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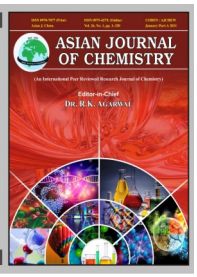

Isolation and Characterization of *Pseudomonas* sp. nai8 Capable of Naphthalene Degradation

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Using the base mineral medium containing 800 mg/L of naphthalene, a degrading bacteria with higher degradation activity was isolated and identified as *Pseudomonas* sp. based on physiological, biochemical characteristics and 16s rDNA sequence. The effects of *Pseudomonas* sp. Nai8's degrading ability, *e.g*., naphthalene concentration, temperature and pH, were investigated. The results showed that under the condition of 30 ºC, pH 7.0 and concentration of naphthalene of 1000 mg/L, the degradation rate in 48 and 96 h was 70.4 and 96.2 %, respectively. *Pseudomonas* sp. Nai8 could also degrade other aromatic hydrocarbons, such as dimethylbenzene, phenol, benzyl acid, utilizing each of them as sole carbon and energy source for its growth, which showed the strain Nai8 has good biodegradation diversity. The kinetics of naphthalene degradation by Nai8 was also calculated and the naphthalene biodegradation constant (k) was 0.038 h^{-1} with R^2 value 0.9679, which showed that the strain nai8 could degrade naphthalene remarkably.

Keywords: *Pseudomonas*, Naphthalene, Biodegradation, Kinetics, Characterization.

INTRODUCTION

Naphthalene is a typical chemical product of polycyclic aromatic hydrocarbons (PAHs), which are widely used as solvents in paints, pesticides and many other industrial products[1]. These have already led to large amount of PAHs release into the environment. Their solubility in water is very low and PAHs are lipophilic, they are often enriched in soils and sediments. PAHs have carcinogenicity and persistent organic pollutant properties[2]. In order to ensure human health and keep agricultural products safety, PAHs in the environment must be removed. In recent years many methods and strategies have been studied to degrade and remove PAHs from the soils, sediments and water, including catalytic ozonation, photocatalysis, volatilization, chemical oxidation, adsorption and biodegradation[3,4]. Biological degradation technology used bacteria or fungi to degrade naphthalene, which is kind to human and environment, nontoxic and no secondary contamination due to natural environment and it is comparatively low cost. Thus biodegradation techniques have received considerable attention[5,6]. Biodegradation and bioremediation offers a cost-effective and environmentally friendly method for remove of naphthalene in industrial effluents and contaminated soils.

Many bacteria and fungi can efficiently degrade naphthalene, such as *Pseudomonas*, *Rhodococcu* sp., *Bacillus* sp., *Mycobacterium sp. Nocardia otitidiscaviarum, Alcaligenes, Corynebacteria* and *Flavobacerium*[7-10]. Bacteria and fungi have naphthalene-degrading gene, including nahAc, nahF, nahV, nahG, nahU, nahH, catA and nahY, which code the naphthalene-degrading enzymes[9]. These degrading enzymes could break the struture of benzene ring of naphthalene, producing phthalic acid, salicylic acid, catechol, *etc*. and these metabolites could be used as energy and carbon source through TCA cycle by bacteria and fungi.

The present study aims to screen and isolate degrading bacteria with high efficiency and study degrading-bacteria characteristics. The degrading strain was employed for study of biodegradation of naphthalene.

EXPERIMENTAL

Naphthalene used for this biodegradation study was purchased from Sinopharm Chemical Reagent Company and all the other reagents used were of analytical grade.

Naphthalene contaminated soils sample and activated sludge samples were obtained from Changzhou chemical industry park and Qingtan wastewater treatment factory in Changzhou, respectively. All the samples were collected in sterile plastic containers.

