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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Isolation and characterization of *Pseudomonas* sp. strain capable of degrading diethylstilbestrol

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Abstract Since diethylstilbestrol (DES) interrupts endocrine systems and generates reproductive abnormalities in both wildlife and human beings, methods to remove DES from the environments are urgently recommended. In this study, bacterial strain J51 was isolated and tested to effectively degrade DES. J51 was identified as *Pseudomonas* sp. based on its nucleotide sequence of 16S rRNA. The quinoprotein alcohol dehydrogenase and isocitrate lyase were identified to be involved in DES degradation by MALDI–TOF–TOF MS/MS analysis. In the presence of 40 mg/l DES, increase of the genes encoding quinoprotein alcohol dehydrogenase and isocitrate lyase in both RNA and protein levels was determined. The HPLC/MS analysis showed that DES was hydrolyzed to a major degrading metabolite DES-4-semiquinone. It was the first time to demonstrate the characteristics of DES degradation by specific bacterial strain and the higher degradation efficiency indicated the potential application of *Pseudomonas* sp. strain J51 in the treatment of DES-contaminated freshwater and seawater environments.

Keywords Diethylstilbestrol · *Pseudomonas* sp. · Degradation · Metabolite · Cytotoxicity

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Introduction

Diethylstilbestrol [DES, 4,4′-(3*E*)-hex-3-ene-3,4-diyldiphenol] is a synthetic estrogen that was first synthesized in 1938 by Leon Golberg, and was approved by the Food and Drug Administration (FDA) in the USA in 1941 (Dodds et al. 1938; Meyers 1983). Its chemical structure was shown in Fig. 1. DES was used as treatments for estrogen-deficiency disorders in veterinary and symptoms associated with menopause, menstrual disorders, postpartum breast engorgement, and primary ovarian failure, and was also used in chemotherapy of advanced breast cancer and prostate cancers from 1940 to 1970 (NIH 1984). DES has been banned since 1970s by the FDA, because it can interfere with hormonal system, and cause adverse effects on the physiology of organisms without inducing immediately toxicological risks (Colborn et al. 1993; Mclachlan and Arnold 1996). DES is one of the endocrine disrupting chemicals (EDCs) that have drawn much attention of the scientific community and the public (Colborn and Clement 1992; Desbrow et al. 1998). DES could be introduced into water and soil environments by chemical discharges, sewage treatment plants, and landfills. For example, the level of DES in the effluent from sewage treatment plants in the Catalonian area was 34 ng/l (Solé et al. 2000; Zhou et al. 2004). Therefore, removal of DES from contaminated environment was highly recommended (Chen et al. 2010).

Advanced oxidation processes (AOPs) are effective for the degradation of a variety of hazardous organic pollutions. AOPs that had been developed to degrade DES, included ultrasonic-induced degradation, electrochemical degradation, photooxidation degradation, UV-light-inducing photodegradation, and ozonation (Abderrazik et al. 2005; Korshin et al. 2006; Lin et al. 2009; You et al. 2006; Zhou et al. 2004). Although these methods could effectively remove DES from the environments, the whole processes needed specific cells in

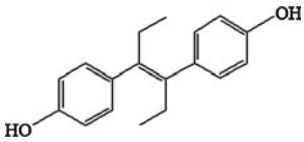

Fig. 1 Chemical structure of diethylstilbestrol

site, such as cells with ultrasound sample, PbO_2 anode, and stainless steel cathode, or certain equipment including high-pressure mercury lamp and ozone generator. Nowadays, transformation of various organic pollutants by microorganisms has been demonstrated to be a more effective and facile, and less expensive way to eliminate pollution in the environments compared to the physicochemical methods (Chang 2008; Singh et al. 2008). Lee and Liu (2002) had reported the degradation of other EDCs, 17β-estradiol and its metabolites, by sewage bacteria. However, till now, there was neither study focusing on DES degradation by bacterial strains, nor study relating to the isolation, identification, and characterization of specific bacterial strain capable of degrading DES.

In our present study, bacterial strain capable of degrading DES was isolated, identified, and characterized. Proteins involved in DES degradation were detected and identified by SDS-PAGE and MALDI–TOF–TOF MS/MS analyses. The degrading metabolites of DES and their cytotoxicity were also determined by HPLC/MS analysis and microscopic observation, respectively.

