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Isolation, Identification and Optimum Conditions of *Pseudomonas* sp. DHS3Y Capable of Efficient Phenol Degradation

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Degradation of increasing phenol and phenolic wastes by some bacteria has become more important these days. 26 bacteria from activated sludge in an insulating material plant of China were isolated to investigate phenol degrading activity in minimal salt medium (MSM) containing 0.5 g/L phenol. 6 isolates showed high phenol activity after 6 cycles each consisting of 120 hours' incubation. The isolate with the highest phenol degrading activity was subsequently identified as *Pseudomonas* sp. DHS3Y based on morphological, physiological, biochemical and 16S rRNA molecular phylogeny. A PCR product encoding the phenol hydroxylase gene was successfully obtained from Strain DHS3Y. The optimum conditions for achieving high phenol degradation were pH 7.5, 2.0 % (w/v) NaCl and temperature of 32°C. *Pseudomonas* sp. DHS3Y was found to degrade phenol of up to 0.9 g/L concentrations under the optimal conditions. The isolation of *Pseudomonas* sp. DHS3Y is a potential alternative for the bioremediation of phenol contaminated environment.

Kay words: Phenol, *Pseudomonas* sp. DHS3Y, Phenol degrading activity, Bioremediation.

Now phenolic contaminants and wastewater are produced by many industries in the manufacturing of products like insulation materials, resins plastics, rubber, lacquers, paint, dyes, disinfectants, soaps, toys and so on. These have been reported as highly hazardous and toxic to living organisms. Due to their potential toxicity and persistence to the environment, rapid removal and detoxification is urgently needed. Many methods have been developed to treat phenols in wastewater including biodegradation[1,2], extraction by liquid membrane[3], chemical oxidation[4], adsorption[5, 6], and membrane separation [7]. The physico-chemical methods have their own limitations due to high energy consumption, insufficient capacity, reaction inefficiency and production of sludge containing iron[8]. However, biodegradation as a technology for decontaminating phenols is gaining attention due to its eco-friendly and low cost-effective characteristics. Future technologies of bioremediation, microbial systems might be the potential tools to deal with the environmental pollutants [9].

For feasible biodegradation of phenol, screening microorganisms capable of degrading phenols needs to be done firstly. Microbial uptake and mineralization of phenol has been studied extensively[10, 11]. The microorganisms that are used in phenol degradation include *Serratia plymuthica*, *Azotobacter* sp, *Pseudomonas* sp, *Candida tropicalis*, *Alcaligenes* sp, *Brevibacillus*, *Acinetobacter* sp. etc[12-14]. The biological

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degradation is accomplished through benzene ring cleavage mediated by intracellular enzymatic reaction[15].

Although there are numerous reports on bacteria with low phenol degrading activity, those describing high phenol degrading activity are generally lacking. In this study, we reported a bacterial strain that is able to degrade high concentrations of phenol. This bacterial strain was subsequently identified as *Pseudomonas* sp. DHS3Y and was able to degrade phenol up to 0.9 g/L. The findings enable the utilization of this strain in the bioremediation of phenol and phenolic wastes in industrial effluents.

MATERIALS AND METHODS

Screening, isolation and purification of phenol degrading bacteria

The activated sludge sample was collected from an insulating material plant of China. The activated sludge sample (1 g) was suspended in 250 mL of minimal salt medium (MSM) containing Na_2HPO_4 (0.5 g), KH_2PO_4 (0.5 g), NaCl (0.5 g), NH_4Cl (1 g), $CaCl_2 \cdot 2H_2O$ (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $MnSO_4$ (10 mg) and $FeSO_4$ (10 mg) dissolved in 1000 mL double distilled water. 0.5 g/L of phenol was used as sole source of carbon and then incubated in 250 mL flask in the 30 °C on rotary shaking incubator with speed of 150 rpm for 120 h[16]. Bacterial cultures were isolated by repeated culturing in a 250 mL conical flask within MSM medium and supplemented with filter-sterilized phenol (0.5 g/L) as a carbon source[17].

