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Effects of Four Metals on the Degradation of Purified Terephthalic Acid Wastewater by *Phanerochaete chrysosporium* and Strain Fhhh

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Purified terephthalic acid (PTA) is one of the most commonly used raw materials for the artificial synthetic products, such as polyester fibers, pesticides, plastics and dyes. PTA wastewater (PTAW) contains benzyl pollutants, which are difficult to be degraded by the native microorganisms. Also, the benzyl pollutants are easy to be transformed into different kinds of phthalic ester chemicals, which could be the causes for various diseases (Kluwe 1982). The cost of treating PTAW is still high (over 10RMB/m^3) in China. The functional strains and effective processes are needed to improve the PTAW treatment efficiency.

The fungus *Phanerochaete chrysosporium* (PC) is effective on the degradation of almost all hazardous persistent organic pollutants (POPs) since it secrets extracellular enzymes, such as manganese peroxidase (MnP) et al. (Bogan et al, 1996). Strain Fhhh is a genetically engineered microorganism strain (GEMs) constructed with the three parental strains, the fungus PC (eukaryote), *Saccharomyces cerevisiae* (SC, eukaryote) and the native bacterium *Pseudomonas* YZ1 (prokaryote), through the protoplast fusion by our research group. Fhhh could integrate the high degradability, the high flocculation and the high adaptability from its three parental strains into its cell demonstrated in the degradation of PTAW (Zhong et al. 2000, Chen et al. 2002). It was because that Fhhh could integrate the functional genes from Fhhh's three parental strains, including encoding *mnp* gene into its cell.

Various metals existing in wastewater could affect the expression level of the peroxidase genes. There are only four metal elements, Mn, Cu, Zn and Se, listed as the limited inorganic pollutants in The National Wastewater Discharge Standard of China (NEPAC, 1996). The four metals might affect the MnP level of the expression of the *mnp* gene of PC and Fhhh, and affect the degradation rate for PTAW. The aim of this study was to test the effects of the four metals, Mn, Cu, Zn and Se, on the expression levels of the *mnp* gene and the degradation efficiency in PTAW. The results would be helpful to control the *mnp* gene expression at a higher level and improve the wastewater treatment efficiency in a pilot study.

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MATERIALS AND METHODS

The first parental strain was *P. chrysosporium* (PC) provided by Professor Wang of NJFU. The second parental strain was the native bacterium strain, *Pseudomonas* YZ1, isolated from the activated sludge of the PTAW treatment plant at NJYZ. The third parental strain is *S. cerevisiae* (SC) obtained from the Dongguan Sugar Mill. Strain Fhhh was constructed by our research group (Zhong et al. 2000). The medium for both PC and Fhhh contained 2% glucose supplemented with 20% potato extract.

The polymerase chain reaction (PCR) was used to demonstrate the three DNA fragments, *mnp* gene from PC, *FLO1* gene from SC and 16S rDNA from YZ1, existed in Fhhh cell. The three pair primers for *mnp, FLO1* and 16S rDNA were synthesized by Bioengineering Company, Shanghai (Godfrey et al. 1990; Teunssion et al. 1993 and Griffiths et al. 2000). DNA markers were 100-3000bp, GeneRuler^{TM}, MBI Fermentas, USA; PCR kit was from the Shanghai Bioengineering Company.

PTAW sample was from NJYZ Co. and the food wastewater (FW) sample was from NTFSM Co. Measurements of the wastewater parameters, COD_{cr} (chemical oxygen demand tested with $K_2Cr_2O_7$), BOD_5 (biochemical oxygen demand in 5 days), TSS (total suspended solid) and biomass, were according to APHA et al. (1992). The metal concentrations were measured with ICP-AES (J-A1100, USA). The concentrations of the four metals in PTAW and FW were regulated to the designed levels with Mn (as $MnCl_2$), Cu (as $CuCl_2$), Zn (as $ZnCl_2$) and Se (as Na_2SeO_3). TN (total nitrogen) and TP (total phosphorus) were measured by the oxidation method of $K_2S_2O_8$ (Qian 1987).

