Isolation and characterization of a newly isolated polycyclic aromatic hydrocarbons-degrading *Janibacter anophelis* strain JY11

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ABSTRACT

The PAHs-degradation bacterium strain JY11 was newly isolated from the polluted soil in Jinan Oil Refinery Factory, Shandong Province of China. The isolate was identified as *Janibacter anophelis* with respect to its 16S rDNA sequence, DNA–DNA relatedness and fatty acid profiles, as well as various physiological characteristics. The strain was Gram-positive, non-motile, non-spore-forming, short rods in young culture, 0.8–1.0 μm in diameter and 1.3–1.6 μm long, and coccoid cells in the stationary phase of growth that are 1.0–1.2 μm in diameter and 1.3–1.5 μm long, occurred in pairs and sometimes in chains or in group, aerobic, oxidase-week positive, catalase-positive. *J. anophelis* strain JY11 can utilize naphthalene, phenanthrene, anthracene, pyrene, xylene, methanol, ethanol and salicylic acid as sole carbon source. The strain could remove 98.5% of phenanthrene, 82.1% of anthracene, and 97.7% of pyrene with an initial concentration of 500 ppm in five days without adding co-metabolism substrates and surfactants.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) may be present in high concentrations at industrial sites associated with the petroleum, coal-tar, gas production and wood preservation industries [1,2]. As toxic, mutagenic and carcinogenic chemicals that are ubiquitous in environment [3], the United States Environmental Protection Agency (EPA) lists 16 kinds of PAHs as priority pollutants [4]. So they arouse considerable environmental concern [5,6] and many researches were carried out in order to eliminate PAHs from environment.

One way to remove PAHs from contaminated sites is to inoculate the site(s) with microorganisms known to metabolize these compounds [7]. Using bioremediation technology to cleanup PAH-contaminated sites has been suggested to be an efficient, economical and versatile alternative to physicochemical treatment [8]. Nowadays, in order to eliminate PAHs from environment by bioremediation, many PAHs-degradation microorganisms were isolated. Most of these microorganisms belong to *Pseudomonas* sp. [9,10], *Alcaligenes* sp. [11], *Mycobacterium* [12,13], *Rhodococcus* [14,15], *Neptunomonas* [16,17], *Stenotrophomonas* [18,19], *Sph-ingomonas* [20,21], *Cycloclasticus* [22,23], *Staphylococcus* [24], *Burkholderia* [25], *Acinetobacter*, *Agmenellum*, *Aeromonas*, *Bacillus*, *Berjerinckia*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Nocardioides*, *Lutibacterium*, *Streptomyces*, *Vibrio*, *Paenibacillus* and some fungi [26–35]. Recently, different aspects such as PAHs-metabolizing of bacteria, PAHs-degradation mechanisms, the way of assimilation into bacteria activities of the degradation-related enzyme and discovering the degradation-related gene has been researched [36–39].

The genus *Janibacter*, established in 1997 by Martin et al. [40], belongs to the family *Intrasporangiaceae* in the order *Actinomycetales*. At present, the genus comprises five species, *Janibacter limosus* [40], *Janibacter terrae* [41], *Janibacter melonis* [42], *Janibacter anophelis* [43] and *Janibacter corallicola* [44]. *Janibacter brevis*, originally described by Imamura et al. [45], was shown to be a heterotypic synonym of *J. terrae* [46]. *Janibacter marinus* had no other report except its 16S partial sequence record in GenBank. According to previous study, the genus *Janibacter* has the ability of degrading fluorene, diphenyl ether, carbazole, dibenzo-*p*-dioxin [47], anthracene, phenanthrene [48], mono-chlorinated dibenzo-*p*-dioxins [49], dibenzofuran [48,50], and polychlorinated biphenyls (Aroclor 1242) [51]. The aim of the present work was to isolate and characterize a PAH-degrading bacterium, *J. anophelis* strain JY11 from contaminated environmental samples, and to evaluate its PAHs-degradation potential.

