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The biological effect of metal ions on the granulation of aerobic granular activated sludge

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ABSTRACT

As a special biofilm structure, microbial attachment is believed to play an important role in the granulation of aerobic granular activated sludge (AGAS). This experiment was to investigate the biological effect of Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and K^{+} which are the most common ions present in biological wastewater treatment systems, on the microbial attachment of AGAS and flocculent activated sludge (FAS), from which AGAS is always derived, in order to provide a new strategy for the rapid cultivation and stability control of AGAS. The result showed that attachment biomass of AGAS was about 300% higher than that of FAS without the addition of metal ions. Different metal ions had different effects on the process of microbial attachment. FAS and AGAS reacted differently to the metal ions as well, and in fact, AGAS was more sensitive to the metal ions. Specifically, Ca^{2+}, Mg^{2+}, and K^{+} could increase the microbial attachment ability of both AGAS and FAS under appropriate concentrations, Cu^{2+}, Fe^{2+}, and Zn^{2+} were also beneficial to the microbial attachment of FAS at low concentrations, but Cu^{2+}, Fe^{2+}, and Zn^{2+} greatly inhibited the attachment process of AGAS even at extremely low concentrations. In addition, the acylated homoserine lactone (AHL)-based quorum sensing system, the content of extracellular polymeric substances and the relative hydrophobicity of the sludges were greatly influenced by metal ions. As all these parameters had close relationships with the microbial attachment process, the microbial attachment may be affected by changes of these parameters.

Introduction

Microbial attachment and biofilm development has always been a hot topic in the field of medicine and food (Berk et al., 2012; Vlamakis et al., 2013). There have also been some studies on microbial attachment of mixed cultures in wastewater treatment, although these experiments have always focused on mitigating biofouling in membrane biological reactor (MBR) systems (Xu and Liu, 2011; Kim et al., 2013).

Aerobic granular activated sludge (AGAS) has been intensively investigated as the self-immobilized aggregates that can hold a wide spectrum of wastewater. Compared with flocculent activated sludge (FAS), AGAS had a compact structure, a high level of biomass concentration, excellent settleability, and greater organic loading capacity (Tay et al., 2005). Studies on the cultivation of AGAS have mostly focused on controlling some physical parameters, for example, the organic loading rate, dissolved oxygen, hydrodynamic shear force, and feeding strategy (Lee et al., 2010; Liu and Tay, 2004). However, the cultivation and maintenance mechanism of AGAS remain unclear and require further investigation, which has greatly inhibited the further development of

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AGAS technology. Hence, it is very necessary to gain a new understanding of AGAS in microbiological aspects, in order to develop a rapid cultivation and stability control strategy.

AGAS shares many features of biofilm systems, in that the granules are formed through self-immobilization of microorganisms by packing together with different bacteria species (Feng et al., 2013). AGAS has always been gradually developed from FAS, and FAS is also a complex aggregated microbiological community. There are actually several steps for bacteria to form a biofilm structure: first of all, bacteria approach a surface or other microbes and slow down; then, they become attached to the surface or other microbes. This process of microbial attachment is important for making it possible to create multiple layers of cells and form a biofilm (Watnic and Kolter, 2000). Therefore, the microbial attachment ability of sludge is thought to have an important and main contribution to the development of AGAS. Ren et al. (2010) observed attached-growth and biofilm formation of cells from AGAS and FAS supernatant in a flow cell. Lv et al. (2014) described the microbial attachment ability of AGAS and FAS, and the results showed that AGAS had a stronger attachment potential.

