Application of the Protoplast Fusion Technique to Engineer a Recombinant Microorganism to More Efficiently Degrade Chlorophenols

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Recombinant strain *Xz6-1* was constructed by the protoplast fusion technique with the goal of endowing it with the ability to efficiently degrade pentachlorophenol (PCP). This compound was considered as a representative of possible compounds that can be obtained during the bleaching of pulp for papermaking. The potential of *Xz6-1* and *Pseudomonas putida* to treat PCP synthetic wastewater was explored. The majority of PCP was removed within the first 20 h; two degradation curves were obtained that followed first-order reaction kinetics. The kinetics data revealed that the rate constant for degradation of PCP for *Xz6-1* was 0.063 h^{-1}, a value that was over 50% greater than that of *Pseudomonas putida* (0.040 h^{-1}). Aerobic granular sludge was highly fortified with *Xz6-1* and *Pseudomonas putida* to provide PCP degradability improvements of 180.9% and 98.3%, respectively, relative to the original sludge. All results demonstrate that the protoplast fusion technique is an effective approach to construct a high-activity chlorophenol-degrading strain.

Keywords: Protoplast fusion technique; Pentachlorophenol; Synthetic wastewater; Biodegradability

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INTRODUCTION

Chlorophenols are in general very toxic and recalcitrant compounds produced from industrial operations. Such compounds are discharged into receiving waters and may gradually accumulate in the environment (Bum *et al.* 1999; Takeuchi *et al.* 2000; Essam *et al.* 2006). They were widely produced in industries such as glue manufacture, pesticides, paint, leather, and in the bleaching of pulp for papermaking (Liu *et al*. 1991; Wang and Chen 2005). Consequently, it is necessary to take steps for the development and application of economic, simple, and efficient methods to eliminate these contaminants.

Worldwide, there are several existing treatment alternatives available for the removal of chloroaromatics from industrial effluents, such as aerobic, anaerobic, electrochemical, photocatalysis, and Fenton treatment (Xu *et al.* 2003; Belmonte *et al*. 2006; Hong *et al.* 2008). The biological oxidation process is often considered as the common option due to its cost-effectiveness, versatility in handling a wide variety of organic pollutants, and it helps to avoid secondary pollution.

Many researchers have focused on the biodegradation of chloroaromatics with single dominant bacteria and have demonstrated that the *Pseudomonas putida* strain is

capable of effectively degrading chlorophenols as sole substrates (Farrell and Quilty 2002; Fakhruddin and Quilty 2007).

The objective of the present investigation was to construct a high-activity pentachlorophenol (PCP)-degrading strain by using the protoplast fusion technique. The improvement of chlorophenol-degrading ability of the recombinant strain was investigated by comparing the degradation properties of the recombinant strain and its parent strain. An engineered microorganism can be of paramount significance for the treatment of industrial wastewaters rich in chlorophenols.

EXPERIMENTAL

Materials

Strains

Pseudomonas putida and *Psathyrella candolleana* were obtained from the State Key Laboratory of Pulp and Paper Engineering, South China University of Technology.

Preparation of PCP synthetic wastewater

PCP synthetic wastewater was obtained by a previously published procedure (Chen *et al*. 2013). Three kinds of effluents from ClO_2 bleaching (D), chelating treatment (Q), and H_2O_2 bleaching (P) were selected and mixed in accordance with the ratio of 1:1:1 to form DQP bleaching effluent. The mixing effluent was diluted for 5 times, and the COD of diluted effluent was observed to be 212 mg/L. PCP synthetic wastewater were prepared by dissolving a certain amount of PCP in diluted DQP bleaching effluent, and the PCP concentration was 100 mg/L.

Methods

Microorganisms cultivation

Pseudomonas putida and recombinant strains were cultivated using the method as follows: One loop of strain from the culture-contained agar was separately transferred to 100 mL of the nutrient medium (beef extract 1.5 g/L; glucose 1.0 g/L; peptone 6.0 g/L; yeast extract 3.0 g/L; pH 7.0.) in a glass flask and activated at 30 °C for 24 h. These activated cells were harvested by centrifugation (5000 rpm for 5 min) as inocula in the late exponential phase, and the cells collected were washed with phosphate buffer solution (PBS, NaCl 8 g/L; KCl 0.2 g/L; KH_2PO_4 0.2 g/L; K_2HPO_4 1.15 g/L.).

