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Genetic analysis of protoplast fusant Xhhh constructed for pharmaceutical wastewater treatment

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ABSTRACT

In order to analyse genetic relationships between functional strain Xhhh previously constructed through protoplast fusion for pharmaceutical wastewater treatment and its parents, random amplification polymorphic DNA (RAPD) and polymerase chain reaction (PCR) were used to investigate genetic similarities among the strains based on genome and functional genes analyses. A total of 739 clear and consistent bands were produced in the RAPD fingerprint analysis with 40 primers. The genetic similarity indices between Xhhh and parental strains PC (*Phanerochaete chrysosporium*), SC (*Saccharomyces cerevisiae*) and XZ (native bacterium *Bacillus* sp.) were 36.21%, 37.73% and 37.48%, respectively. With PCR amplification and DNA sequencing, Xhhh was found containing functional genes of *mnp* and *lip* from PC, *FLO1* from SC and 16S rDNA fragments from XZ. Experimental results of genetic analyses were in accordance with Xhhh biochemical and phenotypic characteristics, and protoplast fusion technique is considered as a promising technique in environmental pollution control.

1. Introduction

Microbiological techniques, including activated sludge, biofilm and anaerobic methods, have been widely used in wastewater treatment plants for the low-cost and high-efficiency characteristics of those methods. However, pharmaceutical wastewater can not been treated efficiently with biological methods as a result of the presence of antibiotic or aromatic pollutants in the wastewater, which not only inhibits microbial growth, but also reduces biodegradability of the wastewater (Zhao et al., 2007). An increasing of concern has been placed on seeking for innovative biological ways to treat pharmaceutical wastewater of high strength and toxicity effectively and economically (Ternes et al., 2002; Snyder et al., 2005). In order to enhance the biodegradation effectiveness in pharmaceutical wastewater treatment, our research group has made considerable efforts to construct a functional strain named Xhhh through inter-kingdom fusion technique (Cheng et al., 2004; Zhang et al., 2004; Zhao et al., 2007). As a fusant, Xhhh inherited simultaneously high capacities of biodegradation, flocculation and adaptation from its three parental strains *Phanerochaete chrysosporium, Saccharomyces cerevisiae* and native bacterium XZ, respectively (Zhao et al., 2007). The related protoplast fusion technique has also been patented (Cheng et al., 2006). The experimental results of biodegradation kinetics demonstrated the functional strain was characterized with high biodegradation capacity (Cheng et al., 2004), which was further confirmed by a pilot study carried out in the wastewater treatment plant of Xuzhou Enhua Pharmaceutical Co. Ltd., China (Zhang et al., 2004). After the phenotypic proofs have been obtained, our next work is to investigate genetic relationship between the fusant and its three parental strains.

It is well known that protoplast fusion has been widely used in planting (Jones et al., 1976; Sheng et al., 2008) and zymurgy (Hashimoto et al., 2006). In the field of environmental pollution control, protoplast fusion is considered as a novel and reformative technique. DNA from parental strains can be integrated into the functional strain with protoplast fusion (Jones et al., 1976; Sivakumar et al., 2004), which is considered as a simple, efficient and useful tool to create new biological species inter-genetically (Richard, 2001). However, the analyses on the integrated genomic DNA in the cells have been puzzling researchers for decades for the complexity and variability of fusant DNA (Jones et al., 1976; Richard, 2001). Combined with similarity coefficient calculation and unweighed pair-group method algorithm (UPGMA), random amplification polymorphic DNA (RAPD) method, a rapid, sensitive and reliable genetic marker system, has been widely used to investigate genetic distance matrix among closely related species of plants and microorganisms (López et al., 2008; Shi et al., 2008). RAPD technology can scan numerous loci in the genome through DNA amplification with several random primers, which makes it particularly attractive for analysis of genetic relationship between species or kingdoms (Yue et al., 2002; Waltenbury et al., 2005; Verma et al., 2007).

