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Characterization of a Newly Isolated Biphenyl-Degrading Bacterium, *Dyella ginsengisoli* LA-4

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Abstract A novel biphenyl-degrading bacterium, *Dyella ginsengisoli* LA-4 was isolated from activated sludge. This isolate could utilize biphenyl as sole source of carbon and energy. The resting cells of strain LA-4 could utilize 100 mg/L biphenyl within 20 h, and they were able to degrade 500 mg/L biphenyl within 40 h. The surfactant, Tween 80, could accelerate the biodegradation process. The increase of NaCl concentration inhibited the biphenyl degradation. No biphenyl degradation was detected when the NaCl concentration exceeds 10%. The effects of metal ions on biphenyl degradation were investigated. The results indicated that metal ions such as Cu^{2+}, Mn^{2+}, and Co^{2+} could completely inhibit the biodegradation of biphenyl, but Mg^{2+}, Ca^{2+}, and Zn^{2+} had no effects on the degradation of biphenyl. The removal rate was about 64% and 37% in the presence of Fe^{3+} and Ni^{2+}, respectively. This study suggested that strain LA-4 could be widely used for bioremediation of soil and wastewater contaminated by biphenyl, NaCl, and metal ions.

Keywords *Dyella ginsengisoli* · Biphenyl · Metal ions · NaCl · Surfactant

Introduction

Biphenyl is a compound in which two benzene rings are connected to each other [1]. It is a natural component of coal tar, crude oil, and natural gas and is present in coking wastewater. Nowadays, it has been widely used in organic synthesis and dyeing of

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polyesters as a fungi stat in the packing of citrus fruits and in food preservatives, heat transfer fluids, a dyestuff carrier, a solvent for pharmaceuticals, and as an intermediate in the synthesis of polychlorinated biphenyl [2–4]. The application of biphenyl has been decreased quickly in recent year, but it still remains in water, soil, and atmosphere.

The use of microorganisms was expected to be an effective tool for bioremediation of polluted environment. Lunt and Evans [5] and Catelani et al. [6] first reported that bacterial isolates could grow on biphenyl as sole source of carbon and energy [5, 6]. From then on, several biphenyl-degrading bacteria have been isolated, and biphenyl degradation by these microorganisms has been widely studied [7].

Biphenyl and other polycyclic aromatic hydrocarbons (PAHs) shared many physical properties, which limited engineered bioremediation processes, and one of these properties was their low solubility and therefore low availability to the degrading microorganisms. As reported, surfactants were used to increase the solubility of hydrophobic environmental contaminants [8, 9] and have been applied to soil washing or soil-flushing technologies for soil remediation [10]. Enhanced water solubility could result in the enhanced bioavailability of contaminants to microorganisms [11]. Nonionic surfactants could be the most logical possibility for enhancing contaminant bioavailability because they were both effective at solubilizing contaminants and were generally less toxic to microorganisms than anionic or cationic surfactants [12]. The surfactants were also applied in solutions of 0.5% and higher in soil-flushing technologies [8] and belonged to environmental pollution. The reports on the co-biodegradation of biphenyl and nonionic surfactant were limited.

Coking wastewater contained the heavy metals and NaCl with high concentration [13]. Meanwhile, heavy metal accumulation in soil has become worldwide concern, particularly in China, with the rapid development of industrialization and urbanization [14]. And, the heavy metal and high concentration NaCl was able to cause the inhibition of most bacterial activities. There were few reports on the study of the effects of the heavy metals and NaCl on the biodegradation of biphenyl. Therefore, exploitation of the salt- and heavy-metal-tolerant bacteria in the biotreatment system would be a great improvement of conventional biological treatment systems and the biotreatment concept.

Recently, a novel biphenyl-degrading bacterium, *Dyella ginsengisoli* LA-4, was isolated from activated sludge. There were no reports on the genus *Dyella* with respect to biodegradation of environmental pollutants. It was necessary to develop this new microbial resource in environmental bioremediation. In this study, the effects of metal ions and chemical compounds on the biodegradation of biphenyl by *D. ginsengisoli* LA-4 were investigated in detail.