Psathyrella candolleana was transferred to 100 mL of the potato dextrose broth medium (PDB = 200 g potatoes were peeled and cut into 1 cm^3 pieces and boiled in 500 mL of deionized water for 20 min; the extract was collected by filtration through gauze, followed by addition of 20 g glucose and water to 1000 mL in total volume of neutral pH value) from potato dextrose agar medium (PDA, 2% agar in PDB) after scattering with a parallel scrambler and cultivation for 72 h. Mycelium was gathered and then transferred to 25 mL of basal medium (glucose 10 g/L; $CaCl_2$ 0.01g/L; KH_2PO_4 2 g/L; $MgSO_4$ 0.25 g/L; ethylenediamine tartrate 0.5 g/L; NH_4NO_3 0.5 g/L; and 10 mL of trace elements solution ($FeSO_4 \cdot 7H_2O$ 7.5mg/L; $MnSO_4 \cdot H_2O$ 2.5mg/L; $ZnSO_4 \cdot 7H_2O$ 2 mg/L; $CoCl_2 \cdot 6H_2O$ 3 mg/L; $CuSO_4 \cdot 5H_2O$ 6 mg/L; Na_2BO_3 3 mg/L)) after scattering. Inoculated media were incubated for 12 h at 30 °C.

Isolation of protoplasts

Pseudomonas putida cells were harvested in a 5 mL tube by centrifugation at 3000 rpm for 10 min, washed with PBS buffer and re-suspended in 1 mL of NSM buffer (NaCl 0.55 mol/L; sodium succinate 0.2 mol/L; $MgCl_2 \cdot 6H_2O$ 0.02 mol/L; pH 6.8) containing 1 mL of lysozyme solution (lysozyme powder was dissolved in 50 mL of NSM solution and filtered with 0.22 μm filter.) and 1 mL EDTA solution (Na_2EDTA 0.13 mol/L; NaCl 0.55 mol/L). The cells were shaken at 150 rpm at 37 °C for 60 min to allow the digestion of the peptidoglycan. The protoplasts were obtained and stained with fluorescein 5-isothiocyanate at the concentration of 50 mg/L in PBS for 3 min.

The mycelium of *Psathyrella candolleana* was harvested in a 5 mL tube by centrifugation at 3000 rpm for 10 min, washed with PBS buffer, and resuspended in 1 mL NSM buffer containing 1 mL mixed enzyme solution (1% cellulase and 0.2% helicase, two enzymes were dissolved in 0.6 mol/L $MgSO_4$ solution) and 1 mL EDTA solution. The mycelium was shaken at 150 rpm at 37 °C for 60 min to begin removal of the cell wall, and the protoplasts were then stained with rhodamine 6B at the concentration of 200 mg/L in PBS for 3 min.

Protoplast fusion

The protoplasts of two parent strains were harvested by centrifugation at 2000 rpm for 10 min at 5 °C, respectively, following by mixing and re-suspended in 1 mL of PEG buffer (30% PEG6000, Sigma Chemicals Co., USA, prepared in STC buffer containing 0.6 M sorbitol; 10 mM Tris–HCl; 10 mM $CaCl_2$, pH 6.5). The fusion mixture was incubated at 37 °C for 5 min, and then diluted with 1 mL of NSM buffer. The hybrid cells with both fluorescent labels were selected using a flow cytometer (Guava easyCyte 8, Merck Millipore Co., USA) and re-suspended in 1.5 mL of NSM buffer.

Immobilization of cells

Pseudomonas putida and *Xz 6-1* were immobilized according to the conventional immobilization procedure using Na-alginate (Nagadomi *et al.* 1999), as follows: The cell suspension in 100 mL deionized water (OD_{600} = 0.4) was mixed with autoclaved alginate solution (6%, w/v) of 100 mL. The mixture was stirred until stable gelatinous liquor was formed, put into an injector, and extruded from the needle into a 2% (w/v) $CaCl_2$ solution to form microspheres of small size (3 to 5 mm). The microspheres were rinsed for three times with sterile water and kept in PBS buffer.

Intensification of granular sludge

The collected cells were transferred into a 300 mL conical flask which contains 100 mL of fresh LB medium (tryptone, 10.0 g/L; yeast powder, 5 g/L; NaCl,10 g/L) and 0.5 g (dry weight) of granular sludge. Microbial cells and sludge were co-cultured with shaking of 150 rpm in 30 °C for 24 h, in order to fix the cells on the granular sludge. Fortified sludge was collected by centrifugation at a speed of 5000 rpm and stored with PBS buffer after washing.

