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Degradation of quinoline by *Rhodococcus* sp. QL2 isolated from activated sludge

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ABSTRACT

A novel aerobic gram-positive bacterial strain capable of utilizing quinoline as sole source of carbon, nitrogen and energy was isolated from activated sludge of a coke plant wastewater treatment process. The isolate was identified as *Rhodococcus* sp. QL2 based on its morphology, physiochemical properties in addition to the results from 16S rDNA sequence analysis. The optimum temperature and the pH for its growth were 35–40 °C and 8.0, respectively. Extra nitrogen sources stimulated the bacterial growth on quinoline. Strain QL2 had strong quinoline degradability, and its degradation kinetics could be described with Haldane's model. Strain QL2 also had a broad range of substrate utilization. Identification of intermediates by GC/MS showed *Rhodococcus* sp. QL2 degraded quinoline via two pathways simultaneously.

1. Introduction

Quinoline, a typical representative of *N*-heterocyclic compounds, is widely used as raw materials and solvents in chemical industry, manufacture of dyes and pharmaceuticals. With an *N*-atom incorporated in the ring system, the water solubility is markedly enhanced; thereby, the bio-availability is increased with an accelerated risk to the environment. Due to its good solubility and low biodegradation, quinoline has become one of the common contaminants in ground water and soil, especially near landfills, coal tar distillation, as well as creosote wood preservation and fossil fuel facilities [1,2]. Many studies have shown that quinoline and its derivatives have toxic, carcinogenic and mutagenic activity to animals and humans [3–5].

In general, the biological treatment has advantages over physicochemical methods for quinoline treatment with less chemical agents, equipments and secondary pollution. Aerobic microbial degradation of quinoline has been studied extensively in the past [6–8]. In most cases quinoline-degrading microorganisms were members of the genus *Pseudomonas*, but other genera such as *Burkholderia* [9], *Nocardia* [10], *Rhodococcus* [11], *Comamonas* [12] have also been reported. Most of the previous studies are related to gram-negative bacteria, but less attention has been paid to gram-positive bacteria.

It is of significance to find more bacterial species with advantages such as wide availability, high environmental endurance and strong degradation capacity. Note that microorganisms in the coke plant biological treatment process suffer quinoline and related compounds chronically, quinoline-degrading bacteria with special characteristics could be expected in the biological process. Hence, activated sludge from a coke plant was chosen to isolate bacteria capable of degrading quinoline. In this study, a novel aerobic gram-positive bacterial strain *Rhodococcus* sp. QL2, which utilizes quinoline as sole source of carbon, nitrogen and energy, was isolated. Optimum growth conditions, substrate utilization and kinetics of quinoline biodegradation were investigated. Quinoline metabolites were analyzed by GC/MS and a unique degradation pathway was observed.

2. Materials and methods

2.1. Chemicals

2-Hydroxyquinoline and 7-hydroxycoumarin were obtained from ABCR. 2,6-Dihydroxyquinoline was purchased from Aldrich. 2,8-Dihydroxyquinoline and 3-(2,4-dihydroxyphenyl)propionic acid were from Fluka. The above chemicals were used as standard controls for identification of quinoline metabolites. *N,O*-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) used for derivatization was from Supelco. Methanol used as mobile phase in HPLC was purchased from Merck. Quinoline used for bacterial growth was purchased from Sigma. All other chemicals used for the preparation

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of media and other biochemical studies were of the analytical grade commercially available.

2.2. Culture medium

The mineral salt medium contained (MSM, $g\,L^{-1}$): 0.79 $K_2HPO_4{\cdot}3H_2O$, 0.2 $MgSO_4{\cdot}7H_2O$, 0.02 $FeSO_4{\cdot}7H_2O$, 1.0 NaCl and 1 mL of trace element stock solution. The trace element stock solution contained ($g\,L^{-1}$): 2.0 $CaCl_2{\cdot}2H_2O$, 0.2 $MnSO_4{\cdot}4H_2O$, 0.1 $CuSO_4{\cdot}2H_2O$, 0.2 $ZnSO_4{\cdot}7H_2O$, 0.09 $CoCl_2{\cdot}6H_2O$, 0.12 $Na_2MoO_4{\cdot}2H_2O$ and 0.006 H_3BO_3.

