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Community analysis and metabolic pathway of halophilic bacteria for phenol degradation in saline environment

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ABSTRACT

A moderately halophilic bacterial enrichment was able to degrade 120 mg/L of phenol in the presence of 1–2 M of NaCl within 3 d or 2.5–3 M of NaCl within 6 d. The optimal degradation was achieved at 1.5 M of NaCl and 350 mg/L of phenol. PCR-DGGE profile of the enrichment showed that the *Acidobacterium* sp. and *Chloroflexus* sp. dominated the community. The phenol-biodegradation pathways consisted of an initial oxidative attack by phenol hydroxylase, and subsequent ring fission by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase. Nuclear magnetic resonance (NMR) spectroscopy profiles showed that ectoine and hydroxyectoine were the main compatible solutes to adjust the bacterial osmotic pressure. This study provides further information on the understanding of phenol-degradation over a wide range of salinity and remediation of phenol as a pollutant in the environment.

1. Introduction

Phenol and phenolic compounds are hazardous pollutants widespread in the environment through wastewater discharges from a variety of industries including phenol-formaldehyde resin, oil refinery, coal conversion, coking plant, leather, textiles, pharmaceutical, etc (Arutchelvan et al., 2006). Because of their toxicity and persistence, they may be bioaccumulated and biomagnified in the food chain, and have adverse effect on aquatic life, plants and humans by inducing carcinogenicity and causing reproductive and developmental toxicity, neurotoxicity and acute toxicity (Paisio et al., 2013). Hence, phenol is one of the most important pollutants in the environment and considered as priority pollutants for control by many countries. In order to removal phenol, many microorganisms have been found to degrade phenol as a carbon and energy source (van Schie and Young, 2000), such as *Pseudomonas putida* (Movahedyan et al., 2009), and *Pseudomonas aeruginosa* (Wang et al., 2011). Moreover, the bioremediation of phenol contaminated sites has been performed with different bacterial species, such as *Acinetobacter* sp. and *Bacillus cereus* (Banerjee and Ghoshal, 2010).

However, when pollution occurred in the high salinity environment, the biodegradation of the phenol often faces greater difficulties due to the high salinity, which inhibits the growth of microorganisms. Halophilic bacteria were isolated for degradation of phenol in the presence of high concentration of salts to improve the microbial activity and degrading efficiency (Afzal et al., 2007). In previous study, some halophilic bacterial were enriched to utilize phenol at concentrations of 320 mg/L in medium containing 10% NaCl (Peyton et al., 2002). Recently, more halophilic bacteria have been isolated from different saline environments to degrade phenol in hypersaline media, such as *Halomonas organivorans*, *Arhodomonas aquaeolei* and *Modicisalibacter tunisiensis* (Bonfá et al., 2013). In particular, moderate halophiles are considered the most versatile group with a great potential for biological decontamination over a very wide range of salinity (de Lourdes Moreno et al., 2011) by members in the genus *Halomonas* (García et al., 2004). Normally, majority of the moderate halophiles employ the compatible solute to maintain the osmotic balance inside the microbial cells in saline environments (Shivanand and Mugeraya, 2011). Compatible solutes consist of a series of organic compounds synthesized and accumulated by halophilic bacteria, including betaine and ectoines

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(ectoine and hydroxyectoine), sugars (trehalose and sucrose), amino acids (glycine, alanine, proline, etc), polyols (glycerol, mannitol, sorbitol) (Brill et al., 2011). They play an important role in the mechanism of salt tolerance and resistance in halophiles to higher concentrations of saline.

Up to now, only a limited reports documented the degradation of phenol by moderately halophilic bacterial community over a wide range of salinity. This study enriched a moderately halophilic bacterial community in degrading high concentration of phenol over a wide range of salinity (1–3 M). In addition, the dominant species of the microbial community, the key functional genes in the phenol-degrading pathway and the molecular mechanism of salinity tolerance of the bacterial community were revealed.

2. Materials and methods

2.1. Acclimating moderately halophilic bacterial community

The upper sediment layers were collected from Qarhan Salt Lake using sterilized spatulas for the aerobic study. The samples were placed in sterile mason jars with ample headspace, and delivered to laboratory at 4 °C. Microcosms were then prepared by using 10 g of sediment sample and 40 mL of autoclaved mineral salts medium (MSM) with pH 6.9 in serum bottles (125 mL capacity), amended with phenol as the sole carbon source in a range of salinity. Bottles were closed with butyl rubber stopper and shaken at 120 rpm in environmental chamber kept dark at 30 °C. The enrichment cultures were propogated after showing significant degradation of the added phenol. The culture mediums were repeatedly amended with phenol 5 times to acclimatize the phenol-degrading bacteria, and the enrichments were transferred 4 times to obtain the sediment-free enrichment cultures. Then 30% of the slurry was aseptically transferred to freshly made and sterile MSM containing 1, 1.5, 2, 2.5 and 3 M of NaCl, respectively.

