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Optimization of Xhhh strain biodegradation with metal ions for pharmaceautical wastewater treatment

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Abstract: *Effects of three ions, Mn^{2+}, Cu^{2+} and Zn^{2+} on biological treatment of pharmaceutical wastewater by a functional strain Xhhh were investigated. Through orthogonal tests, Cu^{2+} was determined to be the most important factor influencing Xhhh biodegradation performance. Biodegradation kinetic experiments demonstrated that with Cu^{2+} concentration at about 2.00 mg l^{-1}, the maximum of specific growth rate and specific degradation rate were obtained to be 0.033 and 0.075 d^{-1}, respectively. The optimal levels of Mn^{2+} (5.00 mg l^{-1}), Cu^{2+} (2.00 mg l^{-1}) and Zn^{2+} (5.00 mgl $^{-1}$) were achieved based on experimental results of their effects on the activities of manganese peroxidase and lignin peroxidase, and biodegradation kinetic parameters. Among three types of biodegradation kinetic models (Monod, Tessier and Contois), Tessier model was found most reasonable for kinetics description of Xhhh growth (R^2 = 0.995) and pollutants degradation (R^2 = 0.970) in the case of metals optimization. Both kinetics evaluation and experimental results demonstrated that optimization with the three metals made a great contribution to Xhhh growth and COD removal for pharmaceutical wastewater treatment.*

Key words: *Metal ions, Functional strain, Pharmaceutical wastewater, Biodegradation kinetics, Peroxidase*
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Introduction

Pharmaceutical industries produce high quantities of wastewater with varying composition and relatively high toxicities (Singh and Singh, 2006; Zounkova *et al*., 2007). The wastewater can not been efficiently treated with biological methods as a result of the presence of antibiotic or aromatic pollutants, which not only inhibit microbial growth, but also lower biodegradability of the pollutants in the wastewater (Schroder, 1999; Singh *et al.,* 2008; Dayaram and Dasgupta, 2008). Increasing concerns have been placed on seeking for innovative biological ways to treat pharmaceutical wastewater effectively and economically (Ternes *et al*., 2002; Snyder *et al*., 2005).

A functional strain (Xhhh) was constructed through protoplast fusion by our research group to improve treatment performance for pharmaceutical wastewater, and the strain has been successfully applied in the wastewater treatment plant (Nhwa Pharmaceutical Co., Xuzhou, China) (Cheng *et al*., 2004; Zhang *et al*., 2004; Cheng *et al*., 2006). As a fusant, Xhhh simultaneously inherited the high capacities of degradation, flocculation and adaptation from its three parental strains *Phanerochaete chrysosporium, Saccharomyces cerevisiae* and native bacterium *Bacillus* XZ, respectively. The related protoplast fusion technique has been patented (Cheng *et al*., 2006; Zhao *et al*., 2007).

Xhhh's degradation capability mainly depends on the expression of its functional enzymes including manganese peroxidase (MnP) and lignin peroxidase (LiP), which are encoded by *mnp* and *lip* gene, respectively and the genes are inherited from the parental strain *P. chrysosporium* (Zhao *et al*., 2007, Zhang *et al*., 2009). MnP and LiP can efficiently catalyze biodegradation of various aromatic compounds, but the catalytic activities can be influenced by some inorganic elements, such as Mn^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+} and Hg^{2+} (Kirk *et al*., 1976; Haapala and Linko, 1993; Mandal *et al*., 1998; Youngs *et al*., 2000).

Among these metals, manganese is a necessary component in protein structure of MnP and plays an indispensable role in its catalytic cycle (Gold and Alic, 1993). Cu^{2+} acts as the assistant factor for the copper radical oxidase to produce extracellular H_2O_2, which is required by LiP and MnP to finish their catalytic effects (Martinez *et al*., 2004). Zinc, an essential nutrient for fungal growth, participates in many diverse cellular processes (Jellison *et al*., 1997). Thus, this study aims to investigate the effects of Mn^{2+}, Cu^{2+} and Zn^{2+} on treatment performance of pharmaceutical wastewater by Xhhh through the orthogonal test and biodegradation

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kinetic experiment and parameters evaluation with kinetic models.

