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Biodegradation of partially hydrolyzed polyacrylamide by bacteria isolated from production water after polymer flooding in an oil field

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ABSTRACT

Partially hydrolyzed polyacrylamide (HPAM) in production water after polymer flooding in oil filed causes environmental problems, such as increases the difficulty in oil–water separation, degrades naturally to produce toxic acrylamide and endanger local ecosystem. Biodegradation of HPAM may be an efficient way to solve these problems. The biodegradability of HPAM in an aerobic environment was studied. Two HPAM-degrading bacterial strains, named PM-2 and PM-3, were isolated from the produced water of polymer flooding. They were subsequently identified as *Bacillus cereus* and *Bacillus* sp., respectively. The utilization of HPAM by the two strains was explored. The amide group of HPAM could serve as a nitrogen source for the two microorganisms, the carbon backbone of these polymers could be partly utilized by microorganisms. The HPAM samples before and after bacterial biodegradation were analyzed by the infrared spectrum, high performance liquid chromatography and scanning electronic microscope. The results indicated that the amide group of HPAM in the biodegradation products had been converted to a carboxyl group, and no acrylamide monomer was found. The HPAM carbon backbone was metabolized by the bacteria during the course of its growth. Further more, the hypothesis about the biodegradation of HPAM in aerobic bacterial culture is proposed.

1. Introduction

Water-soluble, anionic polyacrylamides have recently been widely used to enhance oil recovery in the east oilfields of China. However, partially hydrolyzed polyacrylamide (HPAM) present in production water causes some problems. For example, after polymer flooding, HPAM will remain in the produced water generated by oilfields, increasing the difficulty in oil–water separation. Consequently, the oil content in sewage is greatly increased, and there is a high probability that the wastewater will exceed the local discharge limit. When HPAM enters an oil reservoir with injected water, it can also hardly avoid infiltrating groundwater horizontally in connection with strata configuration. In addition, the costs and difficulties of produced water treatments will be increased because of the high concentration of the HPAM remaining in the waste water.

Furthermore, the residual HPAM in the wastewater can slowly degrade into the toxic acrylamide monomer naturally. The toxicity of acrylamide monomer has been studied by numerous researchers all over the world [1]. Since HPAM can remain in surface water and groundwater for a long period of time, it may endanger human health. Therefore, it is necessary to conduct studies on transforming HPAM into innocuous substance effectively and rapidly. Biodegradation of HPAM may be an efficient way to solve these problems.

Study on HPAM biodegradation is not often seen in previous work, but polyacrylamide (PAM) biodegradation have been studied for a long period. However these studies provided seemingly contradictory results. Suzuki et al. [2] observed that PAM was only slightly biodegradable under aerobic conditions. Kunichika and Shinichi [3] found that while the amide part of PAM could not be utilized by a microbe, the main carbon chain could be partly degraded. Kay-Shoemake et al. [4,5] studied that soil microorganisms could utilize water-soluble PAM as a sole N-source when a supplementary C-source was present, suggesting that the microbes could hydrolyze the amide group but were incapable of cleaving the main C-chain backbone. Haveroen et al. [6] observed that PAM could be added as a nitrogen source to stimulate methanogenesis in anaerobic environments that are rich in fermentable carbon, but lack nitrogen sources. Recently, Wen et al. [7] reported that two strains which were isolated from activated sludge and oil-contaminated soil can use PAM as their sole carbon source. The issue of whether PAM could serve as a main carbon source and/or nitrogen source was still not resolved.

In recent years, PAM has been used to increase aggregation and improve soil physical properties in loamy sand soils. Sojka et al. [8] and Caesar-TonThat et al. [9] studied the effects of PAM on microbes involved in aggregate formation and its biodegradation

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by the microbe consortia. However, they did not report whether PAM could serve as a carbon source or nitrogen source.

The aim of the present study is to investigate the utilization of HPAM by bacteria in aerobic culture and to deduce the mechanism of HPAM biodegradation.

2. Materials and methods

2.1. Sample

The water sample used for the isolation of microorganisms comes from production water after polymer flooding in Shengli oilfield, China.

2.2. Media

The HPAM sample was obtained from Chang'an Polymer Company, Dongying, China. The average molecular weight of HPAM is about 2×10^7 and it is 23% hydrolyzed.

Two different liquid media were used for the isolation, one was beef extract peptone medium (BPM) for enrichment, the other is basic medium (BM) for domestication and screening.