Materials and Methods

Chemicals

Biphenyl (>99% purity) and Tween 80 were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were of the highest purity commercially available and used without further purification.

Media and Culture Conditions

The media used in this study were a defined basal salts medium (DBSM) [15], which (pH 6.2) contained (gram per liter): 0.5 NaCl, 0.5 $K_2HPO_4{\cdot}3H_2O$, 0.5 $MgSO_4{\cdot}7H_2O$, and

0.01 $FeSO_4 \cdot 7H_2O$, and a modified DBSM, which additional contained 2 g L^{-1} of bactotryptone and 500 mg L^{-1} biphenyl. Biphenyl was added from an acetone stock solution (acetone does not support the growth of strain LA-4). Solid media contained 20 g L^{-1} agar in DBSM. Both media were autoclaved at 121 °C for 20 min. The strain was cultivated aerobically at 30 °C and 150 rpm in both media.

Isolation, 16S rRNA Gene Sequence, and Biolog Analysis of *D. ginsengisoli* LA-4

A sludge sample was taken from the wastewater treatment plant of PetroChina Petrochemical Company, Dalian, China. After 2 months of enrichment and screening, the most efficient strain designated as LA-4 was chosen for this study.

The 16S rRNA gene was amplified by PCR using the primers 8F (5′-AGAGTTTGAT CATGGCTCAG-3′) and 1522R (5′-AAGGACGTCATCCAGC CGCA-3′). The 16S rRNA gene sequence of strain LA-4 and related sequences retrieved from GenBank were aligned using Clustal W, and the aligned sequences were used to construct a phylogenetic tree using the neighbor-joining method and Jukes-cantor distance correction matrix method.

Biolog GN2 tests and the manual reading of the plates were performed by BIOLOG (Biolog, Hayward, CA, USA) according to the manufacturer's indications. A pure culture of strain LA-4 was grown on Biolog Universal Growth Sheep Blood agar. And, the bacteria were swabbed from the surface of the agar and suspended to a specified density in GN/GP Inoculating Fluid. Bacterial suspension (150 μL) was pipetted into each well of GN2 Microplate. The Microplate was incubated at 30 °C for 24 h. The color density in the microplates wells was interpreted visually as positive, negative, or borderline.

Preparation of Resting Cells

D. ginsengisoli LA-4 was grown in 100 mL DBSM supplemented with 100 mg L^{-1} biphenyl in 250 mL flasks at 30 °C and 150 rpm. The cells were harvested after 3 days and subsequently transferred into the modified DBSM, until OD_{660} reaching 1.0. Cells were harvested by centrifugation (8,000 rpm, 10 min at 4 °C) and suspended in 0.1 M phosphate buffers (pH 7.4). Then, the cells were washed with the same buffer twice. Finally, the cells were resuspended to a final concentration of 10 mg mL^{-1} (wet cells weight) for various assays or stored at 4 °C.

Biodegradation of Biphenyl by the Resting Cells of *D. ginsengisoli* LA-4

Biodegradation of biphenyl by the resting cells of strain LA-4 was carried out at 30 °C and 150 rpm, when the concentration of biphenyl was from 100 to 1,000 mg L^{-1}. And, the concentration of resting cells was about 20 mg L^{-1} in this experiment.

Effects of Metal Ions and Chemical Compounds

The effects of Tween 80 and NaCl were investigated by adding Tween 80 or NaCl into the modified DBSM. The final concentration of Tween 80 ranged from 0 to 100 mg L^{-1}, and NaCl was from 0% to 15%. The effects of different metal ions on the biphenyl degradation were tested. The metal salts used in this study were as follows: $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeCl_3$, $NiSO_4$, $CoCl_2$, $CuCl_2$, and $MnCl_2$. Stock solutions (1 M) of corresponding metal ions were prepared and then treated by filtration sterilization. The stock solutions were

added to the modified DBSM, and the final concentration of each metal ion was 1 mM. Strain LA-4 grown in DBSM was incubated into the media above for biphenyl biodegradation.