So far, many factors has been confirmed to be responsible for regulation of the microbial attachment process (Hall-Stoodley and Stoodley, 2005; Ansari et al., 2012), and metal ions in the wastewater are one of the most important functional factors. Various kinds of metal ions are often detected in wastewaters (Kadirvelu et al., 2001; Dabrowski et al., 2004). Many of them are harmful or toxic to microorganisms (Fu and Wang, 2011; Sadler and Trudinger, 1967). However, metals ions at lower concentrations have been found to be able to stimulate microbial growth (Ziagova et al., 2014), and some metal ions can even promote the microbial attachment of bacteria at appropriate concentrations. Song and Leff (2006) found that Mg^{2+} could influence the attachment and subsequent biofilm formation or structure of *Pseudomonas fluorescens*. Cruz et al. (2012) also reported that Ca^{2+} in biofilm formation was related to the initial surface and cell-to-cell attachment, and colonization stages of biofilm establishment, which rely on critical functions by fimbrial structures. Kawakami et al. (2007) reported that Ca^{2+} was essential for initiating *Halobacterium salinarum* CCM 2090 cell aggregation, and that Cu^{2+} or Zn^{2+} could replace Ca^{2+}. There have also been some reports stating that metal ions could stimulate the formation of AGAS. Li et al. (2009) revealed that Mg^{2+} augmentation can be beneficial to aerobic granulation, finding that augmentation with 10 mg/L Mg^{2+} significantly decreased the sludge granulation time from 32 days to 18 days. Jiang et al. (2003) examined the effect of Ca^{2+} augmentation on aerobic granulation and found that Ca^{2+}fed granules were denser and more compact, and showed better settling and strength characteristics.

Notably, almost all these studies have focused either on the attached growth of pure culture microorganisms, or have only concerned the long-term and overall effect of metal ions on the AGAS system. The influence of metal ions on the initial formation and microbial attachment of AGAS, which would provide deep insights for a good understanding of the development of the AGAS granulation process, has rarely been discussed. Therefore, it is necessary to examine the influence of metal ions on the microbial attachment of AGAS and FAS.

In this study, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and K^{+}, which are the most common ions present in biological wastewater treatment systems, were chosen to examine the biological effect of different metal ions on the attached growth of FAS and AGAS, which would provide a better understanding for the rapid cultivation and stability control of AGAS in wastewater treatment.

1. Materials and methods

1.1. Source of sludges and pretreatment

Both of the FAS and AGAS sludges were cultivated in a sequencing batch reactor (SBR) with a working volume of 60 L and a volumetric exchange ratio of 67%. The reactor was fed by synthetic wastewater with the composition of 409.6 mg/L CH_3COONa, 152.85 mg/L NH_4Cl, 35.10 mg/L KH_2PO_4, 160.00 mg/L NaCl, 80 mg/L $MgSO_4{\cdot}7H_2O$, and 16 mg/L $CaCl_2$. The SBR was operated on a 6 hr cycle with 5 min feeding, 65 min anoxic phase, 270 min aerobic reaction, 10 min settling and 10 min decanting.

The sludges collected from the aerobic phase were first washed with deionized water 3 times, homogenized at 12,000 r/min for 1 min, and then centrifuged for 5 min at 6000 *g* to harvest the sludge pellets. The pellets were subsequently resuspended in fresh wastewater (CH_3COONa, 500 mg/L, NH_4Cl, 40 mg/L; the composition and concentration of other substances were identical to those in the SBR) with 500 mg dry biomass/L.

1.2. Metal ion test

A total of 6 typical metals ions, including: Ca^{2+} (10, 20, 40, 80, 160 mg/L), Mg^{2+} (10, 20, 40, 80, 160 mg/L), Cu^{2+} (2, 4, 8, 16, 32 mg/L), Fe^{2+} (2, 4, 8, 16, 32 mg/L), and Zn^{2+} (2, 4, 8, 16, 32 mg/L), K^{+} (5, 10, 20, 40, 80 mg/L) were chosen for the study of microbial attachment of sludge; this choice was based on preliminary tests and the possible impacts of these ions. Moreover, the different ranges of the metal concentrations were based on their natural ranges in the wastewater, with a little wider coverage. $CaCl_2$, $MgSO_4.7H_2O$, $ZnCl_2$, $CuCl_2.2H_2O$, $FeSO_4{\cdot}7H_2O$, KCl were used to provide metal ions in this experiment. Glucose and NH_4Cl were used as carbon source and nitrogen source. The mixed liquor containing metal ions and carbon plus nitrogen sources was used as the substrate for the microbial attachment, and the substrate without metal ions was designed as the control (or original value).