The composition of the BPM was 3 g L^{-1} beef extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl, initial pH was adjusted to 7–7.2 by 1 mol L^{-1} NaOH solution [10]. The composition of the BM was 0.1 g L^{-1} yeast extract, 0.3 g L^{-1} HPAM, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4$, 5 g L^{-1} NaCl, initial pH is natural [11]. The solid basic medium (SBM) for agar plate was BM supplemented with 3.5% agar.

In order to investigate the utilization of HPAM by the microorganisms, three different media formulations were tested: (1) HPAM as a sole source of carbon (CM medium); (2) HPAM as a sole source of nitrogen (NM medium); and (3) HPAM as a source of both carbon and nitrogen (CNM medium). The formulations that were developed are the following, CM: basic medium, 0.2 g L^{-1} $NaNO_3$ was added and the yeast was taken out; NM: basic medium, 0.5 g L^{-1} paraffin was added and the yeast was taken out; CNM: basic medium, the yeast was taken out.

The entire media were sterilized in an autoclave at 120 °C before use.

2.3. Isolation of microorganisms

The screening for HPAM-degrading microorganisms was carried out at 37 °C on a rotary shaker at 140 r min^{-1}. 10 mL of water sample was mixed with 100 mL BPM in a 250 mL conical flask. After aerobic cultivation at 37 °C for 2 days, 3 mL of the BPM broth was inoculated into 100 mL of BM containing HPAM in a 250 mL conical flask, and cultivated for 5 days. Then an aliquot of the BM broth was inoculated into a fresh BM and cultivated for another 5 days. These procedures were repeated in 1 month for microorganisms' domestication.

After 1 month domestication, an aliquot of the BM broth was streaked on an agar plate containing SBM, the plates were incubated for several days at 37 °C and a single colony was isolated [11]. These procedures were repeated for the isolation of the microorganism. Strains that showed a good performance for the degradation of HPAM were chosen for further study.

2.4. Preparation of bacterial suspension for inoculation

The HPAM-degrading bacteria after incubated in BPM culture broth was centrifuged at 6150 × *g* centrifugal force to acquire the bacterial cells, these cells were washed by distilled water and centrifuged for 3 times, in order to remove the nutrition in the culture broth. These purified cells mixed with sterile distilled water to get the bacterial suspension, which was 0.4 of absorbance adjusted via absorbance at 600 nm (OD_{600}) [10], then the bacterial suspension was ready for use.

2.5. General identification of bacterial isolates

The bacterial isolates were identified on the basis of amplifying and sequencing approximately 1400 bp of the 16s ribosomal DNA (rDNA) for bacteria [7,12]. Amplification of the bacteria was performed using the universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGC CGCA-3′) [7]. The total DNA was extracted from isolates using a TIANamp Bacteria DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). PCR amplification was performed in a 25 μL reaction mixture containing 2.5 μL of 10× PCR buffer, 0.5 μL of primers 27f, 0.5 μL of primers 1492R, 0.2 mM concentration for each of the four dNTP, 0.25 μL of Taq DNA polymerase (5 U), and template DNA (1 μL). All the reagents were supplied by Tiangen Biotech Co. Ltd., Beijing, China. Amplifications were carried out for 30 cycles (94 °C for 5 min, 61–65 °C for 30 s, and 72 °C for 60 s) in a My Cycler Thermal Cycler (BIO-RAD) with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 10 min. Amplicons were detected by electrophoresis on a 1% agarose gel staining with ethidium bromide (Tiangen Biotech Co., Ltd., Beijing, China). Amplicons were purified using a TIANamp Bacteria DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. The sequencing was completed by Tiangen Biotech Co., Ltd., Beijing, China. The sequences were analyzed with the basic local alignment search tool (BLAST) in national center of biotechnology information (NCBI) of USA for identification of the bacteria.

2.6. Samples after biodegradation pretreatment

The culture broth after biodegradation was centrifuged at 6150 × *g* centrifugal force prior to determining HPAM removal efficiency, in order to get rid of bacterial cells, and it needed further purification prior to SEM, IR and HPLC analysis. In this paper, HPAM solution samples before and after biodegradation means the control and the biodegraded HPAM solution.

2.7. HPAM removal efficiency determination

HPAM concentration was measured by the starch-cadmium iodine method [13]. The HPAM removal efficiency was calculated by the difference of the concentration value between the control and biodegraded sample divided by the concentration value of the control.