The specific degradation rate (SDR) abbreviation is q. q (h^{-1})$=\mu(S_0-S_n)/$ [$X_0(e^{\mu t}-1)$]. SDR is a kinetic parameter used to indicate the degradability of the strains in the wastewater in this research. Here, S_0 is the organic pollution concentration COD_{cr} (mg/L) at the beginning of the reaction; S_n is that at the end of the reaction; μ is the specific growth rate, it is also a kinetic parameter used to indicate the growth ability of the strains. $\mu(h^{-1})= (\ln X_n - \ln X_0)/ t$. Here, X_n is the biomass (mg/L) at the end of the reaction, X_0 (mg/L) is the biomass (mg/L) at the beginning of the reaction, and t is reaction time (h) (Cheng et al. 2000).

The wastewater was centrifuged at 3,000 rpm for 10 min to remove the cells of PC and Fhhh, and then $(NH_4)_2SO_4$ was added to the wastewater until over-saturation for precipitating and collecting the crude protein including MnP fraction. MnP protein was further isolated from the crude protein with 15% SDS-PAGE and measured by the spectrophotometer method of Coomassie blue R-250 (Li et al. 1994).

The specific activity units (SAU) of MnP were tested with 10mM H_2O_2 as the substrate and the buffer was 0.03M KH_2PO_4-Na_2HPO_4, pH 7.0 (Gilbert 1982). The MnP protein

obtained mixed with the reaction solution for exactly 3 min at 25℃. Then 0.5ml 0.6M KI was added in the reacted solution and 8mM $(NH_4)_6MO_7O_{24}$ was used as the indicator. The reaction system was then titrated with 0.0125M $Na_2S_2O_3$. One SAU was defined as the MnP amount for transforming 1 mM of H_2O_2 per minute, [mM H_2O_2 /(min. mg MnP)].

RESULTS AND DISCUSSION

Fhhh contained the three DNA fragments of *mnp* gene from PC, *FLO1* gene from SC and 16S rDNA from YZ1, demonstrated by PCR. But the three parental strains, PC, SC and YZ1, could only contain their own DNA fragment individually (Table 1). It means that Fhhh had genetic stability after isolated and purified over 700 generations. In Table 1, "+" means PCR product existed; "-" means no PCR product existed.

Table 1. The PCR results of the three DNA fragments existing in the four strains.

DNA for PCR	DNA sequences of primers	PCR products			
		PC	SC	YZ1	Fhhh
mnp	5'-ATG GGA GTA GCG GAA GCA-3'	+	-	-	+
(470bp)	3'-CGA GTG CGG TGA AGA GTA-5'				
FLO1	5'-CGG AAT TCC TCC AAC TAC TG-3'	-	+	-	+
(407bp)	3'-CAG AAG CGC AGG CTT AAG GC-5'				
16S rDNA	5'-GGT TAC CTT GTT ACG ACT T-3'	-	-	+	+
(1500bp)	3'-GAC TCG GTC CTA GTT TGG AG-5'				

The qualities of the raw PTAW and FW are shown in Table 2. For the four metal elements, only the concentration of Mn in PTAW was 18.6mg/L being higher than 5mg/L listed in GB3rd (NEPAC, China, 1996).

Table 2. The qualities of PTAW and FW and the standard values of GB3rd.

