Isolation and characterization of organic matter-degrading bacteria from coking wastewater treatment plant

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ABSTRACT

As a step toward bioaugmentation of coking wastewater treatment 45 bacteria strains were isolated from the activated sludge of a coking wastewater treatment plant (WWTP). Three strains identified as Bacillus cereus, Pseudomonas synxantha, and Pseudomonas pseudoaligenes exhibited high dehydrogenase activity which indicates a strong ability to degrade organic matter. Subsequently all three strains showed high naphthalene degradation abilities. Naphthalene is a refractory compound often found in coking wastewater. For B. cereus and P. synxantha the maximum naphthalene removal rates were 60.4% and 79.8%, respectively, at an initial naphthalene concentration of 80 mg/L, temperature of 30 °C, pH of 7, a bacteria concentration of 15% (V/V), and shaking speed of 160 r/min. For P. pseudoaligenes, the maximum naphthalene removal rate was 77.4% under similar conditions but at 35 °C.

Key words | coking wastewater, dehydrogenase activity, naphthalene degradation, organic matter-degrading strains

INTRODUCTION

China is a major coal-producing country, and the pollution caused by coking wastewater poses a severe challenge (Liu et al. 2016). In 2014, the mining and washing of coal was estimated to generate 1,448.26 million ton of wastewater, accounting for 7.75% of total wastewater discharge from industries in China (National Bureau of Statistic China 2015). Coking wastewater contains hundreds of organic and inorganic pollutants such as polycyclic aromatic hydrocarbons (PAHs). Many of these compounds are refractory and mutative (Li et al. 2016; Pueyo et al. 2016).

At present, most coking wastewaters are treated with conventional biological treatment processes known for high removal efficiency and low operational costs (Zhu et al. 2016). The increasing concern for the environment and for effluent management led to the enactment of stricter discharge standards for pollutants from the coking chemical industry by the Chinese government in 2012 (GB16171-2012 2012). A relatively low limit of 80 mg/L was set for chemical oxygen demand (COD) (Dehua et al. 2016). As coking wastewater is known to include several refractory and toxic compounds (e.g., phenols, PAHs, and nitrogen heterocyclic compounds), the conventional biological treatment processes often fail to meet the new discharge standard (Wang et al. 2015; Sasidharan Pillai & Gupta 2016). Therefore, new technologies or improvements in the currently used biological processes are needed.

Bioaugmentation is an improvement in biological treatment processes, using isolated degrading strains (Zhu et al. 2012). Zhu et al. (2015) studied the bioaugmentation strains for phenol, pyridine, quinoline, carbazole, and naphthalene degradation to treat coking wastewater in a membrane bioreactor. These authors reported an additional 10% increase in the removal of COD from the effluent, indicative of the feasibility of using high-efficiency bacteria for coking wastewater treatment. It is necessary to choose the appropriate strains for bioaugmentation. Many studies have identified bacteria from specific agents that provided the ready-made bacteria (Lu et al. 2009), or isolated strains that exhibited high degradation abilities against one or two compounds such as phenol (Zhu et al. 2015), nitrogen (Liu et al. 2015), pyridine, and quinoline (Bai et al. 2010). The presence of large quantities of organic matter in coking wastewater poses problems and difficulties in the treatment process using bioaugmentation. It is
Dehydrogenase (DHA) is an important oxidoreductase in wastewater treatment and acts as a catalyst in important metabolic processes such as the decomposition of organic inputs and detoxification of xenobiotics (Zhang et al. 2011). DHA activity assays have been used to obtain correlative information on the biological activity of microbial populations, including the index of the total microbial activity (Maliszewska-Kordybach & Smreczak 2003). The biological treatment of wastewater involves a complex process of oxidation-reduction by a variety of enzymes produced by microorganisms. Of these enzymes, DHA can activate the hydrogen of oxidized organic and pass it to a specific body. Therefore, DHA activity may serve as an index of the ability of the microorganisms to oxidize organic compounds (Matyja et al. 2016). Meanwhile, naphthalene (C₁₀H₈) is a typical PAH in coking wastewater and consists of two benzene rings. Its stable bi-cyclic aromatic structure makes naphthalene one of the most difficult PAHs for microbial degradation (Chang et al. 2014). Therefore, the naphthalene degradation ability could be studied to investigate the feasibility of bioaugmentation with the isolated bacteria.