Analytical Methods

Biomass concentration was determined by optical density (OD) at 660 nm. And, the concentration of biphenyl was measured by GC. The residual biphenyl was extracted with an equal volume of ethyl acetate. The biphenyl concentration was analyzed via an Agilent 6890 Series Gas Chromatography (Agilent, Inc., USA), equipped with a flame ionization detector and a 30 m×0.32 mm×0.25 μm HP-5 capillary column (Agilent, Inc., USA). The following temperature program was used: initial column temperature 80 °C for 5 min,

Table 1 Utilization of different carbon sources in Biolog GN2 microplates by *Dyella ginsengisoli* LA-4.

Carbon source	Results	Carbon source	Results	Carbon source	Results
Water	−	α-Cyclodextrin	−	Dextrin	−
Glycogen	−	Tween 40	+	Tween 80	+
N-Acetyl-D-galactosamine	−	*N*-Acetyl-D-glucosamine	−	Adonitol	−
L-Arabinose	+	D-Arabitol	−	D-Cellobiose	−
i-Erythritol	−	D-Fructose	+	L-Fucose	−
D-Galactose	(+)	Gentiobiose	−	α-d-Glucose	+
m-Inositol	−	α-d-Lactose	−	Lactulose	−
Maltose	−	D-mannitol	−	D-Mannose	(+)
D-Melibiose	−	β-Methyl-d-glucoside	−	D-Psicose	−
D-Raffinose	−	L-Rhamnose	−	D-Sorbitol	+
Sucrose	+	D-Trehalose	−	Turanose	−
Xylitol	−	Pyruvic acid methyl ester	(+)	Succinic acid mono-methyl-ester	−
Acetic acid	(+)	*Cis*-Aconitic acid	+	Citric acid	+
Formic acid	−	D-Galactonic acid lactone	−	D-Galacturonic acid	+
D-Gluconic acid	+	D-Glucosaminic acid	−	D-Glucuronic acid	+
α-Hydroxybutyric acid	−	β-Hydroxybutyric acid	+	γ-Hydroxybutyric acid	−
p-Hydroxy phenylacetic acid	−	Itaconic acid	−	α-Keto butyric acid	−
α-Keto glutaric acid	(+)	α-Keto valeric acid	(+)	D,l-Lactic acid	+
Malonic acid	+	Propionic acid	(+)	Quinic acid	+
D-Saccharic acid	+	Sebacic acid	−	Succinic acid	+
Bromosuccinic acid	+	Succinamic acid	−	Glucuronamide	+
L-Alaninamide	−	D-Alanine	+	L-Alanine	+
L-Alanylglycine	(+)	L-Asparagine	+	L-Aspartic acid	+
L-Glutamic acid	+	Glycyl-L-aspartic acid	−	Glycyl-L-glutamic acid	−
L-Histidine	+	Hydroxy-L-proline	−	L-Leucine	+
L-Ornithine		L-Phenylalanine	−	L-Proline	+
L-Pyroglutamic acid		D-Serine	−	L-Serine	
L-Threonine	−	D,l-Camitine	(+)	γ-Amino butyric acid	+
Urocanic acid		Inosine	+	Uridine	(+)
Thymidine	−	Phenyethylamine	+	Putrescine	+
2-Aminoethanol	−	2,3-Butanediol	(+)	Glycerol	+
D,L-α-Glycerol phosphate	−	α-d-Glucose-1-phosphate	−	D-Glucose-6-phosphate	−

+ positive, *(+)* ambiguous, − negative

5 °C min^{-1} to 100 °C, 15 °C min^{-1} to 280 °C, followed by a holding time of 5 min. The injector temperature and the detector temperature were all 280 °C. The carrier gas was N_2 at a constant flow rate of 1.0 mL min^{-1}. The injection volume was 1 μL and the injection was split (50:1).