Materials and Methods

Strains and culturing conditions: Xhhh strain was constructed using protoplast fusion technique (Cheng *et al*., 2006). The strain were cultivated at 30°C on potato-glucose media containing 20% potato extract, 2% glucose and 2% agar in mass. Agitation culture was carried out at 150 rpm in 250 ml Erlenmeyer flasks containing 70 ml media without agar. After culture for 3-5 days Xhhh mycelia were harvested for use in orthogonal test and biodegradation kinetic experiment.

Wastewater characteristics: The chemical synthesis-based pharmaceutical wastewater was sampled from a local wastewater treatment plant (Nhwa Pharmaceutical Co., Xuzhou, China) and stored at 4°C. The information about main organic components in the wastewater was kindly provided by the plant, and the characteristics of water quality are given in Table 1. Before orthogonal test and biodegradation kinetic experiment, ratio of chemical oxygen demand (COD_{Cr}), total nitrogen (TN) and total phosphorus (TP) was regulated to be about 100:10:1, and three metal ions (Mn^{2+}, Cu^{2+} and Zn^{2+}) were added into the raw wastewater in the forms of $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$, respectively. All chemicals mentioned above were of analytical reagent grade.

Orthogonal test: The experimental design of L9 (3^3) orthogonal test is shown in Table 2. In the nine cases, three different concentrations were assigned for each metal ion. The low levels were designated as the concentrations of three metal ions in the raw wastewater. The middle levels were determined according to the regulatory discharge limits in China (MEPPRC, 1996), and the high levels were designed to be the optimal concentrations for another functional strain Fhhh (Yan *et al*., 2004), which were above the limits (Table 2). *K* value was determined to be the mean of the three test cases for each factor in the same level. Metal concentration corresponding to the highest *K* value was assigned for the optimal level of each factor. *R* value referred to the result of extreme analysis and the most important test factor was determined corresponding to the highest *R*. The same batch of the wastewater was used in the orthogonal tests to mitigate the impact of the water quality variation and the assay was carried out. Inoculated Xhhh was separated from the cultivation media by centrifugation at 1000 × g for 20 min, and transferred to the diluted pharmaceutical wastewater volumetrically with 90% distilled water and cultured in shaken flasks at the speed of 150 rpm for 7 days. After mixture of Xhhh and wastewater was centrifuged at 1000 × *g* for 20 min, the biomass was collected by washing the residues twice with distilled water and the upper extract was used to determine COD_{Cr}. To further investigate the effects of copper on biodegradation and adaptation ability of Xhhh in pharmaceutical wastewater, Xhhh was cultivated in the wastewater with a series of concentrations of Cu^{2+} (0, 1.00, 2.00, 3.00, 4.00 and 5.00 mg l^{-1}) for 48 hr, and initial and final concentrations for both COD and biomass were detected for the calculation of specific growth rate (μ) and specific degradation rate (q). MnP and LiP activities were also investigated in each reaction liquid with different levels of Cu^{2+}.

Biodegradation kinetic experiment models: Pharmaceutical wastewater with initial COD_{Cr} concentrations ranging from 200 to 5000 mg l^{-1} was used for biodegradation kinetic experiments. The water samples were divided into two groups. In one group, metal regulation was not preformed in raw pharmaceutical wastewater, and in the other, the three metal ions were regulated after dilution to optimal concentrations determined by the orthogonal test. Culture of Xhhh was centrifuged at 1000 × g for 20 min and the residues were washed twice with distilled water. The washed residues were blended in distilled water by a Waring blender for 15 s, and the homogenized microorganism suspension was finally mixed with wastewater of different COD_{Cr} concentrations in 250 ml Erlenmeyer flasks containing 80 ml of the mixture. All the agitated mixtures were cultivated at 150 rpm and 30°C. Initial COD_{Cr} and biomass were assayed before agitation, and final COD_{Cr} and biomass were determined after reaction for 48 hr. All the tests and analyses for orthogonal and biodegradation kinetic experiments were performed in triplicate. Specific growth rate (μ) and specific degradation rate (q) were defined and determined according to Zhang *et al*. (2006a). Three biodegradation kinetic models, Monod, Tessier and Contois, were used for kinetic parameters

