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Full Length Research Paper

Isolation and characterization of naphthalene-degrading strains, *Pseudomonas* sp. CZ2 and CZ5

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Bacterial strains CZ2 and CZ5, isolated from polycyclic aromatic hydrocarbons contaminated sludge in Wuhan, China, can utilize naphthalene as a sole source of carbon. They were both identified as *Pseudomonas* based on Gram staining and 16S rRNA gene sequence analysis. *Pseudomonas* sp. CZ2 and CZ5 can remove 61.8 and 64.3% of naphthalene with an initial concentration of 1 g/L in 48 h, respectively. Addition of glucose as second carbon source, had different effects on strains CZ2 and CZ5 growth. Both strains harbored the same naphthalene dioxygenase genes, and the salicylate route was likely the metabolic pathway for naphthalene degradation. PCR analysis of catechol dioxygenase genes showed both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in strain CZ2, and only catechol 2,3-dioxygenase in strain CZ5. Therefore, strains CZ2 and CZ5 have different metabolic pathways for cleavage of the aromatic ring. Such strains may be useful for bioremediation of PAH-contaminated sites.

Key words: *Pseudomonas* sp., naphthalene, biodegradation, dioxygenase, metabolic pathways.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) consist of fused benzene rings and/or pentacyclic molecules in linear, clustered or angular arrangements (Kotterman and Vis, 1998). PAHs are major components of fossil fuels and widely used as solvents in pesticides, paints and many other industrial products. They can be formed as a result of the incomplete combustion of organic material, and are thus ubiquitous pollutants found in soil, oil contaminated sediments, and both industrial and urban wastewater (Nigam et al., 1998). PAHs have been shown to be highly toxic, carcinogenic and mutagenic, so they present great environmental concerns (Sato and Aoki, 2002). The US Environmental Protection Agency (US-EPA) has designated PAHs as priority pollutants in ecosystems since the 1970s (Mazzera et al., 1999).

Nowadays, different treatment methods are available for the degradation of PAHs, and compared with physico-chemical methods, the biodegradation method has been considered as the most efficient and prevalent way for PAHs contamination due to the lower costs and possibility of complete mineralization (Tay et al., 2005). Bioremediation using various microorganisms is the most efficient and prevalent approach used for the removal of PAH from the environment. In the last three decades, research on the microbial degradation of PAHs has resulted in the isolation of numerous strains of bacteria, and knowledge of bacterial bioremediation has continued to improve (Byss et al., 2008). A number of microorganisms have been reported to be able to degrade PAHs that include members of the species of *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Bacillus* (Peng et al., 2008), *Berjerinckia*, *Burkholderia*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium* (Boldrin et al., 1993), *Pseudomonas* (Jacques et al., 2005; Cao et al., 2008), *Lutibacterium*, *Rhodococcus* (Djefal-Kerrar et al., 2007; Bamforth and Singleton, 2005), *Streptomyces*, *Sphingomonas* (Pinyakong et al., 2003), *Stenotrophomonas*, *Tistrella* (Zhao et al., 2009) and *Vibrio*. Some bacteria from rhizo-

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bia were also reported to utilize PAHs (Loritc et al., 1998). Moreover, some pseudomonad strains are able to biodegrade four-ringed PAHs (pyrene) (McNally et al., 1998). The oxidative pathway of the naphthalene in aerobic bacteria involves the salicylic acid molecule as intermediate which is converted in catechol (Gennaro et al., 2001; Silva et al., 2006), and catechol is further oxidized by catechol 2, 3-dioxygenase or catechol 1,2-dioxygenase to β-ketoadipate (Habe and Omori, 2003), which is then further converted, with two additional steps, to Krebs cycle intermediates.

This paper reports on the isolation of two strains CZ2 and CZ5 with the capability to grow on naphthalene used as a model PAH. We studied naphthalene degradation ability of strains CZ2 and CZ5. Phylogenetic analysis based on 16S rRNA gene was used to reveal genetic relationship of the isolates with other *Puesdomonas* strains. Besides, catabolic pathways were proposed based on metabolites and PCR analysis of dioxygenase genes.