Materials and methods

Chemical regents, medium, and growth condition

Analytical-grade DES, chromatographic grade methanol and ethanol were purchased from J&K Scientific Ltd. (Beijing, China). All other chemicals were purchased from Sangon (Shanghai, China). DES was dissolved in ethanol to prepare DES stock solution of 10 mg/ml. If not specially mentioned, appropriate volume of DES stock solution was added into the medium when DES was needed. Luria-Bertani (LB) medium was prepared as described by Sambrook et al. (1989). Mineral salt medium consisted of 1.36 g/l KH_2PO_4, 1.78 g/l $Na_2HPO_4{\cdot}2H_2O$, 0.50 g/l $MgSO_4{\cdot}7H_2O$, and 0.50 g/l NH_4Cl. The pH of mineral salt medium was adjusted to 7.2 with NaOH, and 1 ml of trace element solution [0.10 g/l $Al(OH)_3$, 0.05 g/l $SnCl_2{\cdot}2H_2O$, 0.05 g/l KI, 0.05 g/l LiCl, 0.08 g/l $MgSO_4$, 0.05 g/l H_3BO_3, 0.10 g/l $ZnSO_4{\cdot}7H_2O$, 0.01 g/l $CoCl_2$, 0.01 g/l $NiSO_4{\cdot}6H_2O$, 0.05 g/l $BaCl_2$, and 0.05 g/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$] per liter was also added (Pandey et al. 2010). 2216E medium contained 5 g/l tryptone, 1 g/l yeast extract, 0.01 g/l $FePO_4$, and 1 l aged seawater. In order to isolate DES-degrading bacteria, seawater collected from coastal zone near the mouth of the river was concentrated and plated onto 2216E medium amended with 1.2 % agar. Pure colonies were examined for their DES degradation ability. To determine the effect of pH on DES degradation by bacterial strain, 10 mg/l DES-containing mineral salt medium of different pHs ranging from 4.0 to 10.0 was used. To prepare the bacterial cells used for inoculation, cells were grown in LB medium to OD_{600} of approximately 1.0, and cell pellet was collected by centrifugation at 10,000×*g* for 5 min. Then cell pellet was washed in sterilized phosphate buffer saline (PBS) for three times, and finally was resuspended in mineral salt medium to an OD_{600} of 1.0. Appropriate volume of the suspended cells was inoculated when the cells were used to degrade DES.

DES degradation by bacterial strain

Suspended cells were inoculated into mineral salt medium containing 10 mg/l DES to 1.0×10^7 colony-forming unit (CFU)/ml. After cultured at 30 °C for different time points, equal volume of ethanol was added into bacterial culture, and the mixture was left at room temperature for 10 min to increase the solubility of DES. The control experiment without inoculation of bacterial cells was performed under the same conditions. Supernatants were obtained by filtering the mixture through 0.45-μm nylon membrane prior to HPLC analysis. To determine whether the reduced DES was due to accumulation in bacterial cells, DES associated with the bacterial cells was detected as described by Zhang et al. (2012) with minor modification. Cell pellet was resuspended in 200-μl protein denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, adjusted pH to 8.0 using NaOH) and lysed for 1 h by gently shaking. Then cell lysate was diluted with 1.8 ml ethanol and the mixture was sonicated using a sonicator equipped with a microtip with 60 2-s bursts at 200 W and a 15-s cooling period between each burst. The lysate was centrifuged at 13,000×*g* for 10 min to pellet the cellular debris and the supernatant was stored for HPLC analysis.

DES degradation by the resting cell system

DES degradation by the resting cells was carried out as described by Masuda et al. (2007) with minor modification. Bacterial cells were cultured in mineral salt medium using ethanol as the sole carbon source at 30 °C for overnight. Cell pellet was collected by centrifugation at 10,000×*g* for 5 min and washed in sterilized PBS for three times. Then the cells were resuspended in sterilized PBS to OD_{600} of 1.0. DES was added into 1-ml cell suspension to a concentration of 10 mg/l. DES that was added into sterilized PBS was used as control. The mixture was incubated at 30 °C on a reciprocal shaker for 12 h, followed by the addition of 1 ml ethanol to dissolve DES. Supernatant was collected after centrifugation and used for HPLC.

Chemical analysis

Suspended cells were inoculated into mineral salt medium containing 10 mg/l DES to 1.0×10^7CFU/ml, and grown at 30 °C. Supernatants were obtained as described previously and further used for HPLC analysis. HPLC analysis was performed using Waters 600 equipped with a 4.6×150-mm reverse-phase C18 column (Hypersil Gold, USA) in conjunction with a UV detector monitoring at 280 nm as described by Lin et al. (2009). The mobile phase was methanol to water at a ratio of 85:15 (*v*/*v*), and the flow rate maintained at 1.0 ml/min.

The degrading metabolites of DES were identified by HPLC/MS, LCQ Fleet high sensitivity multistage ion-trap mass spectrometer system (Thermo Fisher Scientific Corporation, USA). The MS working conditions were as follows: The sheath gas was held at a flow rate of 30 arb and the auxiliary gas was held at a flow rate of 10 arb. The ion transfer capillary temperature was 300 °C. The spray voltage was 5 kV. MS was operated in the electron spray ionization (ESI) mode with a negative polarity, and scanned by normal mass range from 100 to 2,000 *m*/*z*. Characteristic fragment ion was detected in the second-order MS.

Growth of DES-degrading bacterial strain

In order to investigate the growth of bacterial strain in the presence of DES, suspended cells were inoculated into mineral salt medium amended with 1, 4, and 8 ‰ (*v*/*v*) ethanol, and 1, 4, and 8 ‰ (*v*/*v*) DES stock solution to 1.0×10^7CFU/ml, respectively. Bacterial cells were then grown at 30 °C for 84 h and aliquots were taken at different time points for the measurement of absorbance at 600 nm using an UV–VIS spectrophotometer (Beckman, USA).

DNA techniques

Genomic DNA of the DES-degrading bacterial strain was extracted according to the method described by Syn and Swarup (2000). The 16S rRNA gene was PCR amplified with primers 8F and 1492R according to the method described by Lane et al. (1985). Sequencing was carried out by Sunny Bio. Co. (Shanghai, China). Primers used in this study were listed in Table 1 and were synthesized by Sunny Bio. Co. The pairs of primers adhpmF1/adhpmR1, adhpmF2/adhpmR2, adhpmF3/adhpmR2, lypsF1/lypsR1, lypsF2/lypsR2, lypsF3/lypsR3, qadhpsF1/qadhpsR1, qadhpsF2/qadhpsR2, and qadhpsF3/qadhpsR2 were used to amplify fragments of the identified genes.

