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Evolution and functional analysis of extracellular polymeric substances during the granulation of aerobic sludge used to treat *p*-chloroaniline wastewater

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ABSTRACT

Extracellular polymeric substances (EPS) are major high-molecular-weight secretions from microorganisms, binding with cells to form a vast net-like structure for protecting cells against external stress. In this study, the level and function of EPS during the granulation of aerobic sludge used to treat *p*-chloroaniline (*p*-ClA) wastewater were investigated. The content of extracellular proteins (PN) increased relative to the exopolysaccharides (PS) content during the sludge granulation (Stage I), implying that PN contributed significantly to the formation of aerobic granular sludge (AGS). Under the condition of high *p*-ClA shock loading (Stage II), the PS content increased from 122.7 ± 22.0 mg g^{-1} VSS to 482.6 ± 5.4 mg g^{-1} VSS, which might be a regulating mechanism of AGS to address high *p*-ClA loading. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that PN bands increased with the development of sludge granulation, particularly the proteins with molecular weight located at 44.3–97.2 kDa. Fourier transform infrared (FTIR) spectra of inoculated activated sludge and matured granular sludge indicated that microorganisms appeared to aggregate in the presence of tyrosine protein, aspartic acid protein and proteins with secondary structures including 3-turn helix and β-sheets. Five types of proteins were identified by two-dimensional electrophoresis (2-DE), and these proteins were related to the granulation of aerobic sludge and EPS secretion. Furthermore, the analysis of sludge microbial community revealed that the enrichment of *Zoogloea* spp. and *Thauera* spp. as *p*-ClA degrading and EPS secreting microbes was conducive to the formation of granular sludge and degradation of *p*-ClA.

1. Introduction

Aerobic granular sludge (AGS) is a novel wastewater treatment technology developed by Mishima and Nakamura in 1991. Because of its characteristics, such as excellent settling ability, strong impact resistance and smaller occupied area of the bioreactors, AGS has drawn worldwide attention in recent decades [1–4]. Aerobic sludge granulation is a process whereby microorganisms form intact, regular-shaped biofilm through self-flocculation under specific circumstances. Although the AGS formation mechanism remains unclear, the EPS hypothesis appears to be the most plausible [5,6]. Extracellular polymeric substances (EPS) are high molecular weight polymers that are secreted by microorganisms to resist external stress during growth [7,8]. These polymers are primarily exopolysaccharides (PS), extracellular proteins (PN), humic acid and extracellular DNA. Many studies have shown that EPS are keys for cell adhesion and aggregation [9–11].

Most studies suggest that PS and PN are major influencing factors in the formation of biofilms and granular sludge. Previous researches have shown that PS crosslink with each other and form a network that comprises the bulk of granular sludge. High hydraulic force, short sludge settling time and HRT can increase the secretion of PS. They are distributed inside the granules and the gaps between *Zooglea* cells. Different concentrations of EPS lead to changes in the surface character of microorganisms. It has been suggested that PS covering the exterior cell walls can promote granulation by reducing the critical voltage of the cell, and blocked mutants that do not produce PS can barely flocculate [1,12].

In recent years, an increasing amount of research has focused on

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studying PN due to their structural and metabolic functions. McSwain and Wilderer [9] showed that PN were the main component of EPS, with a content of 144–455 mg g^{-1} VSS. The PN/PS ratio was 6.6–10.9. They concluded that electronegative amino acids are primary components of PN that increase cell surface hydrophobicity and also promote the formation of granules via anodic bonds with positive ions. Zhang et al. [13] analyzed the polyacrylamide gelelectrophoresis (SDS-PAGE) strip variation of PN and found that the average molecular weight of PN was 31–97.4 kDa in the EPS of granular sludge. However, questions still remain about the key components of the EPS for the formation and structure stability of aerobic granule.

There are several techniques that are used to analyze protein properties, such as molecular weight and functional groups characterization. The combination of mass spectrometry with 1D- or 2D-electrophoresis (2-DE) is a common and accurate protein identification technique used in proteomics. Park identified many proteins in EPS using proteomics, finding that the EPS of activated sludge contain a large amount of root mucins, specific leucine-binding proteins and flagellin, which are involved in the aggregation and adhesion of bacterial cells [14]. Higgins also found a lectin-like protein with a molecular weight of 15 kDa in PN, and he proposed a model whereby a stable granular network is formed through the combination of PN, PS and divalent cations [15]. However, previous research rarely used proteomics in general, and information regarding the functional proteins in sludge granulation is scarce. On the other hand, *p*-Chloroaniline (*p*-ClA) has been extensively used as an important intermediate in the industrial production of cosmetics, pharmaceutical products, dyes, pesticides and herbicides, which inevitably results in its wide distribution in the environment [16,17]. Due to its environmental toxicity and persistence, economical and effective degradation technologies should be developed. In this study, a sequencing batch reactor was operated to study the variation and effects of EPS on the formation and structure stability of aerobic granular sludge that was used to treat *p*-ClA wastewater. SDS-PAGE and FTIR spectroscopic technologies were used to analyze molecular weights of PN and functional groups characterization of EPS. At the same time, the differential proteins of EPS extracted from inoculated activated sludge and mature aerobic granule were studied by 2-DE to understand the function of the major PN in sludge granulation. Furthermore, pollutants removal performance, sludge characteristics and microbial community structure were investigated during the whole operation process to reveal the toxic effects of *p*-ClA and microbial functions.

2. Materials and methods

2.1. Reactor set-up and operational conditions

A 6-L sequence batch reactor (SBR) with a height/diameter ratio of 5:1 was established in a temperature-controlled room at 25 ± 2 °C (Fig. S1) and the cycle length was 3 h with 10 min of feeding, 162 min of aeration, 5 min of settling and 3 min of withdrawal. For aeration, fine air bubbles were supplied through a porous stone at the bottom of the reactor with a superficial gas velocity of 2.5 cm s^{-1}. Effluent was discharged at a volumetric exchange ratio of 50%. The abiotic loss of *p*-ClA in the SABR was negligible under identical operational conditions.