Results and Discussions

Biolog Analysis of *D. ginsengisoli* LA-4

A novel biphenyl-degrading bacterium, strain LA-4, was isolated from activated sludge, and the 16S rRNA gene sequence of strain LA-4 (GenBank No. EF191354) showed 98.22% homology to that of *D. ginsengisoli* Gsoil 3046 (Fig. 2). Therefore, strain LA-4 was identified as *D. ginsengisoli*. Phylogenetically, the genus *Dyella* clusters within the family *Xanthomonadaceae* of the class *Gammaproteobacteria*. There was little information on the genus *Dyella* with respect to biodegradation of environmental pollutants. The characteristic carbon-source utilization of strain LA-4 was investigated by Biolog Analysis, and the results were shown in Table 1. Numerous carbon sources could be utilized, and totally 35 species of carbon source were readily metabolized. In these carbon sources, Tween 40 and Tween 80, the surfactants could be used by strain LA-4 as the sole source of carbon and energy, which was important for accelerating the biodegradation of biphenyl. According to the results of Biolog analysis, no conclusive identification was obtained for strain LA-4, neither at the genus nor at the species level. The genus *Dyella* strain was first isolated from soil in 2005. And, the genus *Dyella* included four species such as *Dyella japonica*, *Dyella koreensis*, *Dyella yeojuensis*, and *D. ginsengisoli* (Fig. 1) [16–18]. Therefore, the Biolog database could not contain the information of genus *Dyella*.

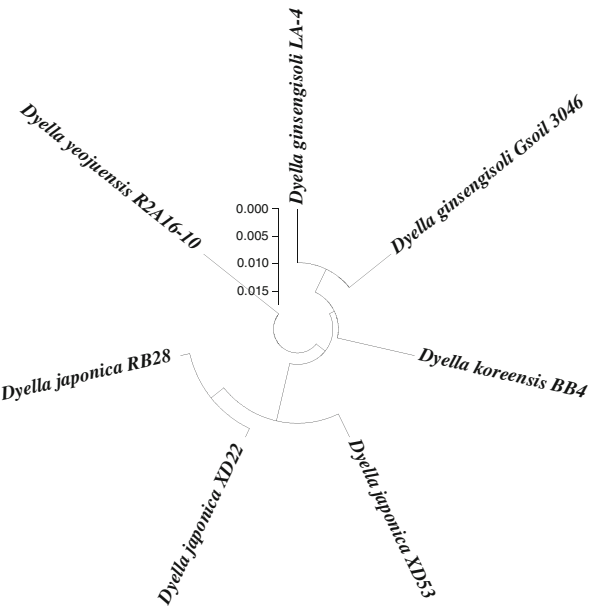

Fig. 1 The phylogenetic relationship of genus *Dyella*. GenBank number as follows: *D. ginsengisoli*LA-4 (EF191354), *D. ginsengisoli* Gsoil3046 (AB245367), *D. koreensis* BB4 (AY884571), *D. japonica* XD22 (AB110497), *D. japonica* XD53 (AB110498), *D. japonica* RB28 (DQ984127), *D. yeojuensis* R2A16-10 (DQ181549)

