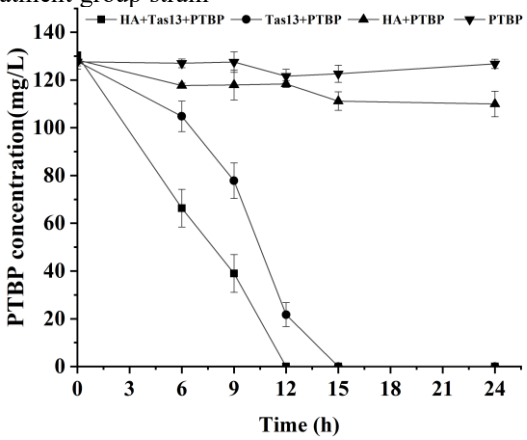

Fig 4. Effect of humic acid on degradation of PTBP by Tas13 strain. The error bars showed the standard error (n=3).

3.2.3 Raman Spectrogram Detection of Strain Tas13

Under the treatment of PTBP, there are obvious vibration peaks at 1576, 1450, 1326, 1218, and 1122 cm^{-1}. Under HA-PTBP treatment, there was some shift in the amide I and amide III modes of the main source and protein [13], indicating that the functional group interaction between the protein and the humic acid polymer was conducive to bacterial growth, and Tas13 grew well in the presence of humic acid. C-H bending at 1450 cm^{-1}, 1326 cm^{-1} due to cellular proteins and lipids [13]. HA-PTBP treatment has shifted to a certain extent, which may be due to the relaxation of humic acids with membrane proteins and lipid monolayers to promote subsequent intracellular metabolism [14]. In addition, a cyclic respiratory pattern of tryptophan (Trp) appeared at 726 cm^{-1}[15]. This indicates that protein interactions are different in the presence of humic acid or PTBP.

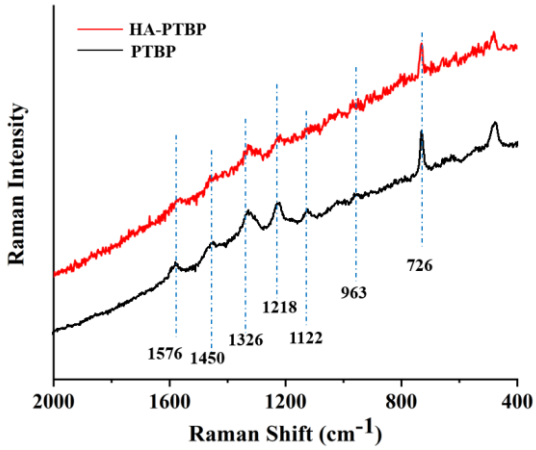

Fig 5. Raman spectra of strain Tas13 treated with HA-PTBP and PTBP.

3.2.4 Effect of humic acid on cell surface zeta potential

The changes in the zeta potential (z) on the cell surface are shown in Table 2. At initial 0 h, the charge on the cell surface is negative. As biodegradation begins, the zeta potential begins to decrease. In the treatment group with humic acid, the zeta potential decreases significantly, reaching a minimum of -23.3 mV, and then tends to stabilize. Humic acids contain a variety of active groups that can interact with bacterial surface functional groups. Denaturated membrane proteins that produce and regulate carboxyl (-COOH), amino (-NH_2), and hydroxyl (-OH) groups, similar to activated sludge, may further increase the negative potential of zeta potential. The decrease in zeta potential of Tas13 may be due to the continuous accumulation of PTBP in the lipid bilayer causing swelling of the Tas13 cell membrane. The addition of HA promotes the transport of PTBP from the outside to the inside of bacterial cells, thereby effectively degrading them.

Table 2. The zeta potential for Tas13 in the PTBP, humic acid and humic acid-PTBP treatment

Zeta (mV)	0h	6h	9h	12h	15h
HA+Tas13	-15.7	-16.2	-15.2	-17.2	-17.1
Tas13+PTBP	-15.7	-17.3	-20.8	-18.2	-15.6
HA+Tas13+PTBP	-15.7	-23.3	-21.4	-17.3	-16.9

3.2.5 Determination of enzyme activity of degradation gene C23O of strain Tas13

At the 6th hour, the Tas13 bacterial concentration in the treatment group with humic acid addition was 0.14 higher than that in the treatment group without humic acid addition. This is the same as the above experimental results. During the experiment, it was detected that when the initial OD_{600}=0.08 and PTBP=200 mg/L, in the Tas13+PTBP treatment group, the Tas13 strain did not grow and the bacterial concentration was lower than the initial bacterial concentration at 3h. This is similar to previous studies. When the initial PTBP concentration was too high, the growth of Tas13 was inhibited for a short time, while the addition of HA reduced the toxicity of PTBP to Tas13, improved the tolerance of Tas13 to PTBP, and promoted the growth of the strain. In addition, humic acid treatment can promote the growth of strain Tas13, and can also upregulate the C23O encoding gene. C23O is an important enzyme in the degradation pathway of aromatic compounds. Many bacteria that degrade aromatic pollutants contain C23O encoding genes

Table 3. Effects of humic acid on C23O enzyme activity of Tas13 strain during biodegradation

Treatment	OD_{600}	C23O (μmol / min)
Tas13+PTBP	0.09±0.00	3.05±0.41
HA+Tas13+PTBP	0.14±0.01	5.66±1.02

4. Conclusion

Based on the results and discussions presented above, the conclusions are obtained as below:

(1) Determination of PTBP by humic acid adsorption.The presence of humic acid can significantly promote Tas13 ccan degrade PTBP

(2) Zeta potential and Raman spectroscopy indicate that the addition of HA significantly decreases the Zeta potential (increases proton permeability). HA interacts with functional groups on the surface of bacteria, producing and regulating transmembrane transport of

carboxylic, amino, and hydroxyl groups, which may further increase the negative potential. Promotes the transport of PTBP from the outside to the inside of bacterial cells, thereby effectively degrading it. This may be due to the relaxation of humic acids and membrane proteins and lipid monolayers to promote subsequent intracellular metabolism.

(3) According to the C23O determination of strain Tas13, humic acid can upregulate the C23O enzyme activity gene to a certain extent, which enhances the gene expression of C23O of the mediated strain Tas13.

Acknowledgments

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