Protein analysis

SDS-PAGE analysis was carried out to explore whether there were any proteins of *Pseudomonas* sp. strain J51 involved in DES degradation. Bacterial cells were separately grown in mineral salt medium containing 2 and 4 ‰ DES stock solution at 30 °C for 12 h, and cells grown in mineral salt medium containing 2 and 4 ‰ ethanol were used as control. Cell pellets were collected by centrifugation at 10,000×*g* for 5 min and resuspended in protein-denaturing buffer and lysed by gently shaking. When the solution became translucent, cell lysate was centrifuged at 13,000×*g* for 30 min at room temperature to pellet the cellular debris and supernatant was collected. The supernatants were mixed with appropriate volumes of 5× sample buffer and were used for SDS-PAGE analysis. The distinct band appeared on the gel after stained with Coomassie brilliant blue was postulated to be the proteins involved in DES degradation. Protein identification by MALDI–TOF–TOF MS/MS was carried out by Bio-Tech Limited Company (Shanghai, China) and was blasted in NCBInr 20120819 (19,922,528 sequences; 6,828,478,126 residues).

Table 1 Primers used in this study

Pairs of primers	Sequence (5′→3′)	Theoretical sizes of PCR product (bp)
adhpmF1	CGCTCGCCCTGATGCT	87
adhpmR1	GGTCGTTGGCAATGTCTTCC	
adhpmF2	CAACCCGCACGATTACGACA	195
adhpmR2	TTGCGGTCAGCGTGGG	
adhpmF3	CCCCGAACGATGCCTGG	106
adhpmR2	TTGCGGTCAGCGTGGG	
lypsF1	GCCAAGGCTGCCGAGAT	217
lypsR1	CGTGCGGAACGGTTACTTTA	
lypsF2	GAAGCGTCTGCCGTCCAA	165
lypsR2	TCGCGGATGCGGTCAA	
lypsF3	ACTGCCGTTTCCGACCTG	603
lypsR3	CTGGAACAGGTTGGACGGCA	
qadhpsF1	TGCGACGTGGTCAACCG	409
qadhpsR1	GGCTCCAGGCTTCCTTCTT	
qadhpsF2	ATCCCAGGCCATCGTCC	533
qadhpsR2	TTGCCGGTCGGGTGGT	
qadhpsF3	ATCTACTTCGGCACCCTCG	350
qadhpsR2	TTGCCGGTCGGGTGGT	
8F	AGAGTTTGATCCTGGCTCAG	1,484
1492R	GGTTACCTTGTTACGACTT	
933F	GCACAAGCGGTGGAGCATGTGG	300
16SRTR1	CGTGTGTAGCCCTGGTCGTA	

Real-time reverse transcriptase PCR assay

Total RNA was extracted from cells separately grown to OD_{600} of 1.0 in mineral salt medium containing ethanol and DES stock solution by using the Trizol Reagent (Invitrogen). DAN interfused with the extracted RNA was digested with RNase-free DNase (Promega). RNA was reversely transcribed

into cDNA using RTase M-MLV (RNase H$^-$) (Takara, China). Real-time reverse transcriptase PCR (RT-PCR) was carried out in an ABI 7500 real-time detection system (Applied Biosystems) by using the Sybr ExScript RT-PCR kit (Takara, China) as described by Zhang et al. (2008). 933F and 16SRTR1 were used to amplify fragment of 16S rRNA, which was used to normalize the mRNA level of each gene.

Cytotoxicity analysis of DES and its metabolites

Cytotoxicity analysis was performed according to the method described by Zhang et al. (2009). Briefly, Hela cells were cultured to confluence in 96-well plates at 37 °C in a humidified atmosphere with 5 % CO_2. One percent (*v*/*v*) of DMSO was added into 10 mg/l DES-containing medium without inoculation of bacterial cells, bacterial culture grown in 10 mg/l DES-containing mineral salt medium, and bacterial culture grown in mineral salt medium with 1 ‰ ethanol respectively to dissolve DES. Then supernatants were collected and ultra-filtered with Amicon Ultra-4 centrifugal filter devices (Millipore) to remove any molecules larger than 10 kDa. The treated supernatants were added to the Hela cells, respectively, and mineral salt medium used as control was also added to the Hela cells. The cells were used for microscopic observation under inverted microscope (Olympus, Japan) after treatment for 8 h, and used for the determination of the viability of cells using the MTT cell proliferation assay after treatment for 48 h. The cytotoxicity of supernatant from bacterial culture was expressed as the percentage of the cytotoxicity of supernatant from DES-containing medium without inoculation of bacterial cells.

Nucleotide sequence number, strain accession number, and statistical analysis

Search for nucleotide sequence similarity was conducted using the BLAST program from the NCBI. The nucleotide sequence of the 16S rRNA gene of J51 had been deposited in the GenBank database under accession number JQ670670. J51 isolate was deposited with the China General Microbiological Culture Collection (CGMCC, Beijing, China) under accession number CGMCC no. 5765. Statistical analyses were carried out using SPSS 15.0 software (SPSS Inc., USA). Differences were analyzed by student's *t* test. The significance level was defined as $P<0.05$.

