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Isolation and Characterization of Two Phenol-Degrading Yeast Strains from Marine Sediment

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Natural phenolic compounds and their derivatives, which are ubiquitous present in the environment, can be exuded from plant roots (Van Schie and Young 1998). They exist in nature as intermediates during biodegradation of natural polymers containing an aromatic ring, such as naphthalene, anthracene, lignin and others from aromatic amino acid precursors. Thus, the pollution related to these chemicals is associated with pulp mills, coal mines, refineries, wood preservation plants and various chemical industries (Van Schie and Young 1998).

Phenol catabolism was a suitable model for analyzing the mechanism of microorganism evolution and understanding the adaptation of synthetic aromatic compound degradation. Phenol itself is not recalcitrant, but many synthetic aromatics have substituted phenol rings. Phenol can be degraded through a variety of pathways. Under aerobic conditions, phenol is usually hydroxylated to catechol and degraded via the *meta* or *ortho* pathway (Hiroyuki et al. 1998). Studies conducted on these pathways dealing with the metabolism and catabolism of microorganisms adapted to ambient phenol concentration, showed that the microorganism can only resist phenol at a certain level. It has been shown that increasing phenol concentration appears to decrease overall phenol biodegradation (Van Schie and Young 1998).

Although many research works reported that some microorganisms have the capability for phenol degradation, most of them were focused on the such as *Pseudomonas sp.* (Banerjee et al. 2001) and *Bacillus sp.* (Duffner and muller 2001). Compared with bacteria, the studies on the phenol-degrading yeasts are very few. Lee et al. (2001) showed that a pure culture of yeast is able to use phenol as a sole source of carbon and energy, such as strain Y103. Another yeast strain called *Candida tropicalis*, which has degraded 1.504 $g \cdot L^{-1}$ of phenol, was isolated from Amazonian soil samples (Bastos 2000). This indicated that the degradation ability of yeast was much better than that of the bacteria. Yeast can be easily reclaimed and is useful for recycling waste. In this paper, the isolation and

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characterization of two phenol-degrading yeasts from marine sediments of Xiamen Western Sea area (Fujian province, P.R. of China) are largely described.

MATERIALS AND METHODS

Phenol-degrading microorganisms used in this study were isolated from marine sediments collected from various geographic locations in Xiamen Western Sea area (Fujian province, P.R.China). These sediments were immediately taken to the laboratory and processed in 24hr for enrichment of the phenol-degrading strain.

A defined mineral salts medium was used for enrichment and isolation of the phenol-degrading strain. Isolation from different locations was carried out using this medium. The isolation and growth medium used for the yeast strain contains (per liter)1.0g of phenol (Purity>99%, Sangon Company in Shanghai, China), 1.0g of $(NH_4)_2SO_4$, 0.8g of Na_2HPO_4, 0.2g KH_2PO_4, 0.2g of $MgSO_4 \cdot 7H_2O$, 0.1g of $CaCl_2 \cdot 2H_2O$, 5.0mg of $FeCl_3 \cdot 6H_2O$ and 1.0mg of $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$. The yeast medium included (per liter) 30.0g of glucose, 5.0g of tryptone, 1.0g of yeast extract and 1L of filtrated seawater. The pH of all media was 6.5, suitable for the growth of yeasts.

The phenol-degrading yeast was isolated after 18 months of enrichment. An aliquot of the culture was inoculated in enrichment medium and plated onto the isolation medium including 1.5% agar and phenol as the sole carbon source. After 2 wks of incubation at 25°C without any sunlight, some of the small colonies that formed on the agar surface could grow on phenol under dark conditions. Some of the colonies were inoculated onto the yeast medium with 1.5% agar. After the colonies were formed through 48h of incubation, they were transferred onto the isolation medium including 1.5% agar and phenol again. This process involved 3-4 repetitions until one pure strain with phenol-degrading ability was isolated. Yeast strains were isolated from the cultures by repeated plating between the isolation medium and the yeast medium with aerobic incubation. The cultures were checked for purity by plating on the yeast medium, as well as by microscopic observation. Pure strains were maintained in cuvettes on agar medium under 4°C conditions. Finally, 7 phenol-degrading yeast strains were isolated from the marine sediment. But in this paper, only strains P2 and P4, with higher phenol degrading ability, are discussed.