Activated sludge from the aeration tank of the Qige wastewater treatment plant in Hangzhou was used as the inoculum of the SBR for cultivation of aerobic granule capable of degrading *p*-ClA, with an initial mixed liquor suspended solids (MLSS) concentration of approximately 6.0 g L^{-1} and an initial sludge volume index (SVI) of approximately 85 mL g^{-1}. The composition of the synthetic wastewater was as follows (mg L^{-1}): sodium acetate, 201; sucrose, 37; NH_4Cl, 96; KH_2PO_4, 22; K_2HPO_4, 28; yeast, 100; peptone, 150; $CaCl_2$, 30; $MgSO_4$, 30; and trace element solution comprised of the following materials: H_3BO_3, 0.05; $CuSO_4{\cdot}5H_2O$, 0.05; $ZuSO_4{\cdot}7H_2O$, 0.05; $AlCl_3$, 0.09; $CoCl_2$, 0.05; $MnSO_4{\cdot}H_2O$, 0.05; $(NH_4)_2Mo_7O_{24}$, 0.05; $NiCl_2{\cdot}6H_2O$, 0.09; and $FeSO_4{\cdot}7H_2O$, 0.05.The target pollutant of the aerobic granular sludge bioreactor was *p*-ClA, with a gradient enhanced influent load during the operation. The ratio of COD: N: P was maintained constantly at 100:5:1.

2.2. Analysis of sludge characteristics and wastewater quality

The biomass concentration including mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) were analyzed according to APHA standard methods. Particle size distribution and sludge morphology were analyzed using an image analysis sensor (Sympatec, QICPIC) and a research microscope (Leica, DMLB + QCOLite), respectively. Granular structure was observed by scanning electron microscopy (SEM) (Hitachi, Model TM-1000, Japan) after sludge samples were dehydrated and coated with gold-palladium, and the method was the same as that described by Zhou [18]. The sludge contact angles were measured using image contact angle measuring instrument (OCA20, DATAPHYSI company, German).

Wastewater quality including chemical oxygen demand (COD), total nitrogen concentration (TN), total phosphorus concentration (TP), and ammonia nitrogen concentration(NH_4^+-N) was measured regularly according to the APHA standard methods after being filtered by 0.45 μm cellulose acetate membrane, and *p*-ClA was measured by liquid chromatographic analysis.

2.3. Extraction and chemical analysis of EPS

Sludge samples were prepared in the late stage of aeration, and the sludge EPS was extracted using a modified heat method according to the previous research [19]. The detailed extraction procedure was as follows: the sludge sample was washed 2–3 times with distilled water, and then the sludge pellet was resuspended with a 0.05% NaCl solution and the suspension subsequently was ground in a glass homogenizer. After that, the sample was soaked in a 60 °C water bath for 45 min, and finally centrifuged at 20,000 rpm for 20 min to collect the supernatant as EPS extracts of the sludge sample.

The PS content was measured using the phenol-sulfuric acid method with glucose as the standard [20], and the PN content was measured using the Lowry method with bovine serum albumin as the standard [21].

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of extracellular protein (SDS-PAGE)

The EPS extracts were concentrated in a rotary evaporator (BUCHI, Switzerland), and the protein was settled at 4 °C for 24 h using trichloroacetic acid as the denaturant. Then, the sample was centrifuged and the supernatant was discarded. The protein sediment was washed with propyl alcohol and ethyl acetate (1:1) for 3 times and then dried with nitrogen. The dry PN sample was stored at −20 °C until use.

The protein samples were reconstituted to 3 mg mL^{-1} in 2-time SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.004% bromophenol) and heated at 100 °C for 7 min. The samples were centrifuged at 12,000 rpm for 5 min and the supernatant was used for analysis by SDS-PAGE on a Mini SE260 electrophoresis cell (Amersham) according to a modified method of Laemmli [22]. Aliquots (10 μL) of protein were loaded into the gel wells. The electrophoresis was performed at 80 V through the stacking gel (5%) and then at 150 V through the separation gel (10%) until the bromophenol blue marker dye reached to within 0.5 cm of the bottom of the gel. Then, the protein bands were stained with Coomassie Brilliant Blue R-250. After staining, gels were destained overnight in a solution containing 7.5% ethanol and 7.5% acetic acid. The following polypeptides were used as the molecular weight markers: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), egg albumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

2.5. Fourier transform infrared spectroscopy (FTIR)

2 mL aliquots of EPS extracts were concentrated using rotary evaporators (BUCHI, Switzerland) and then frozen for 24 h in a −70 °C refrigerator. The frozen EPS extracts were converted into a pellet via freeze-drying. Afterwards, the EPS pellet was mixed with infrared grade KBr at the ratio of 1:100 and homogenised in an agate grinder. 150 mg of the mixture was molded into a disc and then analyzed using a spectrum 8900 IR spectrometer (Shimadzu, Japan). The scanning conditions were as follows: a spectral range of 4000–400 cm^{-1}, 32 scans and a resolution of 4 cm^{-1}.

2.6. Two-dimensional electrophoresis (2-DE) of extracellular protein

10 mL EPS extracts were mixed with 10mL phenol Tris, and then the mixture was oscillated every 5 min (6 times) followed by 30 min centrifugation of 5000 rpm at 4 °C. The upper phase was collected.

The collected protein extracts were repeated above extraction operation twice, after that, adding 5 times of amount of ammonium acetate methanol solution to the final phenol phase. After being oscillated every 5 min (for 6 times), the samples were precipitated at −20 °C overnight, and then the supernatant was discharged after 30 min centrifugation of 5000 rpm at 4 °C. The protein precipitation was washed for two times with methanol and acetone, respectively, and then dried at room temperature. The dry PN sample was stored at −80 °C until use.

2-DE was carried out using a Bio-Rad 2-DE system. Briefly, 150-μg protein samples were used for isoelectric focusing (IEF) with the ReadyStrip immobilized pH gradient (IPG) strips (17 cm, pH 4–7). The strips were placed into a Protein IEF cell and rehydrated at 50 V for 12 h. The proteins were separated based on their isoelectric points according to the following protocol: 250 V with a linear climb for 30 min, 1000 V with a rapid climb for 60 min, 10,000 V with a linear climb for 5 h, and 10,000 V with a rapid climb until 60,000 Vh was reached. After the completion of focusing, the strips were equilibrated by two steps, first in a buffer containing 50 mMTris–HCl(pH 8.8), 30% glycerol, 7 M urea, 2% sodium dodecyl sulfate and 1% dithiothreitol, followed by treatment in a similar buffer (but containing 4% iodoacetamide instead of dithiothreitol). Each step lasted for 15 min. After equilibration, the IPG strips were applied to 12% homogeneous sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels for electrophoresis using a PROTEIN II xi Cell system. The system was run at 10 mA/gel for 30 min followed by 30 mA/gel for 5–6 h until the bromophenol blue line reached the bottom of gels. The gels were then silver stained using Silver Stain Plus kit reagents according to the manufacturer's instructions. The silver-stained gels were scanned using a Densitometer GS-800 and then analyzed using PD-Quest software. The individual protein spot quantity was normalized as follows: the raw quantity of each spot in a member gel was divided by the total quantity of the valid spots in the gel, and normalized spot intensities were expressed in ppm.