1.3. Microtitre plate assay

To test the effect of metal ions on the microbial attachment, Cu^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, K^{+} with different concentrations were added to the resuspended microorganisms. Then 3 mL of the suspension was transferred into each well of a 12-well polystyrene microtitre plate, and 6 replicate wells were used for each assay. The plates were incubated at 30°C for 12 hr without shaking. After incubation, the culture mediums were discarded and the wells were washed 3 times with deionized water to remove the unattached cells, then the plates were dried at 60°C for 10 min. Subsequently, the attached biomass in each well was stained by 200 μL of crystal violet (0.1%). After

20 min of staining, the extra Crystal Violet was removed by washing with deionized water 3 times and the plates were dried at 60°C for 10 min again. Finally, 1 mL of ethyl alcohol was added to each well for decolorization; after 10 min, the optical density at 600 nm of the eluted Crystal Violet was determined to represent the attached biomass.

1.4. Bioassay of acylated homoserine lactones

To prepare the sample for testing, sludges were centrifuged at 3600 ×*g* for 5 min to collect pellets whose dry biomass was equivalent to 10 mg. Then, the pellets were washed with deionized water 3 times and re-suspended in 5 mL of water. Next, the sample was sonicated for 30 min at 20 kHz. After that, the suspension was filtered through 0.45 μm membranes to obtain cell-free culture fluids.

The determination of relative acylated homoserine lactone (AHL) content was carried out based on the method by Singh and Greenstein (2006) with some modifications. The reporter strain KYC55(pJZ384)(pJZ410)(pJZ372) was cultivated to late exponential phase in a rotary shaker (180 r/min, 28°C) in the AT (2 g/L glucose, 10.5 g/L K_2HPO_4, 10.7 g/L KH_2PO_4, 2 g/L $(NH4)_2SO_4$, 78 mg/L MgSO4, 7.6 mg/L $CaCl_2$, 5 mg/L $FeSO_4{\cdot}7H_2O$ and 2.2 mg/L $MnSO_4$; adjust pH to 7.3) supplemented with 0.2 μL/mL of tetracycline, 100 μL/mL of spectinomycin, 6.67 μL/mL of gentamicin. This culture was then diluted in fresh minimal medium containing no antibiotics to an OD_{600} of 0.100 and 50 μL per well was added in 96-well plates that contained 50 μL of cell-free sample fluid in each well. Deionized water was used as the control. Then, the mixture was incubated at 28°C and 180 r/min for 18 hr. At the end of the incubation, an equal volume of X-gal (40 mg/mL) was added, and the plate was incubated at ambient temperature with shaking for 120 min. Finally, the absorbance of the suspension was determined at 635 nm. The relative AHL content was quantified as the absorbance of samples over the absorbance of control. All assays were repeated 3 times.

1.5. Extracellular polymeric substances extraction and analysis

The extracellular polymeric substances (EPS) from the samples were extracted according to the method of Yang and Li (2009) with some modifications. Briefly, 35 mL sludge suspension was first dewatered through centrifugation in a 50 mL tube at 6000 ×*g* for 5 min. The pellet in the tube was washed with 20 mL of deionized water and centrifuged at 6000 ×*g* for 5 min. The washed pellet was diluted with deionized water to its original volume of 35 mL. Then the solution mixture was heated to 80°C for 30 min in a water bath, and centrifuged at 6000 ×*g* for 10 min. Next, the supernatant was filtered through 0.45 μm acetate cellulose membranes. Finally, proteins (PN) were measured by the Coomassie Brilliant Blue method (Bradford, 1976) using bovine serum albumin as the standard. Polysaccharides (PS) were measured by the Anthrone method (Gaudy, 1962) with glucose as the standard.

