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The isolation of a hydrolyzed polyacrylamide (HPAM)-degrading bacterium and its potential application for the treatment of HPAM-containing oilfield wastewater in a UASB reactor

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ABSTRACT

Hydrolyzed polyacrylamides (HPAMs) contained in oilfield wastewater increase the difficulty of oil–water separation and the subsequent wastewater treatment. Besides, the residual HPAMs are detrimental to the nervous and reproductive systems of animals. Biodegradation of HPAMs is a potential solution, which can also increase the efficiency of oilfield wastewater treatment plants and lower their operating costs. This study shows that with the addition of an isolated HPAM-degrading bacterial strain (*Clostridium bifermentans* H5), the viscosity of HPAMs decreased as a result of (1) the biodegradation of their side chains, (2) the change of some functional groups, and (3) the hydrolysis of acylamide into carboxyl groups. The gas chromatograph mass spectrometer analysis showed that besides HPAM fragments with duplet bonds, epoxy, and carbonyl groups, most of the low-molecular-weight degradation products of HPAMs were normal derivatives of acrylamide oligomers. The addition of sodium lactate and sodium sulfate enhanced the removal efficiency of HPAMs. In a batch up-flow anaerobic sludge bed/blanket (UASB) reactor inoculated with pure cells of H5, the removal efficiency of HPAMs, with cane sugar as the carbon source, was competitive given its lower costs when compared with sodium lactate. The results of the polymerase chain reaction–denaturing gradient gel electrophoresis analysis showed that the strain H5 was predominant in the UASB reactor. In conclusion, strain H5 was feasible for the treatment of HPAM-containing wastewater in a UASB reactor.

Keywords: HPAM-containing wastewater; HPAM-degrading bacteria; UASB

1. Introduction

Polymer flooding is an enhanced oil recovery (EOR) process, and water-soluble polymers are often added to floodwater to improve the mobility ratio of the flooding process [1,2]. In the Daqing Oilfield of China, for instance, hydrolyzed polyacrylamide (HPAM) polymers have been widely used in the EOR process given their outstanding properties namely, increase in viscosity and viscoelasticity [3–5]. It has been predicted that Daqing's oil reserves will soon be depleted given that the current oil extractions have significantly higher water content [6–8]. Also, other researchers [9–11] had predicted that in 2010, the demand for HPAM polymers would

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have reached 10–11 t a^{-1} in the Chinese petroleum industry, and 80% of it would be used by the Daqing Oilfield. As the gap between the energy demand and the existing oil reserves increases, the polymer flooding system is increasingly being used in the oil extraction industry. However, after polymer flooding, HPAMs remain in the produced water generated by the oilfield, thereby increasing the difficulty of oil–water separation [12,13]. Besides, the presence of HPAMs also interferes with the oilfield wastewater treatment process and thus increases the operating costs [12]. Furthermore, the residual HPAMs discharged into the environment can be gradually converted into acrylamide monomers [13], which are toxic to the nervous and reproductive system of animals and humans through biomagnification [14]. Given this status quo, the effective decomposition of HPAMs in wastewater is of utmost importance [15–17]. However, reports on the effective and rapid transformation of HPAMs into innocuous substances are rare. Hence, the objective of the present study is to investigate the potential to biodegrade HPAMs for their complete removal from the oilfield wastewater.

The key step in HPAM biodegradation is the acquisition of possible microorganisms, which can lead to the oxidative degradation of HPAMs. In the present study, a sulfate reducing bacterium, strain H5, which had effective degradation ability for HPAMs was isolated from the Daqing Oilfield. Strategies to optimize HPAM degradation by H5, by adding sulfate and extra carbon sources, were investigated. Also investigated is the performance of a lab-scale up-flow anaerobic sludge bed/blanket (UASB) reactor inoculated with anaerobic activated sludge—rich in pure culture of H5—for the treatment of HPAM-containing wastewater. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) analysis was conducted to investigate the stability of H5 in the UASB system. The present study provides useful guidance for the practical application of the biodegradation of HPAMs contained in oilfield wastewater.

2. Materials and methods

2.1. Isolation and identification of the bacterial strain

The bacterial strain was isolated from the aqueous polymer solution (5,000 mg L^{-1}, pH = 7.8–8.0) collected in the curing pot (a national asset) at the HPAM polymer distribution station of the Fifth Branch of Daqing Oilfield, in China. This study is a general scientific research permitted by the regulatory authority, and therefore, no specific authorizations were required. The study field was the inland oilfield, which is free of wildlife, and therefore, there were no concerns of attempts to endangered or protected species. The Hungate Anaerobic Technology, the most probable number, and the roll tube technique were applied for the isolation of the bacterial strain. Pure cultures of strain H5 were obtained after repeated purification process.