Isolation of naphthalene-degrading bacteria: Soil and activated sludge samples were used as inoculum and 1 g of soil or activated sludge was added into 100 mL of sterile modified base mineral medium (BMM) in flasks and naphthalene was added as the sole carbon source to give a final concentration

of 800 mg/L. One liter of basic mineral medium (BMM) containing 5.17 g K_2HPO_4, 1.70 g KH_2PO_4, 1.63 g NH_4Cl and 10 mL of a salt solution. One liter of the salt solution contained 8.5 g $MgSO_4$, 5 g $MnSO_4$, 5 g $FeSO_4$, 0.3 g $CaCl_2$. The initial pH value of media was 7.2[11,12].

The suspension was inoculated in flasks on an orbital shaker at 120 rpm at 30 ºC for 72-96 h. Two milliliters of enriched culture medium mentioned above was transferred into another 100 mL of fresh culture medium and this process was repeated 4-5 times. The obtained suspensions were diluted with steriled water and streaked on the nutrient agar plates. The medium composition is BMM, 800 mg/L of naphthalene and 1.5 % of agar. All the plates were incubated at 30 ºC for 48 h. The morphologically distinct bacterial colonies wan transferred to fresh agar plates several times in order to obtain pure culture. The pure isolated strains were preserved on fresh LB agar plates at 4 ºC.

Characteristics and identification of *Pseudomonas* sp. Nai8: The isolates were selected and streaked on nutrient agar for further purification and study. The purified isolates and cells were microscopically examined to determine shape and gram characteristics. The physiological and biochemical characteristics of the naphthalene-degrading bacteria were carried out according to standard microbiological procedures[13]. Gram stain, catalase- and oxidase-activities, methyl-red, V-P, citrate, gelatin agar, indol and starch hydrolysis tests were investigated. Motility was assessed by testing the ability of the strains to migrate from the point of inoculation through semisolid agar plate[6]. The utilization of some substrates as carbon sources and nitrogen sources by the isolate was performed based on Bergey's Manual of Determinative Bacteriology[14].

Genomic DNA was isolated from the pure culture pellet using consensus primers and partial 16S rDNA genes were amplified by PCR using forward primer (5'-GAGCGGATAACAATTTCACACAGG-3'), reverse primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and internal primer (5'-CAGCAGCCGCGGTAATAC- 3'). The amplified 16s rDNA gene was sequenced by Takara Bio (Dalian, China).

Biodegradation experiments: In order to study the characterization of the selected naphthalene-degrading strain nai8, various factors including temperature (25-40 ºC), pH (5-9) and naphthalene concentration (500-2000 mg/L) were tested while monitoring the growth rate of naphthalene-degrading strain- *Pseudomonas* sp. nai8 and degradation ratio of naphthalene. The effects of these factors were evaluated by measuring the naphthalene degradation rate and all the experiments were carried out in triplicate.

Analysis methods: The naphthalene in the base mineral medium was extracted with 15 mL of *n*-hexane three times. The extracted *n*-hexane was combined and dried with anhydrous sodium sulphate. The dried extracted *n*-hexane was then concentrated through vacuum rotary evaporator at 30 ºC to almost dry and the extracts were dissolved by 5 mL of methanol. The concentration of naphthalene in the extracted *n*-hexane was analyzed using Agilent 7890 GC equipped with FID and capillary column (30 m × 0.32 mm). The initial column temperature was 100 ºC, the detector and injector temperatures were 200 ºC.

RESULTS AND DISCUSSION

Isolation and identification of naphthalene-degrading bacteria: There were 13 morphologically distinct isolates on nutrient agar containing 800 mg/L naphthalene. The isolate 8, designated as nai8, was the one with a larger transparent circle (3 mm) for a colony size of 4-5 mm and was selected for further studies. The strain nai8 had higher degradation activity for naphthalene and could use naphthalene as the sole carbon and energy source to grow.

The naphthalene-degrading strain nai8 was determined to be pure by visual observation of the colonies on nutrient agar, after purification procedures and confirmation by microscopic observation, which demonstrated that both colonies as cells presented uniform morphology.

