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Isolation and characterization of a new *Pseudomonas*-related strain capable of degradation of phenol from oil contaminated soil

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A novel phenol-degrading bacterium named as SKDP-1 was isolated from crude oil contaminated soil, Gudao oil field in the Northeast of Shandong Dongying, East China. The biochemical tests indicated that strain SKDP-1 was Gram-negative, and glucose and citrate could be utilized and starch not gelatin. Both Voges-Proskauer and H_2O_2 enzyme tests were positive. The activities of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) in free cells were measured to be 8 and 91 IU, respectively. 16S rDNA gene sequences of strain SKDP-1 analysis showed the similarity of 98% with *Pseudomonas putida* (AB680847). The phylogenetic tree formed by 16S rDNA sequences from both strain SKDP-1 and its most related bacteria also proved strain SKDP-1 to be one member of the genus *Pseudomonas*. Strain SKDP-1 showed the high phenol-degrading rates from 30 to 99% cultured by mineral salts medium (MSM), which was added with phenol from 100 to 1600 mg/L, respectively. The optimum pH and growth temperature for strain SKDP-1 to remove phenol were about 7.0 and 30°C, respectively. Based on its biochemical properties and high capability of degrading phenol, strain SKDP-1 provided the possibilities of treating phenol contaminated environment in the future.

Key words: Phenol degradation, *Pseudomonas*, *ortho*-cleavage pathway.

INTRODUCTION

Phenol, as an air pollutant, could be emitted from wood and solid waste combustion as well as automobile exhaust and cigarette smoke. It is also responsible for the malodors during the course of decomposition of animal wastes (Zahn et al., 2001). The global production of phenol has been estimated to be about 8.5 million metric tons per annum, and its environmental risk assessments have concluded that phenol has the potential of causing harm both to marine aquatic life and terrestrial biota (Jiang et al., 2002).

Biodegradation of phenol has been extensively studied due to the widespread distribution of phenol as a pollutant in water and soil (Jiang et al., 2005). The biological degradation is accomplished through benzene ring cleavage mediated by intracellular enzymatic reaction (Kumar et al., 2004). In addition, studies on phenol toxicity to bacteria have shown that bacteria can adapt a low level of phenol concentrations, but increasing

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phenol concentrations appeared to decrease overall phenol degradation (Dean and Rahimi, 1995). A range of phenol-degrading microorganisms have been identified, including *Acinetobacter* (Abd et al., 2002), *Bacillus* (Arutchelvan et al., 2005), *Burkholderia* (El et al., 2003), *Pseudomonas* (Whiteley et al., 2001), *Valivorax* (Watanabe et al., 1998), mesophilic and thermophilic *methanogens* (Chen et al., 2008), and the yeast *Candida tropicalis* (Jiang et al., 2005). Many studies proved that phenol-degrading bacteria have been isolated from natural soils (De et al., 2005), plants roots (Wang et al., 2007), root nodules (Wei et al., 2008), rivers (Parvanov and Topalova, 2008), and marine ecosystems (Shashirekha et al., 1997). Oil contaminated soil as the resource of isolating phenol-degrading bacteria has only been reported by Bhavna et al. (2011) until now. The detailed information on those phenol-degrading bacteria in that study such as their phylogenetic analysis was very limited.

Gudao oil field (133.1 km^2) located in the Northeast of Shandong Dongying (37°86'N, 118°78'E), East China, is the biggest production plant of Sinopec Group. Unavoidable oil leak in the processes of oil extraction and transport caused the serious contamination on the soil. The objectives of the present study were: (1) to isolate the bacterium with high phenol-degrading ability from oil contaminated soil in Gudao oil field, (2) to identify the phylogenetic position of this bacterium, (3) to test the isolate on its degrading-phenol capability with the increasing phenol concentration, and (4) to prove the pathway for phenol metabolism of this isolate.