The medium for the isolation of strain H5 was the modified Starkey medium. A volume of 1,000 mL of modified Starkey medium contained 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 2 g Na_2SO_4, 0.1 g $CaCl_2·2H_2O$, 2.0 g $MgSO_4·7H_2O$, 1.0 g yeast extract, and 6 mL of 60% sodium lactate solution. The pH value was adjusted to 7.8. Diazoresorcinal was added to the modified Starkey liquid medium with a 0.2% final concentration. Subsequently, the medium was boiled for complete dissolution with the addition of 0.5 g L^{-1} cysteine. Then, high purity nitrogen was pumped-in to expel oxygen. The medium was autoclaved at 121°C, for 20 min. The sterilized medium was cooled, and 0.1 mL of 3% $FeSO_4·(NH_4)_2SO_4$ was added. Agar (1.6%) was added when the solid medium was prepared before sterilization.

The bacterial strain H5 was Gram-stained after a 48-h incubation at 40°C in the abovementioned sterilized modified Starkey medium. Preliminary morphological observation was conducted by electron microscope (COVER-018, Olympus, Japan). Also, morphological and structural characteristics were observed with a transmission electron microscope (H2600A, HITACHI, Japan).

The fatty acids of strain H5 were analyzed by using a Sherlock Microbial Identification system (MIDI Sherlock, MIDI, USA) equipped with a gas chromatograph (6890N, Agilent, USA) [18].

Identification was also conducted through cloning and sequencing its 16S rDNA sequences. The DNA of strain H5 was extracted with a Bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Primers for 16S rDNA sequencing were universal primers [19]: Eubac27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACG ACTT-3′) (TaKaRa, Dalian, China). The 20 μL reaction mixture for PCR amplification contained 20 ng template DNA, 0.2 U μL^{-1} rTaq DNA polymerase (TaKaRa, Dalian, China), 0.3 mmol L^{-1} dNTPs, 0.1 μmol L^{-1} ITS1 primer, and ITS4 primers. The amplification was performed in a hot lid thermal cycler (PTC-200, MJ-Research, USA), and the procedure was predenatured at 94°C for 5 min, denatured at 94°C for 30 s, annealed at 58°C for 45 s, extended at 72°C for 90 s, and repeated 30 cycles, followed by a final extension of 10 min at 72°C. The amplified PCR product was measured by Agarose/EtBr gel electrophoresis and photographed with a UVP Image Analysis System (EC3, UVP, USA). Target fragments were purified using an Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China) and ligated to a Thymine and Adenine type vector pGEM-T (Promega, Madison, WI, USA). Ligated products were transformed to an *Escherichia coli* chemical competent cell TOP10. Transformed *E. coli* was selected on Luria-Bertani solid culture medium with Amp (50 μg mL^{-1}) and X-gal (200 μg mL^{-1}). The transformed *E. coli* was analyzed using the universal primers T7 and SP6, and sequenced by the Shanghai Sangon Biological Engineering Co., Ltd., China. The sequenced 16S rDNA were compared with a non-redundancy nucleotides database by using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was conducted using BioEdit Version 5.06, and a phylogenetic tree was constructed using the MEGA Software Version 4.1 with a neighbor-joining method.

2.2. HPAM biodegradation tests

To investigate the influences of the addition of extra carbon source and sulfate on the polyacrylamide biodegradation performances of strain H5, HPAM biodegradation tests were conducted in a rotary shaker. HPAM with a molecular weight of 16 million Dalton was used. Pure nitrogen was introduced to create anaerobic conditions when the medium was prepared [20]. The anaerobic modified Starkey medium contained 200 mg L^{-1} HPAM, and the pH was adjusted to 7.8. Then, the medium was divided into 100 mL aliquots

in anaerobic flasks and autoclaved. The pure bacterial cells were obtained by centrifuging the precultivated bacterial suspension at 4,000 rpm. The centrifuged cells were washed repeatedly with deionized water to remove the medium after each centrifugation. Finally, cells were resuspended in 5 mL deionized water and transferred into the anaerobic flasks with a 5% inoculation ratio. There were five groups of captive tests. The first group was taken for blank test without inoculation of strain H5; in the second group, no extra carbon substrate and sulfate were added in the HAPM-containing medium inoculated with the pure cells of H5; in the third group, 0.6 mL sodium lactate was added; in the fourth group, 200 mg sodium sulfate were added; in the fifth group, 0.6 mL sodium lactate and 200 mg sodium sulfate were added simultaneously. Each group contained three parallel samples for consistency. All anaerobic flasks were cultivated at 40°C, 125 rpm in a rotary shaker. The daily polymer concentration was monitored over 21 d.