A volume of 5 ml of enriched media was transferred into freshly prepared MSM media (100 mL) containing 0.5 g/L of phenol and incubated for 120 h at 30 °C with constant shaking (150 rpm). 5 mL of the first culture solution was transferred into 100 mL fresh solution and incubated for 120 h under the same condition. 5 mL of the second culture solution was transferred into 100 mL of inorganic culture solution containing 0.6 g/L of phenol and incubated for another 120 h. Phenol concentrations are 0.8 g/L, 0.9 g/L, 1.0 g/L, 1.1 g/L, 1.3 g/L, respectively. The purpose of several transfers to fresh culture solutions is to dilute and reduce possible carbon sources from the activated sludge sample and at the same time, domesticate phenol degradation bacteria tolerance. The final culture solution was inoculated onto agar-solidified MSM containing 1.3 g/L of phenol. After 120 h incubation at 30 °C, a single colony was selected, suspended in autoclaved ddH_2O, inoculated onto fresh agar-solidified plates and incubated for another 120 h at 30 °C. This step was repeated for once or twice. Plates with separated single colonies were stored at 4 °C for further use.

Morphological, physiological and biochemical characteristics

Colony morphology of the bacterium was determined by cultivating the isolate on minimal salt agar medium supplemented with 1.0 g/L phenol. The colony appearance, bacterium configuration and microscopic structure were observed by naked-eye and microscope, respectively. Some important physiological and biochemical methods were used to analyze Strain DHS3Y, the analysis included gram staining, aerobic test, catalase activity, oxidation zymolysis of glucose, amylase and gelatinase production, citrate utilization, indole test, etc. All tests were done in duplicates. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates[18].

Amplification and phylogeny analysis of 16S rRNA gene

Whole genomic DNA was extracted from bacterial isolate DHS3Y using the protocol of Genomic DNA Extraction Kit (Tiangen, China). Total DNA was diluted to approximate 20 ng/µL for polymerase chain reaction (PCR). PCR amplification was performed using a S1000TM thermoal cycler. The PCR mixture contained 10 × *Ex Taq* buffer 5 µL, dNTP mixture 4 µL, $MgCl_2$ 4 µL, 20 pmol/L forward and Reverse primers 1.75 µ L, E*x Taq* DNA polymerase (TaKaRa, Dalian) 0.25 µL, template DNA 1 µL and ddH_2O was added to a total of 50 µL. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following forward and reverse primers of 16S rDNA, respectively; p27f: 5'-AGAGTTTG ATCCTG GCTCAG-3' and p1492r: 5'-GGCTACCT TGTTACGACTT-3'. PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 94 °C for 30 sec, 55 °C for 50 sec, and 2 steps of 35 cycles of 72 °C for 80 sec and a final extension at 72 °C for 10 min. The PCR products were used for sequencing after verified by agarose gel electrophoresis. All reagents,

primers and sequencing services are provided by Takara Co. (Dalian, China). The sequences were compared with the database in GenBank at http://www.ncbi.nlm.nih.gov/BLAST/.

The phylogenetic position of the phenol degrading bacteria isolated in this study was determined by sequencing analysis of PCR-amplified bacterial small subunit (16S) rRNA gene. The nucleotide sequence from the gene was aligned using CLUSTAL W, version 1.6. A multiple alignment of 12 16S rRNA gene sequences that closely matches isolate DHS3Y were retrieved from the GenBank. We then used maximum likelihood (ML) in MEGA 5.0 to construct the phylogeny of DHS3Y together with the other 12 16S rRNA gene sequences[19]. The confidence level at each branch was evaluated by performing bootstrapping with 1000 replicates in ML analysis[20]. A distance matrix for each replicate was calculated with the Kimura 2-parameter.

Cloning and sequencing of phenol hydroxylase gene

PCR was performed to amplify the phenol hydroxylase structural gene. The sequences of the primers were designed on the basis of *Pseudomonas* sp. Strain CF600 (dmpN)[21], *Acinetobacter calcoaceticus* NCIB8250 (mopN)[22], *Pseudomonas putida* strain H (mopN)[23], *Ralstonia eutropha* E2 (poxD)[24], P1: 5'-AGGCATCAAGATCACCGACTG-3' and P2: 5'-CGCCAGAAC CATTTATC GATC-3'. 50 µL of PCR volume consisted of 5 µL of 10 × PCR buffer, 4 µL of 2.5 mM each dNTP, 1 µL of 20 mM P1 and P2, 0.5 µL of 5U *Ex* Taq polymerase, 1 µL of total DNA and adding ddH_2O making the volume up to 50 µL. The PCR was conducted at 94 °C predenaturing for 5 min and the 35 cycles (94 °C denaturing 30 sec, 58 °C annealing 35 sec and 72 °C extending 75 sec), finally 72 °C extending 7 min again.