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2. Materials and methods

2.1. Media

The basal salts medium (BSM) contained (per liter) 0.8 g KH_2PO_4, 1.2 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 mg $FeCl_3$, 20 mg $CaCl_2$, 1.0 mg $MnSO_4$, 0.2 mg Na_2MoO_4, and the solution pH was adjusted to 7.0. Solid BSM plate was prepared by adding 20.0 g agar into 1000 ml BSM.

Luria–Bertani (LB) medium contained 10.0 g NaCl, 10.0 g peptone, 5.0 g yeast extract, with or without 20.0 g agar in 1000 ml distilled water, and the solution pH was adjusted to 7.0.

All chemicals used were analytical grade. All organic solvents used were high-performance liquid chromatographic (HPLC) grade and were purchased from JK Chemical Ltd., Beijing, China.

Phenanthrene (98%), anthracene (99%) and pyrene (99%) were purchased from Alfa Aesar Ltd., Karlsruhe, Germany. Individual PAH stock solutions were 1000 mg l^{-1} in hexane.

2.2. Isolation of PAHs-degrading bacteria

The soil samples collected from seven sites in Jinan Oil Refinery Factory were suspended in BSM liquid medium supplemented with phenanthrene, anthracene and pyrene crystals, separately (1 g l^{-1}), and incubated aerobically at 25 °C on a reciprocal shaker at 150 rpm. Aliquots were transferred weekly to fresh BSM medium containing corresponding PAH at a concentration of 250 mg l^{-1} as sole carbon and energy source, and incubated under the same conditions; this process was repeated at least three times before the bacterial strains were isolated. Isolation and purification procedures were carried out on BSM agar plates by conventional spread plate techniques. 0.5% (w/v) PAH was dissolved in hexane and sprayed on the surface of the pure culture as the sole carbon source. The size and color of the isolated colonies were recorded. Pure bacterial strains obtained were kept on LB slant culture at 4 °C.

2.3. Morphological and physiological characteristics

The cells morphological properties were examined by light microscopy and transmission electron microscopy (TEM) on LB solid plate. Presence or absence of flagella was examined by TEM using cells from exponentially growing cultures. These were negatively stained with 1% (w/v) phosphotungstic acid, and after air drying, grids were examined with a model H-800 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% p-aminodimethylaniline oxalate. Nitrate reduction, indole production, acid or gas production from D-glucose, sucrose, methyl red and Voges–Proskauer reactions, citrate utilization test and hydrogen sulfide production were tested as described previously [52].

2.4. Lipid analysis

Presence or absence of mycolic acids was determined using the methods of [53]. Menaquinones were extracted twice from lyophilized cells with chloroform–methanol (2:1, v/v) for 25 min, and analyzed by Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA) with a Kromasil 100-C_{18} column (150 mm × 4.6 mm I.D., 5 μm). Further fatty acids isolation and identification was conducted following the instructions [54] of the Microbial Identification System (MIDI, Microbial ID, Newark, Del.).

2.5. Carbon source utilization

Some aromatic compounds were added as sole carbon sources to liquid BSM, the purified strain JY11 were incubated at 30 °C on one of the following compounds at 0.01%: naphthalene, phenanthrene, anthracene, pyrene, benzene, toluene, xylene, phenol, DMSO, ethanol, salicylic acid, Tween 80, cyclohexane and catechol. Growth was measured by the increase of OD_{600} of the culture.

2.6. Determination of G–C content and DNA–DNA hybridization

G–C content was determined by HPLC as described by Tamaoka and Komagata [55], and calculated from the ratios of deoxyguanosine and thymidine. DNA–DNA hybridization to determine genomic relatedness was performed by the method of Ezaki et al. [56] using photobiotin and microimmunoplates. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values for each sample were excluded; DNA–DNA relatedness values are the mean of the remaining three values.