MATERIALS AND METHODS

Media and growth conditions

The minimal medium (MM) was composed of: KH_2PO_4 1 g/L, Na_2HPO_4 1.3 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4 \cdot 7H_2O$ 0.05 g/L, $CaCl_2$ 0.02 g/L, $ZnSO_4 \cdot 7H_2O$ 5 mg/L, $MnCl_2 \cdot 4H_2O$ 5 mg/L, $NaMoO_4 \cdot 2H_2O$ 1 mg/L, $CuCl_2$ 0.5 mg/L. Naphthalene was used as a sole carbon and energy sources. Luria-Bertani (LB) medium was composed of: NaCl 10 g/L, peptone 10 g/L, yeast extract 5 g/L. The medium used for measurement of the antibiotic susceptibility was LB agar medium containing antibiotics in varying concentrations ranging from 10 to 100 mg/L. The plates with microorganisms were checked for growth after 2 days incubation at 37°C (Tao et al., 2007). The following antibiotics were used: Ampicillin, streptomycin, kanamycin, tetracycline, chloromycetin, spectinomycin, and gentamycin.

Isolation and identification of naphthalene-degrading bacteria

The sludge samples were collected from the main pollutant-emission outlets of a chemical plant in Wuhan China. Bacterial strains were isolated by enrichment culture techniques using 5 g of wet sludge as inoculum in 50 ml MM medium containing 500 mg/L naphthalene. Flasks were incubated at 37°C for 4 days. After growth, 5-ml aliquot was transferred to 50 ml of a fresh MM containing the same naphthalene as above and incubated under the same conditions. After four consecutive enrichments, the culture suspension was serially diluted and plated onto plates containing MM mineral medium and naphthalene at the same concentration (Zhao et al., 2008). After incubation at 37°C for 4 days, colonies were selected as the candidate naphthalene-degrading strains. These isolates were further exposed successively to higher concentrations (up to 500 mg/L) of naphthalene. The isolates capable of forming clear zones in MM medium containing naphthalene (1 g/L) at 37°C were obtained and purified.

The chromosomal DNA was isolated by using a method described by Cheng et al. (2006). *Pseudomonas sp.* CZ2 and CZ5 were grown aerobically in LB broth at 37°C. The cells were harvested by centrifugation, lysed by lysozyme and extracted with phenol. Amplification of the 16S rRNA gene was performed by PCR using the following primers of 16S-L (5'AAGGAGGTGATCCAGCC3'), positions 7 to 26 in the *Escherichia coli* 16S rRNA gene, and 16S-R (5'AGAGTTTGATCCTGGCTCAG3'), positions 1541 to 1525. The thermocycling conditions were as follows: 30 cycles each consisting of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, followed by 10 minutes at 72°C and an infinity hold at 4°C. PCR products were obtained and cloned into plasmid vector pMD-18 T (Tarkara, DaLian, China) and then sequenced using an ABI3730 Automated Sequencer by Sunbiotech Company (Beijing, China) (Cheng et al., 2006). Closed relative sequences of 16S rRNA genes of naphthalene-degradation bacteria in gene bank data base were obtained by running BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic relationship tree based on 16S rRNA genes was constructed using the minimum-evolution method of Mega 4.1 software after multiple alignments of data using CLUSTALW multiple sequence alignments (Tamura et al., 2007). Bootstrap values at nodes indicate percentage calculated in 500 replicates.

Naphthalene-degradation assay

The influence of temperature, pH and concentrations on bacterial naphthalene degradation was assessed. For the effect of pH and temperature, the MM medium with 1 g/L naphthalene as sole carbon source was used. The pH value of MM medium was adjusted to 5.0, 6.0 and 7.0, with predetermined amounts of diluted 1 mol/L HCl or 1 mol/L NaOH. Incubation temperature was varied at 28, 37 and 42°C at the optimal pH 6.0. The effect of initial naphthalene concentration on naphthalene degradation was investigated in the range of 100 to 2000 mg/L. Bacterial naphthalene degradation were determined by using the method reported by Riis et al. (1996). The inoculum used was 5% of the total volume. After 2 days of incubation, the naphthalene of the liquid cultures was extracted with cyclohexane and the absorbance measured at 275 nm, uninoculated medium was used as control. The effect of glucose on cell growth was tested at pH 6.0 and 37°C by adding 10 to 500 mg/L into MM medium containing 1 g/L naphthalene. After 2 days of incubation, bacterial cell density was routinely monitored by measuring optical density (OD) at 600 nm (Tao et al., 2007).

