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Characterization and biodegradation kinetics of a new cold-adapted carbamazepine-degrading bacterium, *Pseudomonas* sp. CBZ-4

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Abstract

Carbamazepine is frequently detected in waters and hardly eliminated during conventional wastewater treatment processes due to its complicated chemical structure and resistance to biodegradation. A carbamazepine-degrading bacterium named CBZ-4 was isolated at a low temperature (10°C) from activated sludge in a municipal wastewater treatment plant. Strain CBZ-4, which can use carbamazepine as its sole source of carbon and energy, was identified as *Pseudomonas* sp. by the 16S rRNA gene sequence. The composition and percentage of fatty acids, which can reveal the cold-adaptation mechanism of strain CBZ-4, were determined. Strain CBZ-4 can effectively degrade carbamazepine at optimal conditions: pH 7.0, 10°C, 150 r/min rotation speed, and 13% inoculation volume. The average removal rate of carbamazepine was 46.6% after 144 hr of incubation. The biodegradation kinetics of carbamazepine by CBZ-4 was fitted via the Monod model. $V_{\text{max}}$ and $K_s$ were found to be 0.0094 hr⁻¹ and 32.5 mg/L, respectively.

Key words: carbamazepine; low temperatures; biodegradation kinetics; *Pseudomonas* sp.

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Introduction

Carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide, CBZ), an emerging contaminant from pharmaceutical and personal care products (PPCPs), is frequently detected in the environment, especially in aquatic habitats (Bendz et al., 2005). CBZ is an anti-epileptic drug and widely used to cure trigeminal neuralgias as well as acute mania. Approximately 72% of oral CBZ can be absorbed, metabolized, and excreted with urine, whereas 28% is unchanged and discharged into waters through feces. Therefore, CBZ is usually considered as an indicator of PPCP pollution.

Biodegradation is expected to be an effective method for substantial CBZ removal. However, the average removal rate of CBZ is below 10% by the conventional activated sludge (CAS) process, membrane bioreactors (MBR), and sequencing batch reactors (Zhang et al., 2008). Only 15% of CBZ is reported to be removed by biological transformation (Clara et al., 2004; Joss et al., 2005). The fact that CBZ is fairly persistent and difficult to eliminate effectively by biodegradation can be explained by its properties.

CBZ has active pharmaceutical ingredients and is resistant to biodegradation (Zhang et al., 2008). In addition, it is difficult to attach onto activated sludge and be degraded by microorganisms due to its low distribution coefficient between the aqueous and sludge phases (Ternes et al., 2004).

Previous reports indicate that high temperatures improve the biological transformation of PPCP compounds, whereas low temperatures seriously inhibit the biodegradation and elimination of PPCPs and other refractory compounds (Ternes et al., 1999; Suárez et al., 2008). The average temperature during the long winters of northeast China is approximately –10°C to –20°C, which significantly affects the removal rate of CBZ in municipal wastewater treatment plants (MWTPs). Therefore, a novel cold-adapted CBZ-degrading bacterium needs to be isolated to realize effective environmental CBZ biodegradation.

CBZ exerts relatively limited ecotoxicity on organisms (Ferrari et al., 2003; Andreozzi et al., 2002). However, European legislation has classified CBZ as a harmful substance to aquatic organisms, causing long-term adverse effects in aquatic environments (Marco-Urrea et al., 2010).
Therefore, precautions and effective CBZ removal should be undertaken to avoid potential risks. To date, the reports on CBZ biodegradation are rare and have mainly focused on mesophilic microorganisms (Cui et al., 2009). The objective of this research was to determine the biodegradation characteristics and kinetics of a new cold-adapted CBZ-degrading bacterium for the first time.

1 Materials and methods

1.1 Chemicals

CBZ (CAS 298-46-4, 99%) was purchased from Acros Organics, USA. Methanol (CAS 67-56-1, HPLC grade) was purchased from Sigma-Aldrich, USA. Ethyl acetate (CAS 141-78-6, HPLC grade) was obtained from Amethyst Chemicals, J&K Scientific Ltd., China. The other chemicals were analytical grade and obtained from Kermel Chemical Reagent Ltd., Tianjin, China.