The strain nai8 was a gram-negative, rod-shaped (0.5-0.8 µm × 0.8-1.1 µm, Fig. 1a), facultative aerobe, bipolar flagellation (Fig. 1b) and motile. The colonial morphology on the agar nutrient plate was smooth, wet and orange, after 1 to 2 days of incubation. The physiological and biochemical characteristics were showed in Table-1.

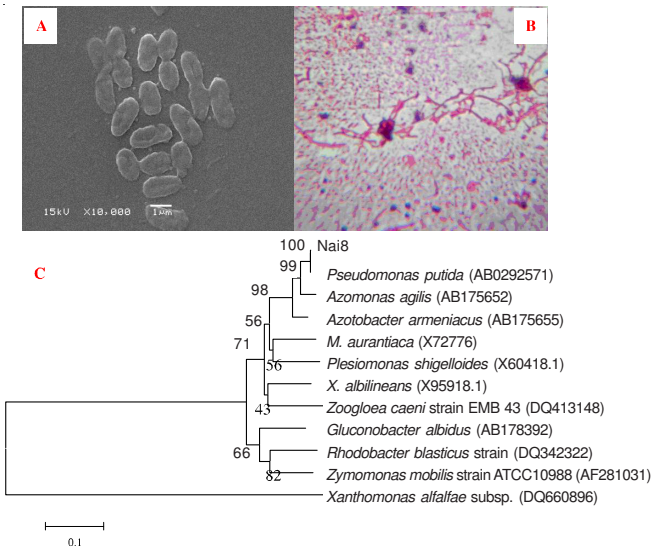


Fig. 1. Electron micrograph (A), flagella graph (B) of *Pseudomonas* sp. nai8 and phylogenetic tree of strain Nai8 based on the 16S rDNA gene homology(C)

TABLE-1
PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF STRAIN NAI8

Test	Results
Gram stain	–
O_2	+
Oxidase	+
peroxidase test	+
Gelatin agar	–
Indol	+
Glucose oxidation	+
Glucose fermentation	+
Nitric acid reduction	+
H_2S production	+
Starch hydrolysis	+
Methyl red	+
V-P	+

*Note: + means positive reaction; – means negative reaction.

Substrate-utilization experiments were performed and showed that the strain nai8 was capable of using a variety of substrates, including phenol, dimethylbenzene, phenol, benzyl acid, 2,4-nitrophenols and salicylic acid , carboxylic acids, aromatic compounds and most inorganic nitrogen sources. The 16s rDNA gene sequences were compared by using BLAST similarity searches and the closely related sequences were obtained from GenBank (Fig. 1c). On the basis of morphological and biochemical analysis in combination with phylogenetic analysis, the strain nai8 was identified as *Pseudomonas* sp. and designated as *Pseudomonas* sp. nai8 (GenBank Accession: N022444).

Carbon-source utilization: Various aromatic compounds were used as the sole carbon source for the *Pseudomonas* sp. nai8. Culture temperature, pH and inoculum were constant at 30 ºC, 7.0 and 5 %, respectively. Various aromatic compounds were added in the BMM as the sole substrate and the $OD_{600\ nm}$ of cell numbers were measured by UV-visible spectrophotometer (Gold Spectrumlab 53, Shanghai, China). The results showed that benzoic acid, phthalic acid, phenol and 1-naththol could be used as the sole carbon and energy source. And the microbial growth and cell density were inhibited by the higher salicylic acid concentration.

Effect of the temperature on naphthalene degradation: Temperature plays an important role in bacteria growth and is one of the most important parameters taken into consideration in the development of biodegradation processes. The temperature range was 25-40 ºC. The results (Fig. 2) showed highest degradation ratio was at 40 ºC after 48 h cultivation. However, naphthalene volatility at 40 ºC is higher than that of at 30 and 35 ºC (Fig. 2) the results of subtracting the amount of naphthalene volatility revealed that the highest naphthalene-degrading ratio by *Pseudomonas* sp. nai8 was 71.21 % at 30 ºC and it was 60.83 % at 40 ºC.