2.3. The lab-scale UASB reactor

A lab-scale UASB reactor was used to investigate the efficiency of H5 for the treatment of HPAM-containing wastewater. The effective volume of the UASB reactor was 100 L with a 36 cm inner diameter and 120 cm height (Fig. 1). The typical HPAM-containing wastewater was collected from the No. 1 Branch of the Daqing Oilfield. The wastewater quality was as follows: HPAM 80–150 mg L^{-1}, COD 192–332 mg L^{-1}, BOD_5 1.7–10 mg L^{-1}, petroleum 3.50–55.0 mg L^{-1}, and pH 7.8–8.2. The wastewater temperature was controlled around 40°C by a thermostat heater. The performances of the UASB reactor under batch and continuous operating modes were investigated.

2.4. PCR–DGGE analysis

Anaerobic sludge samples from the UASB reactor were collected and pretreated with PBS buffer solution before DNA extraction. The DNA extraction was conducted with a bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, China). The V6–V8 region of 16S rDNA genes was amplified by using primers BSF968 (5′-AACGCGAAGAACCTTAC-3′) and BSR1401 (5′-GCTGCCTCCCGTAGGAGT-3′). The final PCR mixture (20 μL) contained 2 μL 10× PCR buffer, 2 μL deoxynucleoside triphosphates (2.0 mmol L^{-1}), 1 μL of each primer (10 pmol L^{-1}), 100 ng DNA extract, 0.3 μL rTaq polymerase, and 2.0 mmol L^{-1} template DNA. The PCR protocol included 9 min initial denaturation at 95°C, 30 s denaturation at 94°C, extension at 72°C for 2 min and repeated 30 cycles, followed by a 10 min final extension at 72°C. PCR products were detected by electrophoresis on a 2% agarose gel. DGGE was performed on a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Volumes of 6 μL of purified PCR products and 6 μL of 10× loading buffer were loaded onto polyacrylamide gels using a denaturing gradient ranging from 40% denaturant at the top of the gel to 60% denaturant at the bottom (100% denaturant contains 7 M urea and 40% [v/v] formamide). Electrophoresis was performed at 60°C and 150 V for 9 h. Finally, gel was silver stained and then photographed by a transillumination scanner (UMAX Power Look 1000, Taiwan, China).

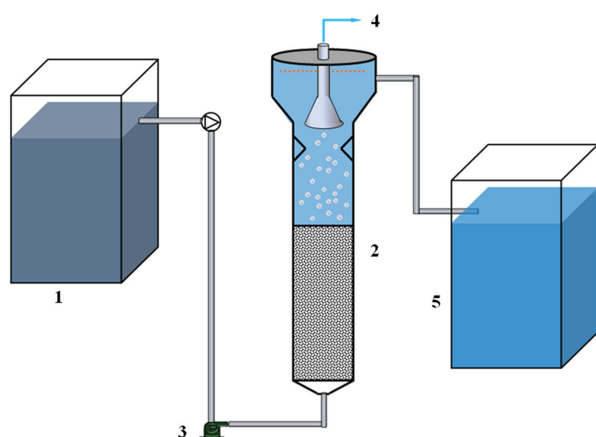

Fig. 1. Schematic diagram of the lab-scale UASB reactor. (1) Stabilizing tank; (2) UASB reactor; (3) peristaltic pump; (4) air vent; and (5) settling tank.

2.5. Analysis methods

Bacterial cultures of the HPAM-containing modified Starkey medium were used to identify the biodegradation metabolites of HPAM after being cultivated at 40°C and 125 rpm in a rotary shaker for 7 d. Methanol, chloroform, hexamethylene, ethyl acetate, and butanol were tested for their extraction capability. It showed that butanol was the best extracting substance. Thus, the upper layer of the butanol phase was collected and concentrated with a rotating evaporator at 45°C. The concentrated sample was then dissolved in 2 mL butanol and filtered for gas chromatograph mass spectrometer (GC–MS) analysis (GCD1800C, HP, USA) [21,22]. Hydrogen sulfide generated during the biodegradation process was analyzed using a gas chromatograph (Sp-2000, Beijing, China) equipped with a flame photometric detector system at 60°C with 25% β-oxydipropionitrile in its stationary phase. The HPAM concentration was detected by the starch–cadmium iodine method [23]. Fourier transform infrared spectrometry (Spectrum One B model, PerkinElmer, USA) was used to analyze the HPAM solid, KBr-squashed dry powder of HPAM solution before and after biodegradation. The viscosity of HPAM was measured by a viscometer (NDJ-1, Shanghai, China) at 45°C.