1.2 Media and culture conditions

The culture medium was modified according to Dominic and Graham’s culture medium (Corti et al., 1995; John et al., 1998) and comprised 1.5 g KH₂PO₄, 0.15 g MgSO₄·7H₂O, 0.5 g (NH₄)₂SO₄, 0.5 g NaCl, 3.5 g K₂HPO₄, 0.1 g CBZ, and 1.0 mL trace elements per liter of solution. The pH was adjusted to 7.0. The screened strains were conserved with the medium containing 2 g peptone per liter used as the screening medium. The trace elements solution contained 0.1 g CuSO₄·5H₂O, 0.3 g MnSO₄·4H₂O, 0.2 g ZnSO₄·7H₂O, 2.0 g NaHCO₃·10H₂O, 0.5 g CoCl₂·6H₂O, 0.05 g CaCl₂·2H₂O, and 0.5 g FeSO₄·7H₂O, 0.02 g (NH₄)₂MoO₄·2H₂O per liter. The media were autoclaved at 121°C for 20 min. The strain was cultivated aerobically at 10°C in the media.

1.3 Isolation and identification of CBZ-degrading strains

Activated sludge was sampled from Harbin Taiping MWTP in China and used as inocula for enrichment cultivation. Enrichment was performed in the CBZ liquid medium (100 mg/L) with 10 mL of activated sludge. After first undergoing aerobic incubation for 12 days at 10°C and rotation speed of 150 r/min in a shaker (ZHWY-2102C, ZHICHENG®, Shanghai Zhicheng Machinery Equipment, Shanghai, China), the 10% enriched culture was inoculated into a fresh medium for a second enrichment. Serial 10-fold dilutions were dispensed on agar medium, and the plates were aerobically incubated at 10°C for 10 days. Different morphologies of colonies were chosen and isolated by repeated streak culturing. The growths of these strains were simultaneously examined with the absorbance at 660 nm, and the most efficient strain was preliminarily selected for subsequent experiments.

The selected CBZ-degrading bacterium CBZ-4 was identified by 16S rRNA gene sequencing. Genomic DNA was extracted using a modified technique bacterial genomic DNA mini kit (TaKaRa, Dalian, China). The 16S rRNA gene was amplified by PCR using the primers 8F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1522R (5′-AAGGACGTCAATCCAGCGCA-3′) (Neilan, 1995). The PCR product was sequenced by GenScript Biotechnology Co. Ltd. (Nanjing, China), and the obtained sequence was compared with sequences in the GenBank database using the BLAST program. The 16S rRNA gene sequence of strain CBZ-4 and the related sequences retrieved from GenBank were aligned using Clustal W to construct a phylogenetic tree with the Jukes-Cantor distance correction matrix method and neighbor-joining method. The branching pattern was checked by 1000 bootstrap replicates.

1.4 Analytical methods

Residual CBZ was extracted with an equal volume of ethyl acetate with vibration for 10 min. The organic phase was collected when the water phase was separated from the upper organic phase. Extraction was carried out again, and the organic phases were combined. The organic phases were evaporated on a rotary evaporator to 5 mL at 40°C and 80 r/min, and then blown to dryness with nitrogen gas using a nitrogen blowing instrument (TTL-DCI, Tongtailian Tech., Beijing, China) at 40°C. The residue was dissolved with 1 mL of methanol and then filtered through 0.45 μm pore size membrane filters for HPLC analysis.

The CBZ concentration was determined using a Shimadzu LC-10AT HPLC instrument (Shimadzu, Japan) equipped with a Venusil MP-C18 chromatogram column (4.5 × 150 mm × 5 μm, Agela Technologies Inc., USA). The mobile phase was a mixture of methyl cyanide and ultrapure water (40/60, V/V). The absorbance of the sample was measured at 285 nm by an SPD-10A ultraviolet detector ( Shimadzu, Japan), and 10 μL of the sample was injected at a flow rate of 1.0 mL/min. The column temperature was maintained at 35°C with a reservation time of 8 min.