Growth on other aromatic compounds

The purified strains were also tested for growth on the following aromatic compounds: anthracene, resorcinol, 2-aminobenzoic acid, 4-nitrobenzoic acid, salicylic acid and phenol (Zhao et al., 2009). MM agar plates containing a given compound with specific concentration were inoculated with the tested strains. After 3 days of incubation at 37°C, the plates were checked for growth.

Amplification of catabolic genes

The *narA* gene encoding for the α and β subunits of the naphthalene dioxygenase was PCR amplified from the isolated DNA with the following primers of Nahaf (5'CACCTGATTCATGGCGATGAA3') and NahAr (5'ACCATCAGATTGTGCGTCTGA3'), the PCR fragment is approximately 1870 bases long (Alquati et al., 2005). PCR products were then cloned into pMD18-T vector and sequenced. The catechol 2,3-dioxygenase gene was amplified using the primers Cat2.3-f (5'TGATCGAGATGGACCGTGACG3') and Cat2.3-r (5'TCAGGTCAGCACGGTCATGAA3'), the PCR fragment is approximately 820 bases long (Alquati et al., 2005). The catechol 1,2-dioxygenase gene was amplified using the primers Cat 1.2-f (5'AAACCCGCGCTTCAAGCAGAT3') and Cat 1. 2-r (5'AAGTGGATCTGCGTGGTCAGG3'), the PCR fragment is approximately 650 bases long. All the positive PCR fragments were further confirmed by sequencing.

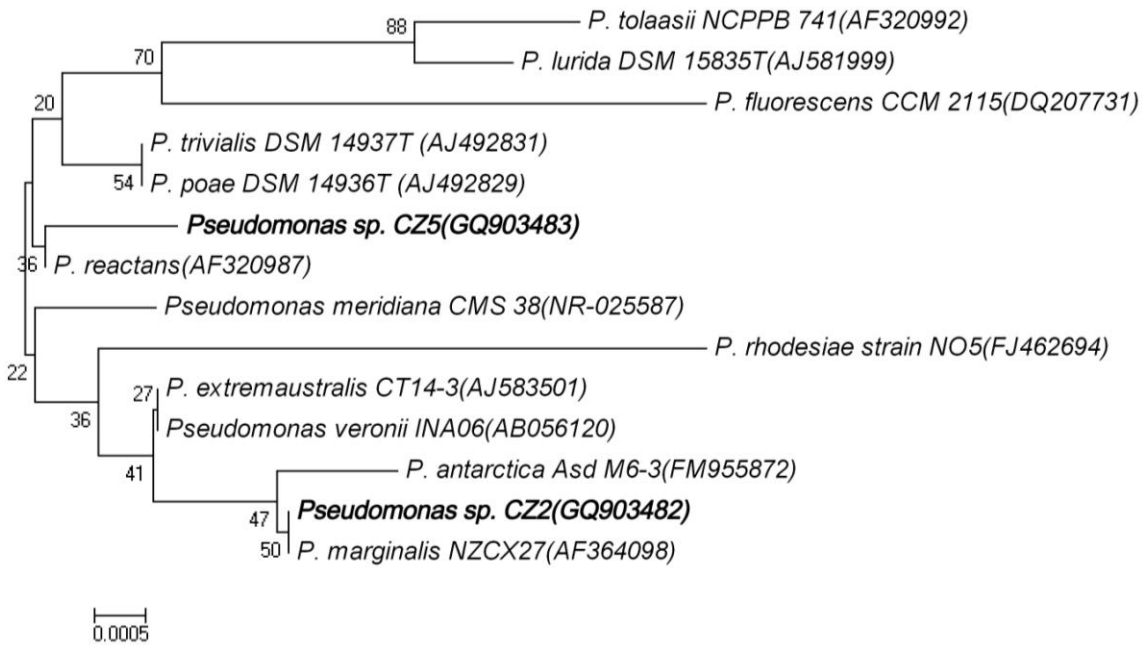

Figure 1. Phylogenetic tree derived from 16S rRNA gene sequences of the naphthalene-degrading stains and related *Pseudomonas* species. Bootstrap values at nodes indicate percentage calculated in 500 replicates. The scale bar corresponds to 0.0005 substitution per nucleotide position. Strains introduced in this study are in bold text.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database. The accession numbers of 16S rRNA genes from strains CZ2 and CZ5 are GQ903481 and GQ903480, respectively. The accession number of gene encoding naphthalene dioxygenases in strains CZ2 and CZ5 are GQ903482 and GQ903483, respectively.