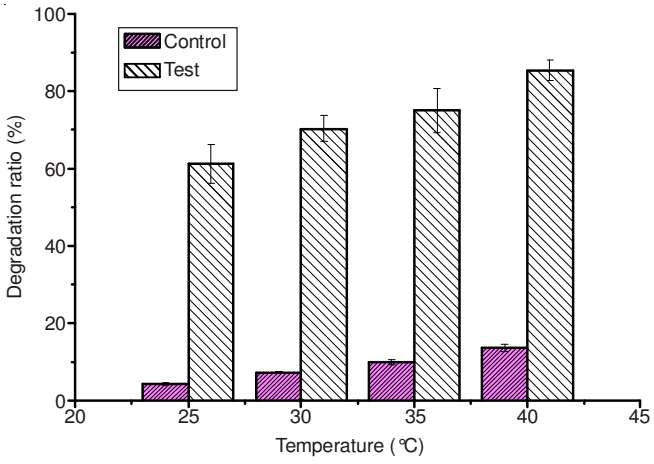


Fig. 2. Effect of temperature on naphthalene degradation ratio by *Pseudomonas* sp. nai8

Effect of initial pH on naphthalene degradation: The initial pH of culture medium affects microbial growth and activity, therefore study the effect of pH on naphthalene degradation by *Pseudomonas* sp. nai8 is necessary. The experiment was carried out as follows: the temperature was maintained constant 30 ºC, for 48 h and pH varied from 5-9. Fig. 3 showed

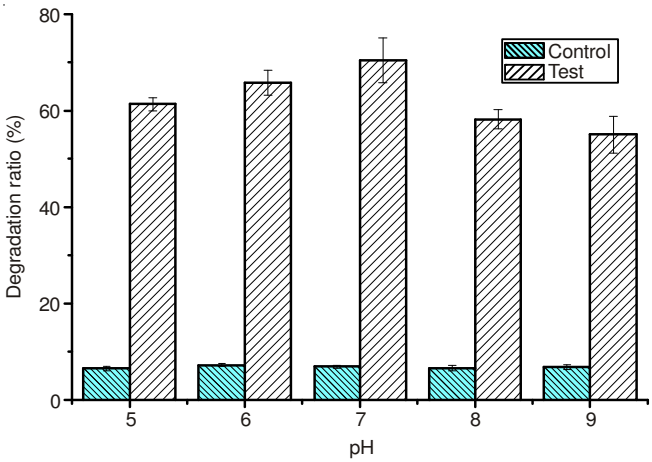


Fig. 3. Effect of initial pH on naphthalene degradation ratio by *Pseudomonas* sp. nai8

that degradation ratio of naphthalene was higher at pH 6-7. The highest degradation ratio could be obtained at pH 7 for 48 h, it was 70.47 %. More than 60 % of naphthalene was degraded when the pH ranged from and the degradation ratio also reached 65.71 and 61.35 % at pH 6 and pH 5.0-7.0, while the degradation ratio was 55.08 % at pH 9. The results of the amount of viable bacteria was also showed that the strain nai8 grew slowly at pH values under 6 and upper 8 and the highest densities of viable bacteria were obtained between the pH range (6-7).

Effect of the substrate concentration on naphthalene degradation: Naphthalene has carcinogenicity and can inhibit microbial activity and growth. For the study of the strain nai8's capacity of tolerate different concentrations of naphthalene, temperature and pH were constant at 30 ºC and 7.0, respectively, for 48 h, while naphthalene varied from 500-1500 mg/L. Fig. 4 shows that degradation ratio decreases with the increasing naphthalene concentration. As the initial concentration of naphthalene in BMM increased from 500 to 1500 mg/L, the degradation ratio decreased from 73.62 to 65.11 % after 48 h. However the total amount of naphthalene degraded by *Pseudomonas* sp. nai8 increased significantly. This means the initial concentration of naphthalene could provide an important driving force to overcome all mass transfer resistances of the naphthalene between the aqueous and solid phases and a higher concentration of naphthalene could enhance the degradation rate[15].