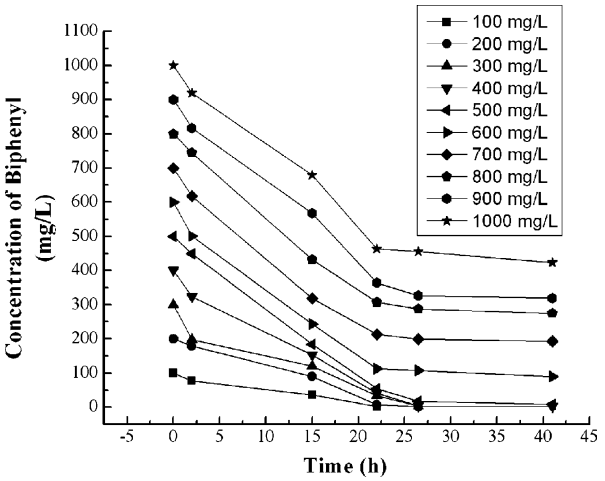

Fig. 2 Time courses of biphenyl degradation by resting cells of *D. ginsengisoli* LA-4

Biodegradation of Biphenyl by the Resting Cells of *D. ginsengisoli* LA-4

In this study, the resting cells of strain LA-4 was able to degrade biphenyl efficiently at 30 °C and 150 rpm. The biodegradation of biphenyl at various initial concentrations was investigated and results were shown in Fig. 2. The resting cells could utilize 100 mg L^{-1} biphenyl within 20 h. And, 500 mg L^{-1} biphenyl was degraded completely within 40 h. However, when the initial concentrations of biphenyl were more than 500 mg L^{-1}, the biodegradation of biphenyl was inhibited. When the initial concentration of biphenyl was 1,000 mg L^{-1}, over 400 mg L^{-1} biphenyl still remained in the culture. And, little biphenyl could be degraded when the inoculation time was more than 25 h.

Effects of Nonionic Surfactant—Tween 80 on the Biodegradation of Biphenyl

The nonionic surfactant could enhance contaminant bioavailability of PAHs, which was hazardous to the environment. According to the Biolog analysis, strain LA-4 could utilize Tween 80 as the sole source of carbon and energy. In this study, Tween 80 was selected to

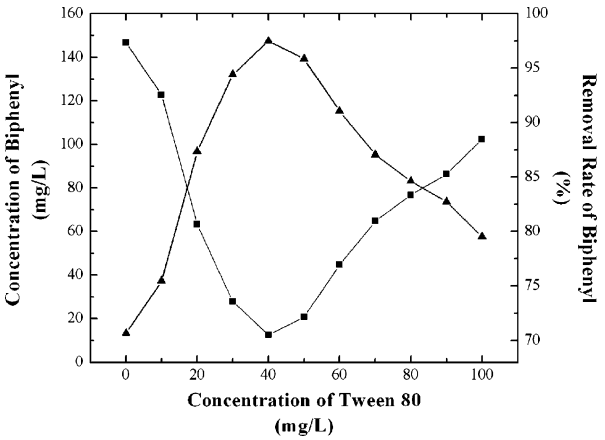

Fig. 3 Effects of nonionic surfactant—Tween 80 on the biodegradation of biphenyl. *Squares* concentration of biphenyl, *triangles* removal rate of biphenyl

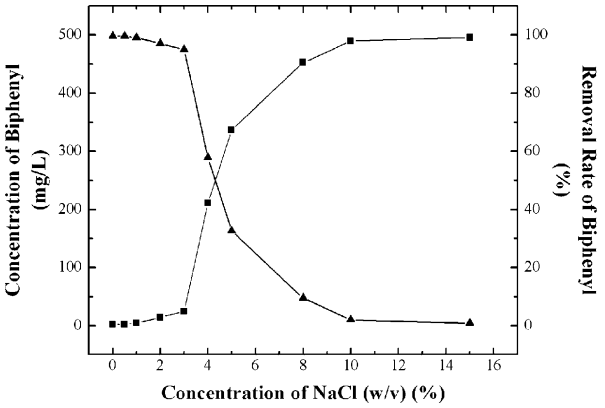

Fig. 4 Effects of NaCl on the biodegradation of biphenyl. *Squares* concentration of biphenyl, *triangles* removal rate of biphenyl