Results

Isolation and genetic identification of DES-degrading bacterial strain

Seawater was concentrated and plated onto 2216E medium supplemented with 1.2 % agar and 10 mg/l DES. After incubation at 30 °C for 2 days, colonies were grown in mineral salt medium amended with 10 mg/l DES, and were examined for their DES-degrading ability. After cultured at 30 °C for 24 h, only one bacterial strain J51 showed obvious growth, and DES concentration in the supernatant from J51 culture was about 80 % lower than DES concentration in the supernatant from DES-containing medium without inoculation of bacterial cells. In order to determine whether J51 could decrease the DES concentration in seawater environment, cells of J51 was inoculated into 2216E medium amended with DES stock solution, and the same significantly decreased DES concentration was also observed. To position the isolate within genus, the 16S rRNA gene of J51 was amplified by PCR. The PCR product was purified and submitted directly for sequencing. Comparison with the known 16S rRNA gene sequence data indicated that the best matches for the 16S rRNA of J51 were those of *Pseudomonas* sp. strains (Fig. 2). With the above results, it could be concluded that J51 was a *Pseudomonas* sp. strain that could grow and degrade DES in both low and high salt medium. However, obvious growth of *Pseudomonas* sp. strain J51 was not observed when solid DES was directly added into mineral salt medium to a concentration of 10 mg/l. While *Pseudomonas* sp. strain J51 grew well in mineral salt medium amended with 1 ‰ ethanol. Therefore, *Pseudomonas* sp. strain J51 might not use DES as the sole carbon source to propagate.

DES degradation by *Pseudomonas* sp. strain J51

To determine the reduced DES was due to degradation rather than simply accumulation in bacterial cells, DES associated with the bacterial cells was detected by HPLC analysis. Only 2.6 % of DES was detected in the cell lysate of *Pseudomonas* sp. strain J51, while 26 % of DES remained in the culture of *Pseudomonas* sp. strain J51. This result confirmed the fact that the significantly reduced

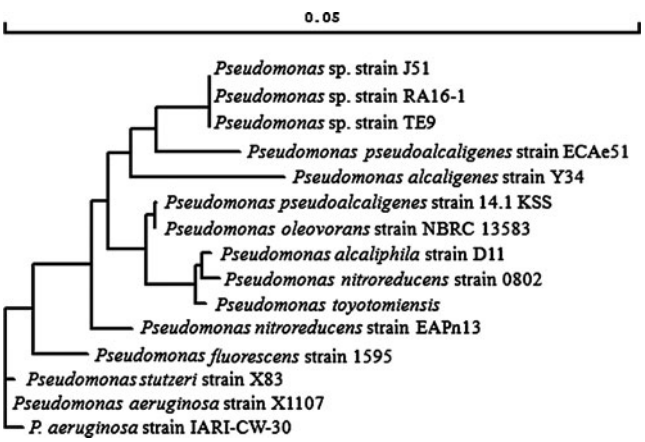

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences showing the position of *Pseudomonas* sp. strain J51

DES was due to degradation by *Pseudomonas* sp. strain J51 rather than simply accumulation.

DES degradation in mineral salt medium by *Pseudomonas* sp. strain J51 with time increasing was investigated. HPLC analysis showed that DES concentration in DES-containing medium without inoculation of bacterial cells decreased slightly during a 6-day observation. Approximately 20 % of DES was naturally degraded and 80 % of DES still remained in the medium at the sixth day. While DES concentration in the culture of *Pseudomonas* sp. strain J51 decreased greatly at the first day, with only 20 % of DES remaining. However, DES concentration in the culture of *Pseudomonas* sp. strain J51 did not drop once the time was longer than 1 day (Fig. 3). These results showed that under the tested conditions, *Pseudomonas* sp. strain J51 could effectively degrade DES in much shorter time compared to the natural degradation. DES degradation was also tested using the resting cell system, and 36 % of DES was removed. pH was found to have a strong impact on DES degradation by *Pseudomonas* sp. strain J51. The optimal pH for DES degradation by *Pseudomonas* sp. strain J51 was centered around 7.0, and deviations from this optimal pH led to drastic diminutions in degradation efficiency. Thus, degradation efficiency of *Pseudomonas* sp. strain J51 was highly pH dependent.

Effect of DES on growth of *Pseudomonas* sp. strain J51

In order to investigate the effect of DES on the growth of *Pseudomonas* sp. strain J51, bacterial cells were inoculated into mineral salt medium amended with different volumes of DES stock solution and ethanol, respectively. *Pseudomonas* sp. strain J51 grew best in the mineral salt medium containing 8 ‰ ethanol and reached maximum cell density after cultured for 48 h, then declined and remained at a stationary phase. *Pseudomonas* sp. strain J51 exhibited similar growth characteristic in mineral salt medium containing DES stock solution and ethanol. DES showed slightly inhibitory effect on the maximum cell density of *Pseudomonas* sp. strain J51, but showed no inhibitory effect on the bacterial growth at stationary phase. The final cell density of culture grown in mineral salt medium amended with DES stock solution was the same as that of culture grown in mineral salt medium amended with corresponding volume of ethanol (Fig. 4).