2.3. Isolation of bacterial strain

The initial enrichment culture was established by inoculating 100 mL sterile MSM containing 0.03% quinoline with 5 g activated sludge (wet weight) taken from a coke plant wastewater biological treatment process. The Erlenmeyer flask was incubated on a rotary shaker at 30 °C and 150 rpm. After one-week's incubation, portions were inoculated into fresh medium with quinoline concentration up to 0.05%, and incubation was continued. After five more transfers at 72 h intervals, the culture was purified by serially streak plating onto solidified MSM containing 0.05% quinoline. Finally, a pure strain was obtained and designated as QL2.

2.4. Identification of bacterial strain

2.4.1. Phenotypic and physiochemical characterization

Optical microscope (Olympus CX31, Japan) and transmission electron microscope (Philips CM10, Netherlands) were used for morphological observation. Physiological and biochemical properties of strain QL2 were determined by the procedures described by Dong and Cai [13].

2.4.2. DNA extraction and sequencing

The DNA of the QL2 culture was obtained using a commercial genomic DNA extraction kit (BioTeke Corp., China). 16S rRNA gene of the strain was amplified from the bacterial genomic DNA by a PCR using universal primers of F27 (5′-AGAGTTTGATCATGGCTCAG-3′) and R1492 (5′- TACGGTTACCTTGTTACGACTT-3′) described by Heuer et al. [14]. PCR amplification condition was as follows: each PCR mixture (50 μL) was composed of 2 μL genomic DNA (≈0.1 μg); 4 μL of dNTP at 2.5 mM; 5 μL of 10 × *Pyrobest* Buffer; 0.25 μL of 5 unit *Pyrobest* DNA Polymerase; 2 μL of the primers and sterile water. The PCR was performed in a PTC-100 Peltier Thermal Cycler (MJ Research, USA) with a hot starting performed at 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

The 16S rRNA gene of about 1.5 kb was purified using a gel extraction kit, and the purified product was ligated with pMD 18-T vector (Takara, Japan). Then the ligation product was transformed into competent bacterial strain JM109 (Takara, Japan), and the positive clones were picked out according to blue/white screening. The recombinant plasmid was extracted from the positive clone according to alkaline lysis method [15] and sequenced by the Chinese National Human Genome Center.

2.4.3. Sequence analysis and construction of a phylogenetic tree

The 16S rDNA sequence of QL2 and its related references retrieved from GenBank were aligned using BioEdit. A phylogenetic tree was constructed according to the neighbor-joining method [16] with the program MEGA 3.1. The accession number of the sequence in GenBank is EF079074.

2.5. The environmental factors analysis

Effects of environmental factors on the growth of QL2 were investigated in 250 mL Erlenmeyer flasks. Each flask was added with 100 mL MSM containing 150 $mg\,L^{-1}$ quinoline and 1 mL of enriched culture with a biomass content of $47.8 \pm 3.3\ mg\,L^{-1}$. Tests on the effect of temperature were first examined at seven levels, 20, 25, 30, 35, 37, 40 and 45 °C, at pH 7. These were followed by tests on the effect of pH at six levels from pH 5 to 10 at the identified optimal temperature. Then four nitrogen-containing compounds, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, and urea were chosen for evaluating the effects of additional nitrogen sources on the bacterial growth. The specific growth rate was defined as an increase in OD_{600} during the logarithmic growth phase.

2.6. Degradation of quinoline and others

Experiments on quinoline degradation were carried at the optimal pH and temperature in 250 mL Erlenmeyer flasks at 150 rpm. Each flask contained 1 mL of enrichment culture ($47.8 \pm 3.3\ mg\,L^{-1}$) and 100 mL MSM with quinoline concentration varying from 60 to 700 $mg\,L^{-1}$. Fermentation broths were taken at intervals of 1–2 h for quinoline and biomass analyses. The experiments were carried out in triplicates, with both uninoculated flasks and flasks without quinoline serving as controls. Uninoculated flasks with quinoline were used for monitoring any abiotic loss of substrates during incubation. The specific degradation rate was calculated from the obtained degradation curves by dividing the slope of the degradation curve by the associated concentration of the biomass.