2.7. Analysis of 16S rDNA sequence and phylogenetic analysis

Genomic DNA was extracted with BS423 genome DNA isolation kit (Sangon Ltd., Shanghai, China), and 16S rDNA was amplified in PCR using the genomic DNA as template and the bacterial universal primers are as follows: F_{27} (5′-CAGCGGTACCAGAGTTT GATCCTGGCTCAG-3′); R_{1492} (5′-CTCTCTGCAGTACGGCTACCTTGTT-ACGACTT-3′). Amplification was as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, a gradient of ±5 °C, and elongation at 72 °C for 2 min, and final elongation at 72 °C for 10 min. The PCR product was purified with a QIAquick PCR purification kit (Qiagen Ltd., Maryland, USA), and the sequence reaction mixtures were electrophoresed by using a model 3700 automatic DNA sequencer (Applied Biosystems Ltd., California, USA).

Multiple alignments of sequences, and construction of neighbor-joining phylogenetic trees [57] were performed with the Clustal X software. Alignment positions which included gaps or unidentified bases were eliminated from the calculations. After the alignment process, sequences of 16S rDNA were adjusted manually. Evolutionary distance matrices were calculated using the algorithm of Jukes and Cantor [58] with the Dnadist program within the Phylip package [59]. The stability of relationships was assessed by a bootstrap analysis of 1000 trials.

2.8. Sequence access number

The JY11 sequence obtained in this study has been deposited in the GenBank database with accession number FJ372723. Other accession numbers for reference 16S rDNA gene sequences used in the phylogenetic analysis are shown in Fig. 2.

2.9. Degradation of PAHs

PAHs-degradation in liquid culture was performed by using washed cell suspensions pre-grown with corresponding PAH. The liquid culture (100 ml of BSM with 50 mg phenanthrene, anthracene, pyrene respectively) was inoculated by transferring 3 ml of pre-culture (BSM medium, OD_{600nm} = 0.25) of strain JY11 (3%, v/v) and the initial pH was adjusted to 7.0. The flasks were incubated in darkness at 150 rpm at 30 °C. Every 24 h, the PAHs-degradation efficiencies were detected by gas chromatography. The rest PAHs were extracted from the liquid culture with hexane four times. Then the organic phase was dehydrated with anhydrous sodium sulfate and then was vaporized in rotary evaporator

to 1–2 ml. Effects of different initial concentrations (100, 500, 1000 ppm), various pH (6.0, 7.0 and 8.0), temperatures (20, 30, 37 °C) and mixed cultivation have also been investigated.

1.0 μl of the organic phase was analyzed by gas chromatography with a flame ionization detector of Agilent GC-6890 (+) system (Agilent Technologies, California, USA). A fuse silica capillary column (30 m × 0.25 mm, I.D., 0.25 μm) was used. The oven temperature was initially at 80 °C for 1.0 min, programmed to 255 °C at a rate of 15 °C min^{-1}, hold for 1 min; and then programmed to 265 °C at a rate 1 °C min^{-1}. The injector and detector temperatures were 250 and 300 °C, respectively. The carrier gas, helium, was adjusted to a linear velocity of 1.1 ml min^{-1}. The PAHs were identified by comparison of the retention times with those of PAH standards.

All biodegradation experiments were performed three times. The sterilize culture without inoculation was used as negative control.