2.8. Biomass determination

The abundance of the bacteria was determined by the MPN method [14]. 1 mL water samples were taken from all flasks and decimal successive dilutions were made in sterile distilled water. Viable counts were made on SBM plates, prepared as was described in Section 2.2. The plates were incubated at 37 °C for 5 days. The number of the strain colonies was recorded. All colonies grown on agar plate were harvested.

2.9. Preparation of solid samples prior to SEM and FT-IR analysis

The liquid samples containing HPAM solution, HAPM solution after sterilizing, HAPM solution before biodegradation (the control) and pretreated HAPM solution after biodegradation were purified with methanol. The sample was added dropwise into a large

excess of methanol (~10) and the remnant polymer in the samples were precipitated [15]. The precipitated polymers were filtered off and washed with methanol (3 times) and then the precipitated samples (and also HPAM solid) were dried in vacuum drying chamber at 50 °C. After preparation, these samples were analyzed by JSM-6700F SEM (Japanese Electronic Co.) and AVATAR 360 FT-IR Spectrometer (Thermo Nicolet, USA).

2.10. Preparation of liquid samples prior to HPLC analysis

Liquid samples containing acrylamide monomer solution, HAPM solution before biodegradation and pretreated HAPM solution after biodegradation were filtered by filterable membrane (0.45 μm) prior to HPLC analysis (LC-6AD HPLC, Shimadzu, Japan). Samples were analyzed using a Simazu HPLC equipped with a Shimpack Vp-ODS column (150 × 4.6 mm). The mobile phase consisted of 50% water and 50% methanol. The flow rate of the mobile phase was 25 μL min^{-1}. A UV–vis detector set to a wavelength of 260 nm was used. Samples were stored at 4 °C prior to analysis.

3. Results and discussion

3.1. Isolation and identification of HPAM-degrading bacteria

Four HPAM-degrading bacteria were isolated from production water after polymer flooding. These strains could grow on media containing HPAM, and among them, strains of PM-2 and PM-3 showed a strong ability to degrade HPAM. In the basic media, after 7 days cultivation at the temperature of 40 °C, the HPAM removal efficiency by PM-2 and PM-3 is 33.7% and 29.1%, the mixed of PM-2 and PM-3 got better removal efficiency, the HPAM removal efficiency is up to 42.1%. These two strains were selected for subsequent studies. DNA was extracted from the pure culture of the two strains and 16S rRNA gene was amplified with primers 27f and 1492R. Partial sequencing with both primers revealed that the closest matches, determined by a BLAST search, corresponded to *Bacillus cereus* strain FM-4 EU794727 (95% similarity, PM-2) and *Bacillus* sp. M7-23 EU706321 (95% similarity, PM-3), respectively. The nucleotide sequences of 16S rDNA of PM-2 and PM-3 determined in this study have been deposited in the GenBank database under accession numbers FJ598436 and FJ598437.

3.2. HPAM biodegradation

In order to investigate the utilization of HPAM by the two bacteria, PM-2 and PM-3, the bacterial suspension containing the mixed two bacteria were incubated with 1% inoculums (volume ratio) in the CM, NM, CNM, respectively, using a rotary shaker (140 r min^{-1}) at 40 °C for 7 days. The results are shown in Fig. 1.

Sutherland et al. [16] studied PAM biodegradation by a White-Rot Fungus and they observed that the degradation of the polymers was occurred only in nitrogen limited cultures. But this study suggested that the two bacteria could grow well in those media containing HPAM, nitrogen limited cultures (NM), carbon limited cultures (CM) and both two limited cultures (CNM). The HPAM removal efficiency (36.3%) was found to be twofold higher in NM medium, when HPAM served as the sole nitrogen source, compared to the removal efficiency in the other two media, CM and CNM, which were 14.7% and 13.6%, respectively. The biomass was also about twofold higher in NM (5.6×10^7 cell mL^{-1}) than in CM (2.8×10^7 cell mL^{-1}) or CNM (3.0×10^7 cell mL^{-1}) (Fig. 1). The HPAM removal efficiency and biomass were similar in CM and CNM. The results of this experiment suggested that the bacteria could partly utilize HPAM as either carbon or nitrogen source, but better biodegradation and biomass were achieved when the bacteria utilized HPAM as the sole nitrogen source. These results supported