Phenol degradation assay

Growth rate of the isolates was routinely assessed indirectly through turbidity measurement at 600 nm. In all the experiments, 30 mL bacterial isolate was inoculated onto 250 mL MSM containing 0.6 g/L phenol and incubated in a shaker incubator at 180 rpm at 32°Cfor over 120 h. The temperature, pH, phenol and NaCl concentrations were varied accordingly in the optimization experiments; range of temperature 15-45, 4.5 - 9.0 pH range in MSM, salinity range set at 0 - 50 g/L of NaCl and with a wide range of phenol concentration (0.3-1.5 g/L), respectively. Values shown in the optimization experiments are mean ± SEM, n=3.

Analytical procedures

Growth of the organisms was recorded by monitoring the optical density (OD) of the culture with an Ultrospec 3300pro spectrophotometer at 600 nm. A Sartorius PP-15E pH meter was used for pH determination. Phenol degrading activity was estimated with the colorimetric assay method for phenol using 4-aminoantipyrene as the reagent[25].

RESULTS

Isolation and identification of Phenol Degrading Bacteria

A bacterium capable of degrading phenol was isolated from the activated sludge sample of an insulating material plant in China. 26 bacterial isolates were found to exhibit phenol degrading activity. It is the most potential source to isolate high performance phenol degrading microorganisms. 6 isolates that were able to efficiently utilize phenol as sole source of carbon were obtained from the enriched population grown in MSM medium, supplemented with phenol. However, only 1 strain capable of utilizing phenol completely at 0.9 g/L within 96 h was characterized using morphological and biochemical properties listed in Table 1. By comparing these characteristics with those mentioned in Bergey's manual of systematic bacteriology, the bacterium was identified as *Pseudomonas*. It is Gram negative, motile, rod, showing growth under aerobic conditions with the optimum temperature of 32 °C. Colonies of *Pseudomonas* on minimal salt agar with phenol appeared yellow color and on nutrient agar colonies were white, round and translucent.

16S rDNA gene phylogeny of DHS3Y

16S rRNA sequences analysis was a fast and accurate method to identify DHS3Y phylogeny position. Part-length (1498 bp) 16S rRNA genes were sequenced (GenBank ID EU545155). We found that Strain DHS3Y was classified in the *Pseudomonas* genera, the similarities between DHS3Y and *Pseudomonas* sp. KHg3 (FJ379321), *Pseudomonas* sp. KI (AJ278107) and *Pseudomonas* sp. JN18_A17_R (DQ168645) were 99.0 %, 98.4 % and 97.7 %, respectively. Combined with the

physiological and biochemical characteristic results, DHS3Y was identified to be *Pseudomonas* sp. (Fig. 1).

Detection of phenol hydroxylase gene in DHS3Y

A PCR product encoding the phenol hydroxylase gene from DHS3Y was successfully obtained with the expected size and cloned into the pMD-19T vector. A recombinant plasmid containing a 684 bp was transformed into *E. coli* DH5α for sequencing. Part-length (about 684 bp) phenol hydroxylase gene was obtained and the results were shown in Fig. 2 (GenBank ID GQ281096).

Phenol biodegradation characteristics

The isolate DHS3Y showed the highest phenol degrading activity. The bacterial isolates were then inoculated onto MSM containing different phenol concentrations and incubated for 120 h. Exposure to increasing phenol concentrations were used to determine the tolerance of phenol. Acclimatization of the microorganisms overcome the substrate inhibition problems that commonly occurred in the biodegradation especially with high concentrations of phenol[26]. The experiment aimed to find the