Bacterial growth was determined by measuring the absorbance at 660 nm (OD₆₆₀ nm) and the protein concentration was measured by the Coomassie Brilliant Blue method using cattle blood serum albumin as the standard. The activity of the CBZ-degrading strain was detected by measuring the electron transport system activity (ET-SA) according to Relexans (1996). The 0.5 mL sample and 1.0 mL of 0.2% iodonitrotetrazolium chloride (INT) solution was injected into a 10 mL EP tube to react for 15–30 min in the dark. A 50-μL of formaldehyde was added to end the reaction. Cells were collected by centrifugation at 10,000 × g for 5 min and the residual was resuspended with 2.5 mL 96% methanol and centrifuged under identical conditions. Absorbance at 495 nm (OD₄₉₅ nm) was monitored with 7 mL of 96% methanol and 2 mL INT as the blank.
ETSA (μg O₂/(g·hr)) was calculated by Eq. (1):

\[ \text{ETSA} = \frac{A \times V \times 32}{15.9 \times 2 \times S \times t} \]  

(1)

where, \( A \) is the OD\(_{95\text{nm}}\), \( V \) (mL) is the methanol volume used in re-suspension, \( S \) (mL) is the sample volume, \( t \) (hr) is reaction time.

1.5 Growth and CBZ-degrading characterizations of strain CBZ-4

Experiments on the growth and CBZ-degrading characterization of strain CBZ-4 were performed to confirm the effects of pH, temperature, inoculation volume, and rotation speed of the shaker on the CBZ-degrading strain growth and degrading characteristics. A certain concentration of cultivation solution was aerobically incubated for 7 days, and each experiment was conducted in duplicate. Under the optimal conditions of biodegradation, the CBZ-degrading strain CBZ-4 was continuously incubated for 144 hr and monitored every 12 hr. The biodegradation curve reflects the change in CBZ concentration, OD\(_{660\text{nm}}\), and ETSA with time.

1.6 Measurement of fatty acid composition and percentage

The fatty composition and percentage were determined by gas chromatographic analysis of fatty acid methyl esters (Agilent 6850, USA). Fatty acids containing 9–20 carbons were analyzed by SHERLOCK® software (MIDI Corporation, USA), and the peaks were automatically named and quantitated by the system. The medium consisted of 30 g trypticase soy broth and 15 g agar. Strain CBZ-4 was aerobically incubated at 28°C with a quadrant streak culture. The sample pretreatment comprised 5 steps: harvesting, saponification, methylation, extraction, and base wash. The sample was detected via gas chromatography (Agilent 6850, USA) using a capillary column (30 mm × 0.25 mm) with an enhanced chemiluminescence detector. The temperature program was ramped from 170°C to 270°C at 5°C/min, followed by a ballistic increase to 300°C for 2 min. The flow rate was 1.0 mL/min using hydrogen as the carrier gas and nitrogen as the complementary gas. The compositions of the samples were compared with database data.

1.7 Growth of strain CBZ-4 on other aromatic compounds

Certain concentrations of different aromatic compounds were added as the sole carbon and energy source in a modified Dominic and Graham’s culture medium without CBZ. The samples were aerobically incubated for 10 days under the optimal degradation conditions. The growth of strain CBZ-4 was determined by its optical density (OD\(_{600\text{nm}}\)) using a scanning spectrophotometer (UV-2550, Shimadzu, Japan). A sample with strain CBZ-4 but without aromatic compound was used as a control.

1.8 Preparation of resting cells of CBZ-degrading bacterium

Strain CBZ-4 was inoculated for 72 hr under the optimal conditions with CBZ as the sole source of carbon and energy. The obtained cells were transferred to 500 mL of conservation medium for subsequent cultivation until OD\(_{660\text{nm}}\) was 1.0. The sample was centrifuged at 4°C and 8000 r/min for 10 min. The residual was washed twice and resuspended with 20 mol/L phosphate buffer (pH 8.0). The resting cells were stored at 4°C for use.