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The objectives of this study are (1) to analyse the genetic relationship between protoplast fusant Xhhh and the parents using RAPD marker system, and (2) to investigate if the fusant has obtained functional genes from its parental strains through PCR assays.

2. Methods

2.1. Strains and antibiotic resistance analysis

Xhhh was constructed and patented by our research group through protoplast fusion with three parental strains as following (Cheng et al., 2006): (1) fungus *P. chrysosporium* (PC), which could improve degradation capacity of Xhhh due to the extracellular enzymes manganese peroxidase (MnP) and lignin peroxidase (LiP) encoded by *mnp* and *lip* genes, respectively (Kumar et al., 2006); (2) yeast *S. cerevisiae* (SC), which could promote flocculation capacity of Xhhh because of the existence of flocculation genes (including *FLO1*) in the cell (Machado et al., 2008); and (3) native bacterium *Bacillus* sp. (XZ), which was isolated from the former pharmaceutical wastewater treatment system to strengthen adaptability of Xhhh in the wastewater. Xhhh and the parents were cultured on SMM medium (3 g/L K_2HPO_4; 1 g/L $K_2H_2PO_4$; 0.5 g/L NH_4NO_3; 0.1 g/L Na_2SO_4; 10 mg/L $MgSO_4 \cdot 7H_2O$; 1 mg/L $MnSO_4 \cdot 4H_2O$; 0.5 mg/L $CaCl_2$; 1 mg/L $FeSO_4 \cdot 7H_2O$; 5.0 g/L yeast extract; 10 g/L tryptone; 10 g/L glucose; 5 g/L CH_3COONa; 2.0% agar; pH 6.5).

All the strains (Xhhh, SC, PC and XZ) were subject to antibiotic resistance tests. These strains were incubated on the media of SMM, IMM1 (SMM medium with 100 U/mL streptomycin), IMM2 (SMM medium with 100 U/mL nystatin) and IMM3 (SMM medium with 100 U/mL streptomycin and 100 U/mL nystatin) for 48 h. The colony formation of all the microorganisms on different media was recorded for analyses.

2.2. Genomic DNA extraction

Following protocol based on the reference method (Zeng, 2003) was used for DNA extraction of fusant, eukaryon and protokaryon cells. Strains were frozen in liquid nitrogen, crushed in an centrifuge tube with extraction buffer (2.8% w/v SDS, 20 mmol/L EDTA, 50 mmol/L Tris–HCl, 10% w/v CTAB, 5 mol/L NaCl, pH 7.2), and incubated at 65 °C for 30 min. Proteinase K (10 mg/mL) (Sigma, USA) was added, and incubated for another 30 min at 65 °C. The mixture was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) by gentle inversion. The precipitate was washed and desalted with chilled 70% ethanol, and the DNA was dissolved overnight in TE buffer (10 mmol/L Tris–HCl; 1 mmol/L EDTA; pH 8.0). The samples were then treated with RNase A (10 mg/mL) (Sigma, USA) for 1 h at 37 °C. Integrity of DNA was confirmed after electrophoresis on 0.8% agarose gels by comparison with a known standard (marker: Lambda DNA/*Hind*III, Takara, Japan). An estimation of the purity of the DNA was spectrophotometrically determined by the ratio of absorbance at 260 and 280 nm.

2.3. 16S rDNA analysis of bacterium XZ

The conserved 16S rDNA was amplified in 25 μl of reaction solution mixture, containing 10 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, and 1.25 U of Taq DNA polymerase (Takara, Japan) with the templates of the bacterium DNAs (0.2 μg) and the primers of the 16S rDNA (0.25 μmol/L each). Primers for PCR amplification and DNA sequencing of the conserved 16S rDNA are shown in Table 1. PCR amplifications were performed using the methodology with 1 cycle at 95 °C for 10 min, 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min before a final extension of 72 °C for 10 min and storage at 4 °C. PCR fragments were cloned directly into pUCm-T vector using a TA cloning kit (Promega Co., USA) and plasmids digested with *Eco*RI to confirm the target DNA insertion. DNA sequencing was performed by automated means at Sangon (Shanghai, China). The 16S rDNA homology searches were performed using the BLAST program at the National Centers for Biotechnology Information (National Institutes of Health, Bethesda, USA).