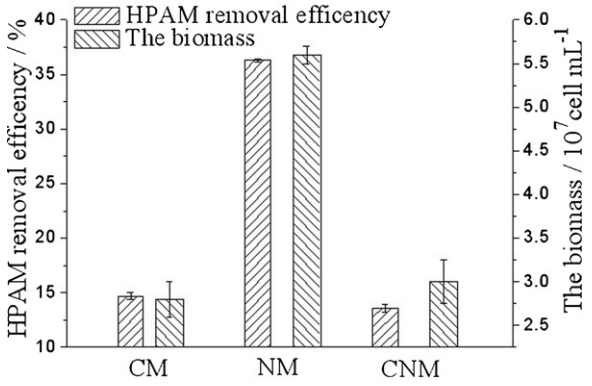

Fig. 1. HPAM removal efficiency and biomass in different media conditions.

the hypothesis that the bacteria could more readily obtain nutrition from the amide groups of HPAM using their amidase enzyme, compared to cleaving the main chain C backbone of HPAM when HPAM served as the sole nitrogen source [4]. The results could also be interpreted as that CNM as a sole carbon and nitrogen source medium inhibits both bacterial growth and HPAM degradation. In this paper, HPAM concentration was measured by the starch-cadmium iodine method [13], actually, starch-cadmium iodine method determines the change of amide group concentration in HPAM, but not the main carbon chain of HPAM. When bacteria grow in NM, HPAM serves as the sole nitrogen source. However, it may not serve as carbon source, because there is other carbon source (paraffin), and paraffin can be more easily utilized by bacteria. When we cultivate bacteria in CM and CNM, HPAM serves as the sole carbon source in these two culture broths, and the main carbon chain of HPAM may be utilized by bacteria. Besides, bacteria do better amide group removal of HPAM in CM (14.7%) than in CNM (13.6%). So we choose CM for further study. 100 mL CM medium which was biodegraded by the bacteria in a rotary shaker (140 r min^{-1}) at 40 °C for 7 days (a sample after biodegradation), and the control (a sample before biodegradation) were ready for SEM, FT-IR and HPLC analysis after pretreatment and purification (followed by Sections 2.9 and 2.10).

3.2.1. SEM analysis

HPAM in different states was studied (solid, autoclaved, after biodegradation) using SEM at 3000× magnification, in order to examine its structure after biodegradation. The SEM photographs are shown in Fig. 2: the HPAM solid is dense with many fine particles of polymer on its surface (Fig. 2a); the HPAM solution after sterilizing in the autoclave at 120 °C (analyzed after dehydration to form a solid sample, Fig. 2b) was also dense with few cavitations; the HPAM sample after biodegradation for 7 days in CM media (analyzed after preparation to form a solid sample, Fig. 2c) had many cavities of different sizes [17]. The biodegraded sample had been get rid of bacterial cells prior to SEM analysis by being centrifuged at 6150 × *g* centrifugal force, so it is hard to see bacteria adhering to the HPAM surface in SEM photograph. The results suggested that bacterial activities might cause the cavities seen with SEM. That is very similar to the studies in Li et al. [17] and Wei and Ma [18].

3.2.2. FT-IR analysis

The FT-IR spectra of different HPAM samples were analyzed, in order to investigate the changes in HPAM molecular structure before and after biodegradation. Fig. 3a shows the spectra of HPAM solid, HPAM solution and HPAM solution after sterilizing. Fig. 3b shows the spectra of HPAM samples before and after biodegradation.