1.9 Biodegradation kinetics of CBZ-degrading strain

Biodegradation tests were performed at different initial concentrations of CBZ and identical volumes of resting cell solutions under the optimal conditions. The CBZ concentration was monitored every 12 hr to determine the biodegradation capability of strain CBZ-4. Based on the experimental data and math software, the Monod model was used to estimate the biodegradation kinetic behavior of the CBZ-degrading bacterium (Eq. (2)).

\[ V = \frac{V_{\text{max}} S}{K_s + S} \]  

(2)

where, \( V \) (hr\(^{-1}\)) is the specific removal rate of substrate, \( V_{\text{max}} \) (hr\(^{-1}\)) is the maximum specific removal rate of substrate, \( S \) (mg/L) is the CBZ concentration, and \( K_s \) (mg/L) is the half-saturation constant or CBZ concentration at half \( V_{\text{max}} \). According to physics, \( V \) was calculated by Eq. (3):

\[ V = \frac{-dS}{dx} = \frac{d(S_0 - S)}{dx} \]  

(3)

therefore

\[ \frac{-dS}{dx} = \frac{V_{\text{max}} S}{K_s + S} \]  

(4)

when \( S \gg K_s \)

\[ V = V_{\text{max}} \]

\[ -\frac{dS}{dx} = V_{\text{max}} x \]

(5)

where, \( S_0 \) (mg/L) is initial CBZ concentration, \( S \) (mg/L) is residual CBZ concentration, \( K_1 \) (L/(mg·hr)) is the reaction rate constant of zero-order, \( x \) (mg/L) is the initial bacterial biomass. Thus, the prediction of substrate degradation has no relation with the organic substrate concentration and resembles a zero-order reaction model. The function above is processed by an integral operation; therefore, \( S \) was calculated by Eq. (6):

\[ S = -mt + n \]  

(6)

where, \( m \) (mg/(L·hr)) is the degradation rate constant of zero-order kinetics, \( t \) (hr) is the degradation time, and \( n \) is a constant.
when \( S \ll K_s \)

\[
V = \frac{V_{\text{max}}S}{K_s} = K_2S, \quad \frac{-dS}{dt} = K_2S
\]  

(7)

which belongs to a first-order reaction model. \( K_2 \) \((L/(mg\cdot hr))\) is the reaction rate constant of first-order. By mathematical calculation of Eq. (8):

\[
\ln S = -kt + b
\]

(8)

where, \( k \) \((mg/(L\cdot hr))\) is the degradation rate constant of the first-order kinetic and \( b \) is a constant. The substrate concentration is the restriction factor of substrate biodegradation.

1.10 Nucleotide sequence accession numbers

The sequences of the 16S rRNA gene from \( Pseudomonas \) sp. CBZ-4 were deposited in the GenBank database under accession number JQ782892.

2 Results

2.1 Isolation and identification of the CBZ-degrading bacterium

Colonies were obtained from the enrichment cultivation solution, and 16 pure strains were repeatedly screened by streak culturing. The strains can grow using CBZ as the sole source of carbon and nitrogen. Strain CBZ-4 grew well and had the highest absorbance ratio by biomass test \((\text{OD}_{660\text{nm}})\) and was thus selected as the experimental strain. The colony of strain CBZ-4 was small, circular, ivory white, slightly rose, and could be picked up easily. The shape of strain CBZ-4 was a short bar, as observed under an atomic force microscope (Fig. 1). Strain CBZ-4 was identified by its 16S rRNA sequence, and had the highest similarity (99%) with \( Pseudomonas \) sp. Nj-59 (accession no. AM491463). A phylogenetic tree was constructed, as shown in Fig. 2. Strain CBZ-4 was deposited in the China General Microorganism Culture Center with accession number 5943.

2.2 Growth and degrading characterization of the CBZ-degrading strain CBZ-4

The impacts on the bacterial growth and CBZ removal rate of the pH, temperature, inoculation volume, and rotation speed of shaker are illustrated in Fig. 3. At \( pH \) 7, strain CBZ-4 grew well and the average CBZ removal rate was above 38%. Therefore, the optimal \( pH \) value was 7. The optimal degradation temperature was 10°C; the removal rate of CBZ was below 20% at 5 or 37°C. When the rotation speed was more than 100 r/min, the degradation rate of CBZ was above 20%. When the rotation speed was increased to 150 and 200 r/min, the removal rate was more than 27%. However, a rotation speed of 150 r/min can realize better removal, and further increase of rotation speed had only slight effect on the biodegradation. Accordingly, the rotation speed of 150 r/min was considered as the optimal speed. For the same reason, an inoculation volume of 13% was determined as the optimal condition.