2.4. RAPD-PCR

RAPD-PCRs were performed using the conditions modified based on previous method (Williams et al., 1993). Primers were chosen at random, with a restriction of C + G content between 50–60%. The sequences of 40 single-chain primers used in this study are shown in Table 1. PCR reactions were performed in a 25 μl volume containing 0.2 mmol/L dNTP (Takara, Japan), 2.5 μl of a ten-fold PCR buffer, 1 unit of DNA polymerase (Takara, Japan), 0.6 μmol/L of each 10-base-pair primer (Operon Technologies, USA), 50 ng of target DNA, 2 mmol/L of $MgCl_2$ and purified water to the final volume. Amplifications were carried out in a Mycycler Thermal Cycler System (Bio-RAD, USA) programmed for RAPD-PCR with 1 cycle at 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min before a final extension step of 72 °C for 10 min and storage at 4 °C. All reactions were repeated to ensure that results were reproducible. Amplification products (5 μl of the total reaction) along with DNA marker (Takara, Japan) were analysed by electrophoresis using 1.5% agarose gels with ethidium bromide in 0.5 × TBE buffer at 100 V for 1 h. Gels were scanned by an automated and computerized microscope gel scanning system (Gel Doc™ XR, Bio-RAD, USA) to give detailed separation and molecular weights of the present DNA bands.

Table 1
DNA sequences of primers used for RAPD and PCR amplifications.

Primer name	Nucleotide sequence (5′ to 3′)	Primer name	Nucleotide sequence (5′ to 3′)
Rapd_1	AGTCAGCCAC	Rapd_25	GGGTAACGCC
Rapd_2	CAATCGCCGT	Rapd_26	TCGGCGATAG
Rapd_3	AGGTGACCGT	Rapd_27	GTTGCGATCC
Rapd_4	GAATGCGACC	Rapd_28	TGAGGGTCCC
Rapd_5	GTGAAGGAGG	Rapd_29	TGCTGCAGGT
Rapd_6	AGCACTGGGG	Rapd_30	ACTGGGACTC
Rapd_7	TCCCAGCAGA	Rapd_31	CACTCTCCTC
Rapd_8	CACTGGCCCA	Rapd_32	CTGGGGCTGA
Rapd_9	CACGCCCTTC	Rapd_33	GTCTTGCGGA
Rapd_10	ATGCAGCCAC	Rapd_34	ACCTGGGGAG
Rapd_11	TGGACCGGTG	Rapd_35	GGCGGATAAG
Rapd_12	GAGAGCCAAC	Rapd_36	GTGACGTAGG
Rapd_13	CAGCACCCAC	Rapd_37	GTCCGTATGG
Rapd_14	GTGTGCCCCA	Rapd_38	GTCCGGAGTG
Rapd_15	AGGGAACGAG	Rapd_39	TGTCATCCCC
Rapd_16	GTCGCCGTCA	Rapd_40	TCTGTGCTGG
Rapd_17	TGTCTGGGTG	Mnp_F	TGGACTTCCAAATCCTGACA
Rapd_18	GGTGACGCAG	Mnp_R	GCACAAACCGAGTCATTGAA
Rapd_19	CGAGTACTGG	Lip_F	GCGCCTGGTTCGATGTCCTC
Rapd_20	TGCCGAGCTG	Lip_R	GACTGTTGTCCACCTGCACTT
Rapd_21	AATCGGGCTG	Flo1_F	AAGTGCGTAGAACAGGTA
Rapd_22	AGGGGTCTTG	Flo1_R	AACGAGCAAGAGTGAAAT
Rapd_23	GGTCCCTGAC	16S_F	AGAGTTTGATCATGGCTCAG
Rapd_24	GAAACGGGTG	16S_R	AAGGAGGTGATCCAGCCGCA