Statistical analysis was performed using Student's *t*-test between protein gels from the sham-operation and IR groups. The significantly differentially expressed the protein spots ($P < 0.05$) with a 1.5-fold or more increased or decreased intensity between the sham-operation and IR groups were selected and subjected to further identification by MALDI-TOF MS/MS. The spot intensities of these spots (possible IR-related proteins) in other groups (IR + SA, IR + NG, IR + CSN) were also examined [23].

2.7. DNA extraction, PCR and 454 pyrosequencing

Genomic DNA of the biomass in the seed sludge and granule samples was extracted using an EZNATM Soil DNA kit (D5625-01, Omega, USA), and then stored at −20 °C. Subsequently, the V1-V3 region of the 16S rRNA gene of bacteria was amplified by PCR with the forward primer (5′-454adapter-mid-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer (5′-454adapter-TTACCGCGGCTGCTGGCAC-3′). The PCR amplification was performed using iCycler thermal Cycler (Bio-Rad, Hercules, CA), and the optimal PCR amplification procedure included: pre-denaturation at 94 °C for 4 min, denaturation 30 s at 94 °C, 45 s annealing at 60 °C, 2 min systhesis at 72 °C, 20 cycles; 7 min extension at 72 °C. Amplification products were tested with 1% agarose gel, and then the PCR-amplified fragments were purified by AMPure Beads.

The amplicon library was quantified on a microplate reader using PicoGreen dsDNA Assay Kit, after that, diluted PCR purified products and mixed them with the library in equal amounts, then the DNA library was captured using DNA Capture Beads and emulsified amplification was performed. Thereafter, amplicon pyrosequencing was implemented using a Roche Genome Sequencer GS FLX Titanium platform (Personalbio™, Shanghai, China).

3. Results and discussion

3.1. Reactor performance and sludge characteristics

The whole operation process was divided into two stages: Stage I - Formation of aerobic granule, and Stage II - Stability of aerobic granule. For the quick startup of the granular sludge process, the sludge settling time was shortened from 10 min to 5 min after 10 days of operation to enhance biological selection force and promote the sludge aggregation at Stage I. Small granules started to appear in the system on Day 28, with the average particle diameter increasing to 182.67 μm, MLSS higher than 6500 mg L^{-1}, SVI lower than 40 mL g^{-1}, the sludge hydrophobic angle of 83.1° and sludge age of 5.652 days (Fig. 1). After Day 45, mature granular sludge was achieved in the system. MLSS stabilized at approximately 6800 mg L^{-1}, SVI decreased to 35 mL g^{-1}, the average particle diameter of the sludge increased to 252.4 μm, and the hydrophobic angle raised to 102.4°.When the *p*-ClA loading increased to 640 g m^{-3} d^{-1} at Stage II, the deterioration of the sludge occurred, but it recovered quickly, showing the excellent shock resistant property of aerobic granular sludge. The granular size decreased and the sludge settleability became worse rapidly when the *p*-ClA loading increased to 800 g m^{-3} d^{-1}, and then the granular size, MLSS and SVI returned to normal level when the *p*-ClA loading was maintained at 640 g m^{-3} d^{-1}. At this phase, the MLSS and SVI stabilized above 6000 mg L^{-1} and below 30 mL g^{-1}, respectively. The sludge age was approximately 6.4 d, the hydrophobic angle was 97.6°, and the average particle diameter was over 330 μm. SEM images showed that mature granules contained compact structure with less cracks, and the microorganisms including coccus and bacillus brevis embedded in the EPS were highly enriched (Fig. S2).

Pollutants removal performance was shown in Fig. 2. At the beginning of Stage I, the system performed under a low *p*-ClA loading, and the *p*-ClA removal efficiency reached 98% after a short period of adaption (Fig. 2a). As the influent substrate was mainly biodegradable sodium acetate and sucrose, the COD removal efficiency stabilize at more than 90% throughout (Fig. 2b). In the first 5 days of operation, ammonia removal efficiency (ARE) and total nitrogen removal efficiency (NRE) were only about 42.4% and 48.8%, respectively (Fig. 2c-d). It is speculated that the activity of ammonia oxidizing bacteria (AOB) was inhibited when suddenly exposed to *p*-ClA. Then, with the gradual adaptation of the system, ARE approached 100% and NRE achieved 85%, respectively. However, the nutrients removal of the system appeared to decline when *p*-ClA loading increased to 96 g m^{-3} d^{-1}, and more severely, ARE, NRE and TP removal efficiency (PRE) (Fig. 2e) decreased to 70.8%, 28.9% and 64.7% respectively at *p*-ClA loading rate 320 g m^{-3} d^{-1}, but it recovered quickly as granular intensity and activity significantly improved in the late stage I.

At stage II, the sludge deterioration was observed along with the *p*-ClA load increasing to 640 g m^{-3} d^{-1}, particularly the pollutant

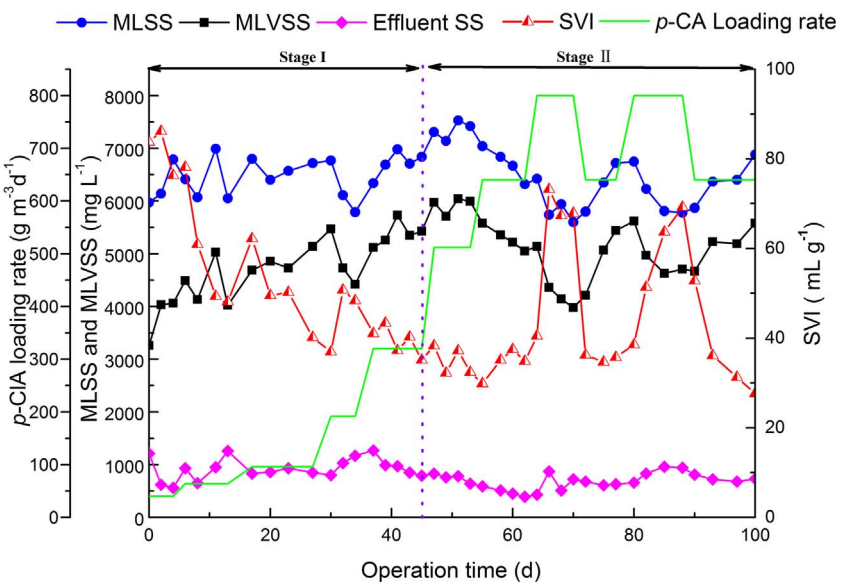

Fig. 1. Variation of the sludge characteristics in different stages.