The growth of strain CBZ-4 over time is shown in Fig. 4. The average CBZ removal rate of two groups of parallel tests reached 46.6% after cultivation for 6 days at 10°C. The \( \text{OD}_{660\text{nm}} \) and ETSA significantly represented the entire growth curve. There was no distinct lag phase in the beginning, and strain CBZ-4 directly went into the logarithmic phase. Strain CBZ-4 arrived at the stationary phase from 5 to 6 days. Later, CBZ-4 started the decline phase but still retained some metabolism activity.

2.3 Growth of strain CBZ-4 on other aromatic compounds

The ability of strain CBZ-4 to utilize 17 other aromatic compounds was determined to confirm the wide range of applications of strain CBZ-4 (Table 1). Evidently, strain CBZ-4 can degrade most compounds with a simple chemical structure, including phthalic acid, salicylic acid, and so on. Strain CBZ-4 can also grow on phenolic compounds such as phenol, \( o \)-cresol, and \( p \)-cresol. Some aniline compounds such as diphenylamine, \( p \)-phenylenediamine, and benzidine can be used as degradation substrates of strain CBZ-4. Strain CBZ-4 can also use some more complicated substances such as the estrogenic hormones 17α-ethinylestradiol, 17β-estradiol, and estrone, which are some of the most common PPCPs. This phenomenon indicated that strain CBZ-4 can degrade not only CBZ
but also other PPCPs. Strain CBZ-4 can be widely applied in the biodegradation of trace refractory organics. Sulfanilamide and sulfamethoxazole were hardly degraded by strain CBZ-4 because they are pharmacologically active compounds with more complicated chemical structures (Carballa et al., 2007).

2.4 Fatty acid composition of strain CBZ-4 at different temperatures

Fatty acids are the most important components of lipids and directly affect the fluidity and mass transfer of cell membranes. The changes in the fatty acid composition and percentage of strain CBZ-4 were the most remarkable adaptation when temperature was suddenly decreased. As shown in Table 2, saturated and unsaturated fatty acids with 10–19 carbons had visible changes at different temperatures (4, 10, 25, and 37°C). The percentage of unsaturated fatty acids increased with decreased temperature, and showed the maximal value at 4°C (Fig. 5).

2.5 Biodegradation kinetics of CBZ by strain CBZ-4

The CBZ-biodegradation behaviors of strain CBZ-4 at initial concentrations of 10 to 160 mg/L are illustrated in Fig. 6a. At 10, 20, 40, and 60 mg/L, CBZ was rapidly degraded within the first 120 hr, and the average CBZ removal rate achieved was approximately 50%. The specific removal rates were 0.0014, 0.0036, 0.0051, and 0.0064 hr⁻¹, which presented a first-order reaction. The biodegradation behaviors resembled a zero-order reaction for the initial concentration of 80 to 160 mg/L, because the specific removal rates had no obvious difference. The first-and zero-order kinetic equations of CBZ degradation by strain CBZ-4 were deduced by their good fit to the CBZ-biodegradation behaviors (Table 3). The change in the specific CBZ removal rate with concentration was obtained using Origin 8.5 software, and the Monod model provided a good fit (Fig. 6b). The $V_{\text{max}}$ and $K_s$ values were 0.0094.
hr⁻¹ and 32.5 mg/L, respectively. The fitted Monod model was 
\[ V = \frac{V_{\text{max}}S}{K_s + S} = 0.0094S, \] 
with \( R^2 = 0.9604 \).

3 Discussion and conclusions

CBZ has pharmaceutical activity at low concentrations and is resistant to biodegradation in the environment. Low temperatures have negative effects on the biodegradation of CBZ. To date, reports on the biodegradation of CBZ at low temperatures are limited. To our knowledge, this report is the first on the biodegradation of CBZ by a cold-adapted CBZ-degrading bacterium.