1.6. Measurement of relative hydrophobicity

The relative hydrophobicity of sludges was measured by the method of Rosenberg (2006) with some modifications. The suspension mixture of sludge with metal ions was cultivated at 30°C for 12 hr; after that, the suspension was shaken for several minutes, and 5 mL of the suspension was placed into a 10 mL tube. Subsequently, 5 mL of *n*-hexane was added to the tube as well. Then the tube was inverted for 15 min, and then left to settle for 30 min. Finally, the OD_{600} of the aqueous phase before and after the treatment with *n*-hexane were marked as *A* and A_0 respectively. The hydrophobicity of the sludge was calculated as:

$$\text{Hydrophobicity} = (A - A_0) / A \times 100\%.$$

2. Results and discussion

2.1. Effect of metal ions on the attachment potential of sludges

AGAS was a special self-aggregated biofilm, and the results showed that after 12 hr cultivation, the biomass of attachment of AGAS was about 300% higher than that of FAS (typically 1.0408 to 0.243), which clearly indicated that AGAS had a stronger microbial attachment ability and that the microbial attachment of AGAS may have an important contribution to the development of AGAS. Such distinctive characteristics also resulted in the different responses of AGAS and FAS to the metal ions. The effect of metal ions on the microbial attachment of FAS and AGAS is summarized in Fig. 1. Cu^{2+}, Fe^{2+}, and Zn^{2+} greatly inhibited the microbial attachment of AGAS at the tested concentrations; the biomass of attachment finally decreased to only 19.28%, 17.66%, 22.97% of the original value at 32 mg/L of Cu^{2+}, Fe^{2+}, Zn^{2+} respectively. However, Cu^{2+}, Fe^{2+}, and Zn^{2+} dramatically increased the microbial attachment of FAS at low concentrations, and the microbial attachment was raised 39.05%, 81.31%, 49.59% at 2 mg/L, but it then dropped to the control level at high concentrations. Actually, cations such as Cu^{2+}, Fe^{2+}, Zn^{2+} are always beneficial to bacterial growth at trace concentrations (Aranha et al., 1982); they are necessary trace elements for the growth of bacteria, which could influence the aggregation of bacteria through biochemical effects. However, these ions might exhibit toxicity at higher concentrations (Tchounwou et al., 2012). In addition, Ca^{2+} and Mg^{2+} could augment the attachment of AGAS at lower concentrations; for example, the biomass of attachment was increased 7.69% and 12.14% when 20 mg/L Ca^{2+} and Mg^{2+} were added respectively. Nevertheless, the microbial attachment was found to be disrupted at higher concentrations; when the concentrations of Ca^{2+} and Mg^{2+} reached 160 mg/L, the biomass of attachment decreased 59.34% and 23.04% respectively. For FAS, however, Mg^{2+} showed a slight positive effect at most added concentrations. Distinctively, Ca^{2+} stimulated the greatest enhancement of the biomass of microbial attachment, from 61.73% to 93.33% in 10–80 mg/L, and still exhibited a 27.65% increase at 160 mg/L. In fact, it was reported that Mg^{2+} and Ca^{2+} could influence biofilm formation directly through their effect on electro-static interactions and indirectly via physiology-dependent attachment processes, by acting as important cellular cations and enzyme cofactors (Fletcher, 1988). Moreover, Ca^{2+}, Mg^{2+}, and Fe^{2+} could bind to negatively charged cells to form microbial nuclei (Mahoney et al., 1987). Interestingly, K^+ exhibited a positive