To test the ability of strain QL2 utilizing various aromatic compounds, phenol, pyridine, indole, phthalate, salicylic acid, naphthalene, phenanthrene, xylene, benzoate, catechol, and protocatechuate were chosen as carbon sources. The MSM was supplemented with 0.1% ammonium sulfate to provide nitrogen source. The isolate was grown in the medium containing one of those substrates. All cultures contained 100 $mg\,L^{-1}$ of the given substrate, with the exception of phenanthrene and xylene added at 50 $mg\,L^{-1}$. Samples from the culture, as well as from sterile controls, were collected and examined for bacterial growth (OD_{600}) after incubation for 2 days except phenanthrene and xylene for 7 days.

2.7. Detection of quinoline and its metabolites

Quinoline concentrations of all samples were analyzed by HPLC system (Agilent 1100 with an UV detector). Samples were prepared by centrifugation, 5 μL of which was injected. Separation was carried out in a ZORBAX SB-C18 reverse-phase column (4.6 mm × 250 mm × 5 μm). The mobile phase was a mixture of methanol and water (60:40, v/v), and the flow rate set at 1 $mL\,min^{-1}$. Quinoline was detected at 275 nm wavelength.

To obtain intermediate products of quinoline degradation, the microorganism was grown in MSM with quinoline at 500 $mg\,L^{-1}$ under the optimum incubation condition. The culture samples were collected every 4 h followed by centrifugation. The supernatants were extracted by ethyl acetate under neutral and acid conditions, with the pH 7.5 and 2.0, respectively. The extracts were dried over anhydrous sodium sulfate, filtered, and subsequently evaporated to dryness under a gentle N_2 stream. The residue was dissolved in ethyl acetate for GC/MS determination. Part of the residue was silylated with BSTFA and analyzed by GC/MS.

GC/MS analysis was performed by an Agilent GC 6890N/MSD 5975 apparatus with a HP-35 fused silica capillary column (30 m × 250 μm × 0.25 μm). The column temperature program was 40 °C isothermal for 5 min and then from 40 °C to 300 °C with an increment of $7\,°C\,min^{-1}$.

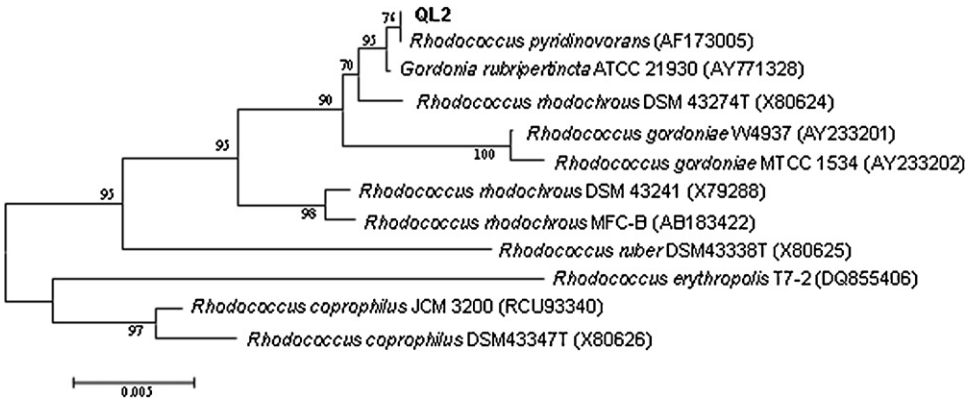

Fig. 1. Phylogenetic tree based on the 16S rDNA sequence of strain QL2 with other reference sequences. The numbers at the forks indicate the bootstrap values in percentage. Bar indicates the nucleotide difference per sequence position. The accession numbers of the sequences are given in parentheses.

3. Results and discussion

3.1. Identification of the quinoline-degrading bacterium

The strain was a gram-positive, non-flagellated, short rod-to-coccus-shaped species of bacteria with a size of (1.1–1.5) μm × 0.9 μm. The colony showed salmon pink, smooth on the surface and trim on the edge. Physiochemical properties of strain QL2 were listed in Table 1. Phenotypic and physiochemical tests suggest that strain QL2 is similar to genus *Rhodococcus*.