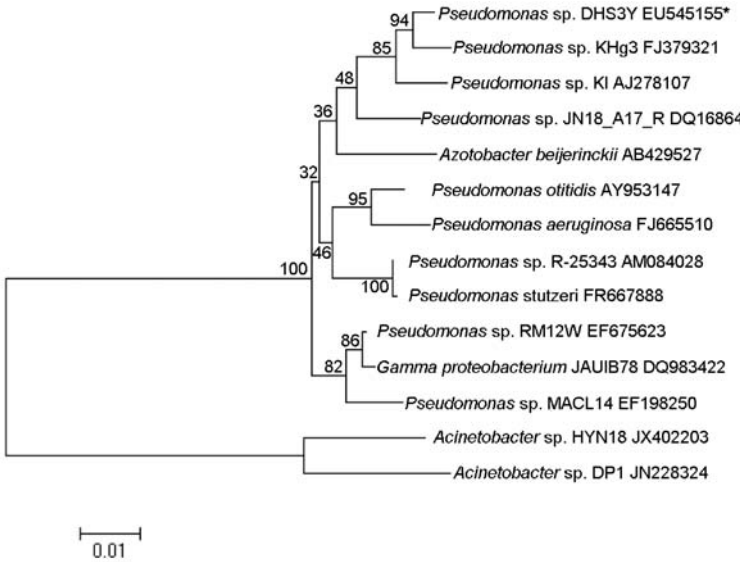

Fig. 1. The phylogenetic tree of Strain DHS3Y and other related reference microorganisms based on 16S rRNA gene sequence. Note: the branch nodes are bootstrap value, and "*" means this study.

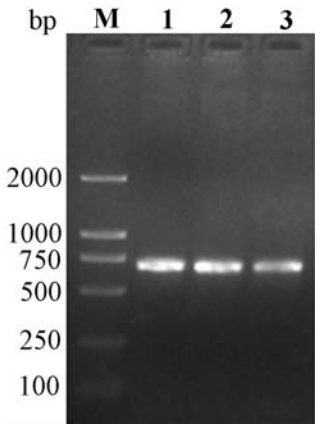

Fig. 2. PCR identification of the phenol hydroxylase. M, DL2000bp Marker ; Lane 1-3, phenol hydroxylase gene.

highest tolerance of phenol. It has found that isolate DHS3Y exhibited phenol degrading activity at 1.0 g/L and it was able to tolerate phenol as high as 1.3 g/L.

In order to obtain the growth and phenol degrading activity of *Pseudomonas* sp. DHS3Y in different phenol concentrations, Strain DHS3Y was inoculated into media containing 0.3 - 1.5 g/L phenol and phenol degrading activity monitored for 120 h (Fig. 3). The bacteria showed different phenol degrading activities and growth with different concentrations of phenol. Phenol degrading activity was higher at lower phenol concentrations but gradually decreased as phenol concentration increased. Phenol degrading activity was observed to taper off to a minimum of 1.3 g/L

phenol and almost no degrading activity above this concentration. Maximum growth was achieved within 60 h of incubation with low phenol concentration of 0.7 g/L, or 96 h of incubation with 0.9 g/L phenol, while a distinct retardation of growth was observed at phenol concentrations higher than 1.1 g/L.

Strain DHS3Y showed high growth rates at pH 6.0 - 8.0. Growth rate was dramatically reduced at pH less than 6.0 and above pH 8.0. Phenol degrading activity showed a similar trend with the optimal phenol degrading activity in the range of pH 6.0 - 8.0. Phenol degrading rate was highest at pH 7.5 (Fig. 4). The effect of temperature on the growth of Strain DHS3Y in 0.9 g/L phenol was studied at temperatures ranging from 15 to 45 °C. The growth of Strain DHS3Y is minimal at temperatures below 20 °C, increased gradually to a maximum at 25 - 30 °C and then decreased gradually until 40 °C after which there is a drastic drop in growth (Fig. 5). Phenol degrading activity was minimal at temperatures below 25 °C, high with temperatures between 25 - 35 °C after which phenol degrading activity dropped drastically and Strain DHS3Y could not grow well. Phenol degrading rate was highest at 32 °C. The effect of salinity on the growth of Strain DHS3Y within 0.9 g/L phenol was studied at NaCl concentrations ranging from 0 to 50 g/L (Fig. 6). The results show that Strain DHS3Y recorded the highest growth with 20 g/L NaCl. Phenol degrading activity was also highest with 20 g/L NaCl. Phenol degrading rate was obviously reduced with concentrations of NaCl less than 0.5 g/L and above 30 g/L.