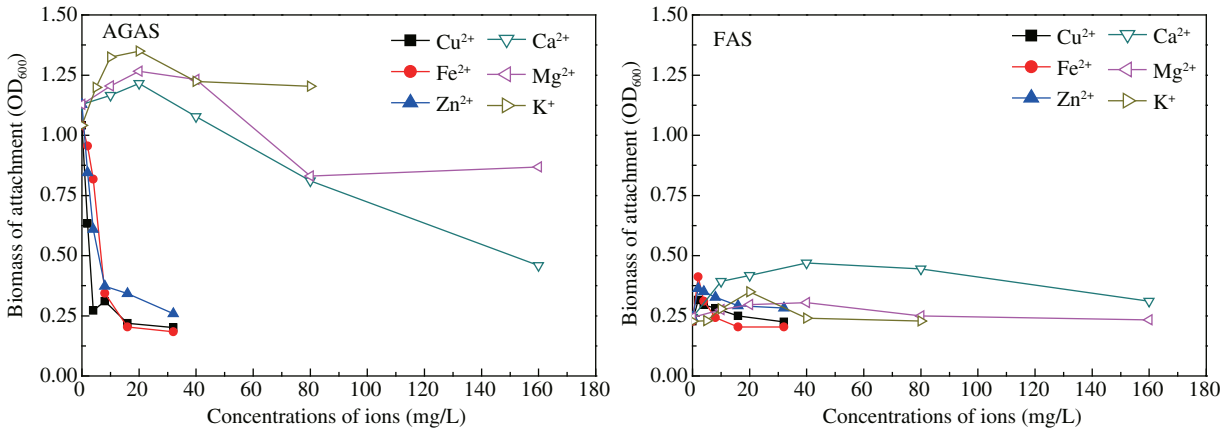

Fig. 1 – The profiles of microbial attachment of aerobic granular activated sludge (AGAS) and flocculent activated sludge (FAS) with different metal ions.

impact on the attachment in both AGAS and FAS, and had no negative effect even at the high concentration of 80 mg/L. The biomass of attachment was increased 29.67% and 53.77% for both AGAS and FAS at 20 mg/L K^+, respectively, which may be because K^+ is the major intracellular cation in bacteria as well as in eucaryotic cells, and bacteria accumulated K^+ by a number of different transport systems that vary in kinetics, energy coupling, and regulation (Epstein, 2013). In fact, it was showed that the amount of biofilm formed by *Xylella fastidiosa* increased 500% with addition of 2.5 mmol/L Ca^{2+}, and the inhibition effect of Fe on biofilm formation was decreased when the concentration of Fe^{2+} was higher than 1.8 mmol/L; in addition, the amount of biofilm was decreased to 50% of the original value with an increase in the concentration of Cu^{2+} and Zn^{2+}, and there were no significant differences in biofilm accumulation when K^{2+} and Mg^{2+} were added. As revealed in the results, AGAS and FAS reacted very differently to the same metal ions: AGAS seemed to be more sensitive to all of the ions than FAS, especially Cu^{2+}, which reduced the biomass of attachment 39.24% even at a very low concentration of 2 mg/L. This difference might come from the changes of microbial community and structural pattern that occur after the transition from FAS to AGAS. Li et al. (2010) reported that the microbial community changed a great deal during the process of granulation. α-*Proteobacteria* and β-*Proteobactria* have commonly been found in conventional activated sludge, and bacteria from *Flavobacteria* have been regarded as dominant in AGAS. It was also revealed by Whiteley and Bailey (2000) that β-*Proteobacteria* and γ-*Proteobacteria* were dominant in AGAS. Because of the differences in microbial communities, the mature AGAS may have a stronger potential of attachment but also be more sensitive to certain types of metal ions. This result showed us that the amount and type of metal ions should be considered when the ions are chosen for application in the rapid cultivation of AGAS.

2.2. Effect of metal ions on the AHL-based quorum sensing system of sludges

Quorum sensing (QS) is an important factor which functions in the process of microbial attachment. QS allows bacteria to communicate with each other through the production and perception of small extracellular signal molecules. Bacteria can utilize QS systems to sense their population density and activate specific gene expression, which enables them to behave in a coordinated manner (Molloy, 2010). Three kinds of QS systems have been identified according to the different types of signal molecules (oligopeptides, AHLs, autoinducer-2). Among the three kinds of QS systems, the AHL-dependent system in Gram-negative bacteria has been best characterized and most studied in the process of biofilm formation. Lynch et al. (2002) found that the ahyI mutant of *Aeromonas hydrophila*, which could not produce C_4-HSL, would be unable to form a mature biofilm. Ren et al. (2012) reported that the AHL-like molecules released by AGAS could promote biofilm formation of *E.coli* K12. In addition, a variety of factors have been considered to have an influence on the quorum sensing system. Paul et al. (2009) reported that biofilm formation on the RO membrane surface with two selected biofouling bacteria, *Aeromonas hydrophila* and *Pseudomonas putida*, would be intensively suppressed with the addition of 0.6 mg/mL Acylase I (the enzyme for AHL). Also, it was revealed by Ponnusamy et al. (2009) that vanillin (4-hydroxy-3-methoxybenzaldehyde) from vanilla beans showed a significant inhibition toward short-chain and long-chain AHL molecules. Hentzer et al. (2002) reported that a synthetic halogenated furanone compound was capable of interfering with AHL-mediated quorum sensing in *Pseudomonas aeruginosa*. All these data offered a good microbiological basis for the investigation of AHLs in AGAS. As a special biofilm structure, AGAS is recognized as a collection of attached-growth microorganisms, for which the variation of cell density is controlled by the AHL-based quorum sensing system. The current study showed for the first time the effect of metal ions on the AHL-based quorum sensing system of FAS. The exact profile of AHL that was influenced by metal ions in AGAS and FAS is shown in Fig. 2. Typically, 6 events of microbial attachment inhibition or enhancement from AGAS and FAS at Cu^{2+} (2 and 32 mg/L), Fe^{2+} (2 and 32 mg/L), K^+ (10 and 80 mg/L) were chosen for the investigation. The results showed that the AHL content of AGAS was significantly higher than FAS, which implied that AHLs played an important role in the formation of AGAS. In fact, aerobic granulation is a process of attachment growth of