MATERIALS AND METHODS

Isolation of phenol-degrading bacteria

Surface soil (area of 13×13 cm; depth of 0 to 8 cm) in Gudao oil field as the sample, was collected. The enrichment operation of bacteria was described as follows: in brief, 1 ml of suspension from 5 g soil sample was incubated in 100 ml of mineral salt medium (MSM). The culture condition was at 30 on rotary shaking incubator at 180 rpm for four weeks. MSM medium contained the following ingredients (L^{-1}): K_2HPO_4 2.75 g, KH_2PO_4 2.25 g, $(NH_4)_2SO_4$ 1 g, $MgCl_2.6H_2O$ 0.2 g, NaCl 0.1 g, $FeCl_3.6H_2O$ 0.02 g and $CaCl_2$ 0.01 g, pH was adjusted to 7.0 (Watanabe, 1998). The medium was autoclaved at 121°C for 15 min for sterilization. Phenol, as the sole carbon source, was separately sterilized and aseptically added into the sterile medium with the concentration of 300 mg/ L.

The purification of strains were summarized as follows: 0.2 ml of the enriched culture obtained from the above steps was streaked onto plates with MSM medium containing 2% agar and incubated at 30°C for one week. The separated colonies were chosen and streaked onto plates for incubation. After four replicates of streaking incubation, the purified isolates were maintained in semi-solid MSM stab cultures supplemented with phenol as sole carbon source at 4°C until further use. One isolate was chosen as the representative bacteria named strain SKDP-1 for the future study.

Phenotypic characterization of isolate

The cell morphology was verified by scanning electron microscope (SEM) (Hitachi, S-4800) and Gram-stain was examined as previously described (Holt et al., 2010).

Physiological and biochemical tests

The physiological and chemical properties were carried out according to Bergey´s Manual of Determinative Bacteriology (Holt et al., 2010).

Phenol removal tests

Strain SKDP-1 was pre-cultured in Luria Bertani (LB) medium (L^{-1}): yeast extract 5.0 g, peptone 10.0 g, sodium chloride 10.0 g, at 30°C with shaking (180 rpm) till logarithmic phase. 3% (v/v) of the above pre-culture was inoculated to MSM medium containing phenol from 100 to 1600 mg/L as the sole carbon source. The growth condition was also at 30°C with shaking (180 rpm). The reaction mixture containing all components but devoid of bacterial inoculums was used as control. The residual phenol concentration present in culture at different incubation period was measured using UV visible spectrophotometer by colorimetric assay 4-amino antipyrene method (Lacoste et al., 1959). In order to test the influences of both pH and temperature on phenol removal for the isolate, series of pH values ranging from 3.0 to 9.0 (intervals of 2) and temperatures from 20 to 40°C (intervals of 5) were made for culturing strain SKDP-1 with 400 mg/L of phenol (according to the above experiments results) contained in MSM medium, respectively. The method of measuring residual phenol in medium was the same as described previously.

Enzyme assays

Strain SKDP-1 was grown on MSM liquid medium added with 400 mg/L of phenol as the sole carbon source for 100 h at 30°C. The above culture conditions were based on the previous experiments (phenol removal tests). Then the cells (strain SKDP-1) were harvested by the ultra filtration method (filter membrane diameter, 0.22 μm) and then washed with phosphate potassium buffer for the following steps (Zhang et al., 2008; Ren et al., 2005).