3.1 Degradation characterization of CBZ by strain CBZ-4

The identification of the degradation characteristics of CBZ had an important impact on strain CBZ-4 growth and CBZ removal. The optimal degradation conditions of pH 7, 10°C, 150 r/min rotation speed, and 13% inoculation volume were obtained by an odd factor experiment. CBZ had a neutral pH value and was more difficult to degrade under acidic conditions (Urase and Kikuta, 2005). Therefore, the characterizations of CBZ determined the optimal removal rate of CBZ and were achieved at pH 7 (Fig. 3a). Strain CBZ-4 can grow better at 8–30°C, but the optimal

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>4°C (%)</th>
<th>10°C (%)</th>
<th>25°C (%)</th>
<th>37°C (%)</th>
<th>Fatty acid</th>
<th>4°C (%)</th>
<th>10°C (%)</th>
<th>25°C (%)</th>
<th>37°C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10:0</td>
<td>0.18</td>
<td>–</td>
<td>0.09</td>
<td>0.09</td>
<td>C16:0 iso</td>
<td>0.12</td>
<td>–</td>
<td>0.21</td>
<td>0.16</td>
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<tr>
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<td>3.51</td>
<td>3.5</td>
<td>2.26</td>
<td>C16:1 w7c</td>
<td>30.74</td>
<td>28.28</td>
<td>25.34</td>
<td>23.84</td>
</tr>
<tr>
<td>C11:0 3OH</td>
<td>0.19</td>
<td>–</td>
<td>0.13</td>
<td>0.13</td>
<td>C16:1 w5c</td>
<td>0.24</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C12:0</td>
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<td>4.21</td>
<td>3.7</td>
<td>3.51</td>
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<td>3.3</td>
<td>5.15</td>
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<td>C13:0 iso</td>
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<td>–</td>
<td>–</td>
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<td>0.22</td>
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<tr>
<td>C13:0 3OH</td>
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<td>–</td>
<td>0.19</td>
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<td>–</td>
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<tr>
<td>C14:0 iso</td>
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<td>–</td>
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<td>0.13</td>
<td>C17:1 anteiso w9c</td>
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<td>0.34</td>
<td>0.61</td>
<td>C17:1 anteiso B</td>
<td>0.15</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C15:1 iso G</td>
<td>0.38</td>
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<td>0.47</td>
<td>0.8</td>
<td>C17:0 cyclo</td>
<td>7.43</td>
<td>16.96</td>
<td>17.35</td>
<td>14.82</td>
</tr>
<tr>
<td>C15:0 iso</td>
<td>7.76</td>
<td>1.41</td>
<td>1.33</td>
<td>6.35</td>
<td>C17:0</td>
<td>0.33</td>
<td>0.31</td>
<td>0.17</td>
<td>0.49</td>
</tr>
<tr>
<td>C15:0 anteiso</td>
<td>0.5</td>
<td>1.15</td>
<td>0.58</td>
<td>2.68</td>
<td>C17:0 iso</td>
<td>0.14</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C15:0 anteiso A</td>
<td>–</td>
<td>–</td>
<td>0.11</td>
<td>0.11</td>
<td>C17:0 iso 3OH</td>
<td>3.64</td>
<td>1.01</td>
<td>0.63</td>
<td>2.97</td>
</tr>
<tr>
<td>C15:1 w6c</td>
<td>0.3</td>
<td>–</td>
<td>0.15</td>
<td>0.34</td>
<td>C17:0 2OH</td>
<td>0.36</td>
<td>0.53</td>
<td>0.22</td>
<td>0.92</td>
</tr>
<tr>
<td>C15:0 iso 3OH</td>
<td>1.27</td>
<td>0.27</td>
<td>0.44</td>
<td>1.08</td>
<td>C18:1 w7c</td>
<td>10.21</td>
<td>13.48</td>
<td>13.21</td>
<td>6.91</td>
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<tr>
<td>C15:0 2OH</td>
<td>0.1</td>
<td>–</td>
<td>0.26</td>
<td>–</td>
<td>C18:0</td>
<td>0.47</td>
<td>–</td>
<td>–</td>
<td>0.3</td>
</tr>
<tr>
<td>C16:1 iso H</td>
<td>–</td>
<td>–</td>
<td>0.2</td>
<td>–</td>
<td>C19:0 iso</td>
<td>0.11</td>
<td>0.29</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>C19:0 cyclo w8c</td>
<td>0.22</td>
<td>–</td>
<td>–</td>
<td>5.53</td>
<td>1.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“-“: not detected.
Table 3 Kinetics equations of carbamazepine degradation by strain CBZ-4