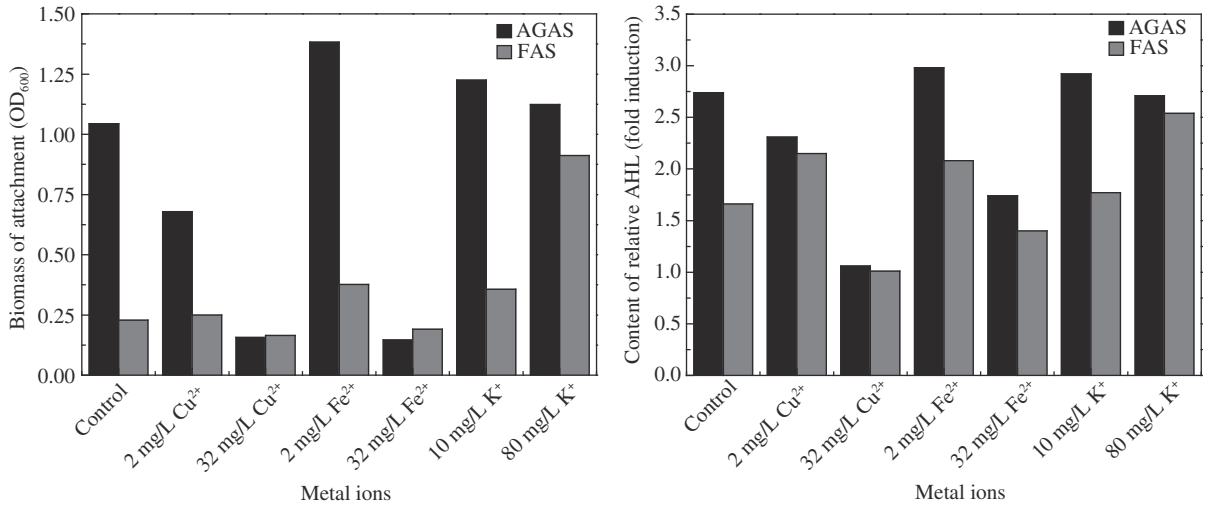

Fig. 2 – The microbial attachment and acylated homoserine lactone (AHL) content of AGAS and FAS with different metal ions.