The cells obtained from the above steps were ruptured for 30 s with ultrasonic processor. The cell debris and undisrupted cells were removed away by centrifuging at 10000 rpm for 20 min. The supernatant obtained was used as free cell extracts (crude enzyme) for enzyme assay. Total protein content in the crude enzyme was measured by the Bradford method using bovine serum albumin as the standard (Ausubel et al., 1995). The reaction mixture of 1.0 mL contained 50 mM sodium phosphate buffer, 50 μg enzyme and 75 μmol of substrate (catechol), and pH was adjusted to 7.0. The activities of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) for strain SKDP-1 were measured by spectrophotometric method. C12O activity was measured as an increase of absorbance at 260 nm by the formation of *cis, cis*-muconic acid (Hegeman, 1966). C23O activity was measured at 375 nm by determining the accumulation of 2-hydroxymuconic semialdehyde (2-HMS) (Feist and Hegeman, 1969). Control experiments (without catechol) were carried out for each assay. Triplicates were done for determining the quantity of *cis, cis*-muconic acid and 2-HMS. The enzyme activities were expressed as μmoles of product formed per min per mg of protein at 30°C.

PCR amplification of 16S rDNA from the isolates

Genomic DNA of the isolate cultured in MSM medium was extracted with TIANamp Bacteria DNA DP302 Kit (Beijing Tiangen Biotech)

Table 1. Morphological and biochemical characteristics of bacteria SKDP-1able to grow on MSM medium with phenol as sole carbon source.

Characteristic	Bacteria strain SKDP-1	Characteristic	Bacteria strain SKDP-1
Gram staining	negative	Citrate	Positive
Motility	Positive	Glucose	Positive
Colony	White slimy	Starch	Negative
Catalase	Positive	Voges-Proskauer test	Positive
Urease	Negative	M.R	Negative
H_2S production	Negative	Aerobic test	Negative
Nitrate reduction	Positive	Starch hydrolyzation	Negative
Indole production	Positive	Gelatin liquefaction test	Negative
Maltose	Negative	Optimum pH	7.0

according to its instruction. PCR amplification of 16S rRNA for the isolate was performed using the primers PF5´-AGA GTT TGA TCC TGG CTC AG-3´and PR 5´-GGY TAC CTT GTT ACG ACT T-3´. PCR reactions contained 100 ng of genomic DNA, each primer at a concentration of 0.4 µM, each dNTP at a concentration of 200 µM. PCR reaction conditions were an initial denaturation step of 3 min at 94°C, 32 cycles of 45 s at 95°C, 45 s at 55°C, with a final extension for 10 min at 72°C. The electrophoresis, the purification of PCR products were essential as described previously (Lu et al., 2008).

Sequence alignment and phylogenetic analysis

The 16S rRNA sequences were determined by the automatic DNA sequencer (ABI Prism Model 3700, CA, USA). The primer used for sequencing was PF as mentioned above. The sequences determined from the isolate were compared with the similar sequences retrieved from DDBJ /EMBAL/GenBank database using the BLAST program. All the obtained sequences were aligned using the CLUSTAL X program (Thompson et al., 1997) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), using the Mega.5 program(Sharma et al., 2002). Bootstrap analysis was performed 1000 times using the same program as above.

Nucleotide sequence accession number

The 16S rRNA sequence for the isolate has been deposited in DDBJ under accession number AB773822.

RESULTS

Isolation and identification of phenol-degrading strain

Soil contaminated with oil was chosen as the source of indigen bacteria isolation in this study. Up to four weeks of treating with phenol as sole carbon source in MSM medium, only phenol resistant strains were left in culture. After four replicates of purification treatments, one isolate was selected as the representative strain and named SKDP-1. Electron scanning micrograph indicated that strain SKDP-1 cultured in MSM medium was slightly curved with rods (sizes, 0.2-0.4 µm wide and 1.0-1.45µm long). The morphological and chemical properties of strain SKDP-1 were listed in Table 1. Comparing with the characteristics of the microorganisms with the ability of degrading phenol mentioned in Bergey´s manual of systematic Bacteriology, strain SKDP-1 was seemed to be one member of the genus *Pseudomonas* sp.