In this study, we aimed to isolate bacterial strains with high degradation abilities to break down organic matter in coking wastewater. Furthermore, we identified these bacteria using colony morphology and Biolog MicroStation with GEN III microplate system. To evaluate the characteristics of the isolated bacterial strains, their DHA activity and naphthalene degradation ability were determined. The results of this work may provide useful information for the removal of organic matter from the coking wastewater.

**METHODS**

**Chemicals and media**

Triphenyltetrazolium chloride (TTC) and tri-hydroxymethyl aminomethane (Tris) were purchased from Shanghai Bioleaf Biotech Co., Ltd (China). Naphthalene was obtained from Tianjin Quartz Clock Bazhou Chemical Factory (China), and ethyl acetate was supplied by Tianjin Kailida Chemical Business Co., Ltd (China).

Six types of media were used in this study. Their components were as follows:

- **Basic medium**: beef extract 3.000 g, peptone 10.000 g, sodium chloride (NaCl) 5.000 g, distilled water 1 L, pH 7.4–7.6.
- **Nutrient agar medium (NAM)**: beef extract 3.000 g, peptone 10.000 g, NaCl 5.000 g, distilled water 1 L, pH 7.4–7.6, agar 15.000–20.000 g.
- **Selective medium**: glucose 1.000 g, peptone 1.000 g, potassium phosphate (K₂HPO₄) 0.500 g, sodium nitrate (NaNO₃) 2.500 g, NaCl 0.500 g, magnesium sulfate (MgSO₄·7H₂O) 0.300 g, ferric chloride (FeCl₃) 0.010 g, coking wastewater 100 mL, distilled water 1,000 mL, pH 7.4–7.6.
- **Beef extract peptone medium**: beef extract 3.000 g, peptone 10.000 g, NaCl 5.000 g, distilled water 1 L, pH 7.4–7.6.
- **Mineral salt medium**: MgSO₄·7H₂O 0.200 g, calcium chloride (CaCl₂·2H₂O) 0.010 g, ferric sulfate (FeSO₄·7H₂O) 0.005 g, potassium bisulfate (KH₂SO₄) 0.400 g, manganese sulfate (MnSO₄·H₂O) 0.020 g, ammonium nitrate (NH₄NO₃) 1.000 g, disodium phosphate (Na₂HPO₄) 0.600 g, distilled water 1 L, pH 7.4–7.6.
- **Mineral salt medium with naphthalene**: naphthalene was dissolved in ethyl acetate (4 g/L) and added to the MSM at a suitable concentration which the naphthalene biodegradation test needed. It could be used until the complete volatilization of ethyl acetate.

All media were sterilized for 20 min under 0.1 MPa.

**Activated sludge sample**

The activated sludge sample was obtained from the aeration tank of a coking wastewater treatment plant (WWTP) located in Shanxi Province, North China. The pH, temperature, and oxygen concentration of the activated sludge in the coking WWTP were 7.0–8.0, 30–35 °C, and 2–6 mg/L, respectively.

**Isolation of bacteria**

To enrich and culture the bacterial strains in the activated sludge, an activated sludge sample from the coking WWTP was added to the selective medium three times and incubated at 30 °C in a rotary shaker at 120 rpm for 7 days. After 7 days of cultivation, 10 mL of the enriched activated sludge was added to 90 mL of sterile water in a conical flask.
containing glass beads, and the flasks were shaken for 20 min to scatter bacterial cells. The samples were serially diluted (10^{-1} to 10^{-6}).

Half a millilitre of each dilution from 10^{-4} to 10^{-6} was spread on nutrient agar medium plates and the plates were incubated at 30 °C for 2–5 days to obtain colonies. Different colonies were selected and transferred onto nutrient agar medium plates and cultivated at 30 °C. To purify the strain, streaking inoculation was repeatedly performed until a single colony was obtained. The purified bacterial strain was inoculated onto agar slants made from nutrient agar medium and incubated for 1–2 days. The bacterial strain was stored at 4 °C and transplanted every 2 months (El-Sayed et al. 2006).

**Colony morphology**

To study colony morphology, the preserved bacterial strains were inoculated on nutrient agar medium plates by streaking and incubated at 30 °C for 48 h. The shape, color, uniformity, transparency, viscosity, and position on the culture medium plate were observed.