<table>
<thead>
<tr>
<th>Initial carbamazepine concentration (mg/L)</th>
<th>Degradation kinetics equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>First order kinetic</td>
<td></td>
<td></td>
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<tr>
<td>9.889</td>
<td>$\ln S = 2.3101 - 0.0066t$</td>
<td>0.9875</td>
</tr>
<tr>
<td>20.008</td>
<td>$\ln S = 2.9234 - 0.0062t$</td>
<td>0.9691</td>
</tr>
<tr>
<td>40.065</td>
<td>$\ln S = 3.6825 - 0.0059t$</td>
<td>0.9829</td>
</tr>
<tr>
<td>60.809</td>
<td>$\ln S = 4.1460 - 0.0052t$</td>
<td>0.9703</td>
</tr>
<tr>
<td>Zero order kinetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80.053</td>
<td>$S = 78.1900 - 0.2476t$</td>
<td>0.9560</td>
</tr>
<tr>
<td>100.389</td>
<td>$S = 104.0096 - 0.2803t$</td>
<td>0.9511</td>
</tr>
<tr>
<td>120.095</td>
<td>$S = 120.6154 - 0.2624t$</td>
<td>0.9891</td>
</tr>
<tr>
<td>139.801</td>
<td>$S = 143.8716 - 0.2418t$</td>
<td>0.9762</td>
</tr>
<tr>
<td>160.998</td>
<td>$S = 163.2317 - 0.2628t$</td>
<td>0.9890</td>
</tr>
</tbody>
</table>

Fig. 5 Change in the percentage of unsaturated fatty acids with temperature.

removal rate was achieved at 10–20°C (Fig. 3b), which demonstrated that strain CBZ-4 was capable of growing over a wide temperature range and appeared to be a psychrotrophic or psychrotolerant bacterium according to the strict division between psychrophiles and psychrotrophs (Morita, 1975).

The growth curve of strain CBZ-4 reflected the status of its proliferation and metabolism, which was the reference index in the actual project to guide the bioaugmentation. Three indicators (OD$_{660\text{nm}}$, protein concentration, and ETSA) were successively monitored under the optimal biodegradation conditions to determine the growth change of strain CBZ-4. The OD$_{660\text{nm}}$ reflected the comprehensive growth condition, including the biomass of dead and live bacteria, and was the most direct and simple indicator. Therefore, the curve of OD$_{660\text{nm}}$ in the decline phase remained at high levels and then slightly decreased compared with the stable phase. The intracellular protein concentration had some positive correlation with the biomass of live bacteria; therefore, it dramatically declined in the late growing stage. The ETSA value was measured at 495 nm following the principle that the dehydrogenase in the respiratory chain can reduce iodonitrotetrazolium chloride into the dark red, fat-soluble substance iodonitrotetrazolium formazan, which can be dissolved by an organic solvent. The ETSA value utilized the activity of dehydrogenase in the respiratory chain to quantify the activity of the electron

Fig. 6 Biodegradation kinetics of CBZ by strain CBZ-4. (a) CBZ-biodegradation behavior of strain CBZ-4 with different initial concentrations; (b) relationship between the carbamazepine concentration and specific removal rate of carbamazepine.
transport system during the metabolism of strain CBZ-4. In the decline phase, the ETSA value of strain CBZ-4 did not decrease notably, and strain CBZ-4 retained its relatively strong metabolism activity and better proliferation capability under the optimal biodegradation conditions. The removal rate of CBZ reached 46.6% after 144 hr of cultivation at a low temperature, better than Acinetobacter sp. HY-7, with degradation rate of CBZ below 50% at 25°C (Cui et al., 2009). This finding can probably explain why strain CBZ-4 can degrade many kinds of substrates at low temperatures. Thus, strain CBZ-4 has a wide range of degradation capability and promising applications in low-temperature wastewater treatment.