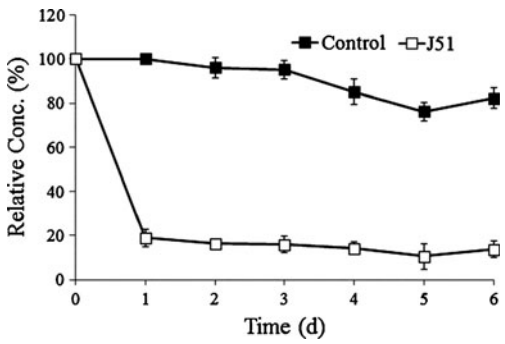

Fig. 3 DES degradation by *Pseudomonas* sp. strain J51 with time increasing. Supernatants from bacterial culture were collected at intervals of 1 day and analyzed for DES concentration. Relative DES concentration was expressed as the percentage of 10 mg/l DES that was initially added into mineral salt medium. Data are the means for three independent experiments and are presented as the means ± SE

Identification of proteins involved in DES degradation

SDS-PAGE analysis showed that none expression of proteins was obviously changed when *Pseudomonas* sp. strain J51 grew in mineral salt medium containing 20 mg/l DES. However, when *Pseudomonas* sp. strain J51 grew in mineral salt medium containing 40 mg/l DES, expression of proteins exhibited similar but distinctly different profile. Expression of proteins with molecular masses of a little more than 60 kDa was significantly enhanced. Through MALDI–TOF–TOF MS/MS analysis and blast in NCBInr 20120819, three putative proteins were identified based on the nine peptides detected. The amino acid sequences of peptides and the basic properties of the putative proteins were listed in Table 2. The three putative proteins were pyrrolo-quinoline quinone (PQQ)-dependent type I alcohol dehydrogenase of *Pseudomonas mendocina* strain ymp, isocitrate lyase of *Pseudomonas stutzeri* strain A1501, and quinoprotein alcohol dehydrogenase, also being the PQQ-dependent type I alcohol dehydrogenase, of *Pseudomonas stutzeri* strain ATCC 14405 (or CCUG 16156). The homology of the two PQQ-dependent type I alcohol dehydrogenase of *P. mendocina* strain ymp and *P. stutzeri* strain ATCC 14405 was 90.89 %, and the difference of the two proteins might be due to the derivation of the PQQ-dependent type I alcohol dehydrogenase from two different

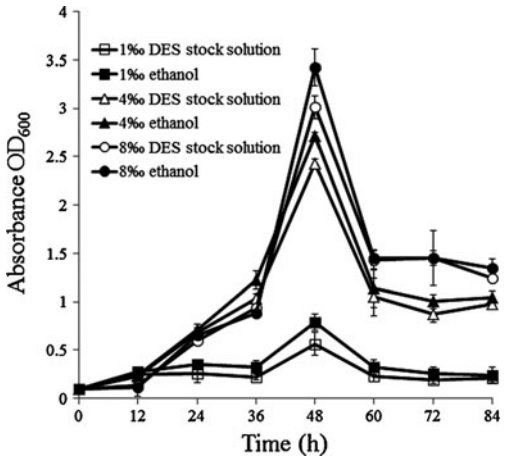

Fig. 4 Growth of *Pseudomonas* sp. strain J51 in mineral salt medium amended with different volumes of ethanol and DES stock solution. Aliquots were taken at different time points for the measurement of OD_{600}. Data are the means for three independent experiments and are presented as the means ± SE

Table 2 The basic properties of the identified proteins

NCBI	Protein name	Score	Theoretical MW (kDa)	Theoretical pI	Peptide
gi|146306985	Pyrrolo-quinoline quinone-dependent type I alcohol dehydrogenase	250	67,951	6.96	LWTYSHR IYFGTLDAR EDYWAEEAHYK APSWPDDPNHPTGK VLLIHGSSGDEFGVVGR GQESQAIIHDGVVYVTGSYSR
gi|146282659	Isocitrate lyase	69	60,671	5.37	RLPSNLFQFR TAVSDLIEELYTFLR
gi|379064735	Quinoprotein alcohol dehydrogenase	64	68,334	6.77	IYFGTLDAR APSWPDDPNHPTGK GQESQAIVHDGVIYVTGSYSR

kinds of bacterial species. Thus it was postulated that quinoprotein alcohol dehydrogenase and isocitrate lyase of *Pseudomonas* sp. strain J51 were involved in DES degradation. Quinoprotein alcohol dehydrogenase of *Pseudomonas* sp. strain J51 possessed the greater similarity to those of *P. mendocina* strain ymp and *P. stutzeri* strain ATCC 14405. Based on the nucleotide sequences of the genes encoding the two proteins, nine pairs of primers were designed. All the primers could amplify their corresponding DNA fragments correctly. Two pairs of primers, qadhpsF1/qadhpsR1 amplified the 409-bp fragment of the gene-encoding quinoprotein alcohol dehydrogenase and lypsF2/lypsR2 amplified the 165-bp fragment of the gene-encoding isocitrate lyase, were chosen for the real-time RT-PCR. Total RNA from cells grown in mineral salt medium with and without DES was extracted and used for real-time RT-PCR to analyze the mRNA levels. The result showed that the presence of DES caused a 5.2-fold increase in the mRNA level of the gene encoding quinoprotein alcohol dehydrogenase and 2.7-fold increase in the mRNA level of the gene encoding isocitrate lyase (Fig. 5).