3. Results and discussion

3.1. Morphological and biochemical characteristics

As Fig. 1 shown, colonies of strain JY11 are cream or pale cream color, opaque and convex with a glistening surface and entire margins on BSM and LB solid plate. Cells are Gram-positive, non-acid-fast and non-mobile, occur singly, in pairs or occasionally in irregular chains at the early stage of growth. Cells are short rods in young culture, 0.8–1.0 μm in diameter and 1.3–1.6 μm long, and coccoid cells in the stationary phase of growth that are 1.0–1.2 μm in diameter and 1.3–1.5 μm long. Optimal growth conditions of strain JY11 are at pH 7.0–8.0 and 28–30 °C. Neither substrate mycelia nor primary mycelia were seen. No growth occurs under anaerobic conditions. Spore formation is not observed. Morphological and physiological properties of strain JY11, together with those of *J.*
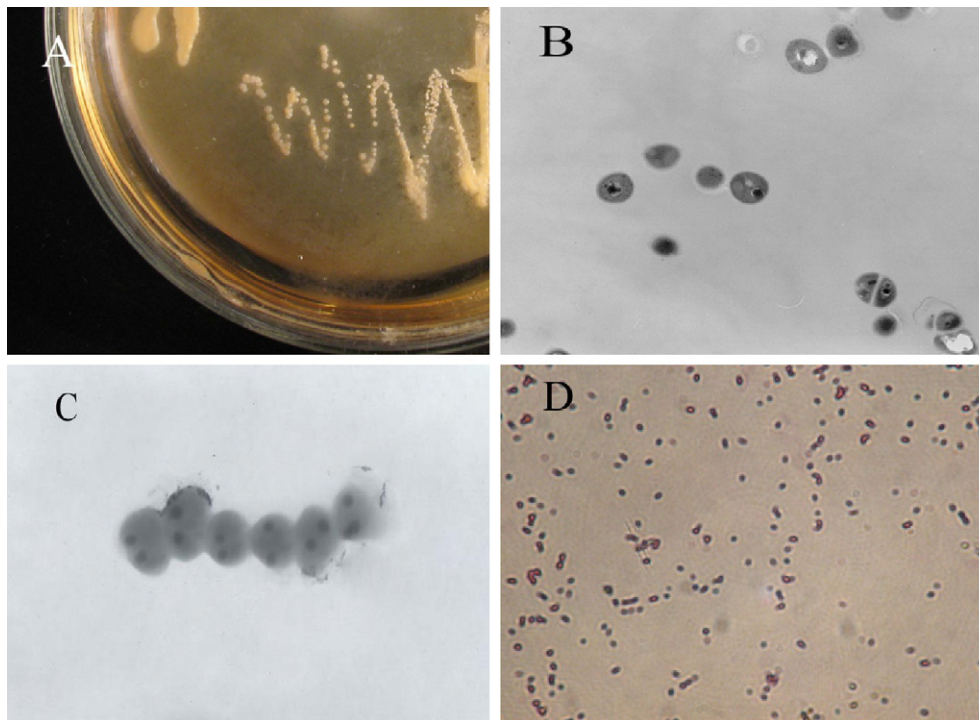

Fig. 1. Photographs of colonies and cells of *Janibacter anophelis* strain JY11: (A) photograph of JY11 colonies on LB media plate; (B) TEM photograph of JY11 (10 × k); (C) TEM photograph of JY11 (16 × k); (D) Gram stain photograph of JY11 (10 × 100).

Table 1
Comparison of morphological and biochemical characteristics of isolated strain JY11 with *J. limosus* DSM 11140[T] and *J. anophelis* CCUG 49715[T].

Characteristic	Strain JY11	*J. limosus* DSM 11140[T]	*J. anophelis* CCUG 49715[T]
Color of colonies	Cream or pale cream	White (cream or pale cream)	Pale cream
Morphology	Coccoid, rod shaped	Coccoid, rod shaped	Coccoid, rod shaped
Motility	−	−	−
Gram straining	+	+	+
Spore formation	−	−	−
Catalase activity	+	+	w
Oxidase test	w	+	+
Nitrate reduction	+	+	+
Indole production	−	−	−
Methyl red	−	−	−
Voges–Proskauer reaction	−	−	−
Citrate utilization test	+	+	+
Hydrogen sulfide test	+	+	w
Acid production from:			
D-Glucose	−	−	−
Sucrose	−	−	−

+, Positive reaction; −, negative reaction; w, Weakly positive reaction. Data for *J. limosus* DSM 11140[T] are taken from Martin et al. [40].

limosus DSM 11140[T] and *J. anophelis* CCUG 49715[T], were shown in Table 1.