Based on the 16S rDNA sequence alignment and phylogenetic tree analyses (Fig. 1), strain QL2 was homologous with *Rhodococcus* spp. The organism that exhibited the highest level of homology (100%) was *Rhodococcus pyridinivorans* (AF173005). Compared with the results presented by the type strain of *R. pyridinovorans* [17], QL2 seems to be a strain of *R. pyridinivorans*, except that it was positive for fermentation of rhamnose and lactose. On the basis of phenotypic and genotypic characteristics, strain QL2 is clearly a member of the genus *Rhodococcus*, as can be substantiated from a variety of biochemical properties and the analysis of the 16S rDNA sequence.

3.2. Effect of environmental factors

3.2.1. Temperature and pH

Fig. 2 reveals that strain QL2 is a mesophilic bacterium. The optimum growth temperature was between 35 and 40 °C. However, specific growth rate decreased sharply above 40 °C and strong growth inhibition of strain QL2 occurred at 45 °C.

The relationship between the specific growth rate and pH is shown in Fig. 3. The specific growth rate of strain QL2 increased quickly when pH increased from 5.0 to 8.0. The highest specific growth rate was observed at pH 8.0. When pH was above 8.0, the specific growth rate decreased gently.

Table 1
Physiochemical properties of strain QL2

Characteristics	QL2	Characteristics	QL2
Oxidase	–	Utilization of sodium lactate	+
Catalase	+	Gelatin liquefaction	+
Hydrolysis of starch	–	M.R. test	–
Nitrate reduction	+	V–P test	–
Fermentation/oxidation		Growth at temperature (°C)	
Rhamnose	+	20–41	+
Lactose	+	Growth at pH	
Maltose	–	5.0–8.0	+
Glucose	–	Growth on NaCl (%)	
Sucrose	–	9.0–10.0	+
Inositol	+	12.0	–

+, positive reaction; –, negative reaction.

3.2.2. Nitrogen source

Extra nitrogen sources all stimulated the growth of the strain. The sequences of their promotional effects were as follows: $(NH_4)_2SO_4$ > NH_4NO_3 > $NaNO_3$ > Urea. Therefore, the strain grows better on inorganic nitrogen relative to organic nitrogen, and better

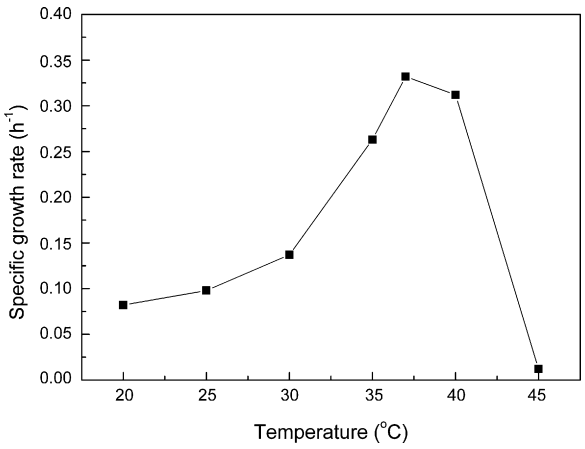

Fig. 2. The specific growth rate of strain QL2 at different temperature (quinoline: 150 mg L^{-1}, pH 7.0, 150 rpm). The specific growth rate was derived from the cell density of OD_{600} and the exponential cells growth period (measured every 2 h).

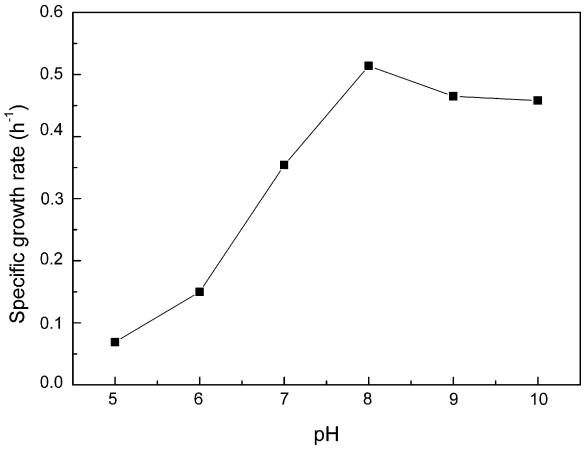

Fig. 3. The specific growth rate of strain QL2 at different pH (quinoline: 150 mg L^{-1}, 37 °C, and 150 rpm).