2.2. Phenol degradation assay

The ability of the consortium to utilize phenol as the sole carbon source was determined by inoculating it into the mineral medium containing different concentrations of phenol from 50 to 350 mg/L. The cell suspensions were separated by centrifugation at 10,000 × *g* for 15 min. The culture supernatant was filtered through a 0.45-μm-pore-size filter for subsequent phenol quantification. The concentration of phenol was quantified by high-performance liquid chromatography (HPLC SHIMADZU) using a C18 column (4.6 × 250 mm). The mobile phase was composed of methanol and water (50:50, v/v) and the flow rate was 0.8 mL/min. Detection was made at 270 nm with a variable-wavelength UV detector, and quantification was made by peak integration using external standards.

2.3. Total DNA extraction, DGGE analysis and sequencing

The total DNA from cells was extracted by a Fast DNA spin kit (ABigen, Beijing, China). The partial 16S rRNA genes were amplified by PCR using the universal primers 968F-GC and 1401R. PCR amplifications were performed in a 50 μL of reaction volume that contained 1 μL of template, 2 μL of each primer, 25 μL of PCR Taqmix, 20 μL of ddH_2O. The PCR condition included: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR reactions were performed with a Mastercyle gradient (Eppendorf, US). The PCR products were run on a 1.0% agarose gel. The digital images were obtained with a G:BOX system (Syngene, Cambridge, UK).

DGGE analysis was performed on a DCode System (Bio-Rad, CA, US). PCR amplicons were loaded onto a 6% polyacrylamide gel with a denaturing acrylamide gradient ranging from 30% to 50%. A 100% denaturant solution was defined as 42 g of urea, 2 mL of 50× TAE buffer and 15 mL of 40% (v/v) acrylamide/bis-acrylamide (37.5:1), in 100 mL of DDW. Zero % denaturant solution was defined as 2 mL of 50× TAE buffer, 15 mL of 40% (v/v) acrylamide/bis-acrylamide, and 83 mL of DDW. The samples were electrophoresed at 200 V and 60 °C for 6 h. The dominant bands were excised with a sterile knife blade and soaked in 40 μL of TE buffer overnight, and then 1 μL solution was used as the template for PCR amplification. The amplicons were purified by the DNA purification kit (V-gene, Shanghai, China) and then ligated to pMD 19-T vector according to the manufacturer's instructions (TaKaRa, Dalian, China). The combined plasmids were transformed into *Escherichia coli* DH5α. The insertion of 16S rRNA gene was retrieved by PCR amplification with the primer set of M13-47 (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) and RV-M (5′-GAG CGG ATA ACA ATT TCA CAC AGG-3′). The PCR products were sequenced (Sangon, Shanghai, China). After editing and checking sequences manually, the typical ones were identified the closest relatives by the BLAST software in GenBank database of NCBI (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed by using the programs Clustal X (1.8) and Molecular Evolutionary Genetics Analysis (MEGA, version 5.05). Robustness for individual branches was estimated by bootstrapping based on 1000 replications.

2.4. Detection of functional genes involved in phenol biodegradation

To detect the presence of three catabolic genes encoding key enzymes of the phenol metabolic pathways, PCR amplification from total DNA was performed using primers for phenol hydroxylase gene (Lph), catechol 1,2-dioxygenase (1,2-CTD) and catechol 2,3-dioxygenase (2,3-CTD). The genes encoding these enzymes were amplified by using the primers sets: Lphf (5′-CGCCAGAACCATTTATCGATC-3′), Lphr (5′-AGGCATCAAGATCACCGACTG-3′) (Xu et al., 2001); 1,2-CTDf (5′-ACCATCGARGGYCCSCTSTAY-3′), 1,2-CTDr (5′-GTTRATCTGGGTGGTSAG-3′); and 2,3-CTDf (5′-GARCTSTAYGCSGAYAAGGAR-3′), 2,3-CTDr (5′-RCCGCTSGGRTCGAAGAARTA-3′) (García et al., 2006).

2.5. Compatible solutes determination with NMR method

The enrichment culture was maintained at 30 °C on MSM containing 0.2% yeast extract, 0.4% glucose and 15% NaCl. Cell growth was monitored by measuring turbidity at 600 nm spectrophotometrically. The cells were harvested by centrifugation (4000 × *g*, 15 min) at late exponential phase and washed with isoosmotic solutions. The wet cell pellets were extracted the intracellular solutes, and the extraction was repeated twice then the solvent was removed by rotary evaporation. The residue was dissolved in $CHCl_3$:CH_3OH:H_2O (5:10:4, v/v) until lipid components were eliminated (Li et al., 2012). The products were purified by a freeze dryer, and then suspended in D_2O for detection of ^1H NMR at 298 K on a Bruker Ultrashield 400 spectrometer operating at 400 MHz, using an inverse multinuclear probehead fitted with gradient along the *z* axis (Motta et al., 2004).

2.6. Nucleotide sequence accession numbers

The GenBank accession numbers for bacterial 16S rRNA gene sequences are KC899112–KC899114, and for functional gene are KC899111, KF017271–KF017272.