DISCUSSION

Bacteria collected from several phenol contaminated sites were reported to exhibit phenol degrading activity. Examples are *Acinetobacter* sp. Strain W-17[1, 27], *Bacillus thermoleovorans*[28], *Burkholderia* sp.[29], *Pseudomonas putida*[30], *Pseudomonas resinovorans*[12], *Alcaligenes faecalis*[17] and *Brevibacillus* sp[12]. *Pseudomonas* sp. DHS3Y was isolated from a contaminated location and had no exception to show high phenol degrading activity.

Pseudomonas sp. DHS3Y is able to degrade phenol up to 0.9 g/L. This is considerably higher than those reported by other groups; *Bacillus stearothermophilus* FDTP-3 degrading up to 0.5 g/L phenol[31], *Acinetobacter* sp. strain PD12 at 0.5 g/L[2], *Xanthobacter flavus* at 0.6 g/L[16], *Pseudomonas putida* Strain DSM 548 at 0.1 g/L[30],

Table 1. Morphological, physiological and biochemical characteristics of isolate *Pseudomonas* sp. DHS3Y

Characteristics	*Pseudomonas* sp. DHS3Y		Characteristics	*Pseudomonas* sp. DHS3Y	
Colony	White slimy		Amylohydrolysis	-	
Colony Shape	Round		Indole Production	-	
Shape (Bacterial cell)	Slightly curved rod		Methyl red test	-	
Colony diameter	0.2~0.3mm		Vogus Proskaur Test	-	
Colony texture	Moist		Citrate Utilization	+	
Endospore	-		Urease	-	
Gram stain	-		esterase	+	
Oxygen Requirement	+		Cellulose hydrolysis	W	
Motility	+		Casein hydrolysis	-	
H_2S Production	-		Gelatin hydrolysis	-	
Catalase	+				
Fermentation Test	Acid production	Aerogenesis	Fermentation Test	Acid production	Aerogenesis
Maltose	+	-	Galactose	-	-
Glucose	+	-	Arabinose	+	+
Lactose	-	-	Sorbic alcohol	-	-
Sucrose	-	-	Rhamnose	+	-
Fructose	+	W	Mannose	+	-