Degradation of phenol by strain SKDP-1

Acclimatization has been regarded as the useful way for microorganisms to obtain the highest resistant capability of phenol concentrations (Lob and Tar，2000). The experiment in the present study aimed to find the highest tolerance of phenol concentration for strain SKDP-1. A series of phenol concentration (as the sole carbon source, 100 to 1600 mg/L) were prepared for culturing strain SKDP-1. The residual of phenol with time changes by strain SKDP-1 under different concentrations phenol in MSM media were shown in Figure 1. Strain SKDP-1 showed the similar capabilities of degrading phenol almost completely (about 94 - 99%) within a relatively short time of 100 h with phenol concentration of 100 to 400 mg/L in MSM medium. Strain SKDP-1 showed the very low removal rates of about 40 and 30% at the time of 100 h when phenol was added up to the concentration of 800 and 1600 mg/L in MSM medium, respectively. The results (Figure 1) prove that 400 mg/L of phenol in MSM medium seemed to be the maximum concentration for strain SKDP-1 to endure and remove. Cells growing at high phenol concentration (800 and 1600 mg/L) showed the longer lag time compared to those growing at low concentration of phenol (data not shown). The same stationary phase at about 100 h for strain SKDP-1 growing at the whole range concentration of phenol (100 to 1600 mg/L) could also be proved by Figure 1.

Based on the previous experiment, phenol concentration of 400 mg/L in MSM medium and 100 h incubation time were used for evaluating the influences of pH and culture temperature on phenol removal for strain SKDP-1.

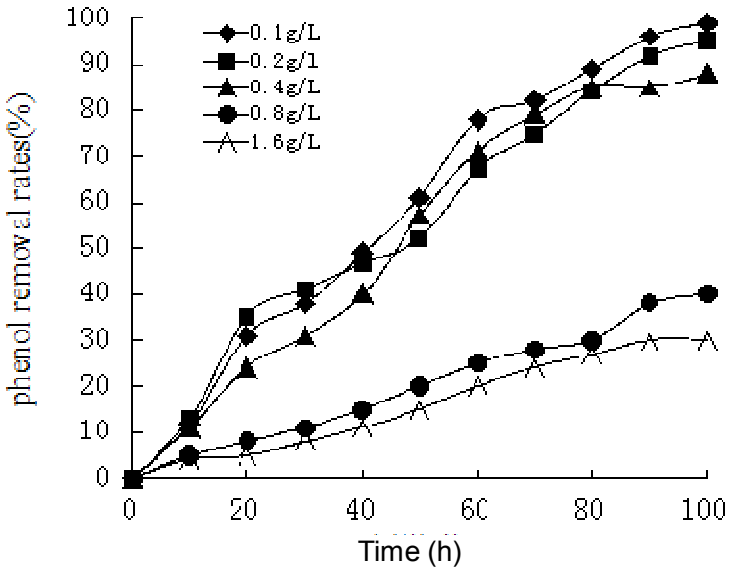

Figure 1. Phenol removal rates of strain SKDP-1 with phenol concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 g/L in MSM medium at 100 h incubation, respectively.

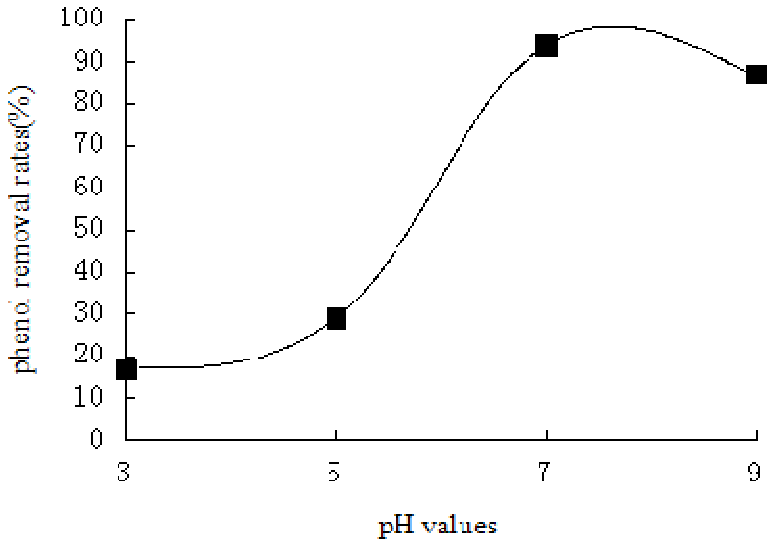

Figure 2. Phenol removal rates of strain SKDP-1 at 100 h incubation in MSM medium with different pH of 3, 5, 7 and 9, respectively.