3.2. Chemotaxonomic characteristics and DNA base composition

The Almost complete 16S rRNA gene sequences of strain JY11 was a continuous stretch of 1437 nucleotides. Subsequent 16S rDNA-based phylogenetic analysis demonstrated that the strain belonged to the genus *Janibacter*. Fig. 2 described the relationship between the isolated strain and the nearest phylogenetic relatives. Similarity calculations after neighbor-joining analysis indicated that the closest relatives of strain JY11 were *J. anophelis* (99.93%), *J. terrae* (98.48%), *J. marinus* (98.38%), *J. limosus* (98.34%), *J. melonis* (98.20%) and *J. corallicola* (97.79%). Strains JY11 was closely related to *J. anophelis* CCUG 49715[T]. Its percentage of the occurrence was 100% in 1000 bootstrapped trees.

The MIDI system was used to determine the cellular fatty acid composition of strain JY11. The result retrieved from the database did not match with any of the species listed in the MIDI library. Strain JY11 contained iso-$C_{16:0}$ (20.60%), $C_{18:1}\omega9c$ (12.66%), anteiso-$C_{17:0}$ (11.19%), iso-$C_{17:0}$ (11.00%), $C_{17:1}\omega8c$ (7.29%), 10-methyl $C_{17:0}$ (7.20%) and $C_{16:0}$ (6.31%) as major components, the detail results were shown in Table 2. They are characterized by branched fatty acids such as iso-$C_{14:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, iso-$C_{18:0}$, iso-$C_{16:1}$ H, iso-$C_{18:1}$ H, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{18:1}\omega9c$ as well as considerable amounts of straight chain saturated ($C_{14:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$ and $C_{19:0}$) and unsaturated ($C_{17:1}\omega8c$, $C_{18:1}\omega9c$ and iso-$C_{19:1}$ I) acid. Some methylated fatty acids were also found, such as 10-methyl $C_{17:0}$ (7.0%) and 10-methyl $C_{18:0}$ (2.08%). Regarding the fatty acid profiles of strain JY11, *J. limosus* DSM 11140[T] and *J. anophelis* CCUG 49715[T], only strain JY11 characteristically contained trace amounts of $C_{14:0}$, anteiso-$C_{17:1}\omega9c$, iso-$C_{18:1}$ H and iso-$C_{19:1}$ I fatty acids, but in particular, $C_{15:0}$ was not found. In addition, strain JY11 was different from *J. limosus* DSM 11140[T] and *J. anophelis* CCUG 49715[T] in the composition of some fatty acids. $C_{17:0}$, one of the major fatty acids in *J. limosus* DSM 11140[T] and *J. anophelis* CCUG 49715[T], was a minor component in strain JY11, and two minor components in *J. anophelis* CCUG 49715[T], anteiso-$C_{17:0}$ and $C_{18:1}\omega9c$, were present in large amounts in strain JY11 (Table 2).

Mycolic acids were absent, the major menaquinone was MK-8(H_4). The DNA base composition of strain JY11 was 71 mol% G + C. The mean level of DNA–DNA relatedness between strains JY11 and

Table 2
Comparison of fatty acid composition (%) of the isolated strain JY11 with *J. limosus* DSM 11140[T] and *J. anophelis* CCUG 49715[T].

	Strain JY11	*J. limosus* DSM 11140[T]	*J. anophelis* CCUG 49715[T]
iso-$C_{14:0}$	0.45	–	4.7
$C_{14:0}$	0.24	–	–
iso-$C_{15:0}$	3.09	0.56	4.4
anteiso-$C_{15:0}$	0.50	–	0.6
$C_{15:0}$	–	0.92	3.3
iso-$C_{16:1}$ H	0.31	–	–
iso-$C_{16:0}$	20.60	12.21	32.9
$C_{16:0}$	6.31	2.12	2.5
iso-$C_{17:1}\omega9c$	–	–	0.4
anteiso-$C_{17:1}\omega9c$	0.27	–	–
iso-$C_{17:0}$	11.00	1.47	3.3
anteiso-$C_{17:0}$	11.19	–	2.0
$C_{17:1}\omega8c$	7.29	26.15	13.8
$C_{17:0}$	2.39	17.79	16.4
10-methyl $C_{17:0}$	7.20	2.84	2.1
iso-$C_{18:1}$ H	0.45	–	–
iso-$C_{18:0}$	4.14	3.36	3.2
$C_{18:1}\omega9c$	12.66	17.48	3.9
$C_{18:0}$	1.29	3.94	2.7
10-methyl $C_{18:0}$, TBSA	2.08	0.78	–
iso-$C_{19:1}$ I	0.17	–	–
$C_{19:0}$	0.39	1.90	1.0
Summed feature 3[a]	2.53	–	–
Summed feature 4[a]	–	1.08	1.2
Summed feature 6[a]	1.74	–	–
Summed feature 8[a]	0.64	7.40	0.9
Summed feature 9[a]	3.09	–	–