Note: Rapd_1 to Rapd_40: Primers of random amplification polymorphic DNA (RAPD) assays; Mnp_F and Mnp_R: PCR primers of *mnp* gene; Lip_F and Lip_R: PCR primers of *lip* gene; Flo1_F and Flo1_R: PCR primers of *FLO1* gene; 16S_F and 16S_R: PCR primers of 16S rDNA fragment.

2.5. Gel scoring and statistical analysis

Only strong and reproducible RAPD bands were scored with the help of Quantity One software 4.5.2 (Bio-Rad, USA). Different patterns observed were scored as discrete variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. Relationships among individuals were determined by the distance matrix method. Nei and Li's Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix (Nei and Li, 1979). A dendrogram was constructed from this matrix with hierarchical cluster analysis, based on the average linkage between groups, such as the unweighed pair-group method algorithm (UPGMA) (Sneath and Sokal, 1973).

2.6. Specific PCR amplifications and DNA sequencing of functional genes

The genomic DNA extraction protocol was similar to RAPD procedure as previously described. According to the sequences of *P. chrysosporium* genes (*mnp* and *lip*) and *S. cerevisiae* flocculation gene (*FLO1*) referring to GenBank (accession number: M60672.1, M92644.1 and X78160, respectively), PCR primers were designed using Primer Premier 5.0 system (Table 1), and synthesized by Sangon Co. (Shanghai, China), to detect the specific DNA fragments of *mnp*, *lip* and *FLO1* genes in the fusant. Amplifications of *mnp* and *FLO1* were programmed for PCR with 1 cycle at 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min before a final extension of 72 °C for 7 min and storage at 4 °C. The methodology of *lip* gene was similar to that of *mnp* with exception of annealing temperature at 56 °C. Primers design and PCR amplification for 16S rDNA of XZ and Xhhh were carried out according to the method of XZ phylogenetic analysis. Negative control of each PCR was designed with no DNA template, but ddH_2O added in the reaction system. After electrophoresis, amplification products of all functional genes were purified with QIAquick PCR Purification Kit (Qiagen, Germany) and confirmed through DNA sequencing by Sangon Co. (Shanghai, China).

3. Results

3.1. 16S rDNA identification of bacterium XZ and antibiotic resistance analysis

The conserved 16S rDNA of XZ was subject to PCR amplification and nucleotide sequencing. By searching in GenBank with BLAST, the closest known relatives were found based on the partial 16S rDNA sequences of bacterium XZ (Fig. 1). To set up a framework for more reliable alignment, all the conserved sequences of 16S rDNA were scored with sequencing lengths, %GC content, and the number of base differences. In the similarity analysis, a 99.06% match was obtained for XZ with *Bacillus* sp. (AMX-4, AH-E-1, NRRL B-23935) and *Bacillus subtilis* strain (BGSC 3A23, CICC 10158, SM98011) (Fig. 1).

Antibiotic resistance tests were performed for Xhhh and its parents cultured on the plates with different media. After culture for 48 h, no cell division, or colony formation were observed on the IMM1 medium for XZ, IMM2 medium for SC and PC, and IMM3 medium for the three parental strains. However, positive results were found on the IMM2 medium for XZ, IMM1 medium for SC and PC. Xhhh can be cultivated on all types of media, including IMM3 containing both nystatin and streptomycin.