The residual phenol was tested at 100 h incubation time with the different pH values (3.0, 5.0, 7.0 and 9.0) and culture temperatures (20 to 40°C) in MSM medium, respectively. The phenol removal dependant changes of pH values and temperatures in MSM medium were shown as Figures 2 and 3, respectively. The highest removal rates of about 99% for strain SKDP-1 with pH value of 7.0 and temperature of 30°C in MSM medium were shown in Figure 2 and Figure 3, respectively.

Figure 1 shows the degradation profile by strain SKDP-1 with phenol concentrations of 100, 200, 400, 800, 1600 mg/L in MSM medium, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the standard deviation less than 5%. Figure 2 shows degradation profile by strain SKDP-1 at 100 h incubation with different pH of 3, 5, 7, 9, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the

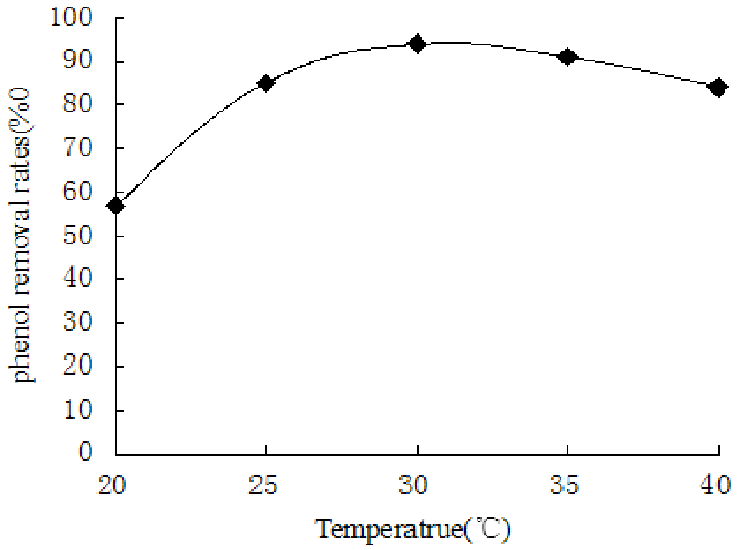

Figure 3. Phenol removal rates of strain SKDP-1 at 100 h incubation in MSM medium with different temperature of 20, 25, 30, 35 and 40°C, respectively.

standard deviation less than 5%. Figure 3 shows the degradation profile by strain SKDP-1 of 400 mg/L at 100 hr incubation with different temperature of 20, 25, 30, 35 and 40°C, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the standard deviation less than 5%.

Enzyme studies

There are two main metabolic pathways (*ortho*- and *meta*-cleavage) for phenol biodegradation which were initiated either by catechol 1,2-dioxygenase (C120) or catechol 2,3-dioxygenase (C230), respectively. In this study, the activities of C120 and C230 in free cells of strain SKDP-1 were found to be 8 and 91 IU, respectively. Therefore, the main way of metabolizing phenol for strain SKDP-1 was *meta*-pathway.