RESULTS

Isolation and identification of naphthalene-degrading strains

With enrichment liquid cultures, two strains, CZ2 and CZ5 were isolated from PAHs contaminated sludge samples in the pollutant-emission outlets of a chemical treatment plant. Both strains were able to grow on MM medium with naphthalene at concentrations of up to 1 g/L as sole carbon source. Strains CZ2 and CZ5 were Gram-negative, motile, rod-shaped, non-sporulating and grew aerobically. Phylogenetic analyses of PCR amplified 16S rRNA gene sequences indicated that both CZ2 and CZ5 strains matched at 99% with the genus *Pseudomonas*.

The phylogenetic tree (Figure 1), comparing the 16S rRNA genes sequences of the naphthalene-degrading strains CZ2 and CZ5 with those of closest *Pseudomonas* species showed that the isolated strains fell into different clusters. Strain CZ2 having 98 to 99% similarity with *Pseudomonas extremaustralis*, *Pseudomonas veronii*, *Pseudomonas marginalis* and *Pseudomonas antarctica*, stood well apart from the cluster of *P. marginalis,* while strain CZ5 having 98 to 99% similarity with *Pseudomonas trivialis*, *Pseudomonas poae* and *Pseudomonas reactans*, stood well apart from the cluster of *P. reactans*.

Antibiotics susceptibility

The reaction to antibiotics was examined for the naphthalene-degrading stains. The results showed that strains CZ2 and CZ5 were both resistant to ampicillin, streptomycin, spectinomycin and intermediately resistant to tetracycline, but they were naturally susceptible to kanamycin and gentamycin. Strain CZ5 was found to be more resistant to ampicillin (15 mg/L) than strain CZ2 (10 mg/L).

Metabolic characterization of strains CZ2 and CZ5

In the pH and temperature range studied, the optimal conditions of naphthalene degradation were determined to be pH 6.0 and 37°C. With pH 6.0 and 37°C, *Pseudomonas* sp. CZ2 and CZ5 could remove 61.8 and 64.3% of naphthalene at initial concentration of 1 g/L, respectively. The naphthalene-degradation bacterial strains CZ2 and CZ5 were exposed to different naphthalene concentrations, and naphthalene degradation was evaluated. Both strains CZ2 and CZ5 can remove more than 60% of naphthalene at any concentration ranging from 250 mg/l to 1 g/L within 2 days. However, in the presence of 2 g/L of naphthalene, naphthalene-degradation activity of strains CZ2 and CZ5 was sharply reduced to 16.8 and 11.4%, respectively.

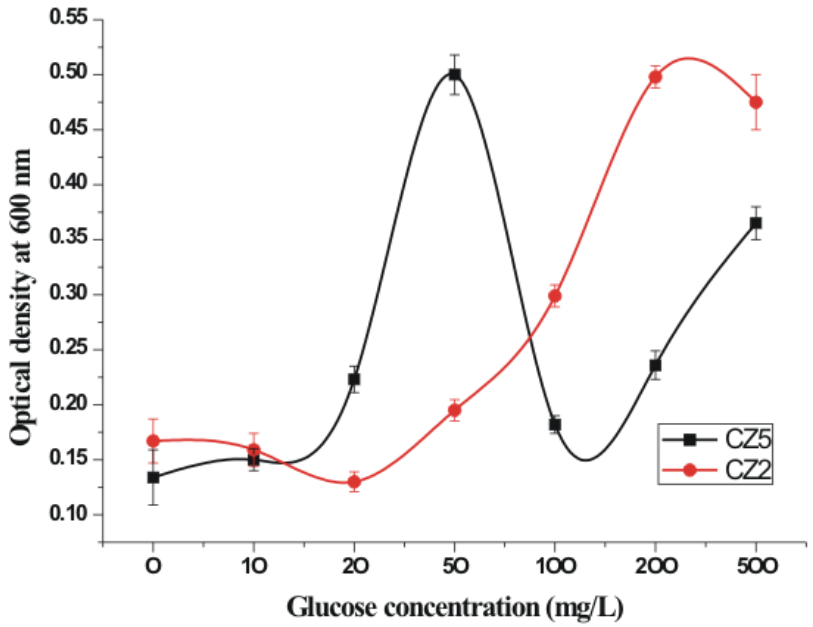

Figure 2. Effect of glucose concentration on cell growth of strains CZ2 and CZ5. Error bars are the single naphthalene degradation means±standard deviations from three independent assays.