removal efficiency dropped. *p*-ClA and COD removal efficiency decreased slightly, down from 99.7% to 92.8% and 98.9% to 90.2%, respectively, and then returned quickly to previous good performance. ARE and NRE declined to 65.2% and 18.9% respectively, but it only took 4 days to recover to the normal level (98.8% of ARE and 77.8% of NRE). Under the twice shock load of 800 g *p*-ClA m^{-3} d^{-1}, only TP removal efficiency decreased from 99.4% to 78.0% at the first load shock and then recovered quickly, while the ammonia and TN removal efficiencies remained generally stable, which showed the excellent shock resistant property of aerobic granular sludge. As the *p*-ClA loading was stabilized at approximately 640 g m^{-3} d^{-1}, the reactor performance remained in good condition, with ammonia and COD removal efficiencies of both higher than 98%, and TN, TP and *p*-ClA removal efficiencies of approximately 80%, 90% and 99%, respectively.

3.2. Variation of EPS contents during aerobic sludge granulation

The changes of EPS content were analyzed (Fig. 3), the results in the start-up stage showed that the PN content increased from 89.36 ± 2.71 mg g^{-1} VSS of seed sludge to 340.06 ± 22.57 mg g^{-1} VSS of initial granular sludge (Day 28). The PS content only slightly increased and PN/PS achieved 5.33. Furthermore, with the increasing degree of granulation, the PN content continued to increase and reached up to 455.48 ± 60.63 mg g^{-1} VSS at Day 37. Consequently, PN had a significant impact on the granulation process, which was consistent with the conclusion that the flocculation ability of sludge increased with an increase in the protein content drew by Wilen et al. [24]. This attributed to the unique charge properties of proteins. The amino groups in proteins are positive and can neutralize the negative charges from carboxyl and phosphate groups, and thus decrease the net negative surface charges of sludge and then promote sludge flocculation and granulation.

At stage II, under the condition of high *p*-ClA shock loading, the PS content increased from 122.7 ± 22.0 mg g^{-1} VSS in the later stage I to 482.6 ± 5.4 mg g^{-1} VSS, whereas the PN content remained stable. At the same time, the PN/PS ratio decreased to 0.7, and a positive correlation was observed between the PS content and *p*-ClA loading (r^2 = 0.85). It is supposed that PS secretion could be a regulating mechanism of aerobic granular sludge to address high *p*-ClA loading. McSwain et al. [9] observed that the cells and carbohydrates were present in the outer layer of aerobic granular sludge. Chen et al. [12] also found that in acetate-fed aerobic granules, protein and β-D-glucopyranose polysaccharides formed the core, whereas the cells and α-D-glucopyranose polysaccharides accumulated in the granule outer layers. Thus, at the shock of high *p*-ClA loading, the protective mechanism of aerobic granular sludge was activated, leading to the mass secretion of EPS, especially PS, as they could cover the outer layer of the granules, and protect microbial cells from the harsh external environment. Subsequently, when the system was acclimated and the structure of granular sludge tended to be more stable, the PS content declined to 333.57 ± 20.18 mg g^{-1} VSS.

3.3. Main component analysis of sludge EPS during aerobic sludge granulation

According to the performance of the reactor at different stages, the sludge samples were selected for studying the key components of sludge EPS during aerobic granulation.

3.3.1. SDS-PAGE analysis of sludge PN

The quantitative analysis results of EPS showed that the PN content played an important role in the granulation of sludge. However, previous studies have shown that PN properties also affected sludge aggregation and cell adhesion [19]. So, the change of molecular weights of PN during granulation and the *p*-ClA stress was further studied. The results showed that the molecular weights of PN in inoculated sludge were mainly located at 14.3–44.3 kDa (Fig. 4a, 1), while the PN bands increased with the granulation of aerobic sludge, particularly the proteins with high molecular weight located at 44.3–97.2 kDa (Fig. 4a, 2–4). Consequently, the molecular weights of PN in stabilized sludge granules were distributed in 20.1–97.2 kDa. It appeared some proteins with high molecular weight over 44.3 kDa tended to exist in the granules, suggesting that these proteins contributed more to the sludge granulation. It may be explained by that PN with high molecular weight could provide more ionic bonding sites or polymer interaction sites, so as to promote microbial flocculation and maintain the stability of the granular structure. When the *p*-ClA loading increased and the granular sludge maintained structural stability at stage II, the number of PN bands increased (Fig. 4b, 1–3), which showed there were more kinds of extracellular proteins produced by microbial secretion for resistance to the adverse environment. Then, as *p*-ClA loading rate was elevated to 800 g m^{-3} d^{-1}, the number of PN bands decreased obviously and the bands located at 44.3–66.4 kDa almost disappeared (Fig. 4b, 4). Meanwhile, the sludge particle size decreased gradually and the disintegration of granular sludge occurred. It could be seen that relatively large molecular PN (44.3–66.4, kDa) is beneficial to the