Values are percentages of total fatty acid content; –: not detected.

[a] Unknown fatty acids; these compounds have no names listed in the peak library file of the MIDI system and therefore were not identified or included in the percentage calculation. Data for *J. limosus* DSM 11140[T] are from Lang et al. [46]; data for *J. anophelis* CCUG 49715[T] are from Kämpfer et al. [43].

CCUG 49715[T] was 76%, when each of their DNAs was separately used as labelled DNA probe.

On the bases of morphological and cultural characteristics, biochemical characteristics, chemotaxonomic characters, DNA–DNA hybridization [60] and the phylogenetic position of 16S rDNA sequences, the isolated strain JY11 clearly belonged to the genus *Janibacter* as a strain of the species *J. anophelis*, but it also showed a strain specificity regarding the fatty acid profile.

3.3. Utilization of carbon source

Fifteen kinds of carbon source were tested as sole carbon substrates of JY11, including various low and high molecular weights of PAHs and some kinds of *n*-alkanes. All of them exist ubiquitously in the crude oil contaminated soil. Strain JY11 was found to have the ability to degrade naphthalene, phenanthrene, anthracene, pyrene, xylene, methanol and ethanol, salicylic acid and Tween 80, which exhibited a very broad substrate profile. The results were shown in Table 3.

3.4. Degradation of PAHs

The degradation test of phenanthrene, anthracene and pyrene was carried out at 30 °C, pH 7.0 and an initial concentration of 500 ppm individually. In the test, none of other carbon sources or surfactants was added to the medium. Fig. 3 presented the degradation results. The strain could remove 98.5% of phenanthrene, 82.1% of anthracene, and 97.7% of pyrene with an initial concentration of 500 ppm in five days without adding co-metabolism substrates and surfactants.
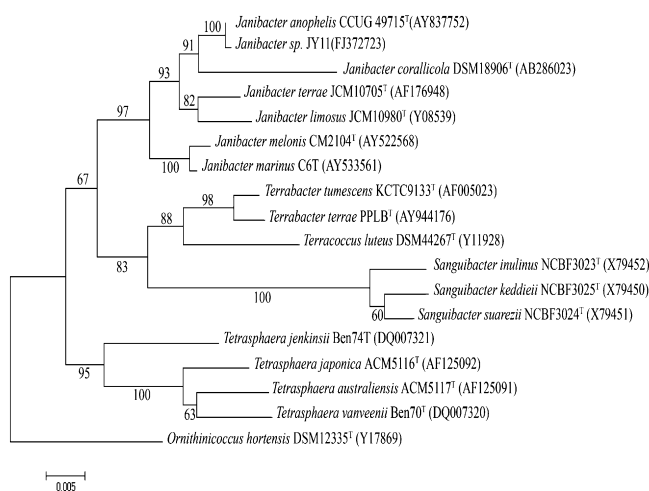

Fig. 2. Phylogenetic tree of *Janibacter anophelis* strain JY11 and related species constructed on the basis of 16S rDNA sequences using the neighbor-joining method. Bar, 0.005 Knuc unit. Bootstrap values (1000 re-samplings) are shown at the branch points.

Table 3
Utilization of carbon substrates by strain JY11.