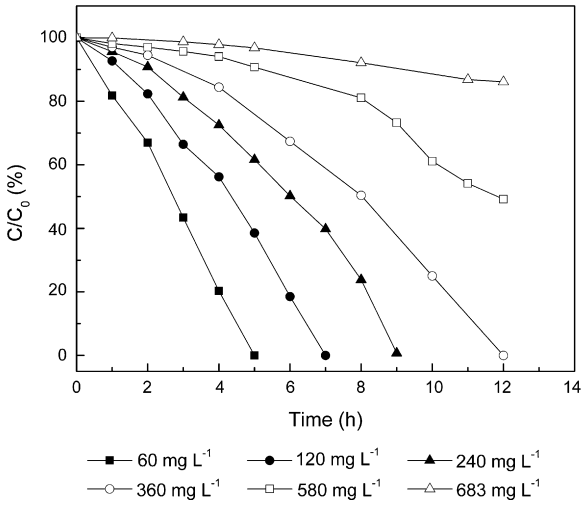

Fig. 4. Quinoline degradation by *Rhodococcus* sp. QL2 at different initial concentrations (37 °C, pH 8.0, 150 rpm). C is actual quinoline concentration, and C_0 is initial quinoline concentration.

on ammonium nitrogen relative to nitrate nitrogen.

3.3. Degradation kinetics analysis

Different initial quinoline concentrations were assessed as shown in Fig. 4. It can be seen that *Rhodococcus* sp. QL2 has strong degradation capacity of quinoline, especially at low substrate concentration (60–120 mg L^{-1}). When the initial quinoline concentrations were 60 and 120 mg L^{-1}, *Rhodococcus* sp. QL2 could degrade quinoline within 5 and 7 h, respectively. No appreciable loss of quinoline was observed in the sterile control. 60–120 mg L^{-1} are quinoline concentrations detected in most contaminated soil and discharged wastewater, so the strain has a great advantage of solving practical quinoline pollution.

When the initial substrate concentration was up to 683 mg L^{-1}, obvious inhibition appeared. The rate of quinoline degradation increased with increasing substrate concentration up to 240 mg L^{-1}, whereas it decreased at higher quinoline concentrations owing to substrate inhibition (Fig. 5). The experimental results could be well described by Haldane's inhibition model (Eq. (1)) [18]

$$\nu = \frac{\nu_{max} S}{K_s + S + (S^2/K_i)} \quad (1)$$

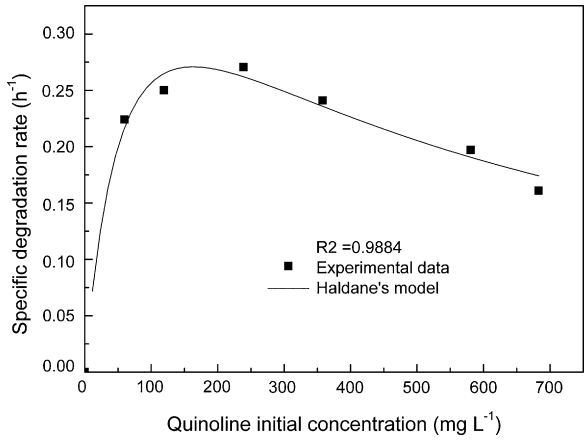

Fig. 5. Calculated and measured specific quinoline degradation rate by strain QL2. The specific degradation rate was derived from quinoline concentration, biomass concentration, and the exponential cells growth period (measured every 2 h).

where ν denotes the specific degradation rate of quinoline (h^{-1}); ν_{max}, the maximum specific degradation rate of quinoline (h^{-1}); K_s, the half-saturation constant for quinoline degradation kinetics (mg L^{-1}); K_i, the inhibition constant for quinoline degradation kinetics (mg L^{-1}).