Item	PTAW	FW	GB3rd	Item	PTAW	FW	GB3rd
Mn, mg/L	18.60	0.04	5.0	COD_{cr}	4,344	750	500
Cu, mg/L	0.01	0.02	2.0	BOD_5	2,828	324	300
Se, mg/L	0.05	0.05	0.5	SS	103	285	400
Zn, mg/L	0.26	0.08	5.0	TP	38.8	1.6	0.3
pH	4.90	6.75	6~9	TN	116.7	4.29	25

In Table 3, the data involving the effects of the four metals on the degradation of FW for PC were listed. They were obtained from a set of 9 group tests of the orthogonal method. The optimal levels of the four metals were 5mg/L (Mn), 5mg/L (Cu), 0.26mg/L (Zn) and 0.05mg/L (Se) listed on the right of Table 3, while the values of the specific degradation rate (SDR) for PC in FW were at the highest levels. When the

concentrations of Mn, Cu, Zn and Se were at the level 1, the values of SDR were the lowest. It is clear that the levels of the four metals in FW (Table 2) were all lower than the optimal levels (Table 3) and they need to be increased in practical treatment.

Table 3. The effects of the 4 metals on SDR for PC in the degradation of FW.

	Tested values						Optimal values	
	Level 1		Level 2		Level 3		Optimal level	
	Metal (mg/L)	SDR (h^{-1})	Metal (mg/L)	SDR (h^{-1})	Metal (mg/L)	SDR (h^{-1})	Metal (mg/L)	SDR (h^{-1})
Mn	10	0.09	5	0.53	0.01	0.23	5	0.53
Cu	10	0.06	5	0.52	0.003	0.21	5	0.52
Zn	10	0.10	5	0.25	0.26	0.51	0.26	0.51
Se	1	0.03	0.5	0.20	0.05	0.23	0.05	0.23

There were three test groups for measurement of the effects of the 4 metals at the optimal levels on the values of SAU and SDR as shown in Table 4. In Raw FW group, the 4 metal concentrations were at their original levels. In GB3rd group, the 4 metal levels were the concentrations as that listed in GB3rd. And in Optimal group, the 4 metal levels were the optimal concentrations according to the results in Table 3. Table 4 shows that the values of SDR and SAU for PC and Fhhh strains were both affected by the 4 metals. And they all reached their highest values while the 4 metals were at their optimal levels in FW.

Table 4. The effects of 4 metals on SAU and SDR for the two strains in FW.

Item	Raw FW group	GB3rd group	Optimal group
Mn (mg/L)	0.04	5.0	5.0
Cu (mg/L)	0.02	2.0	5.0
Zn (mg/L)	0.08	5.0	0.26
Se (mg/L)	0.05	0.5	0.05
SAU(Fhhh),[mM/(min.mg)]	17.04±0.04	14.27±0.03	20.53±0.05
SAU(PC), [mM/(min.mg)]	11.19±0.04	10.18±0.04	13.28±0.04
SDR(Fhhh), (d^{-1})	17.36	16.19	23.76
SDR (PC), (d^{-1})	8.39	8.31	14.95

Table 5 shows that, the values of SAU and SDR for both the two strains in PTAW were affected by the four metal levels. And they reached the highest values at the optimal levels listed on the right of Table 5. The expression of *mnp* gene for Fhhh and PC could reach the best levels (Cheng et al. 2002). The levels of the 4 metals had a significant influence on SAU levels (r=0.998), which in turn affected the SDR values in PTAW. It should be pointed out that the GEMs Fhhh could excrete MnP and degrade wastewater both at higher levels than its parental strain PC.

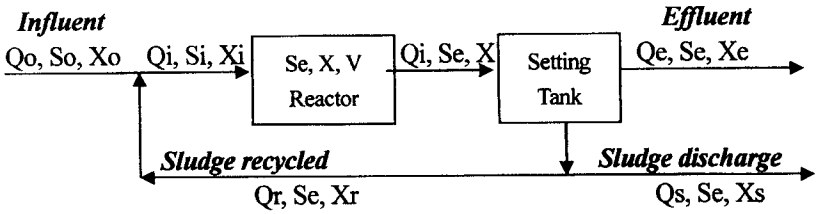

Figure 1. Flow scheme of the activated sludge process

Table 5. The effects of 4 metals on SAU and SDR for the two strains in PTAW.