Substrate	JY11
Naphthalene	++
Phenanthrene	++
Anthracene	++
Pyrene	++
Benzene	−
Toluene	−
Xylene	+
Phenol	−
DMSO	−
Methanol	++
Ethanol	++
Salicylic acid	+
Tween 80	−
Cyclohexane	−
Catechol	−

Growth was followed by measuring the increase of OD_{600nm} for 10 days. (++) Good growth: OD_{600nm} >0.2; (+) growth: OD_{600nm} >0.1; (−) no growth: OD_{600nm} <0.02.

3.5. Effect of environmental conditions on degradation of PAHs

A series of degradation tests were carried out at various pH from 6.0 to 8.0, temperatures from 20 to 37 °C and initial concentrations of each phenanthrene, anthracene and pyrene from 100 to 1000 ppm. As shown in Fig. 4(A–C), the optimal conditions were determined to be at pH 7.0, 30 °C and 500 ppm after five days incubation. The effect of pH and temperature on degradation of PAHs is similar to that reported by Kim et al. [34]. The result also indicated
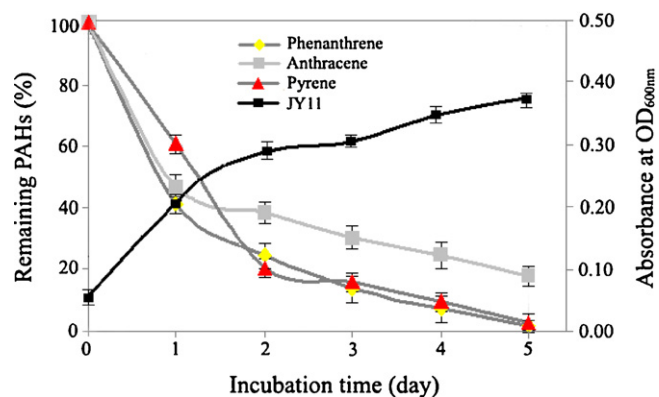

Fig. 3. Degradation of phenanthrene, anthracene and pyrene by strain JY11 in five days. Black square symbol is about the absorbance value at OD_{600nm} which indicates the growth of the strain.

that the degradation rate of *J. anophelis* strain JY11 decreased when the initial concentration of each kind of PAHs is low (100 ppm) or high (1000 ppm). This is mainly because a lower PAHs concentration is not enough for supporting the growth of *J. anophelis* strain JY11. While, higher PAHs concentration will lead to increasing of PAHs metabolites' toxicity.