Phylogenetic analysis

The DDBJ database was used to search for 16S rRNA sequences homologous of all the sequences used in constructing phylogenetic tree (Figure 4). Phylogenetic tree revealed that strain SKDP-1 exhibited a highest similarity (98%) with *Pseudomonas putida* (AB680847) and was belonged to genus *Pseudomonas.*

Figure 4 shows the phylogenetic analysis based on the 16S rDNA sequences of strain SKDP-1 and related species. Neighbour-joining method is used to construct this tree. Bootstrap values obtained with 1000 repetitions are indicated at the nodes. Bar 0.02 substitutions per nucleotide position. Accession numbers from GenBank are in parentheses.

DISCUSSION

It has been found that some bacteria withstand the toxicity from phenol by secreting monocyclic aromatic compounds (MACs) or polycyclic aromatic compounds (PACs) (Hearn et al., 2003; Jiang et al., 2005; Lacoste et al., 1959; Leung et al., 1997). Considering this fact, the residual phenol in culture and the enzyme activities (C12O and C23O) were determined in our present work to find out how strain SKDP-1 tolerate and grow in medium added with phenol as its sole carbon source. The time dependent decrease of residual phenol in MSM medium and the activities of C12O and C23O could prove that the tolerance of the phenol toxicity for strain SKDP-1 was from its high phenol-degrading, not by secreting MACs or PACs (Figures 1, 2 and 3).

In the study of Bhavna et al. (2011), two aerobic bacterial strains OCS-A and OCS-C were also isolated from the oil contaminated soil. The phenol-tolerating and -degrading abilities for strains OCS-A and OCS-C were also tested in that study. Strains OCS-A and OCS-C could only tolerate phenol up to concentrations of 100 mg/L, while strain SKDP-1 in our work showed the much higher phenol tolerance of 400 mg/L (Figure 1). In the same phenol concentration of 100 mg/L in medium, strains OCS-A and OCS-C were only able to degrade about 90% of phenol, while strain SKDP-1 could degrade phenol almost completely (99%) (Figure 1). In addition, comparing strains AT2 and PW3 isolated by Wael et al., (2003) with strain SKDP-1, their optimal temperature (30°C) and pH (7.0) for phenol-degrading were identical.

There are two different pathways for phenol metabolism under aerobic condition, either the *ortho*- or *meta* pathway from catechol in phenol-degrading bacteria, in