Item	Raw PTAW group	Metal concentrations regulated	
		GB3rd group	Optimal group
Mn (mg/L)	18.60	5.0	5.0
Zn (mg/L)	0.26	5.0	0.26
Cu (mg/L)	0.01	2.0	5.0
Se (mg/L)	<0.05	0.5	0.5
SAU(Fhhh),[mM/(min.mg)]	1.38±0.06	1.48±0.10	1.61±0.04
SAU(PC), [mM/(min.mg)]	1.21±0.04	1.31±0.08	1.35±0.09
SDR(Fhhh), (d^{-1})	0.21	0.24	0.34
SDR (PC), (d^{-1})	0.14	0.13	0.27

Table 6. The effects of the 4 metals on the process designation for PTAW treatment.

Parameters in PTAW treatment process	In raw PTAW			At the optimal
	PC	YZ1	Fhhh	Fhhh
Qo, the raw wastewater flow, m^3/d	10,000	10,000	10,000	10,000
So, the raw wastewater COD_{cr}, kg/ m^3	4.344	4.344	4.344	4.344
Se, COD_{cr} of effluent, kg/ m^3	0.01	0.01	0.01	0.01
Xe, biomass of effluent, kg/ m^3	0.0157	0.0036	0.0006	0.0005
X, biomass in reactor, kg/ m^3	0.7601	1.5964	0.2622	0.2465
θ, hydraulic retention time, d	0.17	0.05	0.05	0.04
θ_c, sludge retention time, d	10.26	41.15	39.07	42.49
Xr, recycled sludge concentration, kg/m^3	3.82	3.32	0.56	0.49
Qr, returned flow, m^3/d	2430	9220	8800	10000
q, the specific degradation rate(SDR), d^{-1}	0.2981	0.0304	0.1629	0.1724
μ, the specific growth rate, d^{-1}	0.0975	0.0243	0.0256	0.0235
Vmin, minimal reactor volume, m^3	2632	1785	1781	1655
Cost for reactor Vmin, 10^6	1.3160	0.8925	0.8905	0.8275
Cost for aeration/yr, 10^6	25.6608	6.1495	3.3901	3.0748
Cost for pumping Qo/yr, 10^6	0.7096	0.7096	0.7096	0.7096
Cost for pumping Qr/yr, 10^6	0.1742	0.6543	0.6244	0.7096
Total cost for the first year, 10^6	27. 8588	8.4059	5.6164	5.3295

Fig.1 is the flow scheme of the PTAW treatment process for forecasting the effects of the four metal levels on the treatment process design by use of the Environmental Biotechnological Informatics Software (NJU-Ebis1) (Cheng 2003). All the symbols in Fig. 1 can be found in Table 6.

In Table 6, the cost of electricity consumed was 1.8 RMB/(kw), and the cost for the construction of the wastewater treatment basin was 500 RMB/m^3 used in the calculation with the software of NJU-Ebis1. At the optimal levels of the 4 metals, treating PTAW with Fhhh strain had more advantages than that at the non-optimal levels as the followings:

(1) It could save up to 7.61% of the basin volume, and be equivalent to 286,900 RMB. The money is equivalent of the annual payments of 48 workers in China.
(2) It could save 3,076,400 RMB compared with YZ1. The money is equivalent to the annual payments of 512 workers.
(3) It could save 59.03% of basin volume compared with PC, be equivalent to 22,529,800 RMB. It is equivalent to the annual payments of 3755 workers.
(4) SC cannot grow in PTAW, whose flocculation gene, *FLO1,* was integrated into Fhhh cells and could improve the flocculation of the strain (Chen et al.2002).
(5) Fhhh would be useful in PTAW treatment, but it needs the optimal levels of the 4 metals for the secretion of highly active MnP in order to degrade the wastewater effectively and economically.

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