**Dehydrogenase activity**

We determined the DHA activity by the transformation of TTC to 1,2,5-triphenylformazan. A 2-mL aliquot of Tris-HCl (Tris 0.05 mol/L, hydrochloric acid (HCl) 0.05 mol/L, pH 8.4) buffer solution and 2 mL of the bacterial suspension were added to 2 mL of TTC-glucose (1% glucose and 0.1% TTC) solution in 50-mL glass flasks. The mixtures were shaken and incubated at 37 °C and the coloration timing was recorded. After 2 h incubation, the reaction product was extracted with 5 mL of trichloromethane and centrifuged at 4,000 r/min for 5 min to avoid any interference from the bacterial cells. The absorbance of the sample was measured at 485 nm wavelength. For blank samples, no TTC-glucose solution was added. DHA activity was reported as micrograms TTC per millilitre bacteria per hour (Zhang et al. 2011).

To prepare the bacterial suspension, the preserved strains were inoculated in basic medium and incubated at 30 °C for 48 h. The mixture was centrifuged at 4,000 r/min for 30 min and the solid concentrate was washed three times with distilled water and centrifuged under the same conditions. The solid concentrate was added to sterile demineralized water such that the resulting suspension contained 30 g/L. The suspension was stored at 4 °C before use.

**Bacteria identification**

Biolog (Biolog Inc., Hayward, USA) adapted the principle of substrate utilization by coupling metabolic activity to the simultaneous reduction of a redox dye. Wragg et al. (2014) compared the performance of the Biolog identification system with two other automated systems based on other principles in the identification of isolates of veterinary interest. They found that performance to the species level was similar for all three systems. However, their performance was significantly poorer than a specialist determinative bacteriology laboratory.

The bacterial strains were identified with Biolog MicroStation with GEN III microplate system. The selected single colony was transferred into Biolog Universal Growth (BUG) medium (Biolog) and incubated at 33 °C for 24 h. The colony was emulsified into ‘inoculating fluid A’ (Biolog) and the concentration of the bacterial suspension was adjusted to 90–98% using a turbidimeter, as specified in the user guide, for the subsequent inoculation onto the microplate test plate (Biolog). For each isolate, 100 μL of the cell suspension was inoculated into each well of the microplate using a multichannel pipette and incubated at 33 °C for 24 h. Microplates were read using the MicroStation semiautomated reader and results were interpreted by the identification system’s software (GEN III database, version 5.2.1). Each microplate was read three times (Wragg et al. 2014).

**Naphthalene biodegradation**

The selected strains were incubated in beef extract peptone medium and agitated for 7 days, followed by centrifugation at 4,000 r/min for 30 min. The sediments were repeatedly washed with sterile water to remove the residue, including the substrate and intermediates. The cells were diluted with sterile water to obtain a bacterial suspension with an OD_{600} of 1.0. The suspension was stored at 4 °C for the naphthalene degradation test.

The naphthalene biodegradation test was performed in Erlenmeyer flasks. The selected bacterial suspensions (3, 5, 10, 15 and 20 mL) were added to 100 mL of mineral salt medium containing different concentrations of naphthalene. The pH of the mixture was adjusted to a set value and the mixture was shaken at set conditions (including temperature and rotation speed) for 24 h. The naphthalene degradation potential was evaluated with a controlled trial without naphthalene. The process was terminated with the addition of 10 mL of ethyl acetate to the culture. After extraction,
5 mL of ethyl acetate was added to the separated water phase, which was extracted for the second time. The extract was mixed and diluted with ethyl acetate to make up the volume to 15 mL, followed by the analysis of naphthalene content.

A gas chromatograph (GC-2010 Shimadzu, Germany) was used to measure the concentration of naphthalene. The chromatography analysis was performed using an Rtx®-1 30 m × 0.32 mm × 0.25 μm quartz capillary chromatographic column. The temperature programming comprised a starting temperature of 80 °C for 1 min that increased from 80 °C to 250 °C at a rate of 10 °C/min. The temperature was held for 1 min at 250 °C to complete the run. The sample size used for analysis was 1 μL. The introduction port temperature was 250 °C, while the flow rate of the carrier gas was 1.0 mL/min. The carrier flow of N2 and H2 was 2 and 40 mL/min, respectively, and the air flow was 400 mL/min. The injection temperature was 280 °C, the detector temperature was 280 °C, and the split ratio was 1:20.

RESULTS AND DISCUSSION

Characteristics and DHA activities of the isolated bacteria

Forty-five bacterial strains were isolated from the coking wastewater activated sludge. Table 1 shows the six strains with the highest DHA activity. The highest DHA activity was observed for strains having our numbers JDS19, JDS43, and JDS17. Therefore these three strains were identified and tested for naphthalene removal.

Identification of the bacterial strains

The three selected strains were identified using the Biolog instrument. The results are shown in Table 2. The instrument displays three parameters: probability, similarity and distance. The three are computed using the differences and similarities between the results of the multiple tests performed by the instrument and data from the instrument’s database. According to the manufacturer, in order to identify a strain the similarity should be greater than or equal to 0.5 when the strain is incubated in the instrument for 24 h.