3.2. Identification of inter-kingdom fusant Xhhh by RAPD fingerprint

RAPD assays were carried out for analysis of genetic relationship between Xhhh and its parental strains. RAPD fingerprint analysis with 40 primers generated a total of 739 clear and consistent bands (Table 2). Averagely, 7.78, 7.28, 7.20 and 6.50 bands were produced by PCR with each primer for Xhhh, PC, SC and XZ, respectively. Of total 739 bands, 329 were found to be monomorphic ones shared between Xhhh and its three parental strains. In order to statistically verify the phenogram banding data from the agarose gels, similarity indices were calculated for Xhhh and its parents, and an average linkage dendrogram was constructed (Fig. 2). As expected, the average genetic distances between any two strains of PC, SC, and XZ are very large since they are different species. Fusant Xhhh shared genetic similarity of 36.21%, 37.73% and 37.48% with PC, SC and XZ, respectively (Table 2).

3.3. Identification of inter-kingdom fusant Xhhh by PCRs of functional genes

In order to investigate if the functional genes have been transferred into Xhhh cell during protoplast fusion, the target DNA fragments of *mnp* (439 bp), *lip* (452 bp), 16S rDNA (527 bp) and *FLO1* (427 bp) were subject for PCR amplifications with the designated primers (Table 1). The DNA fragments of the *mnp* encoding MnP and *lip* encoding LiP were observed in the cells of both Xhhh and

Table 2
Fingerprint similarity analysis between the fusant Xhhh and its parental strains based on the results of random amplification polymorphic DNA assays.

Strain/data	Xhhh	PC	SC	XZ
Xhhh	100% (311)	–	–	–
PC	36.21% (109)	100% (291)	–	–
SC	37.73% (113)	11.74% (34)	100% (288)	–
XZ	37.48% (107)	8.35% (23)	9.12% (25)	100% (260)

Note: The data on the brackets mean the number of common bands between the two associated strains, and the percentages represent their genetic similarities.

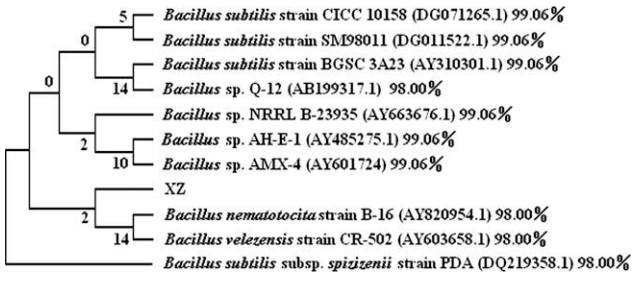

Fig. 1. 16S rDNA phylogenetic analysis of native bacterium XZ. GenBank accession numbers of sequences used in this analysis are given in parenthesis, and the percentages show genetic similarities between XZ and the corresponding strains.

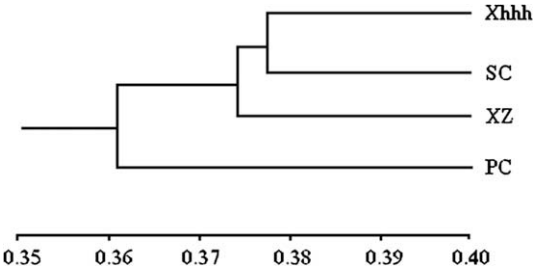

Fig. 2. Phenogram of fusant Xhhh and its parent stains SC, PC and XZ, based on UPGMA cluster analysis and the similarity indices. Scale values along bottom are the UPGMA coefficients, which represent percent similarities between Xhhh and its parental strains.