Biodegradation with free cells

Recombinant strains selected were propagated to the 15^{th} generation and then used to carry out PCP biodegradation experiment, comparing the results with *Pseudomonas putida*. The cells collected were inoculated into PCP synthetic wastewater to give an initial optical density at 600 nm (OD_{600}) of 0.20±0.01. After inoculation, the conical flasks were

capped with cotton plugs and placed in a shaker controlled at 150 rpm and 30 °C. Cell concentration was tested every two hours, and each experiment was stopped when there was no further increase of OD_{600} (stationary phase). The experiments were performed in triplicate.

Biodegradation with immobilized cells

Immobilized cells were inoculated in a glass flask with 200 mL of PCP synthetic wastewater with a volume ratio of immobilized microspheres and wastewater 3:20, and then the mixture was shaken at 150 rpm at the optimal mesophilic temperature range (30±1°C) for 20 h. The experiments were performed in three replicates.

Biodegradation with granular sludge

The various sludges were added separately into a 500 mL glass flask containing 200 mL of PCP synthetic wastewater to give a sludge concentration of 3 g/L (dry weight), and then cultured with shaking (150 rpm) in 30 °C. 10 mL of wastewater was taken from each conical flask for the analysis of PCP every 8 h, and the experiment was terminated when there was no further reduction of PCP. The experiments were performed in three replicates.

Measurement of UV-visible spectroscopy

All spectrophotometric measurements were made with an Aglient-8453 UV/VIS spectrophotometer (Agilent Technologies, USA), equipped with 1.0 cm quartz cells. Distilled water was used as the blank solution. All spectra were recorded from 190 to 1100 nm with 1.0 nm bandwidth and a scan speed of 1000 nm/min.

Measurement of PCP in wastewater

PCP in synthetic wastewater was quantified based on a UV-vis spectroscopy method, as has been proposed in a previous study (Chen *et al*. 2013).

RESULTS AND DISCUSSION

Protoplast Fusion

Fusion between *Pseudomonas putida* and *Psathyrella candolleana* was performed to construct high-activity chlorophenol-degrading strains. Twelve recombinant strains were isolated based on the morphological characteristics of colonies and an antagonism phenomenon (Table 1), whereas four strains were verified for chlorophenol-degrading capability (Fig. 1).

Figure 1 shows a characteristic UV-Vis peak at 321 nm due to the PCP in wastewater (Chen *et al.* 2013). The absorbance of original PCP synthetic wastewater was 2.64, and it dropped by varying extents when treated with *Xz 6-1*, *Xz 6-3*, *Xz 6-5*, *Xz 8-2*, and *Pseudomonas putida*. The PCP degradation rates were obtained using a PCP determination method proposed in a previous study (Chen *et al.* 2013), and are shown in Table 2. It was shown that the PCP degradation of *Xz8-2* and *Xz6-5* were less than the parent strain (*Pseudomonas putida*), while *Xz6-1* and *Xz6-3* degraded PCP more effectively than *Pseudomonas putida*. *Xz6-1*, especially, showed a remarkable removal rate of 77.1%, a value that improved by 22.9% over *Pseudomonas putida*, and improved by 14.1% than the encapsulated cells method reported in the literature (Cassidy *et al.* 1997).

Thus, *Xz6-1* was further used as a recombinant strain with high-activity chlorophenol degradation capacity.

Table 1. Colonial Morphologies of Recombinant Strains and Parent Strains

Strains	Colonial morphology
Pseudomonas putida	Circular shape, white, smooth surface, soft texture and with a bulge in the colony
Psathyrella candolleana	White, circular shape, rough surface, mycelium, hard texture
X_Z8-1	Radial distribution, yellow-white, smooth surface and soft texture
X_Z3-3	Circular shape, white, smooth surface, soft texture and with a bulge in the colony
X_Z8-2	Radial distribution, yellow-white, smooth surface and soft texture
X_Z6-1	Radial distribution, yellow-white, smooth surface and soft texture
X_Z6-2	Circular shape, white, rough surface, hard texture and with a bulge in the colony
X_Z6-3	Radial distribution, yellow-white, smooth surface and soft texture
X_Z6-5	Radial distribution, yellow-white, smooth surface and soft texture
X_Z6-6	Circular shape, yellow, smooth surface, soft texture and with a bulge in the colony
X_Q3-2	Radial distribution, yellow-white, smooth surface and soft texture
X_Q8-3	Circular shape, white, smooth surface, soft texture and with a bulge in the colony
Z_Q6-1	Circular shape, white, rough surface, hard texture and with a bulge in the colony
Z_Q3-4	Radial distribution, yellow-white, smooth surface and soft texture