enhance water solubility and accelerate the biodegradation process (Fig. 3). The critical micelle concentration (CMC) of Tween 80 was 36.7 mg L^{-1}, which was taken from the comprehensive study of Patist et al. [19]. When the concentration of Tween 80 was less than CMC value, the removal rate of biphenyl was increased with the increase of Tween 80 concentration. It indicated that Tween 80 accelerated the biodegradation process of biphenyl. However, when the Tween 80 concentrations were greater than the CMC value, the removal rates of biphenyl was decreased. The reason was that surfactants reduced the actual concentration of biphenyl in the aqueous phase because of concentrating biphenyl in micelles [20]. Meanwhile, polycyclic aromatic hydrocarbon substrates in nonionic surfactant micelles have previously been shown not to be bioavailable [21]. The concentration of Tween 80 contained was 2 g L^{-1} in GN2 Microplate [22, 23] and was much higher than that used for accelerating the biphenyl degradation in this study. Consequently, successful integration of biphenyl and nonionic surfactant degradation was achieved by strain LA-4.

Effects of NaCl on the Biodegradation of Biphenyl

Effects of NaCl on the biphenyl degradation were determined, and the results were shown in Fig. 4. Strain LA-4 could grow on and degrade biphenyl in the medium containing 3%

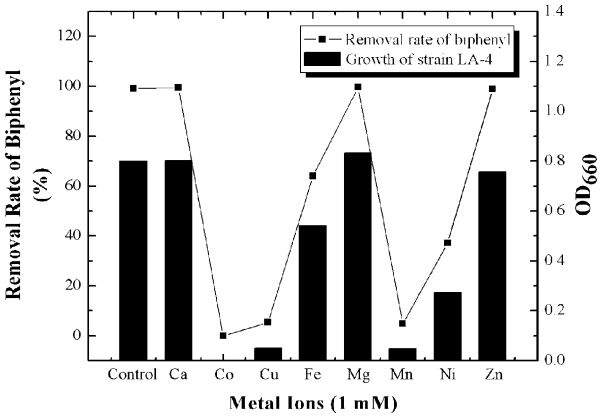

Fig. 5 Effects of metal ions on the biodegradation of biphenyl and growth of strain LA-4

sodium chloride (*w*/*v*). The removal rate of biphenyl was decreased rapidly when the concentration of NaCl was over 3%. And strain LA-4 could not degrade biphenyl when the concentration of NaCl exceeded 10%.

Effects of Metal Ions on the Biodegradation of Biphenyl

Study on the effects of metal ions on the biodegradation of biphenyl was necessary because metals have been widely distributed in soil and wastewater [13, 14]. The result indicated that the metal ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} had no effects on the biodegradation of biphenyl (Fig. 5). The biodegradation of biphenyl was partially inhibited in presence of Fe^{3+} and Ni^{2+}, and the removal rate was about 64% and 37%, respectively. However, other metal ions, especially Co^{2+}, can significantly inhibit the biodegradation. It demonstrated that strain LA-4 could be used to degrade biphenyl when the wastewater or soil polluted by some heavy metals. As shown in Fig. 5, effects of metal ions on the biodegradation of biphenyl were consistent with that on the growth of strain LA-4.

Conclusions

The present study confirmed that the use of the newly isolated *D. ginsengisoli* LA-4 for biodegradation of biphenyl was efficient. The effects of some factors (surfactant, NaCl, and metal ions) on biphenyl were investigated. The results showed that Tween 80 could accelerate the biodegradation of biphenyl, when the concentration of Tween 80 was less than CMC value. When salinity fluctuations were between 0% and 3%, strain LA-4 was able to degrade biphenyl efficiently. Meanwhile, the presences of 1 mM Ca^{2+}, Mg^{2+}, and Zn^{2+} had almost no effects on the microbial growth and biodegradation. And, biphenyl could be partially degraded in presence of 1 mM Fe^{3+} and Ni^{2+}. It suggested that *D. ginsengisoli* LA-4 could hold potential for field application.

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