The optimal kinetic parameters calibrated from experimental data in terms of Eq. (1) were $\nu_{max} = 0.499\ h^{-1}$, $K_s = 68.7$ mg L^{-1}, and $K_i = 387.1$ mg L^{-1}. Fig. 5 shows that the substrate inhibition model reasonably describes the relationship between the specific degradation rate and the quinoline concentration. Similar substrate inhibition model was also found in quinoline degradation by *Burkholderia picekttii* [19].

3.4. Substrate utilization of Rhodococcus sp. QL2

There are many contaminants existing together with quinoline in the environment. Therefore, microorganisms, which have a broad range of substrate utilization are of significance in practical applications. Utilization of different aromatic compounds as sole substrates by strain QL2 was evidenced by significant increase of cell optical densities in the test flasks after 2 days inoculation compared with the initial cell optical densities. The results showed that strain QL2 could use pyridine, indole, phenol, benzoate, phthalate, protocatechuate, catechol, salicylic acid, and naphthalene as the sole carbon source except xylene and phenanthrene. In general, strain QL2 has a strong environmental adaptability with a broad range of substrate utilization. There are differences between QL2 and previous quinoline-degrading *Rhodococcus* species. Strain QL2 was isolated from activated sludge of a coke plant in China. *Rhodococcus* Q1 reported by O'Loughlin et al. [11] was isolated from soils in the United States, and it could not utilize phthalate. The three *Rhodococcus* species reported by Schwarz et al. [7] were isolated from activated sludge of a sewage treatment plant in Germany, and none could utilize protocatechuate and naphthalene.

3.5. Identification of intermediates

Pigment production during quinoline biodegradation has been observed in previous quinoline-degrading *Rhodococcus* species. In this study, the fermentation broth produced a pink pigment, and

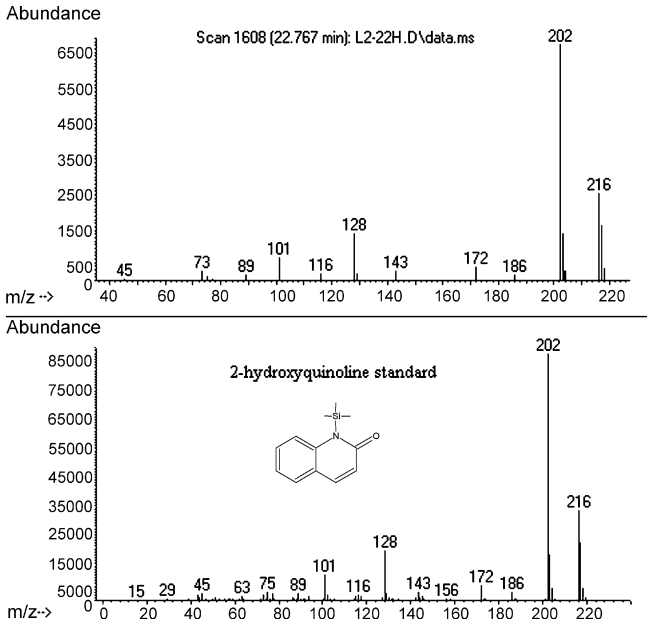

Fig. 6. Comparison of MS data of a metabolite of quinoline produced by *Rhodococcus* sp. QL2 with authentic 2-hydroxyquinoline.

then color turned to brownish-red gradually. Sometimes color became green rapidly after the pink pigment appeared, followed by a dark green pigment. Similar results were observed by *Rhodococcus* sp. B1 reported by Schwarz et al. [20]. But other reported *Rhodococcus* species exhibited different phenomena, i.e. Q1 reported by O'Loughlin et al. [11] produced green to greenish-brown pigments when grown on quinoline, but did not produce a pink pigment. These phenomena indicated that several intermediates were produced during the biodegradation of quinoline.

In the present study three intermediates were identified by GC/MS. They were 2-hydroxyquinoline, 2,6-dihydroxyquinoline and 8-hydroxycoumarin, respectively. These metabolites were not detected in sterile controls. 2-Hydroxyquinoline was mainly found in extracts of neutral fraction, and it was the first intermediate, which was identified with silylation (Fig. 6) and without silylation (not shown).