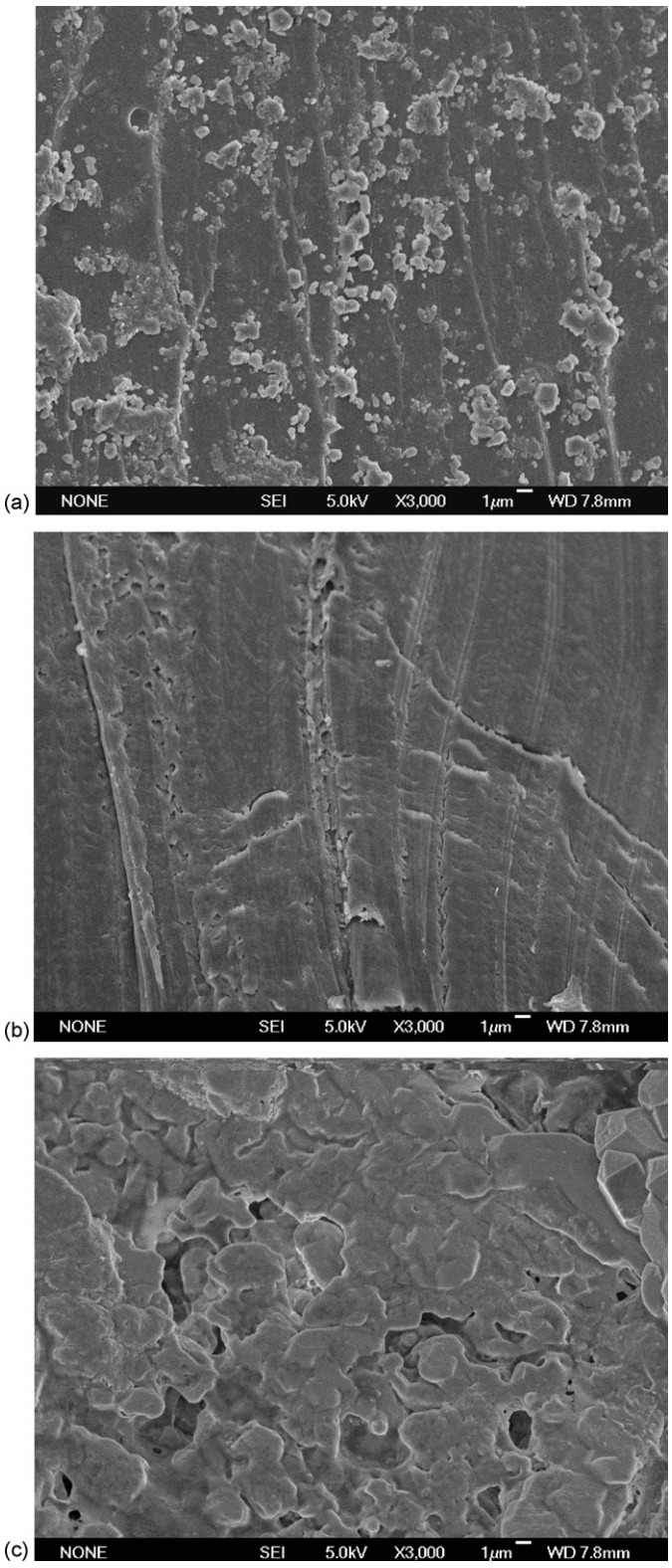

Fig. 2. Scanning electronic microscope photos of different HPAM samples (3000×). (a) HPAM solid. (b) HAPM solution after autoclaving at 120 °C. (c) HAPM sample after biodegradation.

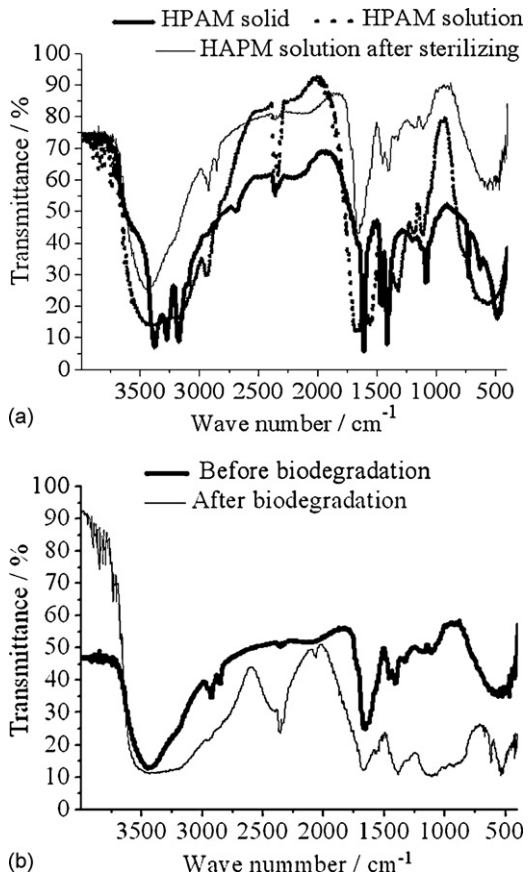

Fig. 3. FT-IR analysis of HPAM under different conditions. (a) Effect of hydroxylation on HPAM IR spectra. (b) Effect of biodegradation on HPAM IR spectra.