Identification of the degrading metabolites of DES

HPLC analysis revealed the presence of one new peak with the retention time of 4.52 min in supernatant from culture of *Pseudomonas* sp. strain J51 (Fig. 6). However, this new peak could not be found in the supernatants from culture grown in mineral salt medium without DES, and from DES-containing medium without inoculation of bacterial cells. The peak area increased as the culture time reached 12 h, but disappeared as culture time rose to 24 h. The peak with the retention time of 4.52 min represented a major degrading metabolite of DES, which actually had a lower hydrophilicity than DES. In the standard MS operated in the ESI mode with a negative polarity, two prominent molecular ions at *m*/*z* 267 and *m*/*z* 326.8 were found in the product with retention time of 4.52 min (Fig. 7a). The degrading metabolite with molecular ions at *m*/*z* 267 was identified as DES-4-semiquinone, according to its *m*/*z* appeared in MS and in the light of the referenced literatures. Considering the fact that acetic acid with a molecular mass of 60 was ubiquitously presented in the bacterial culture, the molecular ions at *m*/*z* 326.8 was postulated to be *m*/*z* $[M+CH_3COOH–H]^-$ (M= DES-4-semiquinone). To verify this speculation, the fragment ion peak of *m*/*z* 326.8 was analyzed in the second-order MS. The spectrum of *m*/*z* 326.8 in the second-order MS indeed exhibited the molecular ion *m*/*z* 267 of DES-4-semiquinone (Fig. 7b). Along with the disappearance of the peak with the retention time of 4.52 min on HPLC, the disappearance of *m*/*z* 267 and *m*/*z* 326.8 in the MS detection was also observed. The other peaks with the retention time of 3.25 and 1.78 min were also explored for the degrading metabolites, but none was identified under the current conditions.

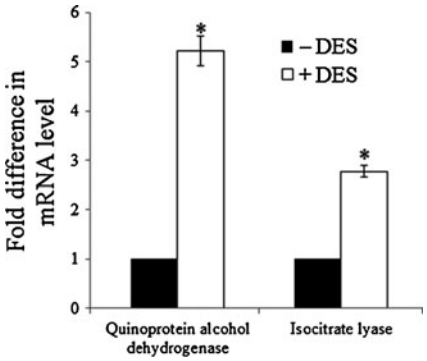

Fig. 5 mRNA levels of the genes encoding quinoprotein alcohol dehydrogenase and isocitrate lyase in the presence of DES. Cells of *Pseudomonas* sp. strain J51 were grown in mineral salt medium amended with DES stock solution and ethanol, respectively. Total RNA was extracted and used for real-time RT-PCR. The mRNA level was normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means ± SE. *$P<0.05$

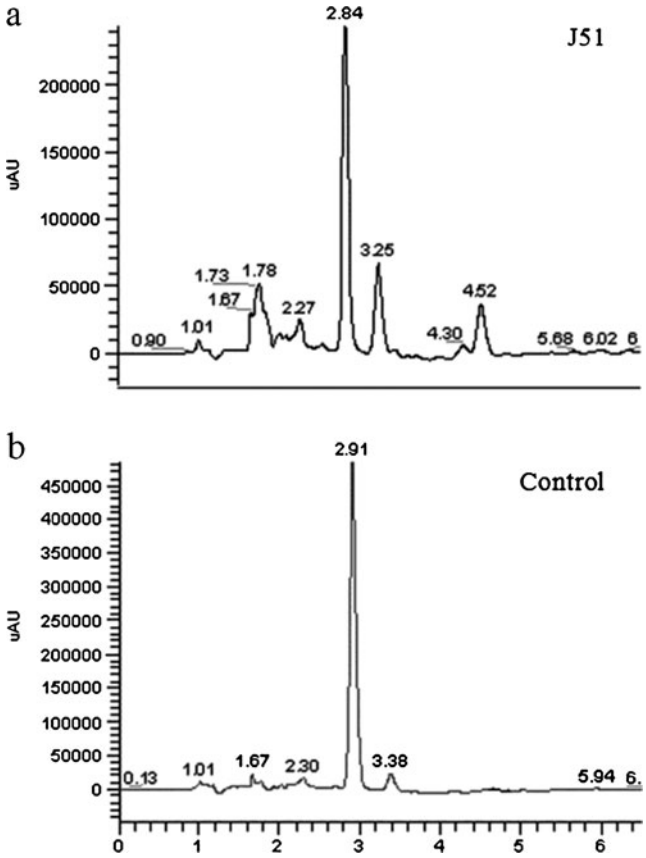

Fig. 6 HPLC detection of metabolites appeared during the DES degradation by *Pseudomonas* sp. strain J51. **a** Supernatant was from culture of *Pseudomonas* sp. strain J51 grown in medium containing 20 mg/l DES. **b** Supernatant was from 20 mg/l DES-containing medium without inoculation of bacterial cells

Cytotoxicity analysis of the degrading metabolites of DES generated by *Pseudomonas* sp. strain J51

Cytotoxicity analysis was carried out to determine whether the metabolites of DES generated by *Pseudomonas* sp. strain J51 had any reduction in the cytotoxic effect. The supernatants from 10 mg/l DES-containing mineral salt medium without inoculation of bacterial cells, culture of *Pseudomonas* sp. strain J51 grown in mineral salt medium containing 10 mg/l DES, and culture of *Pseudomonas* sp. strain J51 grown in mineral salt medium containing 1 ‰ ethanol were applied to the cultured Hela cells. Hela cells treated with mineral salt medium were used as the control cell. From the MTT cell proliferation analysis, supernatant from bacterial culture grown in mineral salt medium containing 10 mg/l DES remained only 30 % of cytotoxicity compared to the supernatant from the 10 mg/l DES-containing mineral salt medium without inoculation of bacterial cells. Consistently, microscopic observation showed that supernatant from 10 mg/l DES-containing mineral salt medium without inoculation of bacterial cells imposed a profound damaging effect on Hela cells and led to complete destruction of cell morphology after treatment for 8 h; however, supernatants from cultures of *Pseudomonas* sp. strain J51 grown in mineral salt medium containing DES stock solution and ethanol, respectively, were found to exert almost the same effect on Hela cells, having far less negative effect on the Hela cell and maintaining the normal morphology (Fig. 8). Considering the fact that the supernatant from culture of *Pseudomonas* sp. strain J51 still contained 19 % of DES, its smaller negative effect on Hela cells was postulated