3. Results and discussions

3.1. Morphological observation and identification of strain H5

Strain H5, which can use HPAM as its sole carbon source and has strong sulfate reduction ability, was isolated. Strain H5 was rod shaped, gram negative, movable, 0.32–2.9 μm in width, and 0.53–5.12 μm in length with gemma (Fig. 2). Strain H5 was a strictly anaerobic bacterium, which used sulfate, sulfite, and sulfide as its electron acceptors and reduced them to H_2S. H5 used a variety of substrates as its carbon sources, such as sodium lactate, sodium formate, malic acid, glucose, succinic acid, yeast extract, peptone, and cane sugar. The growth rate of H5 varied for different carbon substrates. The highest growth rate was obtained in

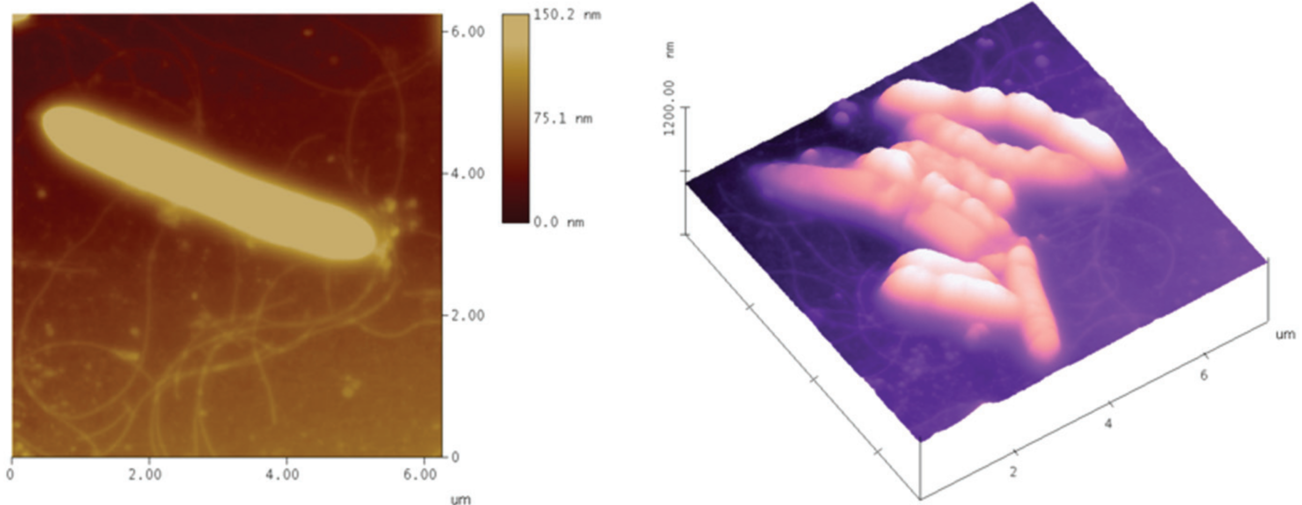

Fig. 2. AFM images of the strain H5.

yeast extract. Ammonia sulfate and carbon amide were used by H5 as nitrogen sources. Strain H5 strain was mesophilic and grew at 10°C–55°C. The optimum temperature for H5 was 40°C. The pH tolerance range for H5 was 6.0–11 and the optimal pH was 8.0.

After sequencing the 16S rDNA sequences of H5, 1483 bp 16S rDNA sequences were obtained. The nucleotide sequences of 16S rDNA of H5 determined in this study were deposited in the GenBank database under accession numbers DQ011236. Representative strains that had high homology with H5 were selected and their phylogenesis tree was constructed. The average genetic distance of the 13 strains was 0.061 in the phylogenesis tree (Fig. 3). The resemblance between strain H5 and *Clostridium bifermentans* (accession no. AY781385) was 99%.