Effect of inoculum on naphthalene degradation: Inoculum can affect the long or short time of lag inoculation, which also effects the naphthalene degradation ratio. The results of the effect of inoculum on the naphthalene degradation showed in Fig. 5 the naphthalene degradation ratio increased from 61.24-81.47 % with the inoculum concentration increased. When the inoculum concentration was above 8 %, the naphthalene degradation ratio at 48h had no significantly difference.

Naphthalene degradation and the strain nai8 growth: Fig. 6a showed the relationship between growth of the strain nai8 in BMM and the degrading naphthalene, naphthalene was a sole carbon and energy source in the best conditions, temperature, pH, naphthalene concentration and inoculum were

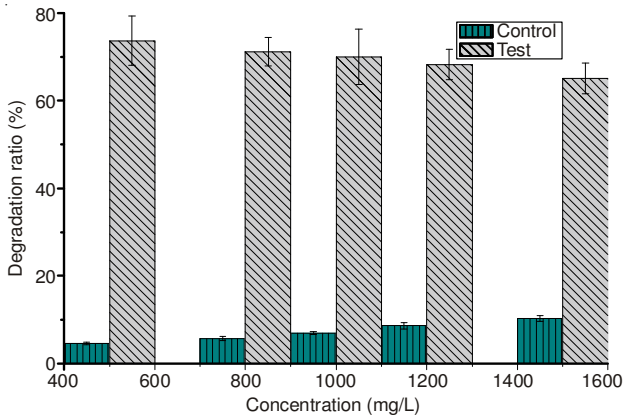


Fig. 4. Effect of initial concentration on naphthalene degradation ratio by *Pseudomonas* sp. nai8

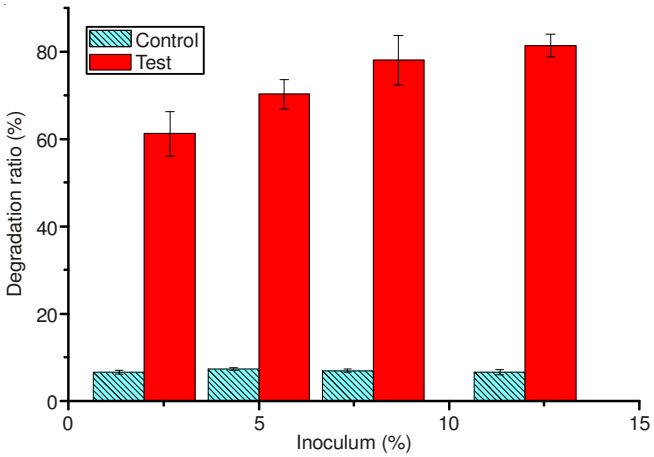
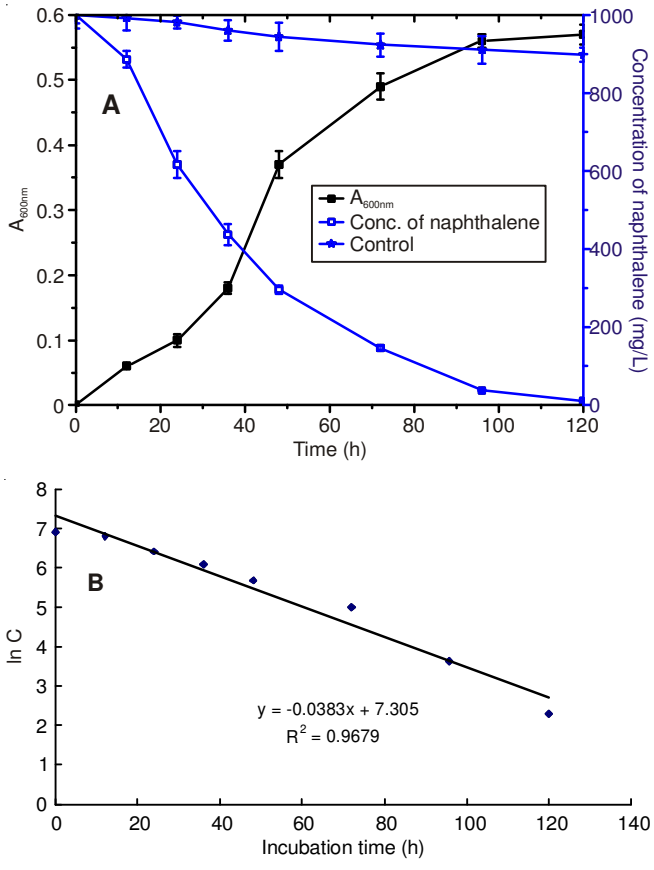