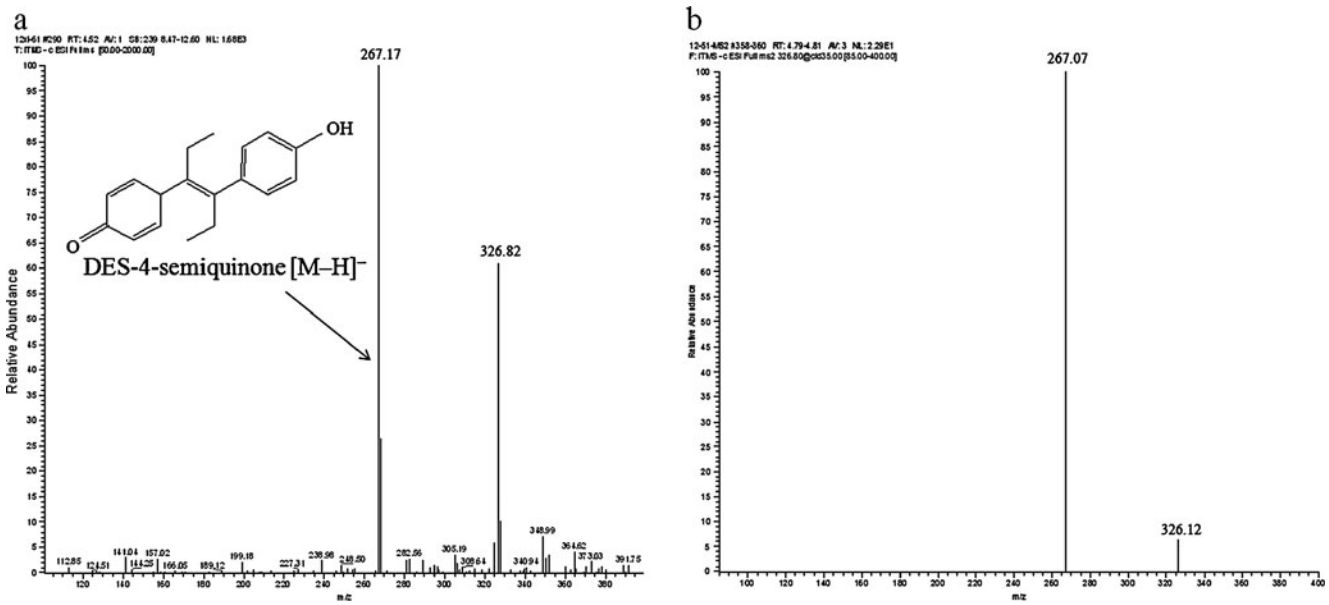

Fig. 7 **a** MS detection of metabolites appeared during DES degradation by *Pseudomonas* sp. strain J51 in the light of the referenced literature (Zhou et al. 2004). **b** The fragment ion peak of second-order MS of *m/z* 326.8