2,6-Dihydroxyquinoline was found only in extracts of acid fraction and by derivatization. This intermediate cannot be extracted by ethyl acetate in neutral solution possibly due to formation of an ionized state. It is supposed that the molecular intermediate was initially formed during degradation, followed by ionization in aqueous solution. Retention time of authentic 2,6-dihydroxyquinoline and 2,8-dihydroxyquinoline was found very close, but the mass spectrum of derivatized compounds could be quite different, so the metabolite in our work could be identified through mass spectrum. MS data comparing the metabolite with authentic 2,6-dihydroxyquinoline and 2,8-dihydroxyquinoline is shown in Fig. 7. Through comparisons of characteristic fragment ions and peak abundances, the metabolite was identified as 2,6-dihydroxyquinoline.

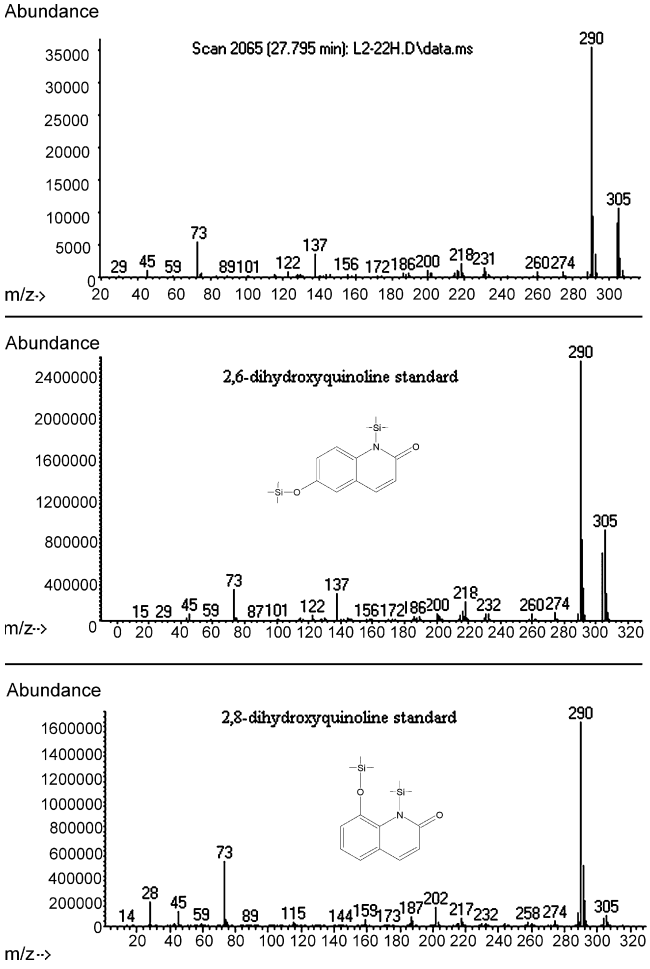

Fig. 7. Comparison of MS data of a metabolite of quinoline produced by *Rhodococcus* sp. QL2 with authentic 2,6-dihydroxyquinoline and 2,8-dihydroxyquinoline.