The probability parameter numbers in Table 2 indicate some uncertainty in the identity of each strain. However, there is other evidence to support these three identifications.

- Huang et al. (2016) reported that Pseudomonas has a high phenol degradation coefficient.
- Banerjee & Ghoshal (2004a, 2004b) isolated Bacillus cereus from oil refinery and exploration sites and demonstrated that B. cereus had high phenol degradation ability. They also showed that B. cereus exhibited cream-colored, opaque and round colony morphology (Banerjee & Ghoshal 2004a), which was consistent with the results in Table 1.
- The morphological characteristics of P. synxantha colonies were similar to those pictured in Cai et al. (2016).

### Table 1 | Morphological characteristics and dehydrogenase activity of the strains separated and purified in nutrient agar medium

<table>
<thead>
<tr>
<th>Our ID no.</th>
<th>Color</th>
<th>Shape</th>
<th>Uniformity</th>
<th>Position in NAM</th>
<th>Transparency</th>
<th>Viscosity</th>
<th>Dehydrogenase activity (µg/(mL·h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>JDS2</td>
<td>Milk white</td>
<td>Irregular</td>
<td>Smooth</td>
<td>Surface</td>
<td>Opacity</td>
<td>A bit dry</td>
<td>9.263 ± 0.169</td>
</tr>
<tr>
<td>JDS5</td>
<td>White</td>
<td>Ellipse</td>
<td>Smooth</td>
<td>Surface</td>
<td>Opacity</td>
<td>Sticky</td>
<td>6.513 ± 0.061</td>
</tr>
<tr>
<td>JDS16</td>
<td>Ivory yellow</td>
<td>Round</td>
<td>Smooth</td>
<td>Surface</td>
<td>Opacity</td>
<td>Non-sticky</td>
<td>9.098 ± 1.108</td>
</tr>
<tr>
<td>JDS17</td>
<td>White</td>
<td>Round</td>
<td>Not smooth</td>
<td>Surface</td>
<td>Opacity</td>
<td>Non-sticky</td>
<td>17.925 ± 0.844</td>
</tr>
<tr>
<td>JDS19</td>
<td>Milk white</td>
<td>Round</td>
<td>Not smooth</td>
<td>Surface</td>
<td>Opacity</td>
<td>Non-sticky</td>
<td>27.651 ± 2.653</td>
</tr>
<tr>
<td>JDS43</td>
<td>Milk white</td>
<td>Round</td>
<td>Smooth</td>
<td>Surface</td>
<td>Transparent</td>
<td>Non-sticky</td>
<td>21.816 ± 0.901</td>
</tr>
</tbody>
</table>

### Table 2 | Identification result of the selected bacteria

<table>
<thead>
<tr>
<th>Our ID no.</th>
<th>Probability</th>
<th>Similarity</th>
<th>Distance</th>
<th>Incubation time (h)</th>
<th>Strain identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>JDS17</td>
<td>0.631</td>
<td>0.631</td>
<td>5.372</td>
<td>24</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>JDS19</td>
<td>0.704</td>
<td>0.704</td>
<td>4.249</td>
<td>24</td>
<td>Pseudomonas synxantha</td>
</tr>
<tr>
<td>JDS43</td>
<td>0.660</td>
<td>0.660</td>
<td>4.878</td>
<td>24</td>
<td>Pseudomonas pseudoalcaligenes</td>
</tr>
</tbody>
</table>
Austin & Stobie (1992) described the morphology of *P. pseudoaligenes* colonies, which agree with our observations.

**Biodegradation of naphthalene**

**Effects of different initial concentrations of naphthalene**

The effects of different initial concentrations of naphthalene were investigated with the rotation speed of 120 r/min, temperature of 30 °C, pH of 7.0, and initial bacterial concentration of 10%. Results are shown in Figure 1.

Figure 1 shows that all three bacterial strains had the highest naphthalene degradation efficiencies at an initial concentration of 80 mg/L. The degradation efficiency of naphthalene reached 36.7%, 46.0%, and 42.3% in the presence of *B. cereus*, *P. synxantha*, and *P. pseudoaligenes*, respectively. The decrease in degradation efficiency at the highest initial concentration of naphthalene may be due to naphthalene suppression of enzyme activity. On the other hand, the carbon source needed for bacterial growth was scarce when the initial concentration of naphthalene was low, resulting in slower bacterial growth. *P. synxantha* exhibited the highest degradation efficiency. This observation is consistent with DHA activity results (Table 2). This suggests that *P. synxantha* is more suitable for bioaugmentation compared with the other two strains.