+: Positive, -: Negative, W: Weak response

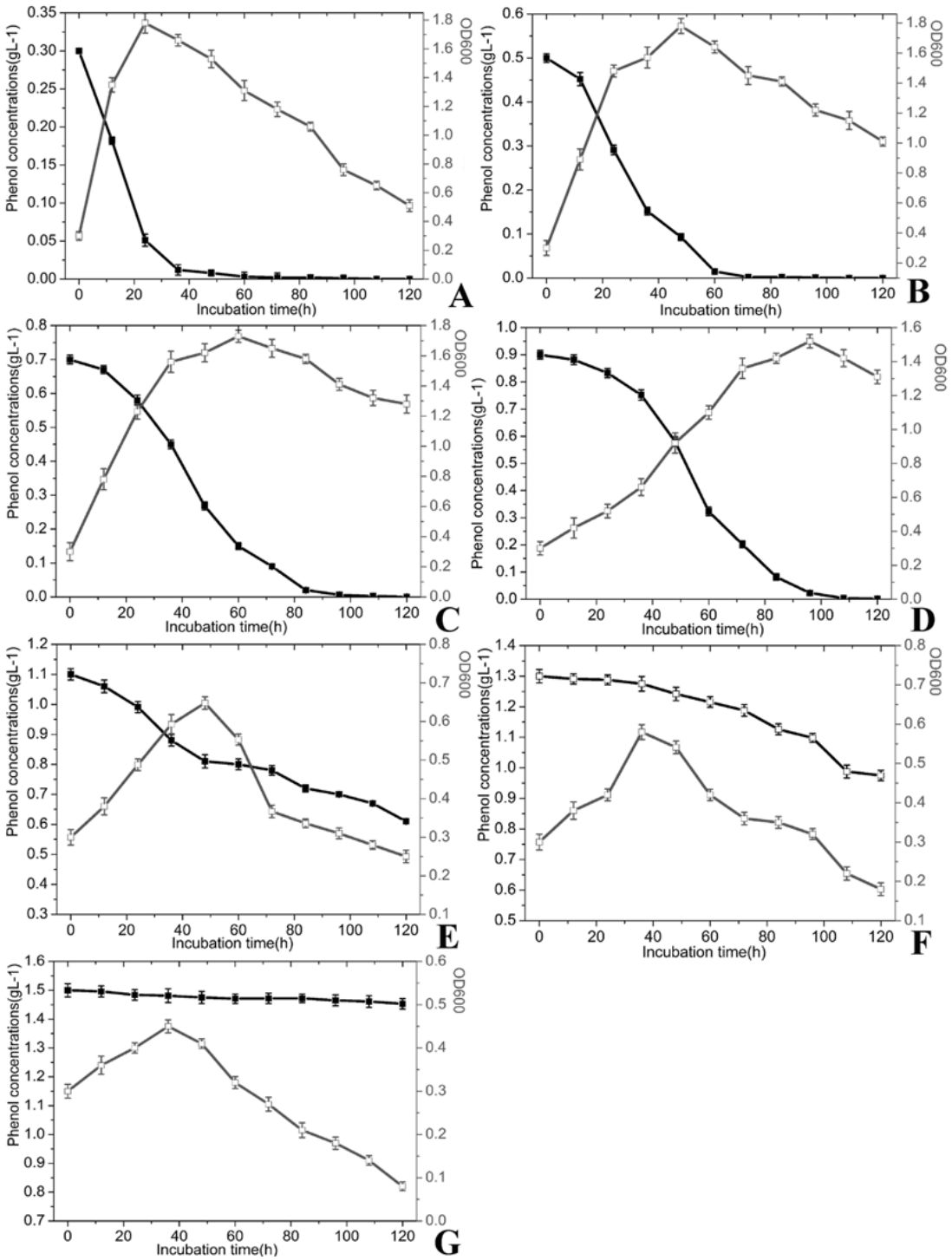

Fig. 3. Growth and phenol degrading activity of *Pseudomonas* sp. DHS3Y in different concentrations of phenol. A, 0.3 g L-1; B, 0.5 g L-1; C, 0.7 g L-1; D, 0.9 g L-1; E, 1.1 g L-1; F, 1.3 g L-1; G, 1.5 g L-1; □, Bacterial growth; ■, phenol degrading activity. Values shown are mean ± SEM, n=3.

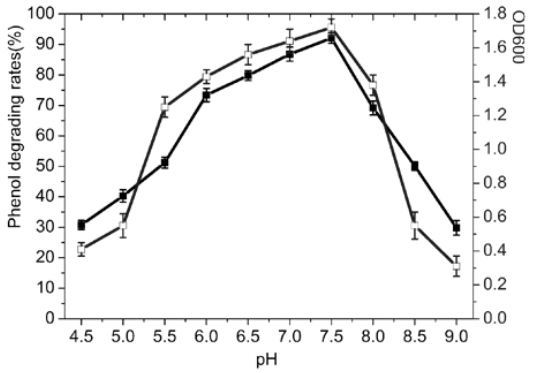

Fig. 4. The effect of pH on growth and phenol degrading activity of *Pseudomonas* sp. DHS3Y.
□, Bacterial growth; ■, phenol degrading activity. Values shown are mean ± SEM, n=3.

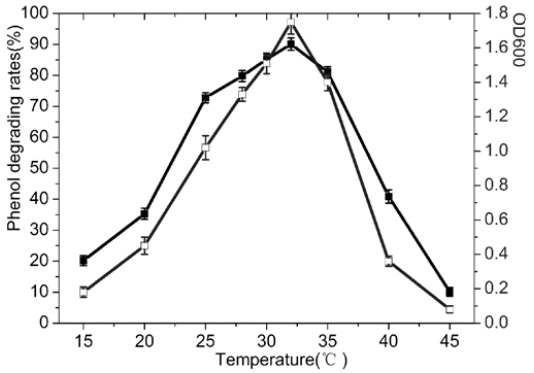

Fig. 5. The effect of temperature on growth and phenol degrading activity of *Pseudomonas* sp. DHS3Y.
□, Bacterial growth; ■, phenol degrading activity. Values shown are mean ± SEM, n=3.

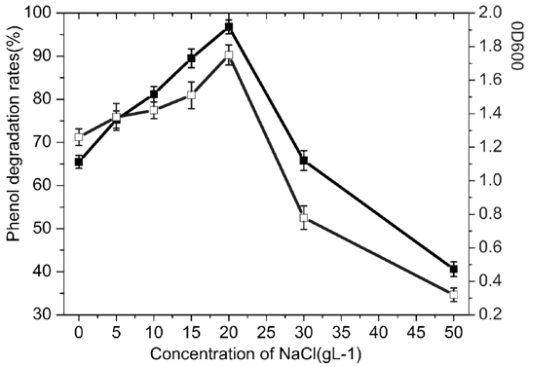

Fig. 6. Phenol degrading activity and growth of *Pseudomonas* sp. DHS3Y in different concentrations of sodium chloride.
□, Bacterial growth; ■, phenol degrading activity. Values shown are mean ± SEM, n=3.