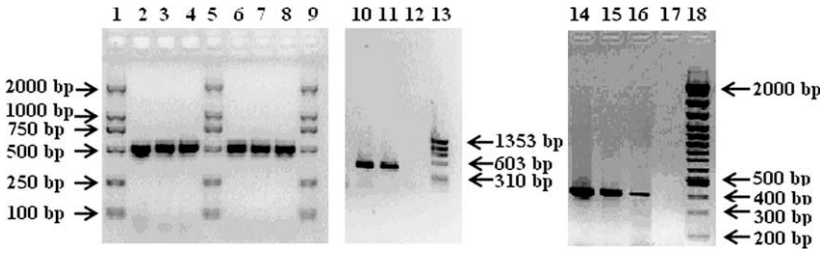

Fig. 3. PCR amplifications of target genes *mnp* (Lane 2–4), *lip* (Lane 6–8), 16S rDNA (Lane 10–12) and *FLO1* (Lane 14–17) in Xhhh and its parental strains. Lane 1, 5, 9, 13 and 18: Molecular weight marker; lane 2 and 6: PC; lane 10: SC; lane 16: XZ; lane 3, 4, 7, 8, 11, 14 and 15: Xhhh; lane 12 and 17: Negative control (using ddH_2O as PCR templates in reaction system). The PCR results were confirmed through DNA sequencing, and the GenBank accession numbers are M60672.1 (*mnp*), M92644.1 (*lip*) and X78160 (*FLO1*).

its parental strain PC (Fig. 3). Additionally, flocculation gene *FLO1* of parental strain SC and 16S rDNA fragments of parental strain XZ were found in Xhhh cell by specific PCR (Fig. 3). Each PCR product was further confirmed through DNA sequencing, and nucleotide sequences of PCR products were found to be highly consistent with those from GenBank, with accession numbers of *mnp*, *lip* and *FLO1* as M60672.1, M92644.1 and X78160, respectively. In one word, the specific PCR results show that Xhhh cell simultaneously contain the functional genes of *mnp* and *lip* (from PC), *FLO1* (from SC) and 16S rDNA fragments (from XZ).

4. Discussion

The results of antibiotic resistance tests demonstrate that both SC and PC are sensitive to nystatin and resistant to streptomycin, and the antibiotic characteristic of bacterium XZ is just contrary to that of PC and SC. Xhhh's resistance to both nystatin and streptomycin indicates that the fusant has inherited the antibiotic characters from its parental strains. Antibiotic resistance genes of parental strains can be transferred to hybrids through cell fusion, and some fusants are capable to inherit antibiotic properties from parental strains (Beltrametti et al., 2007).

RAPD DNA marker system including RAPD-PCR, similarity coefficient calculation and UPGMA has been proved to be a convenient and reliable method for genetic distance analysis of different species (Novak et al., 2004; Waltenbury et al., 2005; López et al., 2008). In this study, the results of RAPD fingerprint analysis account for recombination of the genomes from three strains in the fusant cell (Richard, 2001). Appearance of different bands in gels gives the proof of obvious polymorphism between Xhhh and the parental strains. The genetic similarity indices between Xhhh and its parental strains ranged from 36.21% to 37.73% (Table 2), which indicates that Xhhh obtained nearly equivalent genetic information from each parental strain. By detailed analysis of the fingerprint, however, Xhhh inheritance degrees of the bands were found to be variable among the three parents. For primer Rapd_32, Xhhh inherited more DNA information from XZ than those from SC and PC, while for Rapd_29, Xhhh inherited more fragments from SC (Fig. 4). Additionally, genetic materials loss and genome recombination have taken place in the course of protoplast fusion since a high percentage of 'unique' bands (50–60%) appeared in both Xhhh and its three parents (Waltenbury et al., 2005; Verma et al., 2007).

Although morphological and physiological features can be preparatory signs for primary screening of the fusants, genomic makeup analysis is one of the decisive means to identify the aimed hybrids (Novak et al., 2004), since DNA fragments based marker are conservative at the level of microorganism division. RAPD-PCR has been proved as a reliable method for genomic analysis, and widely used in the genetic investigation for protoplast fusants (Yang et al., 2007; Sheng et al., 2008). It has been found in previous