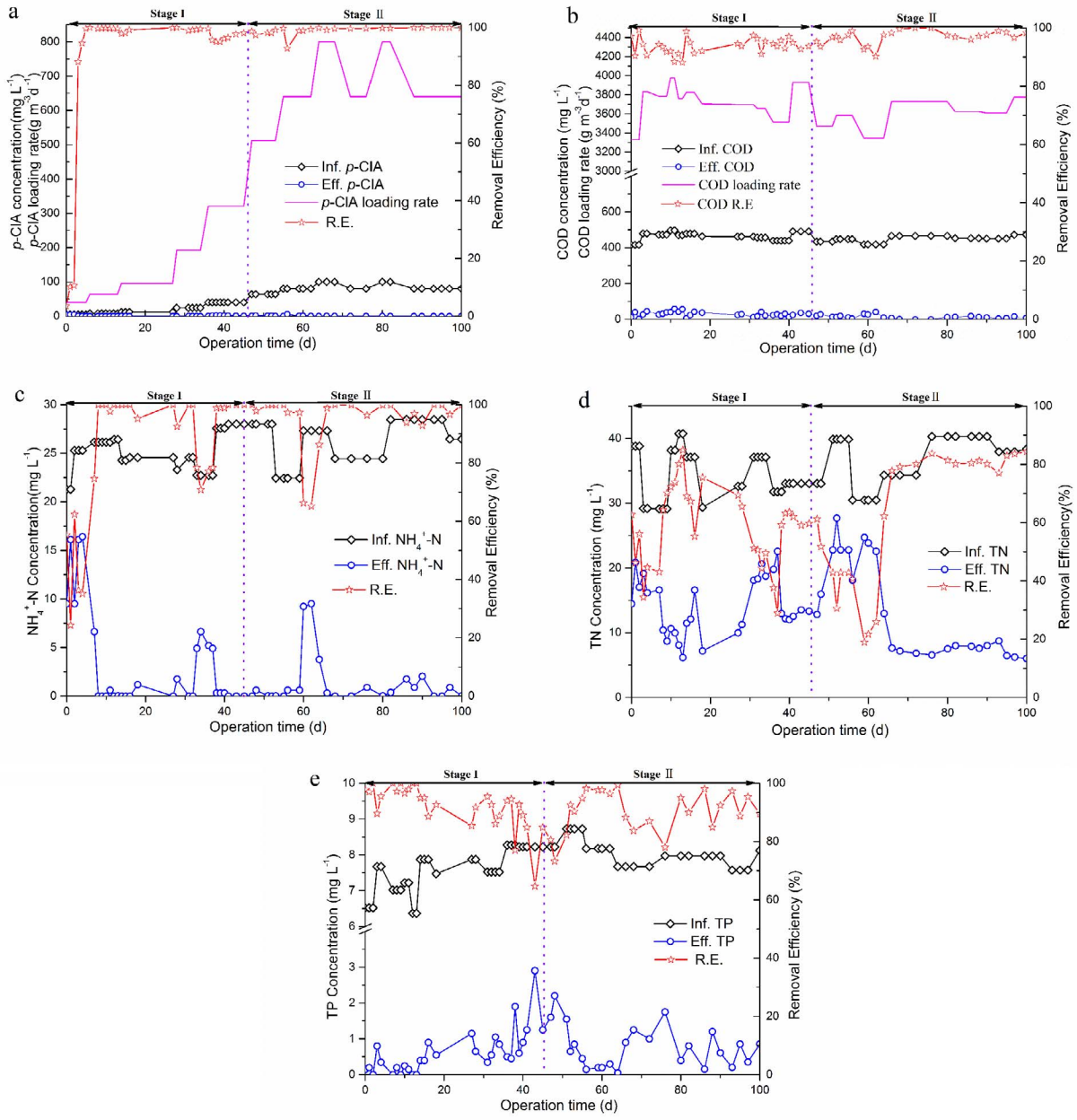

Fig. 2. Pollutants removal performance during the operation of granular sludge reactor: (a) *p*-ClA, (b) COD, (c) NH_4^+-N, (d) TN, (e) TP.

stability of granular sludge. Afterwards, *p*-ClA loading rate was declined to 640 g m^{-3} d^{-1}, and the number of PN bands increased (Fig. 4b, 5) and the stability of granular sludge recovered progressively.

3.3.2. FTIR analysis of sludge EPS

Based on the above analysis results, the FTIR analysis of the sludge EPS on 0 d (inoculated activated sludge) and 45 d (mature granular sludge) was carried out to clarify the roles of functional groups and protein structures in granulation of aerobic sludge (Fig. 5). Spectra showed that the predominant bands containing several characteristic functional groups were as follows: 3458–3454 cm^{-1} (hydroxyl functional groups), 2362–2341 cm^{-1} (CO_2 in air), 1662–1626 cm^{-1}(Amide I), 1558 cm^{-1}(Amide II), 1517 cm^{-1}(Tyr sidechains), 1400 cm^{-1} (carboxylic group), 1084–1080 cm^{-1} (polysaccharides), 993–984 cm^{-1} (nucleic acids) and 900–600 cm^{-1} (fingerprint region) [25]. The region among 1800–900 cm^{-1} was analyzed in detail, because the major bands of amide, carboxylic, and carbohydrate functional groups were located in this zone.

The two peaks at 1662 cm^{-1} and 1626 cm^{-1} existed in the sludge EPS sample on day 45 were associated with the C═O stretching vibration of 3-turn helix and β-sheets in secondary protein structure, which favored bio-flocculation [26]. Comparatively, there was only one absorption peak at 1647 cm^{-1} assigned to random coil structure in the sample on day 0, so the ability of cell adhesion and aggregation was weaker. The shoulder peak at 1517 cm^{-1} was associated with ring vibration in the phenols of the tyrosine side-chains [27] and only existed in the sludge EPS sample on day 45, indicating that tyrosine protein might be a structural component of mature granular sludge, which was in accordance with previous studies. The peak at 1400 cm^{-1} caused by C═O symmetric stretching vibration of deprotonated carboxyl group in aspartic acid [28] consisted in both EPS sample, however, the absorption peak intensity was obviously enhanced from day 0 to day 45, which coincided with the variation of protein contents, denoting that aspartic acid protein played an important role in the granulation process. Peaks at 1084 and 1080 cm^{-1} corresponded to multiple C—O asymmetric stretching vibration in polysaccharide [29], and the lower

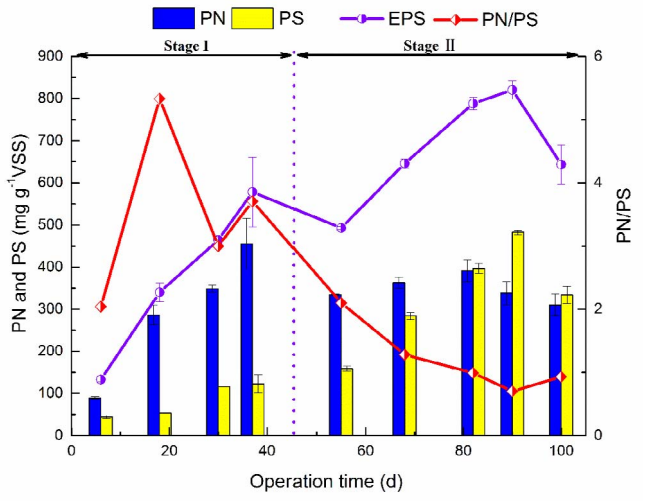

Fig. 3. The changes of EPS content during aerobic sludge granulation and *p*-ClA biodegradation.

peak intensity in the mature granular sludge EPS sample implied that the polysaccharide was not conducive to microbial aggregation. Weak vibrations from phosphodiester bonds were detected at 984 and 991 cm^{-1} indicating low amounts of extracellular nucleic acids [26], since it could serve as an indicator for quality of the sludge in terms of the health of the microbial population and biomass, lower extracellular nucleic acid content showed higher biological activity in mature granular sludge.