Table 1. Growth of bacterial strains CZ2 and CZ5 on different aromatic compounds.

Substrate	Concentration (mg/L)					
	Pseudomonas sp. CZ2			*Pseudomonas* sp. CZ5		
	50	100	200	50	100	200
Anthracene	++	+	+	+	+	-
Naphthalene	++	++	++	++	++	++
2-Aminobenzoic acid	++	++	++	++	+	+
4-Nitrobenzoic acid	++	++	++	++	++	++
Resorcinol	-	-	-	+	+	-
Salicylic acid	++	++	++	++	++	++
Phenol	++	+	+	++	++	++

Annotation: (++) Well growth; (+) normal growth; (−) no growth.

The effects of extra carbon source on cell growth were also tested by adding glucose at 10 to 500 mg/L into naphthalene-containing MM. As shown in Figure 2, the growth of strain CZ5 was enhanced by adding glucose at concentrations of 10 to 50 mg/L, but inhibited at higher concentration of glucose (>50 mg/L). However, the cell growth of strain CZ2 was always enhanced by adding glucose at all tested concentrations.

Growth on various aromatic compounds

Bacterial strains CZ2 and CZ5 were tested for utilization of different aromatic compounds as sole substrate. As shown in Table 1, both strains CZ2 and CZ5 could utilize anthracene, naphthalene, 2-aminobenzoic acid, 4-nitrobenzoic acid, salicylic acid and phenol as the sole carbon source. Strain CZ5 could grow on 1,3-benzenediol, but no growth of strain CZ2 was observed with resorcinol even at the lowest concentration of 50 mg/L. In addition, both strains were able to utilize salicylic acid, which was considered as intermediate product of naphthalene degradation via salicylate route (Zhao et al., 2008).

Genotyping of dioxygenase

Pseudomonas sp. CZ2 and CZ5 were analysed for the naphthalene dioxygenase genes, key enzymes in naphthalene degradation. Almost complete open reading fra-