The analysis of fatty acids showed that the total response of strain H5 was 391,634, which met the demand of phylogenesis identification. The similarity index was 0.008, much less than 0.3000. As the strain did not exist in the MIDI database, the system automatically provided a similar strain, *Propionibacterium acnes*. The fatty acid response was distributed around $C_{10:0}$–$C_{20:0}$; the main fatty acids consisted of iso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1\omega7c}$, and iso-$C_{17:0}$, which accounted for 39.4%, 7.38%, 10.09%, 8.75%, and 7.11%, respectively.

Consequently, based on the results of morphological observation, physiological tests, fatty acids, and 16S rDNA analyses, strain H5 was initially identified as *Clostridium*. It could be a new species, and thus was provisionally named *Clostridium bifermentans* H5.

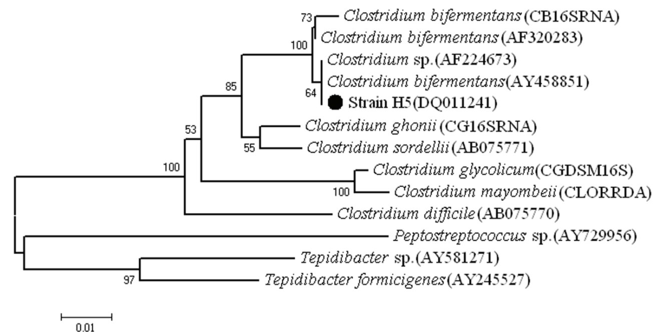

Fig. 3. The neighbor-joining phylogenesis tree based on 16S rDNA sequence of the strain H5 and the related strains.

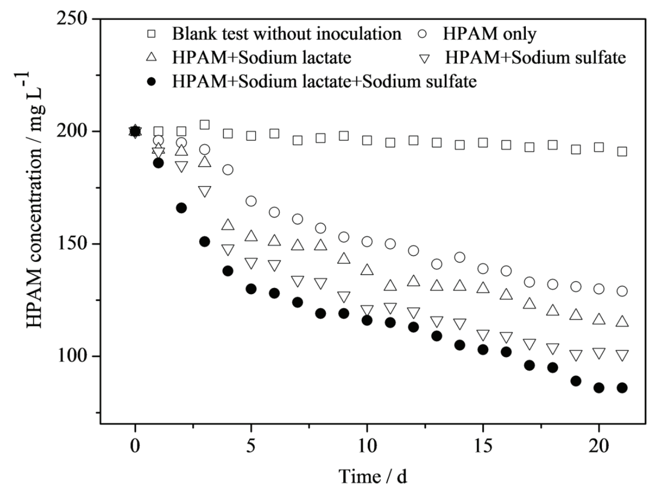

Fig. 4. Influences of extra carbon source and sulfate on the HPAM biodegradation of bacterial strain H5.

3.2. Influences of the extra carbon source and sulfate on HPAM biodegradation

The HPAM removal efficiency was about 31.0% for the HPAM solution without the addition of an extra carbon source and sulfate (Fig. 4). The removal efficiencies with the addition of sodium sulfate and sodium lactate increased to 38.0% and 45.0%, respectively. It suggested that sodium lactate was more beneficial for HPAM biodegradation than sodium sulfate. The highest removal efficiency was 52.5% when sodium lactate and sodium sulfate were added together. It was estimated that in the initial growth phase of H5, sodium lactate was used as its carbon source. The biodegradation of HPAM occurred when sodium lactate was exhausted. Thus, the presence of suitable extra carbon sources and sulfate was

essential for the growth of anaerobes and biodegradation of polymers. Wen et al. [18] also indicated that the addition of a low concentration of glucose improved the biodegradation of PAM with the stimulation of microbial growth and production of enzymes. However, the concentration of extra nutrients should be limited to avoid their excessive utilization instead of HPAM, which consequently leads to the deterioration of HPAM biodegradation [19,24].