F1 ATCC 17484 and Q5 at 0.2 g/L[32, 33], CCRC14365 at 0.6 g/L[34]. The optimal pH ranges of between 6.0 - 8.0 of Strain DHS3Y is similar to other phenol degrading bacteria which has alrealy reported, *Candida tropicalis* at pH 6.5[35], *Acinetobacter* sp. strain RD12 at pH 7.2[2], *Alcaligenes faecalis* at pH 7.2[17], *Pseudomonas putida* at pH 8.0 and an indigenous mixed microbial consortium at pH 7.0[34, 36]. Information on the pH optimum for growth and phenol degrading activity would help in designing an effective bioremediation strategy. Additionally, temperature has certain effect on strain growth and phenol degradation. Several other groups had also reported that bacterial strains had optimum growth and phenol degrading activity in the range of temperatures between 25 - 37 °C[16, 17, 34, 36]. The long period of high or low temperature could inhibit the strain growth and then influence phenol degradation efficiency.

In 2007, Bai et al had reported that phenol-degrading *Alcaligenes faecalis* has the optimum NaCl concentration of 0.1 g/L while *Alcaligenes faecalis* isolated from Amazonian rain forest exhibited optimum growth with 56 g/L NaCl[17, 37]. Eduardo et al demonstrated a bacterium *Alcaligenes faecalis* and yeast *Candida tropica* could degrade the phenol and still had a high salt concentration at 150 g/L[38]. In contrast, *Pseudomonas putida* has the lowest optimum NaCl concentration at 0.015 g/L[34]. Detection of salinity range provides the potential advantages of bioremediation[39]. With the optimum salinity of 20 g/L shown by Strain DHS3Y, it is more suitable to apply DHS3Y in complicated contaminant conditions. Overall information on the optimized conditions for *Pseudomonas* sp. DHS3Y in growth and phenol degradation will be of great help in realizing its potential usage in the bioremediation of phenol.

Morphological, physiological and biochemical and 16S rRNA molecular phylogeny of strain DHS3Y were identified as *Pseudomonas* sp. Strain DH3Y could tolerate phenol up to 1.3 g/L. The results of purified bacteria culture in MSM with 5.0 - 1.3 g/L of phenol showed that Strain DH3Y can remove phenol up to 0.9 g/L completely after 96 hours incubation. The isolate with the highest phenol degrading activity was subsequently detected with specific primers. The phenol hydroxylase fragment (684bp) was amplified

by PCR technique (Fig. 2). Sequence alignment showed that amplified fragment (684bp) from Strain DHS3Y was part of phenol hydroxylase gene sequence (Data not shown). The phenol hydroxylase is responsible for converting phenol to catechol, which is the initial and rate-limiting step in phenol-degrading pathways[40]. Both single-component and multicomponent types of this enzyme have been identified and multicomponent enzymes are considered the major ones in the environment[40, 41]. It is clear that dominant phenol-degrading bacteria such as Strain DHS3Y can lead to the promotion of phenolic wastewater treatment. Therefore, expression of phenol hydroxylase genes in *E. coli* or other *Pseudomonas* strains is required for further applications in bioconversion reactions of phenolic compounds.

Several works are in progress to isolate new and efficient microbial strains, which are capable of degrading phenol and phenolic contaminants. We report here a bacterial strain DHS3Y, isolate as a potential selected strain to utilize phenol as sole source of carbon and energy. The degradation ability was checked up to 0.9 g/L. DHS3Y belonged to the *Pseudomonas* genus and was closely related to *Pseudomonas* sp. KHg3 according to physiological, biochemical characteristics and 16S rRNA gene analysis. One phenol hydroxylase gene was found in its genomic DNA. The phenol degradation rate was 96.8 % with an initial concentration of 0.9 g/L, pH 7.5, 32 °C within 96 h. This paper provides a useful guideline in evaluating potential phenol biodegradations isolated from environment.

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