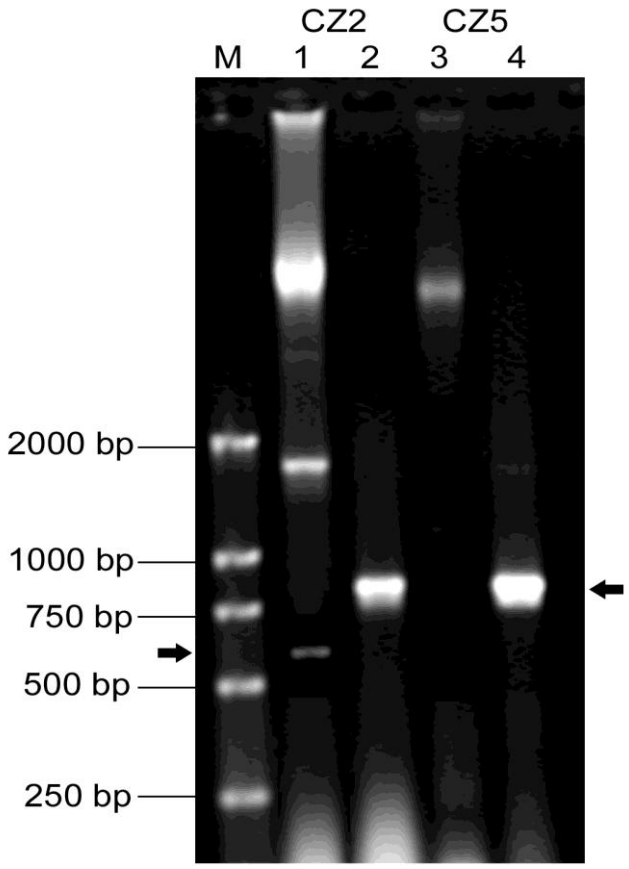

Figure 3. PCR amplification of catechol dioxygenase genes from *Pseudomonas* sp. CZ2 and CZ5. Lane M, DNA ; Lanes 1 and 3, catechol 1,2-dioxygenase gene, the rightwards arrow is showing the band at approximately 650 bp resulting from the amplification of catechol 1,2-dioxygenase gene; Lanes 2 and 4, catechol 2,3-dioxygenase gene, the leftwards arrow is showing the band at approximately 820 bp resulting from the amplification of catechol 2,3-dioxygenase gene.

mes of the naphthalene dioxygenase genes were amplified by PCR, using primers designed according to the known *nahAc* and *nahAd* sequences from *Pseudomonas putida*. Both strains had the same nucleotide sequences of naphthalene dioxygenase genes, which showed 100% identity with the *nahAc* and *nahAd* genes of *P. putida* KTSK2 and *Pseudomonas fluorescens* PC20.

From *Pseudomonas* sp. CZ2 and CZ5, PCR amplification reactions were also performed to detect genes coding for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase involved in naphthalene metabolism. All the PCR products were further sequenced and identified as specific to the dioxygenase genes. The amplified fragments of catechol 1,2-dioxygenase gene and catechol 2,3-dioxygenase gene were about 650 and 820 bp, respectively. As show in Figure 3, the catechol 2,3-dioxygenase gene was amplified in both strains, but the amplified fragment of catechol 1,2-dioxygenase gene was only found in *Pseudomonas* sp. CZ2.

DISCUSSION

In this study, we described the isolation and characterization of two naphthalene degradation strains. Both CZ2 and CZ5 strains belonged to the genera *Pseudomonas*, but were related to different species. Many strains from genus *Pseudomonas* which belong to *γ-Proteobacteria* have been known to be common naphthalene-degraders (Nievas et al., 2006). They were both resistant to ampicillin, streptomycin, spectinomycin and naturally susceptible to kanamycin and gentamycin. Antibiotics (such as penicillin, tetracycline, macrolides and cephalosporin) are widely used and distributed in the pollution environment, so the capacity to resist them may provide advantage of PAHs-degrading engineering technology.

The optimal conditions for naphthalene degradation were determined to be at pH 6.0 and 37°C. This result is not similar to the strain *Arthrobacter* sp. P1-1, which occurred at pH 7.0 and 24 to 30°C (Seo et al., 2006). Both strains were shown to be of a high efficiency in naphthalene degradation. *Pseudomonas* sp. CZ2 and CZ5 could rapidly degrade 61.8 and 64.3% of naphthalene at initial concentration of 1 g/L, respectively. But the degradation rate decreased sharply as the concentration of naphthalene increased to 2 g/L, which is similar to that reported by Pathak et al. (2009).

Significant differences were observed in growth by adding glucose into naphthalene-containing MM. The growth of strain CZ2 was always enhanced with glucose concentration in the range of 10 to 500 mg/L. This is consistent with results of Tian et al. (2003) who also reported that addition of glucose at concentrations of 10 to 100 mg/L could enhance the growth of strain *P. mendocina.* However, the growth of strain CZ5 was inhibited at higher concentration of glucose (>50 mg/L). Tao et al. (2007) also found such inhibitory effect, and suggested the addition of appropriate concentration of glucose when adding it as a co-metabolic carbon source to enhance the degradation of phenanthrene.

Pseudomonas sp. CZ2 and CZ5 were able to tolerate as high as 2 g/L of naphthalene, which is more than other strains of *Pseudomonas* sp. Which have been known to tolerate usually not more than 2 g/L. However, *Pseudomonas* sp. HOB1 from sediments of a polluted canal can tolerate very high level of naphthalene at the concentration of 60,000 ppm (Pathak et al., 2009). The ability to tolerate very high concentrations of naphthalene makes them potential isolates for the bioremediation of the soil massively contaminated with naphthalene.