After incubation by yeast medium to the late exponential phase, 200μL portions of the cultures were added to 20mL portions of growth medium with phenol in 50mL bottles. The bottles were closed with sterilized seal-film. Phenol was added at a starting concentration of $1.0g \cdot L^{-1}$ after sterilizing. Cultures were incubated in an aerobic chamber at optimal conditions (pH 6.5, temperature about 25°C), which is

most close to the marine environment. The shaking speed was 150rpm. Phenol-degrading cells in late exponential phase were immobilized by FA solution (Formaldehyde: Acetic acid = 4:10) for 1~2 hr. The suspension was dehydrated gradually by ethanol and photographed on the JSM-6360A scanning electron microscope (JEOL, Japan). Phenol concentrations were proportionally diluted by deionized water and measured as depicted by Gambaro et al. (1992). Yeast growth in the liquid medium was monitored by measuring the optical density at 460nm with a UV-2602 spectrophotometer. All cultivations were performed with three different replicates, and the purity of a culture that exhibited growth was checked microscopically (Van Schie and Young 1998; Yoshifumi et al. 2000).

The yeast cultures were incubated in the growth medium with phenol ($1.0g \cdot L^{-1}$) for 72h. After being washed with PBS buffer twice, the yeast cells were broken down and the crude enzymes were collected. The activity of dioxygenase was determined by measuring catechol production in a cell suspension containing 30μL of 30mM catechol, 300μL of 40mM EDTA, 2.07ml of PBS buffer (pH 7.0) and 600μL of the crude enzyme extraction, as described by Spain and Nishino (1987). The reaction was allowed to proceed for 30 min at 25°C, and measured at 260nm (C12O) or 375nm (C23O) by the UV-2602 spectrophotometer (Liu et al. 2002; Strachan et al. 1998).

Total genomic DNAs were prepared from the phenol-degrading yeast strains by the improved extractive method of NaCl-CTAB by using 3d cells grown aerobically on the growth medium with $1.0g \cdot L^{-1}$ phenol. A portion of each DNA (ca.100ng) was used in the PCR to amplify the 18S ribosomal RNA (rRNA) gene with oligodeoxynucleotide primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACC TACGGA-3'), which were designed to anneal to conserved regions of the yeasts' 18S rRNA (White et al. 1990). The PCR conditions used were as follows: 5 min of activation of polymerase at 95°C, followed by 35 cycles consisting of 30 s at 95°C, 50 s at 55°C and 2.5 min at 72°C and finally by 10 min of extension at 72°C. The PCR products were electrophoresed through a 1.0% (wt/vol) agarose gel in TAE buffer. The PCR amplified products were purified by E.Z.N.A Cycle-Pure Kit and then linked to the T-vector by T4 ligase before sequencing (Sambrook and Russell 2001). The 18S rRNA gene sequences of isolate P2 and P4 were deposited at GenBank under accession number DQ177817 and DQ187955, respectively.

RESULTS AND DISCUSSION

It is not surprising that phenolic compounds are present in many different environments, including the ocean as a special environment with several extreme conditions and many unknown organisms. The enrichment cultures that degraded

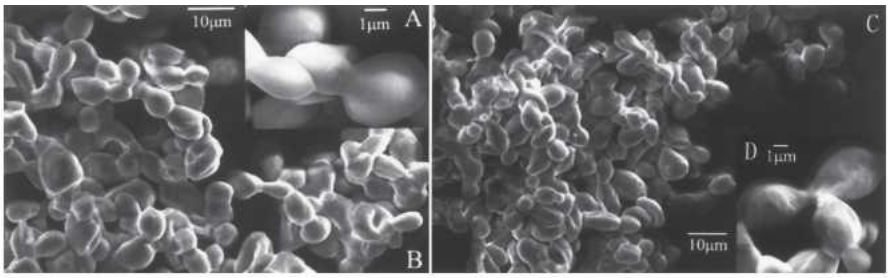

Figure 1. Scanning electron microscopic photograph of the strain P2 and P4 (A, B: P2; C, D: P4)

phenol were established in a continuous-feed fermentor by using sediments collected from Xiamen Western Sea area, Fujian province, P.R.China, as the source of yeasts. Initially, 7 yeast strains were isolated from different coastal zones, and they all maintained the ability to use phenol as a sole source of carbon and energy under aerobic conditions. Among these strains, only two yeast (P2 and P4) with relatively high degradation ability were selected and discussed in this work. As the characteristics show (Table 1-2), these two strains were quite similar with only limited differences as strain P2 formed mycoderm when grown in stationary medium, while strain P4 did not. The morphology of both isolates were circle-like or ellipse, and approximately 6 by 4 μm with ascospores. Growth of these two strains in the enrichment or isolation medium was slow. Then, these stains just formed pinpoint colonies in 2 weeks or more. But when growing on yeast medium, the strains formed colonies in only 24hr and produced an alcoholic scent as well. Under electron microscopy, similar morphologies of strains P2 and P4 were detected (Figure 1).