Table - 1: Characteristics of raw pharmaceutical wastewater

Composition	Concentration (mg l^{-1})	Composition	Concentration (mg l^{-1})
COD_{Cr}	8200-16700	Mn	0.08-0.13
BOD_5	430-920	Cu	< 0.004
Total nitrogen	670-1450	Zn	0.08-1.12
Total phosphorus	6.8-10.5	Se	< 0.15
Tetrahydrofuran	1480-3210	Cd	< 0.004
Toluene	438-1103	Pb	< 0.05
Methylamine	312-863	Fe	0.12-0.36
pH	7.0-8.1	Ba	0.06-0.10

COD_{Cr} = Chemical oxygen demand; BOD_5 = Biological oxygen demand

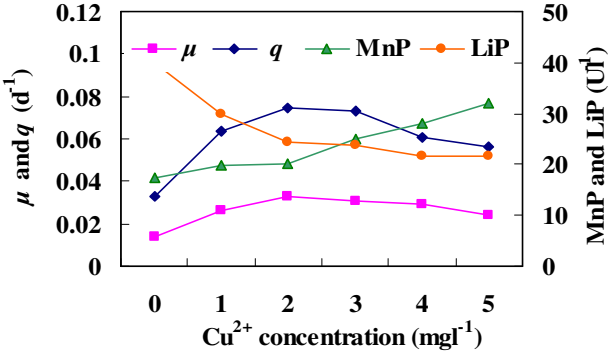

Fig. 1: Effects of Cu^{2+} on the activities of manganese peroxidase (MnP) and lignin peroxidase (LiP), as well as specific growth rate (*i*) and specific degradation rate (*q*) for pharmaceutical wastewater treated by Xhhh

evaluation, and the models were previously described in detail (Tessier, 1942; Monod, 1949; Contois, 1959). To estimate the kinetic parameters based on experimental data, the suitability of the models was determined by the regression coefficient R^2, the sum of squares of differences and *F* statistic test with SPSS 13.0. The degree of convergence was determined by both the convergence criterion and error squared criterion. The sum-of-squares convergence and the parameter convergence were set to 10^{-8}. Least-squares method was used for parameter estimation, and the iteration process was performed based on Levenberg-Marquardt algorithm (Norusis, 2005).

Enzymatic and chemical analysis methods: MnP and LiP activities were measured every 24 hr during the 7 days cultivation. MnP activity was determined spectrophotometrically at 30°C with 2, 6-dimethylphenol (DMP) as a substrate which was oxidized to coerulignone (Wariishi *et al*., 1992). Reaction mixtures contained 100 mM sodium tartrate (pH 4.5), 0.1 ml of crude enzyme, 0.5 mM DMP, 1 mM $MnSO_4$ and 0.1 mM H_2O_2 in a final volume of 1 ml. The reaction was initiated by adding H_2O_2. The extracellular fluid was obtained by centrifuging the culture from the Erlenmeyer flasks at 1000 × *g* for 20 min and used as the crude enzyme. The appearance of coerulignone was monitored at 469 nm wavelength for 5 min. LiP activity was determined spectrophotometrically at 30°C with veratryl alcohol (VA) as a substrate which was oxidized to veratraldehyde (Tien and Kirk, 1988). Reaction mixtures contained 0.2 ml of crude enzyme, 50 mM sodium tartrate (pH 2.5), 1 mM VA, and 1 mM H_2O_2 in a final volume of 1 ml, in which H_2O_2 was added to initiate the reaction. The appearance of veratraldehyde was monitored at 310 nm for 2 min. One unit (U) of enzymatic activity was defined as the amount of the enzyme needed to transform substrate to 1 μM product per minute. Concentrations of the metals were determined using ICP-AES (J-A1100, USA). COD_{Cr}, BOD_5 (biological oxygen demand in 5 days), pH, TN, TP and biomass were analyzed according to the standard methods (APHA, 2005).