Fig. 5. Effect of inoculum on naphthalene degradation ratio by *Pseudomonas* sp. nai8



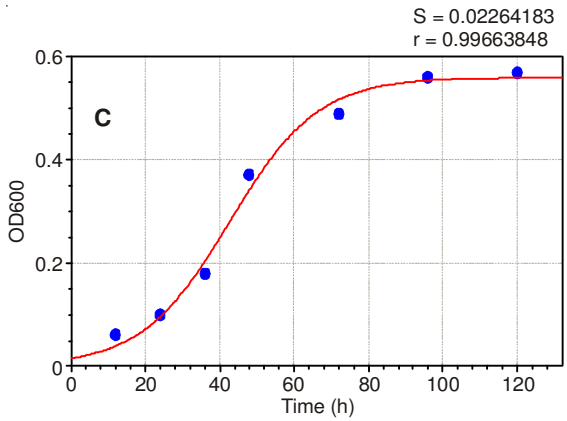


Fig. 6. Utilization of Naphthalene as sole of carbon for growth and biodegradation by *Pseudomonas* sp. nai8. (a) Experiment curve; (b) and (c) kinetic model)

30 ºC, 7.0, 1000 mg/L and 8 %, respectively. Naphthalene concentration and cell numbers in the BMM were monitored every 12 h. The results showed that the concentration of naphthalene in BMM decreased with the increased cell numbers. In the initial 12 h after inoculum, the degradation rate was relatively slow, from 1000 to 886.73 mg/L and the degradation rate of naphthalene was rapidly increased after 12 h, which meant the lag phase was short. After 72 and 96 h of incubation, naphthalene concentration in BMM was 147.26 and 38.07 mg/L, respectively.

PAHs are used as solvents in many area, they are difficult to degrade and accumulate in environment. In the last decade, PAHs, especially naphthalene biodegradation has been attracted much attention in the world. There are many research reported many naphthalene-degrading bacteria such as *Pseudomonas*, *Rhodococcu* sp., *Bacillus* sp., *Mycobacterium sp., etc*.[8,10]. However naphthalene has carcinogenicity and toxicity, it can inhibit bacteria growth and degradation rate. In present study, a strain of naphthalene-degrading bacteria was isolated and designated as *Pseudomonas* sp. nai8. The strain nai8 has higher naphthalene degradation rate and higher naphthalene tolerance[1,10,16-18]. Under the condition of 30 ºC, pH 7.0 and concentration of naphthalene of 1000 mg/L, the degradation rate in 48 and 96 h was 70.4 and 96.2 %, respectively. *Pseudomonas* sp. Nai8 could also degrade other aromatic hydrocarbons, such as dimethylbenzene, phenol, benzyl acid, 2,4-nitrophenols and salicylic acid, utilizing each of them as sole carbon and energy source for its growth, which show the strain Nai8 has good biodegradation diversity.