Pseudomonas sp. CZ2 and CZ5 could use several aromatic compounds as the sole carbon source. It was reported that bacteria catabolize PAHs via either salicylate or phthalate pathways (Rodrigues et al., 2005; Iwabuchi and Harayama, 1997). The growth on salicylic acid suggests that the strains CZ2 and CZ5 degraded naphthalene via salicylate route. Both strains harbored the same naphthalene dioxygenase genes involved in the first steps of naphthalene degradation. The naphthalene

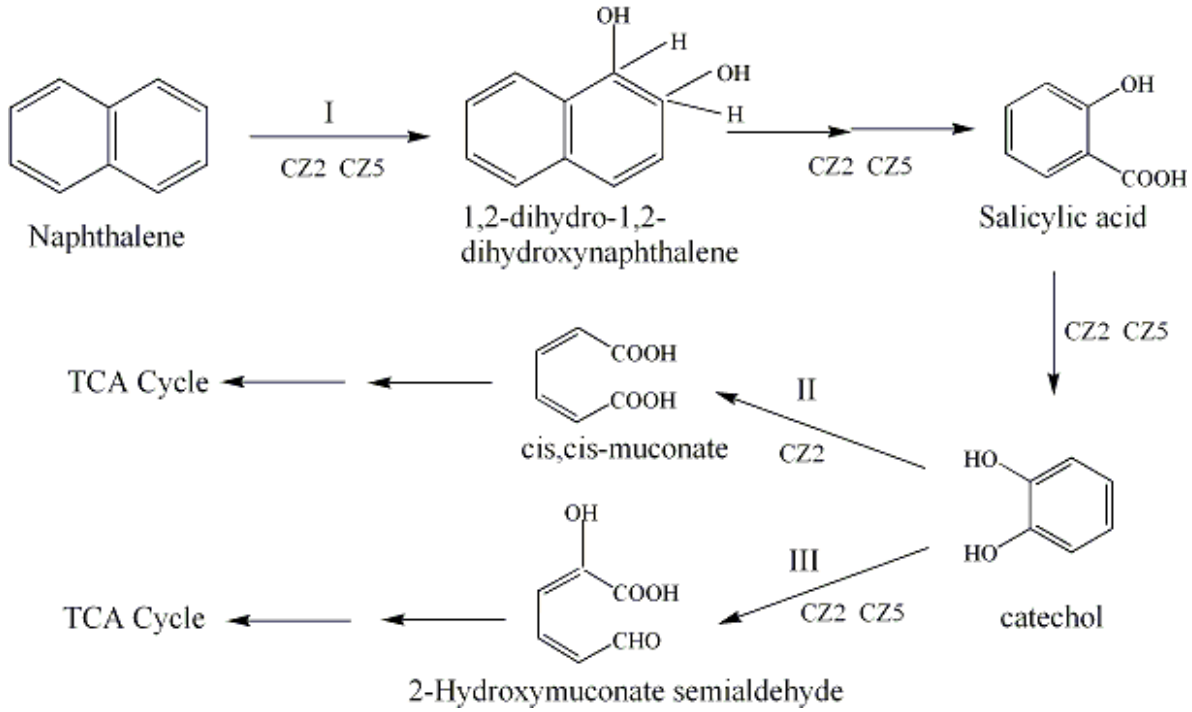

Figure 4. Proposed pathway for the degradation of naphthalene by strains *Pseudomonas* sp. CZ2 and CZ5. I, Naphthalene dioxygenase; II, catechol 1, 2-dioxygenase; III, catechol 2, 3-dioxygenase. CZ2, *Pseudomonas* sp. CZ2; CZ5, *Pseudomonas* sp. CZ5.

dioxygenase genes present in most *Pseudomonas* suggests that these genes originated from a common ancestor (Habe and Omori, 2003). PCR analysis of *Pseudomonas* CZ2 and CZ5 strains were performed to detect dioxygenase genes coding for catechol 2,3-dioxygenase and catechol 1,3-dioxygenase, with catabolize catechol via the α-ketoacid and β-ketoadipate pathways, respectively (Dunn and Gunsalus, 1973). Both strains carry the catechol 2,3-dioxygenase gene, while only strain CZ2 have the catechol 1,2-dioxygenase gene. These results suggested that both the α-ketoacid and β-ketoadipate pathways are present in strain CZ2, but only the α-ketoacid pathway is present in strain CZ5. Based on the above findings, the probable pathway of naphthalene degradation was proposed (Figure 4).

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