**Effects of different pH values**

Figure 2 shows the effect of different pH values on the naphthalene degradation efficiency at an initial concentration of 80 mg/L, rotation speed of 120 r/min, temperature of 30 °C, and initial bacterial concentration of 10%. The highest degradation efficiencies for *B. cereus*, *P. synxantha*, and *P. pseudoaligenes* were observed at pH 7.0 (40.9%, 62.9%, and 42.3%, respectively). At pH >7.0, the degradation efficiency decreased. These results are consistent with those reported in previous studies, wherein microbial growth was favored at pH levels ranging from 6.0 to 8.0 (Xu et al. 2015). As mentioned above, the pH of the activated sludge in the coking WWTP was 7.0–8.0. If it is possible to keep the pH closer to 7.0 than 8.0, our results indicate that this could improve plant performance.

As shown in Figure 2, the naphthalene degradation ability was higher for *P. synxantha* and *P. pseudoaligenes*. Ma et al. (2006) isolated PAH-degrading bacterial strains using naphthalene as a sole carbon source and found that most of the strains isolated belonged to *Pseudomonas* spp. This is consistent with the results of our study.

**Effects of initial bacterial concentration**

The effects of the initial bacterial concentration of each strain were investigated at a rotation speed of 120 r/min, temperature of 30 °C, pH of 7.0, and initial naphthalene concentration of 80 mg/L. Results are shown in Figure 3.

The degradation efficiency increased with an increase in bacterial concentration from 3% to 10%. The maximum degradation efficiency was achieved with an initial bacterial concentration of 15%. However, any further increase in the inoculation dosage resulted in a decrease in the degradation efficiency. This may be due to insufficient nutrients, thereby restraining bacterial activity.
Effects of different temperatures

Figure 4 shows the effects of different temperatures on the naphthalene degradation efficiency at an initial concentration of 80 mg/L, rotation speed of 120 r/min, pH 7.0, and initial bacterial concentration of 15%. The incubation time was 6 days.

The degradation efficiency initially increased and then decreased with an increase in temperature from 20 °C to 40 °C for all three bacterial strains. The optimal temperature for B. cereus and P. synxantha strains was 30 °C, while that for P. pseudoaligenes strain was 35 °C. The temperature of the activated sludge in the coking WWTP was between 30 °C and 35 °C so the strains are well adapted to their environment and therefore possible candidates for bioaugmentation.

Effects of different rotation speeds

Figure 5 shows the effects of different rotation speeds on the naphthalene degradation efficiency at an initial concentration of 80 mg/L, temperature of 30 °C for B. cereus and P. synxantha and 35 °C for P. pseudoaligenes, and initial bacterial concentration of 15%.

The naphthalene degradation efficiency of the three strains increased as the rotation speed increased from 80 to 160 r/min. It is probable that the dissolved oxygen content gradually increased with an increase in the rotation speed, leading to a rise in the naphthalene degradation efficiency. The maximum degradation efficiencies were 60.4%, 79.8%, and 77.4%, for B. cereus, P. synxantha, and P. pseudoaligenes, respectively. The degradation efficiencies decreased as the rotation speed increased beyond 160 r/min. This may be due to friction between the microbial cells and the walls of the reaction bottle, resulting in mechanical damage to the cells. The oxygen concentration at the coking WWTP varied between 2 and 6 mg/L. Our results indicate that plant performance could be improved by finding and maintaining an optimum oxygen concentration.

CONCLUSION

Three bacterial strains displayed good naphthalene degradation abilities among the 45 bacteria strains isolated from a coking WWTP. The three strains were tentatively identified as B. cereus, P. synxantha, and P. pseudoaligenes.

The naphthalene degradation efficiencies of B. cereus, P. synxantha, and P. pseudoaligenes were 60.4%, 79.8%,...
and 77.4% respectively under optimal conditions. Therefore all three are candidate strains for the bioaugmentation of coking wastewater treatment.

The DHA activities of the three were 17.925, 27.651, and 21.816 µg/(mL·h), respectively. This observation indicates that there is a relationship between DHA activity and the degradation ability of the strain. Therefore it is possible that strains can be selected for the treatment of refractory organic wastewater based on their DHA activities.

Furthermore the results of this study indicated that the pH and the oxygen concentration in the activated sludge of the coking WWTP might be controlled to improve the degradation of refractory compounds.

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