3.3.3. Proteomics analysis of sludge EPS

Based on the comparison of 2-DE patterns of PN in inoculated activated sludge and mature granular sludge (Fig. 6), more than 20 protein spots highly deregulated were found. Among them five protein spots were confirmed to be functional during sludge granulation according to previous researches: 1. Chaperonins (Molecular chaperone-htpG, GroEL): These proteins maintain the folding of nascent chains, polymer assembly and protein transport by regulating and adjusting the network structure system and also affect protein transfer, location and secretion directly. Researches have shown that the existence of

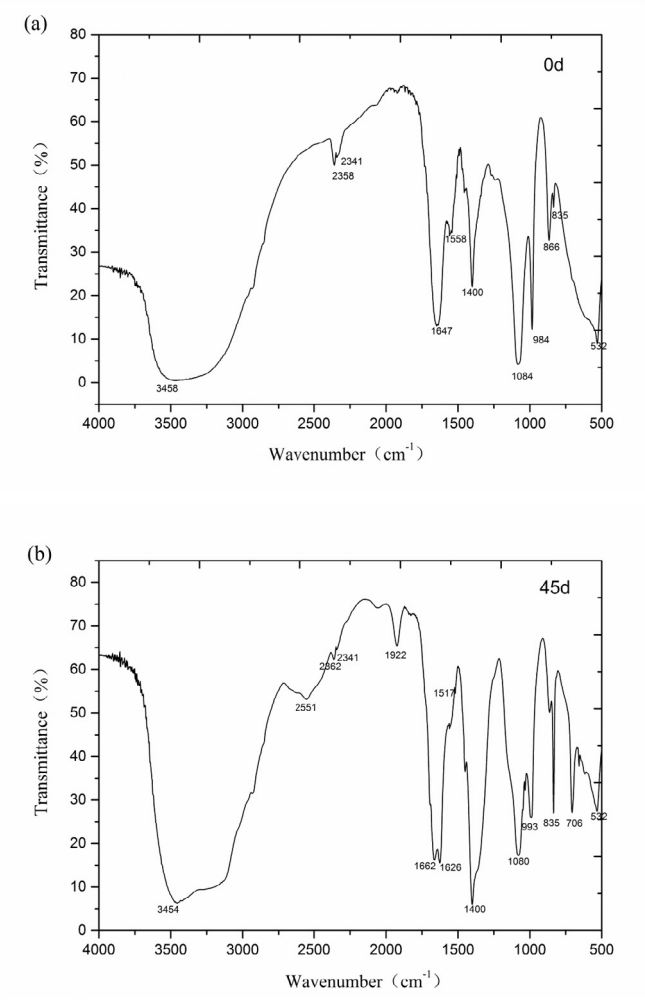

Fig. 5. FTIR spectra of sludge EPS during granulation process of aerobic sludge for treating *p*-ClA wastewater: (a) inoculated activated sludge, (b) mature granular sludge.

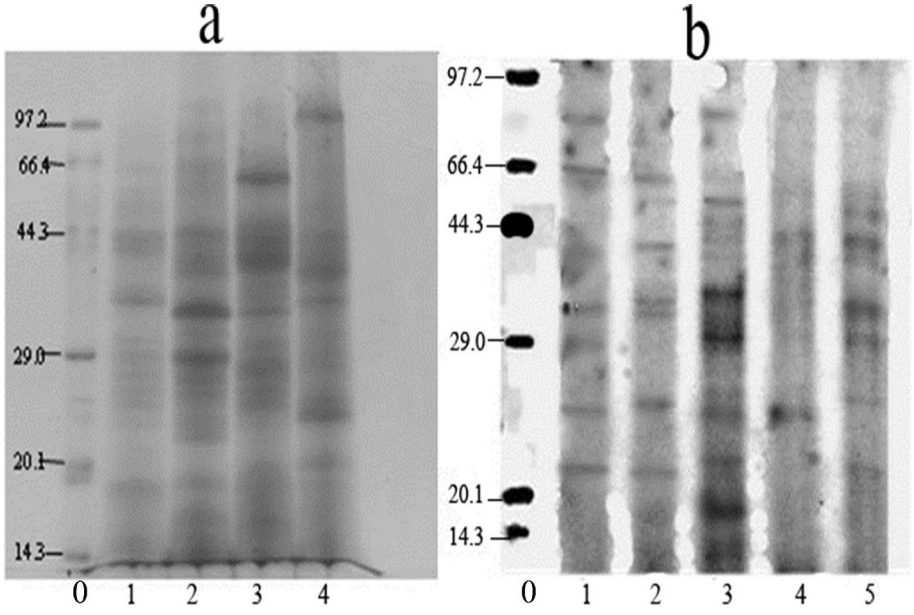

Fig. 4. SDS-PAGE of PN from sludge samples: (a) the change in Stage I (0-the molecular weight markers, 1–4 referred to the sample of 1, 18, 30, 37 d); (b) the change in Stage II (0-the molecular weight markers, 1–5 referred to the sample of 55, 68, 82, 90, 100 d).