In Fig. 3a, some differences in the spectra of the three samples were observed at 3100–3500 and 1500–1700 cm^{-1}, which was due to the different degree of hydrolysis of the amide groups. In the spectra of HPAM solid, the peaks of 3381 and 3273 cm^{-1} are the characteristic absorbing peaks of amide group. The peaks of 1615, 1415 and 1475 cm^{-1} indicated the existence of C=O, C–N and N–H bonds [19]. Compared with HPAM solid, the absorbance peaks caused by the C=O stretching vibration in the spectra of HPAM solution and HPAM solution after sterilizing, moved to the position of higher wave number, at 1668 and 1670 cm^{-1}. Regarding the effect of the carboxyl, O–H stretching vibration, the large absorbing peaks of the amide group (3381 and 3273 cm^{-1} in the HPAM solid spectrum) were absent in these two samples. However, the peaks due to C–N stretching vibration (around 1415 cm^{-1} in the spectra of these two samples) and the N–H flexing vibration (around 1475 cm^{-1}) remained in the two samples, which indicated that the amide groups were still present in the two samples, and were only partly and not completely hydrolyzed. However, few previous studies focus on FT-IR spectra changes of HPAM due to hydrolysis.

As shown in Fig. 3b, the absorbance peak caused by the C=O stretching vibration in the spectrum of the HPAM sample after biodegradation, also moved to the position of higher wave number compared with the same peak in the spectrum of the HPAM sample before biodegradation. The peaks due to the C–N stretching vibration (1415 cm^{-1} in the spectrum of HPAM sample before biodegradation) and N–H flexing vibration (1475 cm^{-1} in the spectrum of HPAM sample before biodegradation) had disappeared, and the peak at 3100–3500 cm^{-1} had become wider [20]. This indicated that the amide group had degraded into a carboxyl group. This showed a consistency results with Liao et al. [21]. Wen et al. also did FT-IR analysis of PAM before and after the degradation, and it indicated that the amido groups of the PAM were picked off by the microorganisms from the main chain of the PAM [7].

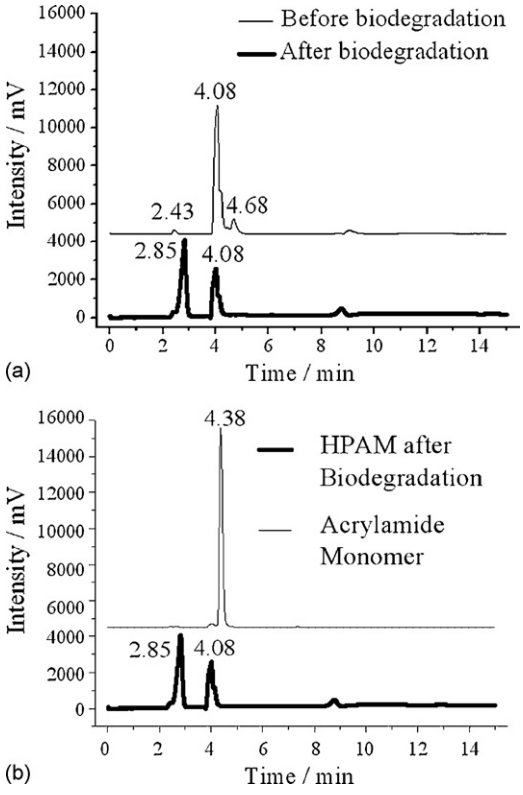

Fig. 4. HPLC analysis of HPAM samples and acrylamide monomer. (a) HAPM before and after biodegradation. (b) HAPM after biodegradation and acrylamide monomer.

3.2.3. HPLC analysis

In order to determine whether acrylamide monomer exists in an HPAM sample after biodegradation, HPAM samples before and after biodegradation were studied using HPLC. And a standard acrylamide monomer sample was also included in the analysis. Before biodegradation, there were three peaks in the HPAM liquid chromatogram with retention times (r.t.) of 2.43, 4.08 and 4.68 min, respectively. In the liquid chromatogram of the HPAM sample after biodegradation (Fig. 4a), besides the first two peaks (r.t. 2.43, 4.08 min, respectively), there was another peak with r.t. 2.85 min. The r.t. of the acrylamide monomer was 4.38 min, and there was no peak at this retention time in the liquid chromatogram of the HPAM sample after biodegradation (Fig. 4b). These results indicated that there was no acrylamide monomer in the HPAM sample after biodegradation. And FT-IR analysis of PAM biodegraded samples in Wen et al. also indicated that metabolism products other than acrylamide were formed in the biodegradation [7]. The compound corresponding to peak found at r.t. 2.85 min in the sample after biodegradation may be polyacrylic acid, which was one kind of metabolism products of HPAM. However, further analysis about it was not done. And the difference between liquid chromatograms of the HPAM sample before and after biodegradation need further exploration in later work.