Results and Discussion

Effects of metal ions on biodegradation capacity of Xhhh: The results of orthogonal test and extreme difference analysis are presented in Table 2. *R* values of the three metals for the specific degradation rate *q* and specific growth rate μ followed the same sequence: $Cu^{2+} > Mn^{2+} > Zn^{2+}$, indicating that Cu^{2+} was of the greatest importance for Xhhh growth and COD_{Cr} removal. It should be noted that Cu^{2+} is necessary for the copper radical enzyme (glyoxal oxidase), which can produce extracellular H_2O_2, and the later is required by LiP and MnP for in vivo catalytic activity (Martinez *et al*., 2004). Although Mn^{2+} was recognized as the important metal ion to one of Xhhh's parental strain, *P. chrysosporium* (Urek and Pazarlioglu, 2008), in this study Cu^{2+} was found to contribute more to enhance LiP and MnP activities of Xhhh.

Table - 2: Orthogonal test L9 (3^3) analyses for the effects of Mn^{2+}, Cu^{2+} and Zn^{2+} on specific growth rate (μ), specific degradation rate (*q*), and the activities of manganese peroxidase (MnP) and lignin peroxidase (LiP)

Trial No.	Factors and levels			Parameters			
	Mn^{2+} (mgl^{-1})	Cu^{2+} (mgl^{-1})	Zn^{2+} (mgl^{-1})	*q* (d^{-1})	μ (d^{-1})	MnP (Ul^{-1})	LiP (Ul^{-1})
1	10.00	5.00	10.00	0.035	0.013	27.7	15.0
2	10.00	2.00	5.00	0.070	0.031	16.7	16.7
3	10.00	< 0.004	0.11	0.018	0.008	24.0	51.7
4	5.00	5.00	5.00	0.089	0.039	36.7	25.0
5	5.00	2.0	0.11	0.107	0.047	21.3	25.0
6	5.00	< 0.004	10.00	0.037	0.021	18.3	36.7
7	0.10	5.00	0.11	0.043	0.019	31.3	25.0
8	0.10	2.00	10.00	0.047	0.021	22.7	31.7
9	0.10	< 0.004	5.00	0.043	0.016	10.0	31.7
K		Mn^{2+}	High	0.041	0.017	22.8	27.8
			Mid.	0.078	0.036	25.4	28.9
			Low	0.044	0.018	21.3	29.5
		Cu^{2+}	High	0.056	0.024	31.9	21.7
			Mid.	0.075	0.033	20.2	24.5
			Low	0.033	0.014	17.4	40.0
		Zn^{2+}	High	0.040	0.018	22.9	27.8
			Mid.	0.067	0.029	21.1	24.5
			Low	0.056	0.025	25.5	33.9
R		Mn^{2+}		0.037	0.018	4.1	1.7
		Cu^{2+}		0.042	0.018	14.5	18.4
		Zn^{2+}		0.027	0.011	4.4	9.4

K = Average value of the three test cases for each factor in the same level, *R* = Extreme analysis result of *K*

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Table - 3: Kinetic evaluation with various models based on the experimental results before and after optimization with Mn^{2+}, Cu^{2+} and Zn^{2+}

Model	Before or after optimization		μ_{max} or q_{max} (d^{-1})	K_S (mg l^{-1})	X (mg l^{-1})	R^2
Monod	Before	μ	0.025	384	-	0.648
		q	0.408	6173	-	0.968
	After	μ	0.254	3137	-	0.994
		q	0.689	2170	-	0.954
Tessier	Before	μ	0.035	1000	-	0.883
		q	0.414	5366	-	0.928
	After	μ	0.172	2238	-	0.995
		q	0.487	1667	-	0.970
Contois	Before	μ	1.264	558	100	0.984
		q	4.139	476	100	0.927
	After	μ	2.276	558	100	0.810
		q	0.689	541	4	0.954

μ = Specific growth rate (d^{-1}), q = Specific degradation rate (d^{-1}), μ_{max} = The maximum specific growth rate (d^{-1}), q_{max} = The maximum specific growth rate (d^{-1}), X = Biomass, K_S = The substrate-affinity constant (mg l^{-1})