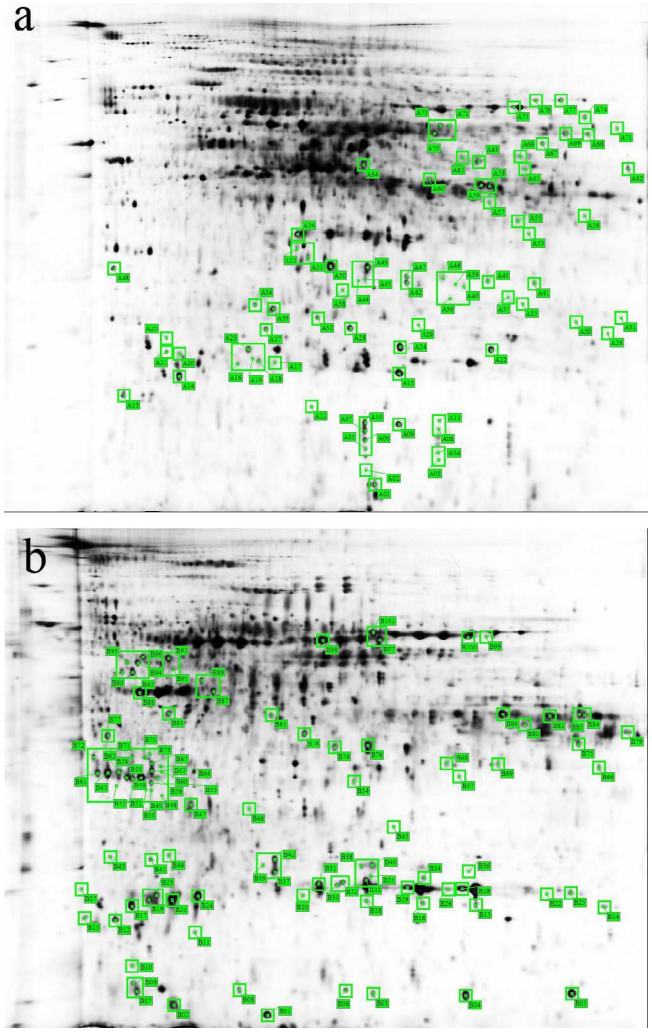

Fig. 6. 2-DE spectrometry of extracellular proteins in inoculated activated sludge (a) and stable granules (b).

chaperonins can influence the aggregation of microorganisms [30]. 2. Binding proteins (Binding Protein-bztA, Extracellular solute-binding protein, Outer membrane porin protein): Researches have shown that binding proteins are relevant to microbial colonization, adhesion and reunion [15]. These two types of proteins are enriched in granular sludge EPS and benefit sludge granulation by cell communication. 3. Metabolism-related proteins (Succinyl-CoA ligase [ADP-forming] subunit alpha, ATP synthase subunit beta, Aldehyde dehydrogenase, Malate dehydrogenase and 6-phosphofructokinase): These proteins participate in glycolysis and tricarboxylic acid cycle processes. Their existence could enhance the removal efficiency of organic pollutants [31]. 4. A-synthesis proteins (DNA-directed RNA polymerase subunit alpha, Glycyl-tRNA synthetase alpha subunit, Exodeoxyribonuclease seven large subunits and 50S ribosomal protein L25): Extracellular eDNA participates in the formation of biofilm, and the appearance of relative DNA-synthesis proteins is noteworthy [32]. 5. Other functional proteins (A-type flagellin protein): These proteins could activate signaling molecules, promoting the microbial aggregation [33].

The proteomics analysis showed the clear differences in the PN composition between seed sludge and granular sludge, which corroborates the SDS-PAGE and FTIR results. Compared with floc sludge, granular sludge is formed through a specific process, where flocs attached on inorganic nuclei and grew on the core. During this process, substances like EPS are synthesized and absorbed and energy is generated through metabolism. From the spectrometry of granular sludge, functional proteins that can achieve these objectives were identified, whereas none was present in inoculated activated sludge. Chaperonins, binding proteins and metabolism-related proteins, contain Amide III and secondary structures, which were crucial for sludge granulation. Additionally, PS did not obviously increase during sludge granulation, but PN increased greatly. It is clear that functional PN-secreting microorganisms were enriched during this process. Further analysis concerning these PN will hopefully reveal the stabilization and regulation strategy of aerobic granular sludge through structural components and metabolism.

3.4. Evolution of microbial community structure

The aerobic granular sludge reactor was started up by both stepwise increasing *p*-ClA loading and enhancing biological selective pressure to achieve mature and stable structure of granular sludge, and enrich *p*-ClA degrading bacteria in the system at the same time. Thus, the samples from inoculated sludge and mature granular sludge were collected and sequenced, respectively, and the OUT obtained by sequencing were clustered and statistically analyzed to investigate the evolution of microbial community structure.

The sequencing results showed that 17,136 and 32,094 dominant sequences were obtained from inoculated activated sludge samples and steady-state granular sludge samples. The study found that steady-state granular sludge had lower microbial biomass coverage than that of inoculated sludge (Coverage: 0.9882–0.9191), while the richness index (Chao index: 1453–9002) and diversity index (Shannon index: 4.8212–6.1669) raised considerably, suggesting microbial community structure changed severely during aerobic sludge granulation. The Veen map (Fig. S3) also showed that two of the samples jointly contained only 5.89% of the total amount of microbes measured in aerobic granular sludge. The disappearance of several microbes and the emergence of new strains showed that the screening and enrichment of key functional microbes occurred in the gradient increase of *p*-ClA loading and granulation of aerobic sludge.

As shown in Fig. 7, further analysis of sludge microbial community revealed that the predominant microbes in the inoculated sludge were mainly *Zoogloea* spp. (20%), *Thauera* spp. (19%), *Haliscomenobacter* spp. (11%), *Niabella* spp. (7%), *Nannocystis* spp. (6%), *Microlunatus* spp. (6%), *Gemmatimonas* spp. (5%), *Rhodobacter* spp. (3%), *Micropruina* spp. (3%), and *Tetrasphaera* spp. (2%). After the formation of aerobic granular sludge, the dominant microbes were mainly *Thauera* spp. (45%), *Zoogloea* spp. (10%), *Haliscomenobacter* spp. (5%), *Microlunatus* spp. (5%), *Niabella* spp. (5%), *Gemmatimonas* spp. (3%), *Amaricoccus* spp. (2%), *Leadbetterella* spp. (2%), *Runella* spp. (2%), and *Sphingopyxis* spp. (2%).