The temperature, pH, concentration of naphthalene and inoculum was investigated. The results showed these factors influence the biodegrading process by inhibiting growth of the pollutant-degrading microorganisms, these was in accordance with previous reports[19,20]. Temperature and pH can influence the degrading enzymes activity, higher or lower temperture/ pH will lead to the lower enzyme activity. There was higher naphhtalene degradation ratio in the lower pH BMM, which means *Pseudomonas* sp. Nai8 has higher growth activity in the lower pH, which is not accordance with the previous reports[7-10]. *Pseudomonas* sp. Nai8 also has higher naphthalene tolerance. In the different naphthalene concentration from 500-1500 mg/L, degradation ratio was above 65 %. With the initial

concentration of naphthalene increased, the degradation ratio decreased, however the total removal amount of naphthalene increased significantly, which showed that the initial concentration of naphthalene could provide an important driving force to overcome all mass transfer resistances of the naphthalene between the aqueous and solid phases and a higher concentration of naphthalene could enhance the degradation rate[15]. Higher tolerance to naphthalene due to *Pseudomonas* sp. Nai8 can also degrade naphthalene metabolites, such as dimethylbenzene, phenol, benzyl acid, 2,4-nitrophenols and salicylic acid *etc*., which can eliminate these metabolites toxic effects. The toxic metabolites can be accumulated in the BMM[21], thus inhibit the bacteria growth and decrease the degradation rate. The strain nai8 can utilize each of these toxic metabolites as carbon and energy source.

The kinetics of naphthalene degradation was analyzed based on the naphthalene degradation and nai8 growth described in Fig. 6. The degradation rate constant was calculated through the followed equation.

$$-\ln\frac{c}{C_0} = kt$$

In the equation C_0 is the initial concentration of naphthalene (mg/L), C is the real-time concentration of naphthalene in BMM (mg/L), t is time (h) and the k is the reaction rate constant for the degradation of naphthalene (h^{-1}) . According to the data shown in Fig. 6, the kinetics was calculated as the following logistic equation:

$$\ln C = -0.038\ t + 7.305$$

The biodegradation constant was 0.038 h^{-1} with R^2 value 0.9679 (Fig. 6b), which showed that the stain nai8 could degrade naphthalene greatly and the first-order rate model fitted welll the biodegradation of naphthalene (1000 mg/L).

The relationship between cell growth and incubation time was often described with logistic models, which could be proposed as followed[17,21]:

$$C_t = \frac{a}{1 + b \times e^{-kt}}$$

C_t is the real-time bacterial concentration (OD_{600}); t is time (h); a is the maximum population of cell; b is parameter related to the intercept of y axis; k is the reaction velocity constant for bacterial growth in BMM. The model prediction was compare with experimental data and the kinetic constants in this model were calculated by Curve Expert 1.4. The input values of logistic model were a = 0.560, b = 36.762, c = -0.0847, respectively and the value of R^2 equal 0.997 (Fig. 6c). The logistic equation was as followed

$$C_t = \frac{0.560}{1 + 36.762 \times e^{-0.0847t}}$$

Biodegradation kinetics can illustrate the mechanism of microbial degradation and the prospect of its application. The naphthalene-degrading constant of *Pseudomonas* sp. Nai8 was 0.038 h^{-1} with R^2 value 0.9679, which showed that the rate of degrading naphthalene using *Pseudomonas* sp. Nai8 was very fast. Therefore *Pseudomonas* sp. Nai8 is a potential candidate for degrading and bio-remediating NAHs, especially naphthalene polluted soils.

Conclusion

In this study, a naphthalene degrading bacteria was isolated and identified. The effects of *Pseudomonas* sp.Nai8's degrading ability, were investigated. under the condition of 30 ºC, pH 7.0 and concentration of naphthalene of 1000 mg/L, the degradation rate in 48 and 96 h was 70.4 and 96.2 %, respectively. The kinetics of naphthalene degradation by Nai8 was also calculated and the naphthalene biodegradation constant (k) was 0.038 h^{-1} with R^2 value 0.9679, which showed that the strain nai8 could degrade naphthalene greatly. The next step we will clone naphthalene degrading gene and construct gene-engineering bacteria with higher naphthalene-degradation ratio, which may open new possibilities for its biotechnological applications and allow the use of *Pseudomonas* sp. Nai8 in the treatment and bioremediation of PAHs contaminated soils and industrial effluents.

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