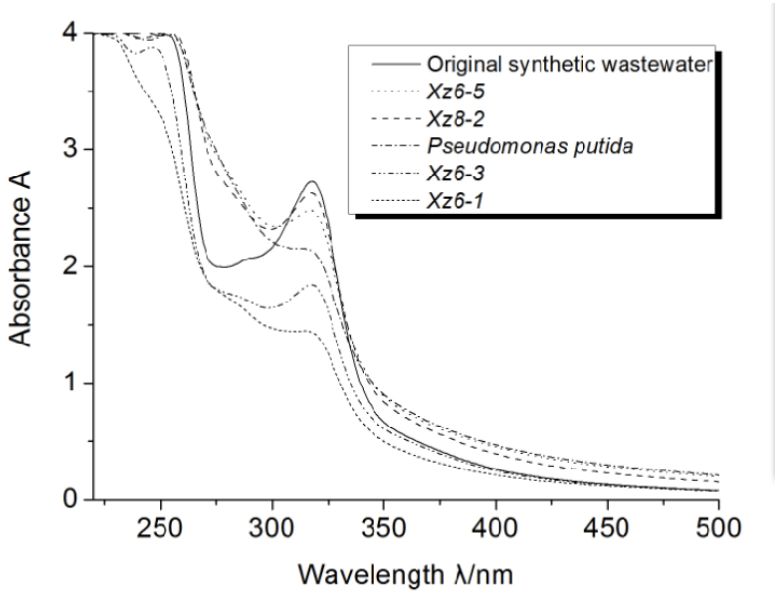

Fig. 1. UV-visible spectra of synthetic wastewaters treated by different strains

Table 2. PCP Removal Rates of the Different Degraders

Degraders	PCP Removal Rate %
Pseudomonas putida	54.12
Xz6-1	77.06
Xz6-3	61.15
Xz6-5	8.07
Xz8-2	21.16
Immobilized *Xz6-1*	82.72
Immobilized *Pseudomonas putida*	62.14
Original sludge	10.69
Spp*	21.20
Sxz*	30.03

* Spp is the sludge fortified with *Pseudomonas putida*, Sxz is the sludge fortified with *Xz6-1*.

Degradation Kinetics of Non-Immobilized *Pseudomonas putida* and *Xz6-1* for PCP

The degradation curves for the non-immobilized cells were plotted and are shown in Fig. 2.