It was found that *Proteobacteria* increased from 50% of the inoculated sludge to 66% of granular sludge, among which the succession of *Thauera* spp. was most obvious, and its proportion raised from 19% to 45%. The role of *Thauera* spp. in degrading aromatic compounds and denitrifying for nitrogen removal in biological treatment systems has been reported [34]. Under aerobic conditions, *Thauera* spp. can hydroxylate the aromatic hydrocarbons by using molecular oxygen as the substrate and then break the aromatic ring under the action of monooxygenase or double oxygenase [35]. In this study, *p*-ClA loading increased stepwise, as a result of enrichment of *Thauera* spp. as *p*-ClA degrading bacteria, and they might control the degradation process of *p*-ClA by encoding key enzymes such as aniline monooxygenase and C23O enzyme. In addition, *Thauera* spp. also have the ability of denitrification with denitrifying gene of *nirS/nosZ* [36]. So, high TN removal efficiency of 80% in the later stage II was mainly attributed to the increase in the proportion of *Thauera* spp. More importantly, EPS biosynthesis gene cluster could be found in many of *Thauera* spp, and EPS synthesis and floc formation have been described previously in *Thauera* strains [37]. Consequently, the enrichment of *Thauera* spp. in this study supported the sludge granulation and was conducive to the

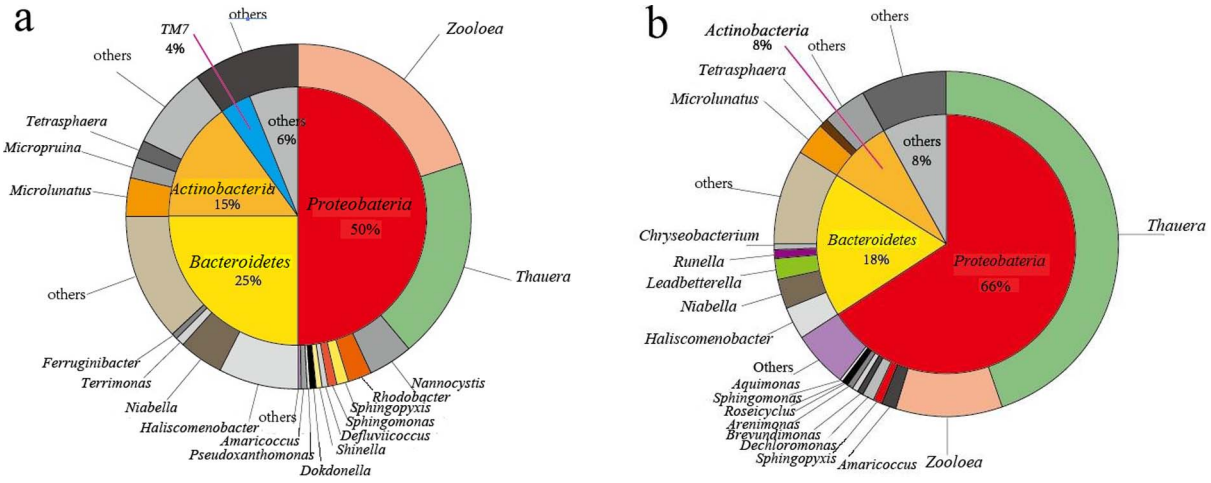

Fig. 7. Species distribution of seed sludge (a) and aerobic granular sludge degrading *p*-ClA (b).

stability of granular sludge since this genus produces EPS responsible for the cell aggregation.

It is also noteworthy that *Zoogloea* spp. accounted for 10% of total bacteria in aerobic granular sludge, and its richness was only next to *Thauera* spp. Previous studies have shown that large gene cluster encoding glycosyltransferases and other proteins involved in biosynthesis and export of EPS existed in *Zoogloea* [37]. These microbes played a key role in the formation of matrix of multicellular conglomerates and have been considered to be the typical microorganism responsible for the formation of sludge flocs. Thus, the presence of *Zoogloea* might play an important role in the further cohesion of sludge flocs, as well as the formation of granular sludge. In addition, as revealed by the result of 454 pyrosequencing, the proportion of *Zoogloea* spp. in mature granular sludge (10%) was lower than that in inoculated activated sludge (20%), which may attribute to high influent *p*-ClA loading. The results were similar to previous conclusion that *Zoogloea* was not the predominant species in the industrial wastewater-treating activated sludge [38].

3.5. Importance of EPS to aerobic granulation

Granule formation was usually rather difficult for high-strength industrial wastewater, despite of strong shear force and selective discharge of loose flocs adopted. So, the underlying mechanism of granulation and the potential role of EPS were preliminarily explored in the treatment of wastewater containing *p*-ClA in order to gain popularity in real industrial or municipal wastewater treatment. The study found that mainly due to the stimulation of *p*-ClA, more EPS was accumulated on the surface of bacteria, especially the PN with high molecular weight, and the EPS in microbial aggregates had many charged groups (e.g., carboxyl, hydroxyl and amino groups) and apolar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates), which contributed to decrease the net negative surface charges of sludge and then promote sludge flocculation and granulation. Jorand et al. [39] used XAD resin to separate the hydrophilic and hydrophobic EPS fractions and found that the hydrophobic fraction mainly comprised proteins, whereas the hydrophilic fraction mainly consisted of carbohydrates, so the increased PN/PS ratio at stage I improved the hydrophobicity of sludge and then promoted the formation of microbial aggregates. Furthermore, specific proteins (e.g., chaperonins and binding proteins) secreting by functional microorganism (e.g., *Thauera* spp. and *Zoogloea* spp.) were crucial for sludge granulation. Consequently, the EPS, a complex high-molecular-weight mixture of polymers excreted by microorganisms, were a major component in microbial aggregates for keeping them together to form aerobic granular sludge with three-dimensional matrix and stable structure.

4. Conclusion

Aerobic granular sludge is a novel sludge morphology in biological wastewater treatment, and the formation and structure stability of AGS is of great significance for engineering application. Results showed that the PN content of aerobic sludge increased with the sludge granulation, and it has a clear positive correlation with sludge characteristics. SDS-PAGE results indicated that PN with high molecular weight favored the formation and structural stability of AGS, and FTIR spectra showed that tyrosine protein, aspartic acid protein and other specific proteins with protein secondary structures including 3-turn helix and β-sheets could promote microbial aggregation. At the same time, five types of proteins related to microbial aggregation and EPS secretion were identified in mature granular sludge by 2-DE spectrometry analysis, especially, chaperonins and binding proteins both containing Amide III and secondary structures, which were crucial for sludge granulation. According to the analysis of microbial community, the enrichment of *Zoogloea* spp. and *Thauera* spp. for *p*-ClA degradation and EPS biosynthesis was conducive to the aerobic sludge granulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2017.07.174.

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