3.3. Preliminary study of HPAM biodegradation mechanism

Anionic polyacrylamide is a negatively charged high molecular weight polymer and strongly resists biodegradation, even after ozone degradation (decreasing the molecular weight from 280,000 to 840 g mol^{-1}) [2]. This is not only due to its high molecular weight but also the molecular structure of the PAM "backbone" linkage [22]. Bacteria possess a net negative charge at the pH value found in most natural habitats [23]. In the current culture broth for HPAM degradation, all of them are near-neutral pH solution,

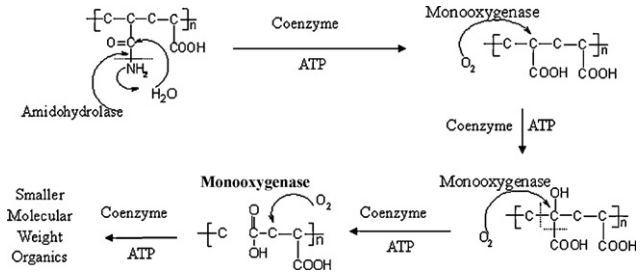

Fig. 5. Hypothetical mechanism of HPAM biodegradation in aerobic condition.

thus, these bacteria get negative charge. The carboxylic groups (COO^-) on the side chains of the HPAM molecule are also negatively charged. Therefore, we hypothesize that because of static rejection of negative charge on bacteria and COO^-, bacteria have difficulty in contacting the main chain of the HPAM molecule. The amide group, which is the side chain of the HPAM molecule, can be readily attacked by amidase enzymes. Amidase activity has been identified in numerous genera of bacteria, including *Rhodococcus*, *Bacillus*, *Mycobacterium*, *brevibacterium*, *Alcaligenes*, *Pseudomonas*, etc. [5].

The following hypothesis to explain the mechanism of biodegradation of HPAM in aerobic culture is proposed (Fig. 5). (1) The bacteria first used HPAM as their sole source of nitrogen. The nitrogen was obtained by hydrolysis of the amide groups from HPAM, using an amidase enzyme produced by the bacteria. The C–N in the amide group of HPAM was cut, –OH in aqueous solution replaced $–NH_2$, $–NH_2$ was released and –COOH was produced; (2) catalysis by monooxygenases oxidation on the main HPAM carbon chain occurred, which was similar to the α-oxidation of fatty acid [24]. The α-[$–CH_2–$] was first oxidized to –COH–, transferred to –CHO, and then oxidized to –COOH [25,26]. During this process, the main carbon backbone was cleaved and the HPAM was transformed into smaller uncharged molecules, which could then serve as the carbon source. Additional bacterial metabolism and physical shaking in the bacterial incubator caused further breakdown of the main carbon chain of HPAM into smaller pieces. During this biodegradation progress, large amounts of ATP and coenzymes were needed, thus, enough phosphorus should be available from the medium.

4. Conclusions

Two HPAM-degrading bacterial strains, named PM-2 and PM-3, were isolated from the produced water of polymer flooding. They were subsequently identified as *B. cereus* and *Bacillus* sp., respectively. Growth and biodegradation of HPAM by bacteria was explored. The results showed that bacteria could partly utilize HPAM as an either sole nutrient source of carbon or of nitrogen plus carbon; however, twofold higher HPAM removal efficiency and bacterial biomass production were obtained when nitrogen was provided as the sole nutrient source for bacterial growth. Based on the SEM, IR and HPLC analysis of HPAM samples before and after biodegradation, a hypothesis about the mechanism of biodegradation of HPAM in aerobic culture is proposed that suggests that HPAM undergoes hydrolysis of its amide side groups by bacterial amidase enzymes under conditions where nitrogen is provided as the sole nutrient source. As a consequence of bacterial growth and metabolism and mechanical shearing processes, the main carbon backbone of the HPAM molecule is cleaved into smaller molecules which can then provide a more accessible carbon source for the bacteria, and which can be degraded further by bacterial monooxegenase enzymes.

Acknowledgements

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