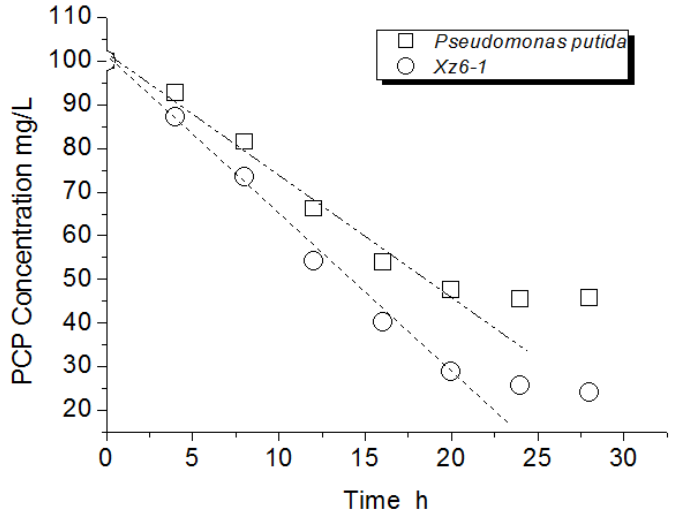

Fig. 2. Degradation curves of PCP by *Pseudomonas putida* and *Xz6-1*

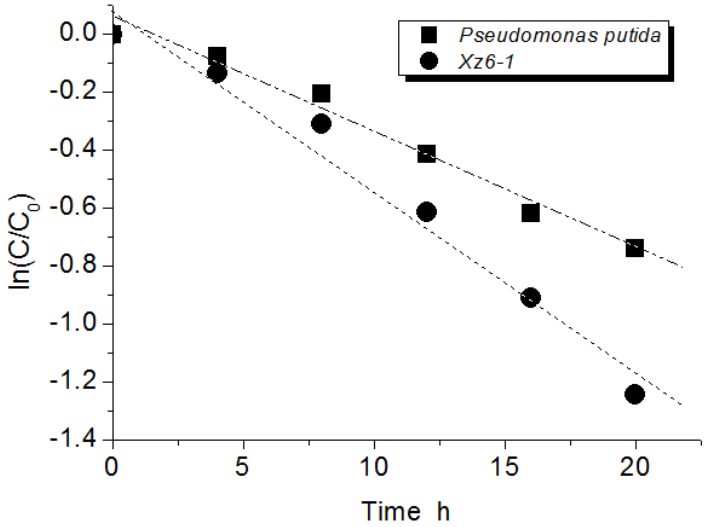

Fig. 3. Degradation kinetics of PCP by *Pseudomonas putida* and *Xz6-1*

The degradation pattern of *Xz6-1* is similar to *Pseudomonas putida*. PCP was degraded within the first 20 h at high rates and was then slowed down significantly to 71.2% and 52.4% within 20 h, respectively. The degradation kinetics curves of the two strains within 20 h are shown in Fig. 3, which showed the variation of the log of (C/C_0) as a function of time (where C is the residual PCP concentration at a specific processing time; C_0 is the original PCP concentration in synthetic wastewater, and t is time). For both strains, the kinetics showed that there is a good linear relation between ln (C/C_0) and t within 20 h indicating that the PCP degrading processes of *Pseudomonas putida* and *Xz6-1* followed first order reaction kinetics models.

The first order reaction kinetics equation can be described as:

$$-\frac{dC}{dt} = kC \quad (1)$$

Equation (1) was integrated to obtain Eqs. 2 and 3,

$$\ln C = -kt + \ln C_0 \quad (2)$$

$$\ln(C / C_0) = -kt \quad (3)$$

where k is the reaction rate constant or the negative slope of the kinetics curves from Eq. (3). Therefore, PCP degradation kinetics models of *Pseudomonas putida* and *Xz6-1* were established (Table 3). The value of k can reflect the PCP biodegradability by the strains. As previously demonstrated, a k value of 0.063 h^{-1} was obtained for *Xz6-1* at a half-life ($t_{1/2}$) = 17.33, a half-life that is significantly much higher than that of *Pseudomonas putida* (k = 0.040 $h^{-1}$and $t_{1/2}$ =10.98).

Table 3. PCP Degradation Kinetics Models

Strains	Kinetics model	k (h^{-1})	Half-life（ln2/k）
Pseudomonas putida	$C=C_0\exp(-0.040)t$ $R^2=0.9802$	0.040	17.33
Xz6-1	$C=C_0\exp(-0.063)t$ $R^2=0.9758$	0.063	10.98

PCP Degradation by Immobilized Cells

The UV-Vis spectra of different wastewater samples treated by various immobilized cells are shown in Fig. 4. Peak absorbance values at λ＝321 nm dropped to 1.11 and 1.54 after treatment with immobilized *Xz6-1* and immobilized *Pseudomonas putida*, respectively.

The PCP removal rates of immobilized cells were obtained based on precedent (Chen *et al.* 2013). For *Xz6-1*, the removal rate was 82.7%, which is much higher than the 62.1% removal rate attributed to *Pseudomonas putida* (Table 2), indicating that the ability of immobilized *Xz6-1* to degrade PCP is also superior to that of its immobilized parent strain. Meanwhile, a contrasting analysis was performed between immobilized cells and free cells for *Xz6-1*, which showed that the removal rate improved from 77.1% to 82.7% upon cell immobilization.

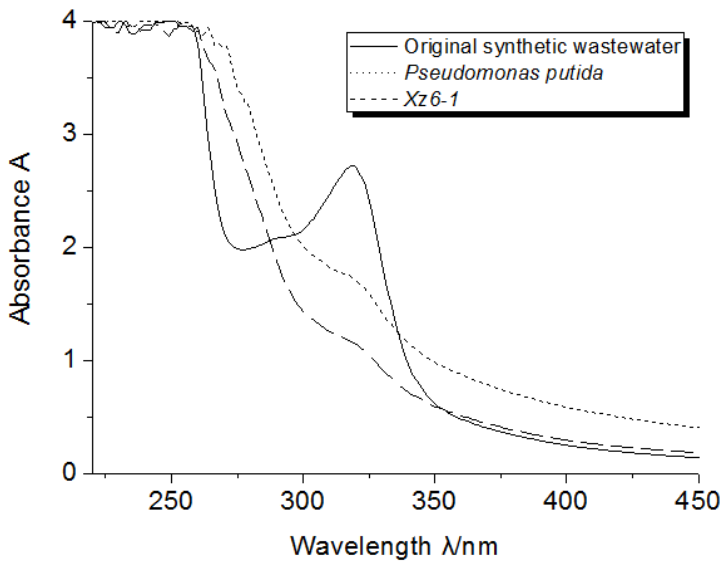

Fig. 4. UV-visible spectra of synthetic wastewater treated by immobilized strains