Table 1. Characterization of two phenol-degrading yeast strains P2 and P4.

Strain	Morphology	Length (μm)	Width (μm)	Ascospores	Phenol tolerance ($g \cdot L^{-1}$)	Mycoderm [a]
P2	Circle-like	5.5	4.1	+	1.0	+
P4	Ellipse	6.1	3.9	+	1.0	-

[a] Form mycoderm when grown in stationary, aerobic cultures.

Figure 2 shows growth curves and substrate consumption for cultures yeast strains P2 and P4 in growth medium containing phenol as the sole carbon source under aerobic conditions. In the two phenol-degrading yeast strains, degradation of phenol was observed along with reduction of phenol; these results were similar to results obtained with known aromatic compound-degrading microorganisms. Reduction of phenol was measured during the growth of these two yeast strains. It was found that strain P4 could degrade about $1.0 g \cdot L^{-1}$ phenol after 72 h of

incubation in growth medium, while strain P2 could do it after 96 h. Over 95% of the phenol could be used up as the sole carbon source after cultivation of strains.

Table 2. Growth of strains P2 and P4 on various carbon sources under aerobic conditions.

Substrate	Utilization by [a]:		Substrate	Utilization by [a]:	
	P2	P4		P2	P4
Aromatic Compounds			Adonitol	+	+
Hydroquinone	+	+	Lactose	−	−
Resorcinol	+	+	Plant Indican	+	−
Catechol	+	+	Organic acids		
Benzoic acid	Weak	Weak	Urea	−	−
Naphthalene	+	Weak	Malonate	−	−
o-Phthalic acid	Weak	+	Esculin	−	−
3,5-Dihydroxytoluene	Weak	Weak	Citrate	+	+
p-Nitrophenol	−	−	Amino acids		
p-Dichlorobenzene	Weak	Weak	Phenylalanine	−	−
Diphenylamine	−	−	Lysine	−	−
Sugars			Ornithine	−	−
Mannose	−	−	Arginine	−	−
Xylose	+	+	p-Coumarinc	+	+
Raffinose	−	−	Enzyme and others		
Ribose	−	−	Oxidase	+	+
Maltose	+	+	DP-300	−	−
Sorbitol	+	+	Acetamide	−	−
Mannitol	+	+	Polymyxin B	−	−
Sucrose	+	+	H_2S	−	−
Fructose	+	−	ONPG	−	−
Rhamnose	−	−	Glucose Oxidative	+	+
Arabinose	−	−	Glucose fermentative	+	+
Inositol	−	−	Growth Control	+	+

[a] +: growth, -: no growth.

Table 2 shows the growth of two yeast strains P2 and P4 on various carbon sources under aerobic conditions. Some organic acid, sugars and amino acids could support the growth of these strains, but some differences between strain P2 and P4 were found. Stain P2 could use plant indican and fructose as the carbon source for growth, while strain P4 cound not. When cultured on the isolate medium with different benzene homologous carbon sources, both strains P2 and P4 utilized hydroquinone, resorcinol, catechol, etc. as a sole source of carbon and energy, but did not utilize p-nitrophenol and diphenylamine. In addition, strain P2 grew better

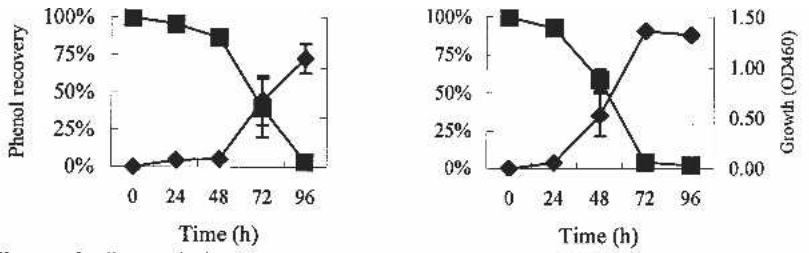

Figure 2. Degradation of phenol by strains P2 (Left) and P4 (Right) under aerobic conditions. ◆: growth; ■: phenol recovery.