When the mixture (500 ppm each) of phenanthrene, anthracene and pyrene were degraded by JY11, the removal of each compound tended to decrease. The result was shown in Fig. 4 (D). This may be due to one substrate inhibit the degradation of another. In addition, the reduction of degradation could be a result of the high concentration of PAHs. As mentioned above, the degrada-
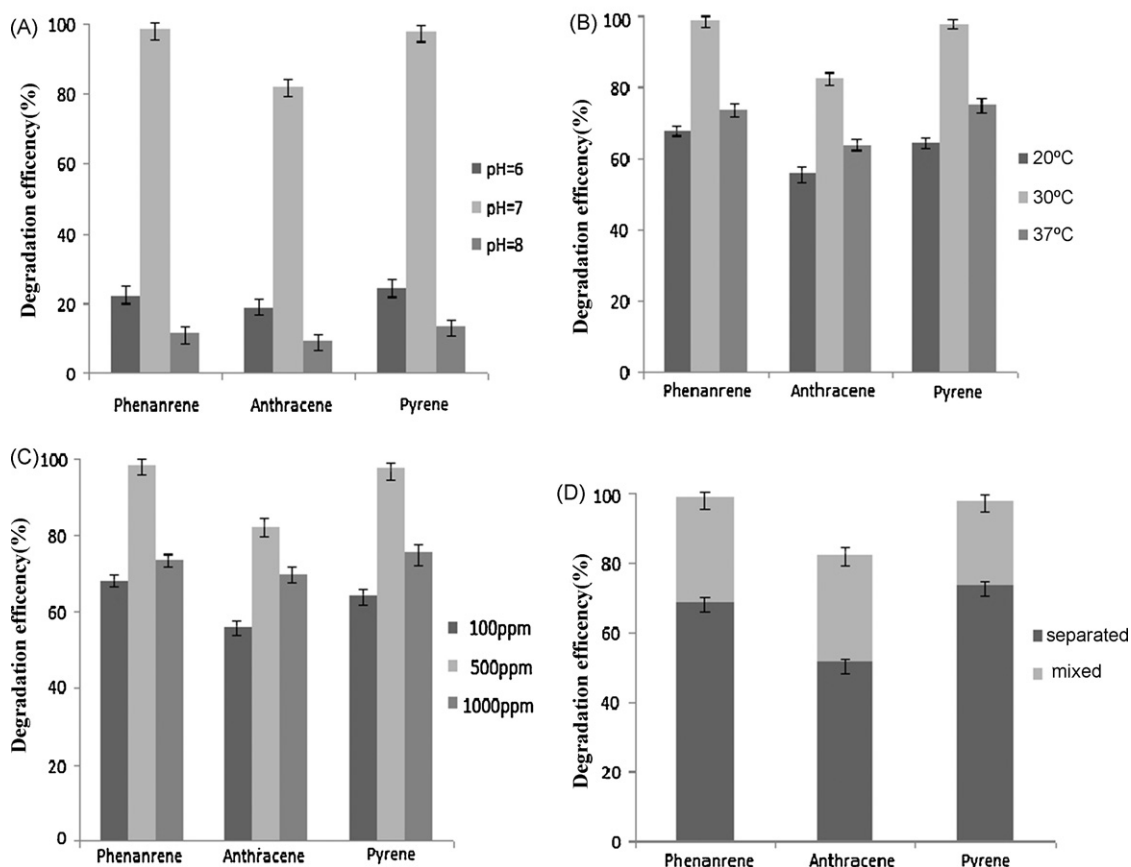

Fig. 4. Effects of condition changes on biodegradation of PAHs by *Janibacter anophelis* strain JY11: (A) pH; (B) temperature; (C) initial concentration; (D) mixed PAHs-degradation. All data are resulted from five days incubation.

tion rate was also decreased in 1000 ppm of a single PAH, and in the mixed PAHs-degradation the total concentration of PAHs was 1500 ppm.

Sierra et al. [51] reported that MS3-02, belonging to the genus *Janibacter* sp., was able to degrade polychlorinated biphenyls (Aroclor 1242) between 70% and 100% after seven days of incubation under laboratory scale. Also, strain JY11 can degrade polychlorinated biphenyls in transformer oils by 50% in 10 days, and the study is undergoing further research. So, the PAH degradation capabilities of *J. anophelis* strain JY11 can be exploited further for the development of effective bioremediation technology for environmental cleanup.

4. Conclusion

Bacteria strain JY11 was isolated and characterized belonging to the genus *Janibacter* according to its 16S rDNA, fatty acid profiles, as well as biochemical characteristics. DNA–DNA relatedness indicates that strains JY11 and *J. anophelis* CCUG 49715^T are members of the same genomic species. It is Gram-positive, non-motile, non-spore-forming, short rods in young culture, 0.8–1.0 μm in diameter and 1.3–1.6 μm long, and coccoid cells in the stationary phase of growth that are 1.0–1.2 μm in diameter and 1.3–1.5 μm long, occurred in pairs and sometimes in chains or in group, aerobic, oxidase-week positive, catalase-positive. *J. anophelis* strain JY11 can utilize naphthalene, phenanthrene, anthracene, pyrene, xylene, methanol, ethanol and salicylic acid as sole carbon source. Strain JY11 has a high ability to degrade phenanthrene, anthracene, pyrene in a wide pH, temperature and initial concentration range. The degradation efficiencies were examined by GC-FID and the result showed that the isolate could remove 98.5% phenanthrene, 82.1% anthracene, and 97.7% pyrene in five days at 30 °C pH 7.0 when the initial concentration of substrate is 500 ppm.

Acknowledgement

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