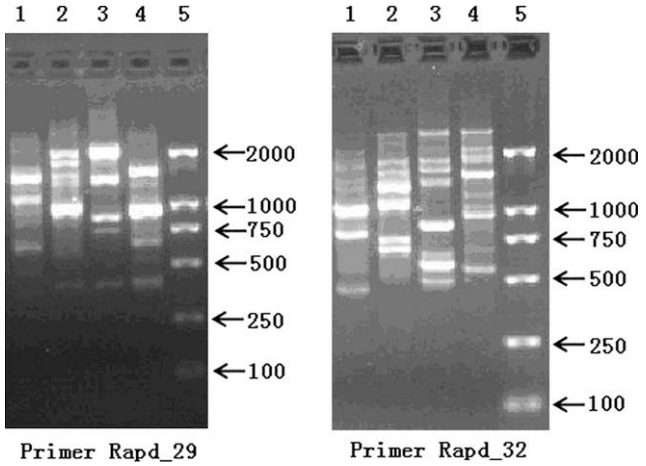

Fig. 4. Amplification profile of Xhhh strain and its parental genotypes with the primers of Rapd_29 and Rapd_32. Lane 1: PC; lane 2: SC; lane 3: XZ; lane 4: Xhhh; lane 5: Molecular weight marker.

studies that genetic information in inter-kingdom fusant cell obtained from one parental strain is usually much more or less than that from the other strain, by the evidence of great variance in the genetic similarity index detected with RAPD (Chakraborty and Sikdar, 2008). However, the similarity indices in this study varied slightly (Fig. 2). The inconsistency of the results may be due to the different procedures of the protoplast fusion applied, or partly as a result of the genetic distances of the parental strains used for fusion.

Through 16S rDNA phylogenetic analysis, the closest relative of native bacterium XZ was determined to be *Bacillus* sp. In previous studies, it has been reported that *B. thuringiensis* can be cultivated in medicine manufacturing wastewater, and the strain is capable of transferring pollutants into biopesticides (Yezza et al., 2006). The presence of XZ 16S rDNA fragments and SC flocculation gene *FLO1* in Xhhh cell demonstrates that the fusant has obtained genetic materials from the two parental strains. MnP (*mnp*) and LiP genes (*lip*) were observed in both Xhhh and its parental strain PC, indicating that the two functional genes of PC have been successfully transferred to Xhhh cell through protoplast fusion. MnP and LiP are major components of nonspecific extracellular degradation system produced by PC, which can efficiently catalyze biodegradation of a broad range of substrates, including lignin (Zhang et al., 2006a). MnP and LiP enzymes with high activity were previously detected in the culture solution of Xhhh by enzymatic assays (Zhao et al., 2007), thus the two functional genes present in Xhhh cell can be normally transcribed and translated. The high biodegradation ability of Xhhh is probably associated with the two functional genes inherited from PC.

Combined with antibiotic biochemical test, RAPD-PCR of genome and specific PCR of functional genes make the viewpoint more reliable that gene resources of the three parental strains have been transferred into the Xhhh cell, and were partly recombined or lost. The results of genetic analyses provide a strong evidence for Xhhh phenotypic characters (Zhang et al., 2004). The similar characters of genotype and phenotype were previously found in another functional strain Fhhh, which was constructed with protoplast fusion in our laboratory to improve the biodegradation efficiency of petrochemical wastewater and has been successfully applied in wastewater treatment plant (Zhang et al., 2006a, 2006b).

Protoplast fusion in the same genus is feasible, and has been widely used for breeding of plants and microorganisms (Hashimoto et al., 2006; Prabavathy et al., 2006; John et al., 2008). This technique may also be introduced to produce hybrids between taxonomically distant species (Xia et al., 1998). Interestingly, in this study, the protoplast fusion across kingdoms was used to construct a functional strain to improve biodegradation efficiency of environmental pollutants. Inter-kingdom fusion extends the possibility for breeding by utilizing effective techniques to create new hybrids (Jones et al., 1976), which will achieve promising applications in environmental science.

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