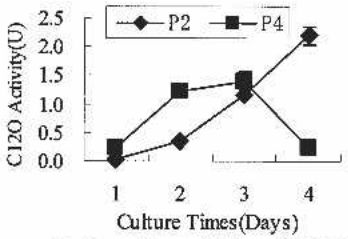

Figure 3. Specific activity of C12O of strains P2 and P4 on the growth medium.

in the naphthalene–containing medium, while strain P2 could grow in the presence of o-phthalic acid. The results showed that strains P2 and P4 had some differences in utilization of the aromatic compounds.

The degradation of phenol by yeast or other microorganisms may utilize many enzymes such as hydroxylase, monooxygenase and dioxygenase. Phenol was usually hydroxylated to catechol by hydroxylase and then degraded via the *meta* or *ortho* pathway by dioxygenase. The activities of the enzymes for phenol degradation, catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O), were measured in the two yeast strains. Almost no C23O activity was detected when strains P2 and P4 were grown in the growth medium for more than 72h (data not shown), while C12O activities were found when the two strains were grown with phenol as the sole carbon source (Figure 3). Strain P2 expressed the highest C12O activity, which had the enzyme activity of 2.338U (1U means the production of muconic acid catalyzed by 1 mg enzyme extraction in one minute). Compared with strain P2, the C12O activity of strain P4 was low and had 1.572U. These results suggested that both strain P2 and strain P4 oxidized phenol by the ortho type of ring cleavage, but not by the meta type of ring cleavage. Most of these enzymes could be induced by its degradable substrates. The hydroxylase, catechol 2,3- dioxygenase from strain TA441 and the catechol 1,2-dioxygenase have been cloned by Hiroyuki et al. (1998) and Khomenkov et al. (2005), respectively.

Just like the 16SrDNA used in the phylogenetic analysis of prokaryotes, eukaryotes, especially yeast microorganisms were analyzed based on 18S rRNA sequences. The nucleotide sequences of 18SrRNA gene of strains P2 and P4 were amplified by PCR and determined by automated fluorescence sequencing. To be consistent with their morphology and metabolic characteristic, they appeared to be very similar at the 18SrRNA level as well. The GeneBank database was used to search for 18SrRNA sequences homologous to 18SrRNA sequences of these new two isolates. Almost complete sequences of the 18SrRNA of strain P2 (1,719 bases) and strain P4 (1,686 bases) were determined. The taxonomic positions of the strains inferred from these sequences are presented in Figure 4. The results showed that the 18S rRNA of strain P2 and P4 appeared to be more than 98% concordant to the 18SrRNA of *Candida tropicalis* small ribosomal subunit RNA gene (Bastos et al. 2000). Thus, strain P2 and P4 were classified as a member of this species and able to use phenol as a sole source of carbon and energy. Other researchers had isolated some *Candida sp.*, which were also capable of growth with some monoaromatic compounds (Vallini et al. 1997; Corvini et al. 2004; Tanghe et al. 1999). Hence, *Candida sp.* might be providing the important monoaromatic-degrading yeast to the natural environment. Considering the characteristics of the two strains, the exact classification of them will not be authenticated until more strains of this kind and other *Candida sp.* strains have been isolated and characterized by other method such as the analysis of GC content or DNA/RNA hybridization.

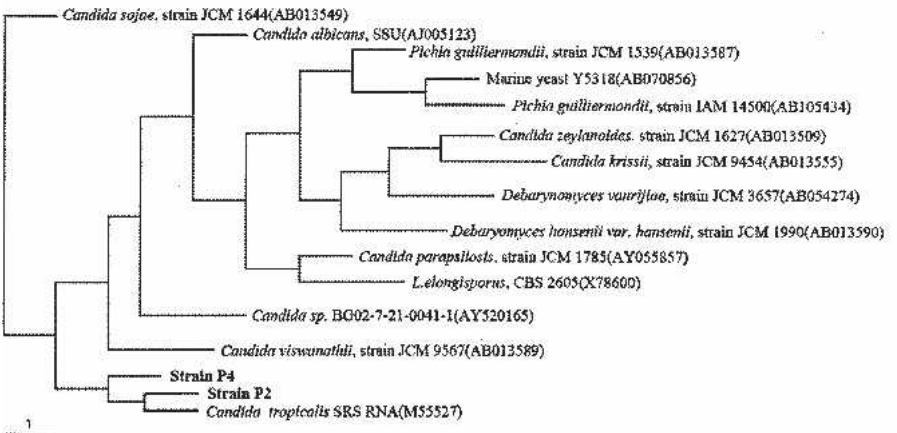

Figure 4. Phylogenetic relationship of strains P2 and P4 in the class *Candida* based on 18SrRNA sequence comparison.

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