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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.