PCP Degraded by Granular Sludges

Figure 5 shows the UV-Vis spectra of synthetic wastewaters treated by different granular sludges, including the original sludge, the sludge fortified with *Pseudomonas putida* (abbreviated as Spp), and the sludge fortified with *Xz6-1* (abbreviated as Sxz). As can be seen in Fig. 5, the PCP degradability of the original sludge was the lowest. A slight reduction of the absorbance value at 321 nm from 2.64 to 2.39 was observed.

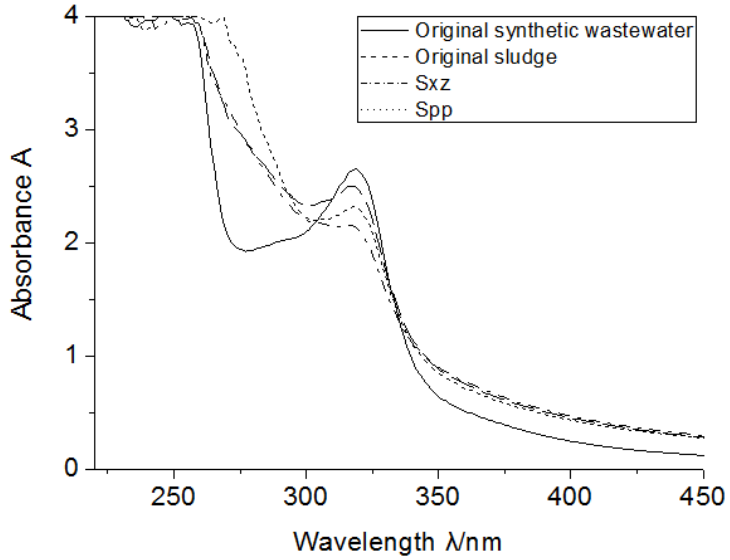

Fig. 5. UV-visible spectra of synthetic wastewaters treated by granular sludge.

Bioaugmentation improved PCP degradability by introduction of exogenous bacteria into the original sludge. For synthetic wastewaters treated by Spp and Sxz, the absorbance values (321 nm) dropped to 2.24 and 2.05, respectively. Table 2 summarizes the removal extents of PCP in synthetic wastewater by different granular sludges. The value is just 10.7% for the original sludge, while the removal rates of Spp and Sxz were 21.2% and 30.0%, which means the PCP degradability of two fortified sludges were

enhanced by 98.3% and 180.9%, respectively, relative to the original sludge. It also indicated that the fortification by the recombinant strain (*Xz6-1*) is superior to that of parent strain (*Pseudomonas putida*). van Limbergen *et al.* (1998) reported that few reports existed in the literature describing the augmentation of activated sludges with microorganism possessing catabolic activities, resulting in improved degradation of xenobiotics. However, our research have proved the bioaugmentation of activated sludge can result in enhanced degradation of chlorophenols.

CONCLUSIONS

1. A new strain (*Xz6-1*) with the ability to degrade chlorophenols efficiently by protoplast fusion between *Pseudomonas putida* and *Psathyrella candolleana* is herein described. During PCP degradation using *Xz6-1* and *Pseudomonas putida*, most of the PCP was removed within the first 20 hours following first order reaction kinetics. The kinetics models of two strains have been established. The kinetic parameter k of *Xz6-1* was 0.063 h^{-1}, while it was 0.040 h^{-1} for *Pseudomonas putida.*
2. The PCP removal rates reached to 82.7% and 62.1%, when the PCP synthetic wastewaters were treated with immobilized cells of *Xz6-1* and *Pseudomonas putida*, respectively.
3. Granular sludge was fortified with *Xz6-1* and *Pseudomonas putida*, respectively. The PCP removal rate for the original sludge was only 10.7%, while the removal rates were 21.2% and 30.0%, respectively, when Spp and Sxz were used. The PCP degradability of two fortified sludges was improved by 98.3% and 180.9% over the original sludge.

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