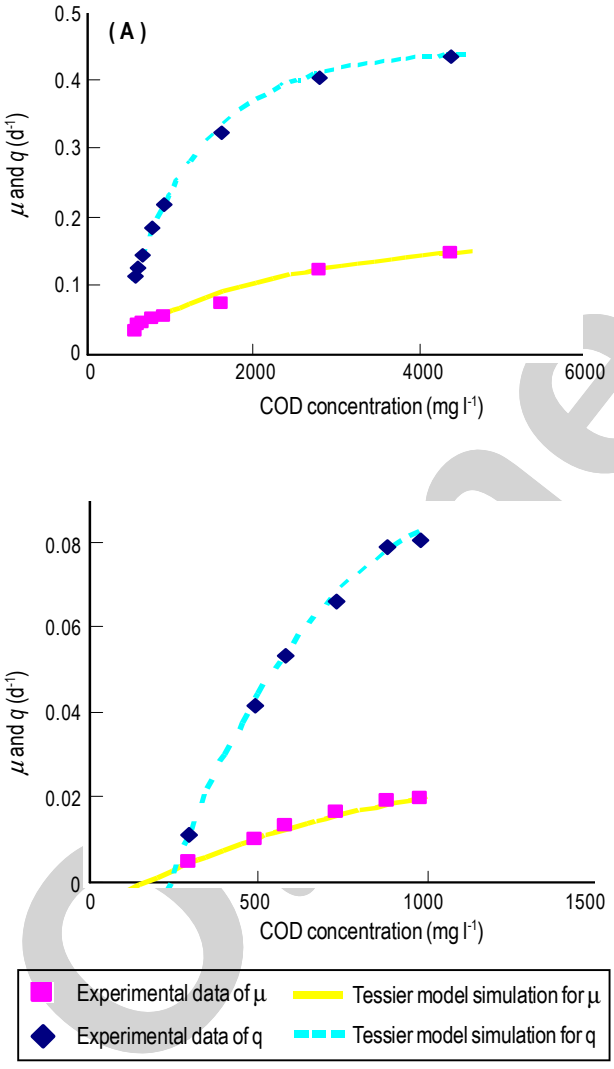

Fig. 2: Biodegradation kinetic simulation for specific growth rate (μ) and specific degradation rate (q) based on the experimental results with (a) or without (b) optimization of metal ions

To further study the effects of Cu^{2+} on Xhhh performance for pharmaceutical wastewater biodegradation, variation of μ, q and MnP and LiP activities were investigated for Xhhh with the increasing of Cu^{2+} concentration. As shown (Fig. 1), with Cu^{2+} in the wastewater ranging from 0 to 5.00 mg l^{-1}, MnP activity was increased correspondingly, but a contrary result was achieved for LiP ($p < 0.05$). However, the peak values of μ (0.033 d^{-1}) and q (0.075 d^{-1}) were achieved at the middle concentration of Cu^{2+} (2.00 mg l^{-1}). Copper, an essential micronutrient for fungal growth, functions as a metal activator of several fungal enzymes (Haapala and Linko, 1993). The level of copper in the environment is critical for equilibrium maintenance of cellular metabolism and the survival of the organisms (Jellison *et al*., 1997). In one word, copper was found to be the most important determinant of degradation performance of Xhhh strain.

No statistical correlation was found between the specific degradation rate and the activities of MnP or LiP for Xhhh cultivated in pharmaceutical wastewater ($p > 0.05$). However, it was previously reported that there were positive correlations ($p < 0.05$ or 0.01) between MnP activities and removal efficiencies for some aromatic pollutants in purified terephthalic acid manufacturing wastewater (Zhang *et al*., 2006b). The specific degradation rate was also found to be directly related to MnP and LiP activities in the petrochemical wastewater (Sun *et al*., 2005; Zhang *el al*., 2006a). MnP and LiP contribute greatly to removal of certain aromatic compounds (Demir *et al*., 2007; Pant and Adholeya, 2007; Pant *et al*., 2008), but complexity in the components of pharmaceutical wastewater makes the biodegradation more dependent on the jointly effects of a variety of catalytic enzymes (Veeresh *et al*., 2002; Matamoros *et al*., 2008). Therefore, it is reasonable and acceptable that the activity of particular enzyme (MnP or LiP) performs no direct correlation to COD removal in the wastewater.