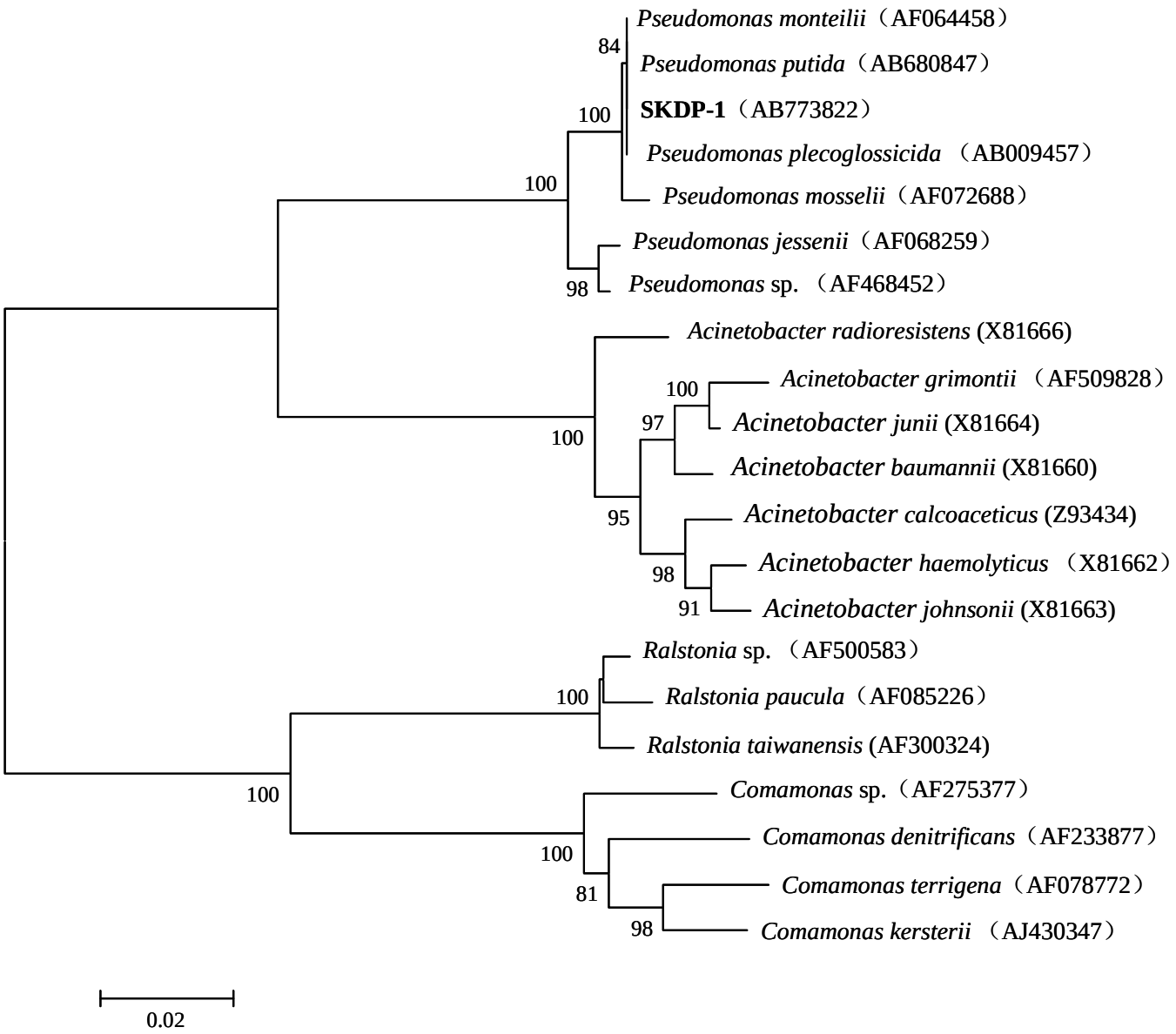

Figure 4. Phylogenetic analysis based on the 16S rDNA sequences of strain SKDP-1 and related species. Neighbour-joining method is used to construct this tree. Bootstrap values obtained with 1000 repetitions are indicated at the nodes. Bar 0.02 substitutions per nucleotide position. Accession numbers from GenBank are in parentheses.

which *meta* cleavage of catechol for phenol metabolism was found to be predominant in nature. Our results also reveal that phenol was mainly degraded by strain SKDP-1 through *meta* pathway and was consistent with the report by Dong et al. (2008).

The microorganism isolated from oil contaminated site used phenol as sole carbon source. This was identified and characterized as strain SKDP-1 in our study. These mesophilic bacteria showed optimal growth at 30°C and at pH of 7.0. According to Bergey's manual, strain SKDP-1 seemed to be one member of *Pseudomonas* genus with its morphological and biochemical properties.

Further identification of phylogenetic position (Figure 4) indicated that strain SKDP-1 was most related to *Pseudomonas putida* (AB680847) with a high similarity of 98%, therefore strain SKDP-1 was certainly one member of *Pseudomonas* genus. Further characterization including DNA-DNA homology assay, G+C contents determination and fat acids composition tests will be needed for strain SKDP-1(AB773822). Additionally, an on-site determination of phenol-degrading capability for strain SKDP-1 (AB773822) in the oil contaminated site will also be needed for obtaining the more exact reflection of its phenol degrading properties.

The utilization of biological systems on bioremediation is more cost-effective than traditional cleaning techniques such as waste incineration; possible savings have been estimated to 65-85%. This work could provide a useful guideline in evaluating potential phenol microorganisms living at the polluted environments and a possibility for using the bacterium to decontaminate phenolic and hydrocarbon wastes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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