3.3. The molecular structure changes of HPAM

Compared with the HPAM solid and HPAM solution before biodegradation, some peaks disappeared after biodegradation (Fig. 5). For instance, peaks due to the stretching vibration of C–C bond (2,942.4242 cm^{-1}) and C–N bond (1,560.6061 cm^{-1}), in the spectrum of HPAM solution before biodegradation disappeared. The peak at 3,387.0996 cm^{-1} had become wider, which indicated that the amide group had been degraded into a carboxyl group. Besides, new peaks at 624.1556 and 862.4242 cm^{-1} suggested the existence of different benzene rings and other bacterial metabolized products. Previous studies pointed to the fact that microorganisms used extracellular amidase to disconnect the side chain of HPAM, which could been used as nitrogen source. Due to their macromolecular weight, it was difficult for microorganisms to incorporate HPAMs into their cells for further biodegradation [25–28]. Even if the molecular weight was insignificant, the HPAMs showed strong resistance to biodegradation due to the static rejection of negative charges on bacteria and carboxylic groups [13]. It demonstrated that the bacterial strain isolated in the present study used HPAMs as its sole carbon and nitrogen sources, which led to the breakage of HPAMs by the degradation of their side chains and conversion of some functional groups, while the viscosity of the solution also decreased.

3.4. The biodegradation products of HPAM

Comparisons of the GC–MS spectrum of the HPAM solution before and after biodegradation indicated that compounds involving $C_{16}H_{33}N$, $C_8H_{18}O$, and $C_{13}H_{24}O_4$ were possible C–C bond breaking products of HPAMs. There were also 13 other metabolites detected during the bacterial degradation of HPAMs. Most of the biodegradation products were normal derivatives of acrylamide oligomers. Others were HPAM fragments with duplet bonds, epoxy, and carbonyl groups [22]. Khan et al. [29] also indicated that the parts of the products from the HPAM degradation were amino acids, which were easily reused by microorganisms [30]. However, the HAPMs in the oilfield showed differences from those low molecular polymers, although they showed similarities as well.

3.5. Performances of the UASB reactor

During the start-up period, 30 L of anaerobic sludge (dry mass = 3,050 mg L^{-1}) and pure bacteria of H5 were added to the UASB reactor. The final amount of H5 was about 0.8 × 10^5 mL^{-1}. In the first 10 d, the reactor was operated under a condition of continuous flow with 10 h of HRT and 4–5 L h^{-1} of flow rate. The HPAM concentration did not significantly decrease during this period (Fig. 6). Subsequently, the 6-d cycle batch flow mode was adopted, including the daily addition of 30 mL of sodium lactate and 10 g of Na_2SO_4. The HPAM concentration decreased significantly simultaneously with the sludge domestication and bacterial growth from the second cycle. The highest removal efficiency of HPAM was up to 50.89%. Granulated sugar was used as carbon source instead of sodium lactate in an attempt to reduce the costs. The removal efficiency when using granulated sugar reached 43.55% at the end of the second cycle, which was competitive considering the higher price of sodium lactate in real life application; it indicated that the use of H5 in the UASB reactor resulted in stable and efficient treatment of the HPAM-containing wastewater.

3.6. The bacterial community variations

Table 1 summarizes the studies of the sample collection protocol for DNA extraction. The targeted fragments of 16S rDNA were about 500 bp and showed great specificity for amplification, which was suitable for DGGE analysis (Fig. 7).

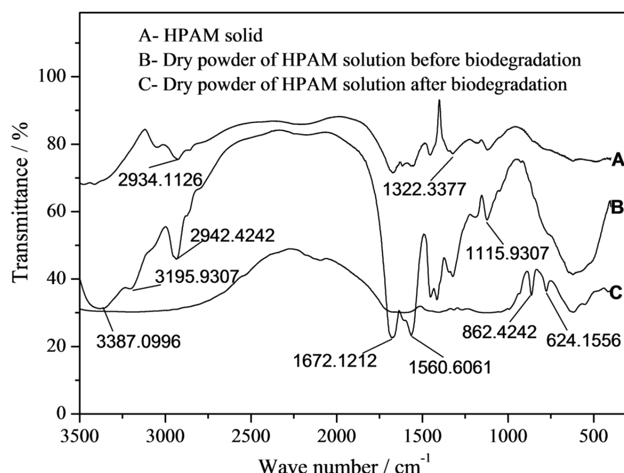

Fig. 5. Infrared spectrums of HPAM before and after biodegradation.

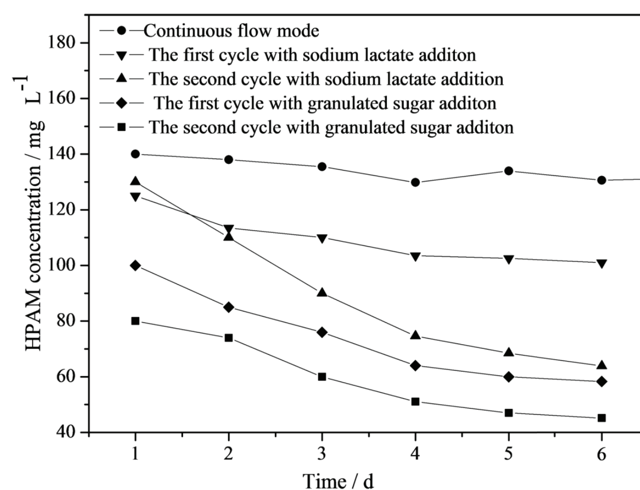

Fig. 6. Biodegradation of HPAMs in the lab-scale UASB reactor.