Optimal concentrations of metal ions: According to K values in Table 2, both the specific growth rate μ and the specific degradation rate q were significantly inhibited when the concentration of each metal ion was above middle levels. The activity of LiP achieved its

maximum with both Cu^{2+} and Mn^{2+} at low levels, and Zn^{2+} at middle level. MnP activity reached the peak with Cu^{2+}, Mn^{2+} and Zn^{2+} at high, middle and low levels, respectively. Consequently, considering metal concentrations of regulatory discharge limits (MEPPRC, 1996), the optimal levels of the three ions were determined to be 5.00 mg l^{-1} of Mn^{2+}, 2.00 mg l^{-1} of Cu^{2+} and 5.00 mg l^{-1} of Zn^{2+}.

Yan *et al*. (2004) found MnP secreted by PC and its biodegradation performance arrived at the highest level when Mn^{2+}, Cu^{2+} and Zn^{2+} in petrochemical wastewater were regulated to be 5.00, 5.00 and 0.26 mg l^{-1}, respectively. Mn^{2+} acts as a key role in MnP catalytic system (Sundaramoorthy *et al*., 2005). MnP requires Mn^{2+} to complete the catalytic cycle, especially when degrading non-phenol pollutants under aerobic condition. Mn^{2+} can be oxidized to Mn^{3+}, whose chelate causes one-electron oxidations of various pollutants and produce multifarious radicals. These radicals are thought to be a source of peroxides that are generated via autocatalytic reactions and can be used by MnP or LiP in the absence of external H_2O_2, which are capable of one-electron oxidations of more substrates (Hofrichter, 2002). Therefore, MnP secreted by Xhhh contributed much to efficient degradation of the pollutants in pharmaceutical wastewater, and it is very important to maintain key metallic ion (Mn^{2+}) at appropriate concentrations for MnP expression (Zhang *et al*., 2006a).

Evaluation of biodegradation kinetic parameters: Evaluation of kinetic parameters was conducted based on the experimental results from biodegradation test for pharmaceutical wastewater before and after optimization with the metal ions. All the experimental data were simulated by Monod, Tessier and Contois models (Fig. 2). Table 3 shows the kinetic constants obtained from nonlinear regression analysis. According to the R^2 in Table 3, each of the three models gave a good fit to the experimental data whether the metal ions were regulated or not. Comparatively, Tessier type was found to be the most reliable one among the three models for the kinetics descriptions of both Xhhh growth (R^2 = 0.995) and substrate degradation (R^2 = 0.970) in the case of metal optimization. However, for Xhhh cultivated in the pharmaceutical wastewater without metals regulation, Monod was considered as the most reasonable model in biodegradation simulation (R^2 = 0.968) and Contois model was thought preferentially suitable for microbial growth description (R^2 = 0.984).

As a result, calculated by each model, the maximum specific growth rate μ_{max} of Xhhh in the optimal conditions was found to be higher than that without the metal regulation. The maximum degradation rates q_{max} obtained by both Monod and Tessier model were achieved to be at higher levels than those before optimization. According to evaluation results of biodegradation kinetic parameters, it was found that optimization of the metal ions made a great contribution to both Xhhh growth and COD removal for pharmaceutical wastewater treatment, which is in accordance with the previous study (Zhao *et al*., 2007).

In conclusion, during the course of biological treatment of pharmaceutical wastewater by the functional strain Xhhh, influencing extents of the three metals on the specific degradation rate q and specific growth rate μ followed the same sequence: $Cu^{2+} \geq Mn^{2+} > Zn^{2+}$. Cu^{2+} was found to be the most important metallic factor for Xhhh growth and COD_{Cr} removal. Through orthogonal test, the optimal levels of Mn^{2+}, Cu^{2+} and Zn^{2+} were determined to be 5.00, 2.00 and 5.00 mg l^{-1}, respectively. There were no positive correlations found between MnP or LiP activities and COD removal for pharmaceutical wastewater ($p > 0.05$). Whether metal ions were regulated or not, each of the three biodegradation kinetics models (Monod, Tessier and Contois) gave a good fit to the experimental data. In the case of metal optimization, Tessier type was comparatively found to be the most suitable kinetic model for the descriptions of Xhhh growth and pollutants degradation in pharmaceutical wastewater. Experimental results and model evaluation demonstrate that keeping metal ions at optimal levels is crucial for Xhhh growth and COD removal in pharmaceutical wastewater treatment system, and the regulation strategy can be used as a guide for Xhhh's application to wastewater treatment plant and subsequent optimization of operational process.

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