The cold-adaptation principles of psychrophiles and psychrotrophs have been widely studied, and the accepted viewpoints include the lipid composition and fluidity of the cell membrane, cold-shock or cold-induced proteins, and cold-adapted enzymes (Hébraud and Potier, 1999; Morita, 1975). As shown in Fig. 5, the percentage of unsaturated fatty acids increased with decreased temperature, and a possible explanation for this phenomenon may be the need to maintain the degree of fluidity in the lipid phase of the cell membrane to adapt to the temperature shift (Gounot, 1986; Diniz-Mendes et al., 1999; Margesin and Schinner, 1994; Chintalapati et al., 2004). Such a change was observed in a number of psychrophiles, including Pseudomonas, Listeria, Bacillus, and Brevibacterium, and so on (Canillac et al., 1982). Wada et al. (1994) have pointed out that when the temperature suddenly decreases, the lipid membrane needs to recover its fluidity by transforming a large proportion of its fatty acid composition. The most notable change is the increase in the ratio of unsaturated fatty acids to short chain, branched fatty acids (Gounot, 1986). The length of the fatty acid chain is also shortened, methyl branches increase, the ratio of cyclic fatty acids decreases, and so on (Li et al., 2006). These results are expected in response to the regulation of lipid composition and membrane fluidity at low temperature, which agrees with one of the recognized cold-adaptation mechanisms (Hébraud and Potier, 1999). This result successfully demonstrates the cold-adaptation and metabolism principles of most cold-adapted microorganisms, laying theoretical foundation for research on psychrophiles and psychrotrophs.

3.2 Biodegradation kinetic analysis of CBZ by strain CBZ-4

Nine concentration gradients from 10 to 160 mg/L were used in the kinetic experiments. The obtained results reflected the dynamics of degrading strain growth and CBZ metabolism when exposed to pure cultures of the CBZ-degrading strain. There was no obvious phenomenon indicating that CBZ inhibited its own biodegradation. Thus, the Monod model was used to fit the results because the Monod equation establishes a basic theoretical frame of growth-linked substrate utilization and is widely used to fit pure culture systems under a single controlled condition (Naziuruddin et al., 1995; Smith et al., 1997; Robinson and Tiedje, 1983). The Monod equation is also more adapted to environments with slow growth and low density of cells (Wang and Zhu, 2007).

At low CBZ concentrations (10 to 60 mg/L), the degradation rates of CBZ were approximately proportional to the CBZ concentration, increasing with increased substrate (first-order equation). From the aspect of mass transfer, the active locations in the enzyme system of strain CBZ-4 were not saturated by the substrate at low concentrations and the concentration gradient was small during degradation. Consequently, the reaction impulse was poor and the removal rate was constrained by the CBZ concentration. At high CBZ concentrations (80 to 160 mg/L), the rates were independent of the substrate concentration (zero-order equation). Based on the analysis above, the reaction impulse increased with increased concentration. Strain CBZ-4 was saturated by substrate and degraded CBZ at the maximum removal rate. Thus, the degradation rate can increase by improving the biomass. After mathematical integration, the nonlinear Monod equation was expressed in the linear form with better fitting, and the overall dependence change of CBZ depletion with time (Table 3) was slow. $V_{max}$ and $K_s$ were only 0.0094 hr$^{-1}$ and 32.5 mg/L, much less than 0.081 hr$^{-1}$ and 60.7 mg/L for phenol biodegradation (Mamma et al., 2004), 0.50 hr$^{-1}$ and 25.2 mg/L for aniline biodegradation (Han et al., 2007), and 0.23 hr$^{-1}$ and 84.31 mg/L for 4-aminobenzenesulphonate biodegradation (Wang et al., 2009). To some extent, $V_{max}$ was determined by the microbial species and $K_s$ were controlled by the substrate. This finding signified that CBZ was resistant to biodegradation especially at low concentrations, as supported by many research results (Stamatelatou et al., 2003; Joss et al., 2006).

In summary, Pseudomonas sp. CBZ-4 had a certain ability to degrade CBZ and some other aromatic compounds at low temperatures. This expands the application range of psychrotrophs in the biodegradation of PPCP pollutants. Future research on the biodegradation mechanism and routes of CBZ, as well as on related biodegradation genes, will be useful to improve biodegradation of environmental CBZ and its application in biotechnology.

Acknowledgments

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