The dominant bands, A, B, and C were present in the entire operation of the UASB system (Fig. 8). Differences between bacterial community of the first and 12th cycles with the addition of sodium lactate were insignificant. This suggested that the bacterial community was relatively stable. The dominance of bands B and D increased at the end of the 12th cycle. For the bacterial community with the addition of granulated sugar, the changes were insignificant, but there were no remarkable dominant bands [24–26].

Dominant bands on the DGGE gel were recycled, and the phylogenesis tree was constructed after sequencing (Table 2). The dominant species represented by bands B and G belonged to *Clostridium bifermentans*, which was the

Table 1
Samples collection protocol for DNA extraction in the UASB reactor

No.	Substrate	Cycle number
1	Raw wastewater	
2	Sodium lactate	1
3	Sodium lactate	12
4	Granulated sugar	1
5	Granulated sugar	2

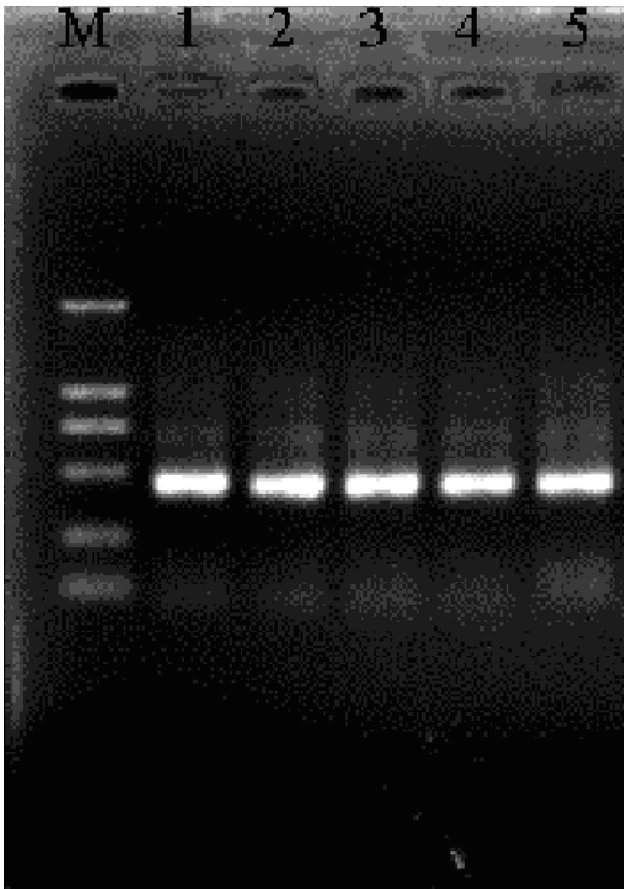

Fig. 7. Electrophoresis profile of PCR products (M: DL2000).