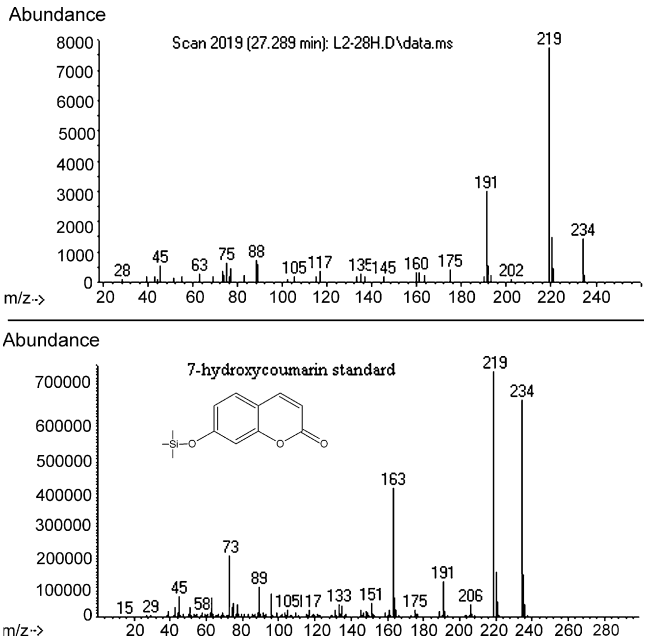

Fig. 8. Comparison of MS data of a metabolite of quinoline produced by *Rhodococcus* sp. QL2 with authentic 7-hydroxycoumarin.

8-Hydroxycoumarin was found only in neutral extracts and by derivatization. Because 8-hydroxycoumarin is not commercially available, 7-hydroxycoumarin was used as a standard in the experiment. The metabolite had a somewhat shorter retention time in GC analysis than 7-hydroxycoumarin, but it yielded a mass spectrum identical to that of 7-hydroxycoumarin. According to the mass spectrum (Fig. 8), it was identified as 8-hydroxycoumarin. The molecular ion was *m*/*z* 234 [M^+], and other main peaks were 219 [M^+-CH_3], 202 [M^+-2CH_3-2H], 191 [M^+-3CH_3+2H], 160 [$M^+-Si(CH_3)_3-H$], 145 [$M^+-OSi(CH_3)_3$], 117 [$M^+-OSi(CH_3)_3-CO$], and 88 [$M^+-OSi(CH_3)_3-CO-CHO$].

The microbial degradation of quinoline has been widely investigated by different researchers [20–22]. In all cases, 2-hydroxyquinoline was found to be the first intermediate in the degradation pathway. Subsequent degradation may go through two pathways by different bacteria. "5,6-Dihydroxy-1*H*-2-oxoquinoline pathway" involves preferential cleavage of benzene ring [12,20,23], and "8-hydroxycoumarin pathway" involves cleavage of *N*-heterocyclic ring prior to benzene ring cleavage [20,22]. Interestingly, from previous researches, 2,6-dihydroxyquinoline and 8-hydroxycoumarin belong to different pathways, but they both existed in the degradation of quinoline by strain QL2. Based on the previous results, a unique metabolic pathway for the degradation of quinoline by *Rhodococcus* sp. QL2 is proposed that quinoline degradation is initiated by hydroxylation at position 2 to form 2-hydroxyquinoline, which is then oxidized to 2,6-dihydroxyquinoline and 8-hydroxycoumarin, respectively via two pathways. As no other intermediates were observed in the experiments, the sequential cleavage was still unclear.

4. Conclusions

Strain QL2 capable of degrading quinoline was isolated from activated sludge in a coke wastewater treatment plant. According to the morphological, physiochemical characteristics, and sequence analysis of 16S rDNA, the strain was identified as *Rhodococcus* sp.

The optimal growth temperature of strain QL2 is 35–40 °C, and the optimal pH is observed at 8.0. The strain grows better on inorganic nitrogen than that on organic nitrogen, and better on ammonium nitrogen than that on nitrate nitrogen. The kinetic analysis showed that quinoline degradation by strain QL2 could be well described by the Haldane's inhibitory model with kinetic parameters such as $\nu_{max} = 0.499\ h^{-1}$, $K_s = 68.7\ mg\ L^{-1}$, and $K_i = 387.1\ mg\ L^{-1}$.

Rhodococcus sp. QL2 has a broad range of substrate utilization, capable of degrading pyridine, indole, and other aromatic compounds. Special pigments were produced in the process of quinoline biodegradation, whilst three metabolites were identified through silylation followed by GC/MS analysis. Production of 2,6-dihydroxyquinoline and 8-hydroxycoumarin indicated that quinoline degradation pathway by *Rhodococcus* sp. QL2 was unique, via 5,6-dihydroxy-1*H*-2-oxoquinoline and 8-hydroxycoumrain pathways simultaneously. In summary, *Rhodococcus* sp. QL2 could be used as a potential candidate for the bioaugementation process of quinoline-containing wastes because of its high degradation efficiency and diverse capacity of aromatic ring degradation.

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