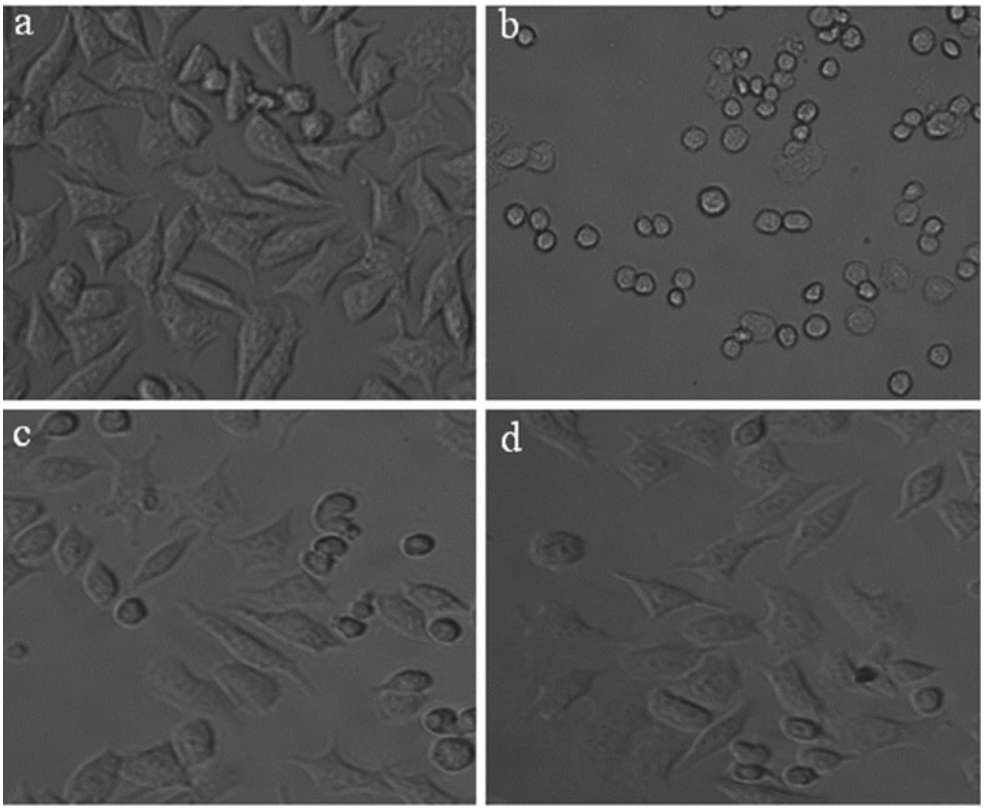

Fig. 8 Examination of cytotoxic effects of different supernatants on cultured Hela cells by inverted microscope. Hela cells were treated for 8 h with **a** mineral salt medium; **b** supernatant from 10 mg/l DES-containing mineral salt medium without inoculation of bacterial cells; **c** supernatant from culture of *Pseudomonas* sp. strain J51 grown in mineral salt medium containing 10 mg/l DES; **d** supernatant from culture of *Pseudomonas* sp. strain J51 grown in mineral salt medium containing 1 ‰ ethanol. Images are taken at ×400 magnification

to be caused by the remaining DES in the supernatant. Together, these results demonstrated that *Pseudomonas* sp. strain J51 degraded DES to metabolites with far less cytotoxicity compared to DES.

Discussion

The phenolic estrogen DES containing two monohydroxylated benzene rings is often considered as one of the EDCs that were discharged into the environment and had caused adverse effects on human health (Chen et al. 2010; Potter 2011). This study was the first to investigate DES degradation by specific bacterial strain. A *Pseudomonas* sp. strain J51 was isolated, identified, and characterized to possess the ability to degrade DES. *Pseudomonas* sp. strain J51 degraded DES in both mineral salt medium and 2216E medium, indicating that it should possess the ability to remove DES from both freshwater and seawater environments. This phenomenon was consistent with the fact that *Pseudomonas* sp. strain J51 was an isolate from the interaction region of river and sea. DES degradation by *Pseudomonas* sp. strain J51 was different from DES degradation by oxidation methods. pH was determined to have strong effect on the degradation efficiency of *Pseudomonas* sp. strain J51. Different from the optimal pH at 3.5 for DES photooxidation and the optimal pH at 9.0 for DES ozonation, the optimal pH for DES degradation by *Pseudomonas* sp. strain J51 was at 7.0, which was the mildest method among all of the developed strategies to eliminate DES till now (Lin et al. 2009; Zhou et al. 2004).

Two proteins, quinoprotein alcohol dehydrogenase and isocitrate lyase of *Pseudomonas* sp. strain J51 were determined to be involved in the DES degradation. Quinoprotein alcohol dehydrogenase, which usually occupies PQQ as a cofactor and belongs to the family of PQQ-dependent type I alcohol dehydrogenase, participates in five well-known metabolic pathways (Anthony 2001). In this study, the gene-encoding quinoprotein alcohol dehydrogenase of *Pseudomonas* sp. strain J51 was stimulated by 40 mg/l DES in both RNA and protein levels, suggesting that quinoprotein alcohol dehydrogenase was involved in DES degradation and may also occupied PQQ as its cofactor. Moreover, another gene-encoding isocitrate lyase of *Pseudomonas* sp. strain J51 was also stimulated in both RNA and protein levels. As isocitrate lyase is an enzyme in the glyoxylate cycle that catalyzes the cleavage of isocitrate to succinate and glyoxylate (Beeching 1989; Tanaka et al. 1990), the result obtained in this study suggested that further DES degradation may occur, leading to the formation of acetate. Degrading metabolite of DES generated by *Pseusomonas* sp. strain J51 was different from that generated by photooxidation and ozonation (Lin et al. 2009; Zhou et al. 2004). After degraded by *Pseudomonas* sp. strain J51, only one major metabolite DES-4-semiquinone was detected and identified. Another metabolite DES-*o*-quinone which was generated by photooxidation and could turn the sample of DES from colorless to yellow during the concentration process

(Zhou et al. 2004) was not detected. According to the present study, degradation pathway from DES to DES-4-semiquinone was postulated to be catalyzed by quinoprotein alcohol dehydrogenase. The identification of the single metabolite DES-4-semiquinone suggested that only one degradation pathway from DES to DES-4-semiquinone existed in *Pseudomonas* sp. strain J51. DES degradation pathway in *Pseudomonas* sp. strain J51 was more specific compared to the two photooxidation pathways proposed by Zhou et al. (2004). The peak of DES-4-semiquinone on HPLC disappeared as the culture time reached 24 h, suggesting that DES-4-semiquinone could be further metabolized through ring cleavage or polymerization according to the previous reports (Johansem and Moeller 1977; Liehr 1991; Zhou et al. 2004). Considering the fact that isocitrate lyase was proved to be involved in DES degradation, it was more convincing that DES-4-semiquinone was further metabolized through ring cleavage. But there still needs efforts to study the separation and detection conditions for the identification of more degrading metabolites.

In previous studies, there was no attempt to study the cytotoxicity of degrading metabolites of DES. In our study, supernatant from the culture of *Pseudomonas* sp. strain J51, which contained the remaining DES and its degrading metabolites, was determined to be far less cytotoxic compared to the supernatant from DES-containing medium without inoculation of bacterial cells. To the best of our knowledge, it was the first time to study the cytotoxicity of the degrading metabolites of DES, and consequently the cytotoxicity of the degrading metabolites was much attenuated compared to the cytotoxicity of DES. Combining the results obtained in this study, it could be concluded that DES degradation by *Pseudomonas* sp. strain J51 was a mild and environmentally friendly method to effectively remove DES and could be potentially applied for the treatment of DES-contaminated freshwater and seawater environments.

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