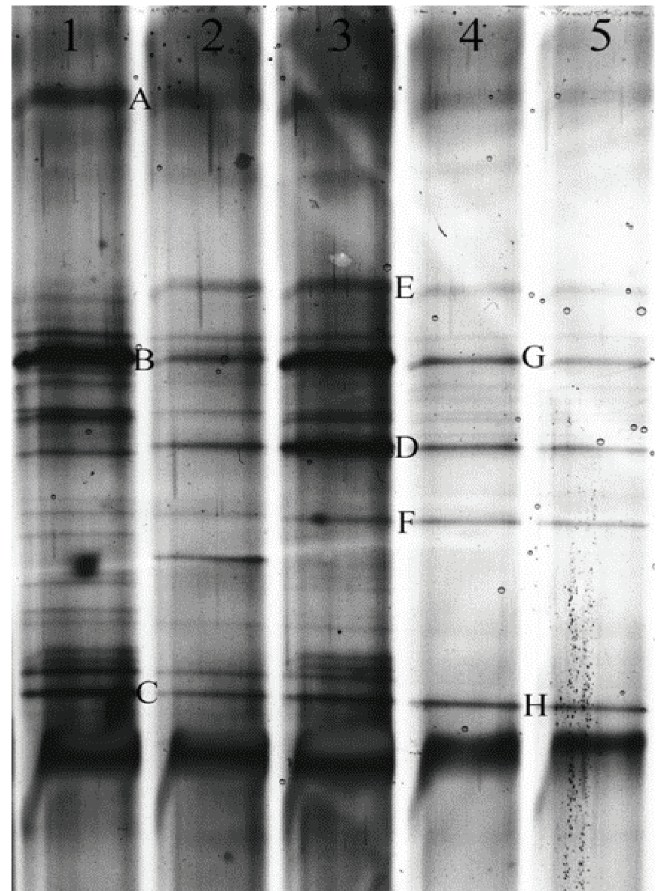

Fig. 8. DGGE profiles of V6–V8 region of 16S rRNA gene in the bioaugmented UASB reactor.

Table 2
Sequences of predominant DGGE bands of 16S rDNA V6–V8 region

Band	The most similar bacteria of the 16S rRNA sequences	Similarity (%)	Sources
A	*Desulfovibrio* sp. (DQ839140)	91	Fuel disposal conditions
B	*Clostridium bifermentans* (AY781385)	98	–
C	*Pseudomonas* sp. (EU180220)	99	Degradation of 1-naphthol
D	*Arcobacter cibarius* (AJ607391)	98	–
E	*Thauera* sp. (AJ315678)	98	Degradation of aromatic compounds
F	*Hydrogenophaga* sp. (DQ986320)	99	Soil and sediments
G	*Clostridium* sp. (DQ839378)	100	–
H	*Pseudomonas* sp. (AM402949)	99	Indian Ocean

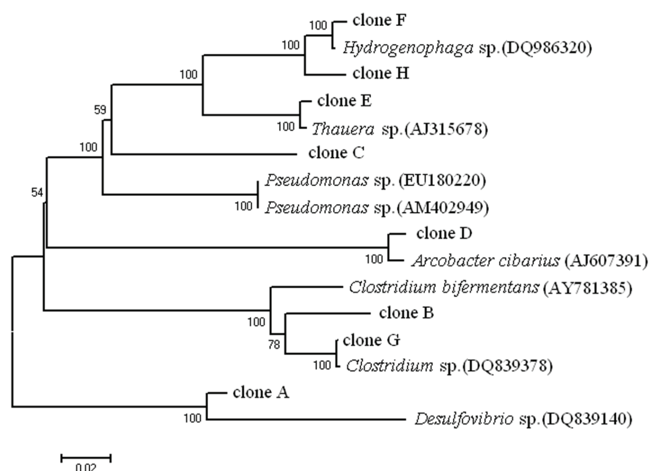

Fig. 9. The phylogenesis tree of the dominant bands with their most similar sequences in GenBank.

same as H5 (Fig. 9) [23]. Bands C and H belonged to the same species, *Pseudomonas* sp. [18]. The dominant species did not change, but was reinforced during the entire operation of the USAB reactor inoculated with H5. It indicated that stable wastewater treatment bioreactors have stable microbial community structures under normal operating conditions and are able to adapt in response to perturbations to sustain a high effluent quality [28,31,32]. Thus, both the stability and flexibility of the bacterial community were crucial for the favorable performance of the biological systems [33,34].

4. Conclusions

This study found that:

- Strain H5, which used HPAMs as the sole carbon source, was isolated. The strain, which could be a species, was temporarily named *Clostridium bifermentans* H5.
- After the biodegradation of strain H5, the side chains of HPAMs were degraded, some of its functional groups were converted, and the acrylamide groups were hydrolyzed to carboxyl groups. As a result, the viscosity of the HPAM solution decreased. Besides the fractured HPAMs with duplet bonds, epoxy, and carboxyl groups, most of the biodegradation products of HPAMs were derivatives of normal acrylamide oligomers.
- The addition of other carbon sources and sulfate benefited the bacterial growth and HPAM biodegradation.
- The batch-operated UASB process was preferable to the continuous process for the treatment of HPAM-containing wastewater. Sodium lactate was more suitable for HPAM degradation than granulated sugar as a carbon source. However, granulated sugar was competitive considering the higher price of sodium lactate in real applications.
- Stain H5 was dominant throughout the operation of the UASB reactor, and its use was feasible for the treatment of HPAM-containing wastewater. Further studies will be conducted for the optimization of bacterium and the treatment system.

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