microorganisms. In this process, the biomass density would certainly be increased; consequently, more AHLs would be released, and when the content of AHLs reached a certain threshold, the expression of some genes would be initiated to promote mutual attachment among the microorganisms. Jiang and Liu (2012) confirmed that the AHL content of AGAS remained at a low level in the initial stage of granulation, but the content would rise up with the increase of biomass density. And Li et al. (2014a, 2014b) reported that aerobic granules showed higher AHL-based QS than flocculent sludge, and that inactivation of AHLs by AHLs-acylase could weaken the stability of aerobic granules. It was also found that inactivation of AHLs led to a reduction of extracellular polysaccharides and protein (PN), especially PN, and induced damage to the extracellular polymeric substance matrix. The results also revealed that the metal ions could have an influence on the AHLs of sludges; specifically, with the addition of 10–80 mg/L K^+ and 2 mg/L Fe^{2+}, the AHL content of AGAS was increased. Moreover, when the AHL content of FAS was increased to a higher level with the treatment of 2 mg/L Cu^{2+}, 2 mg/L Fe^{2+}, 10 mg/L and 80 mg/L K^+, the biomass of attachment of FAS was enhanced as well. Actually, ions like Cu^{2+}, Fe^{2+} and K^+ at low concentrations could be important components of the enzymes, playing crucial roles in the cells of bacteria, and thus would be beneficial to the growth and biological activity of bacteria. Hence, the addition of these ions might increase the secretion of AHLs, enhancing the quorum sensing system, to make the bacteria in the sludges aggregate and attach to the surface. However, when the attachment biomass of AGAS was decreased at 2–32 mg/L Cu^{2+} or 32 mg/L Fe^{2+}, the AHL content appeared to be lower than the control. Likewise, with 32 mg/L Cu^{2+} and Fe^{2+}, the content of AHLs was significantly decreased; and consequently, the biomass of attachment of FAS dropped. In these situations, the concentrations of the cations became too high, the bacteria might suffer damage, and the quorum sensing system would certainly be depressed. All the conclusions could be confirmed again by the positive relationship between the biomass of attachment and content of AHL ($p < 0.01$) (Fig. 3), which indicated that the metal ions could affect the microbial attachment through regulating the quorum sensing system.

2.3. *Effect of metal ions on the EPS production and relative hydrophobicity of sludges*

The EPS is believed to be functional in the microbial attachment and granulation of AGAS. It is mostly composed of proteins and polysaccharides. The measurements of EPS for AGAS and FAS are shown in Fig. 4. Overall, the EPS of AGAS was higher than that of FAS. When the microbial attachment of both AGAS and FAS was greatly inhibited at the exposure to 32 mg/L Cu^{2+} or Fe^{2+}, the content of proteins was dramatically reduced to a very low level, while polysaccharide was sharply increased, *i.e.*, a very low protein/polysaccharide (PN/PS) ratio was produced. This meant that a low PN/PS ratio would lead to the unattachment of biomass, and that more proteins were beneficial for biomass attachment. Many reports have

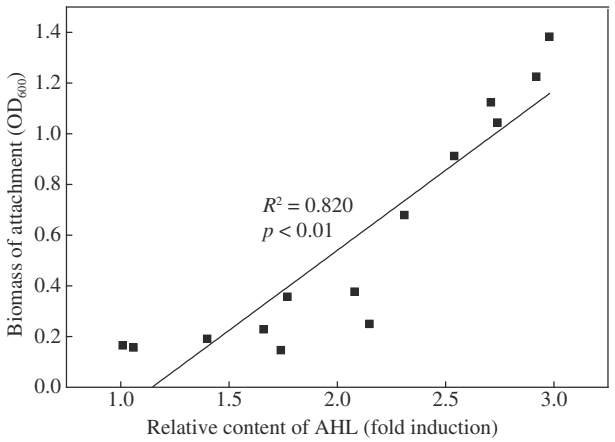

Fig. 3 – The relationship between microbial attachment and AHL content of AGAS and FAS.

supported this idea, and Morohoshi et al. (2004) also revealed that the expression of a 55 kDa protein in the fish pathogen *Edwardsiella tarda*, which was reported as a virulent-strain-specific protein, was controlled by AHLs. Campisano et al. (2006) indicated that PslD is a secreted protein required for biofilm formation of *P. aeruginosa*. In the current study, when the amount of proteins was increased at 2 mg/L Fe^{2+}, the microbial attachment of AGAS was also enhanced. However, with 10 and 80 mg/L K^+, the content of proteins remained nearly the same as the control, while the biomass of attachment was slightly increased. Moreover, it seemed that the content of polysaccharides of AGAS exhibited no notable variation or just showed a slight increase in the other different conditions, including the treatment with 2 mg/L Fe^{2+} or 10 and 80 mg/L K^+, except for the significant increase at the exposure to 32 mg/L Fe^{2+} or Cu^{2+}. However, the content of proteins in FAS was increased under different conditions, including the exposure to 2 mg/L Cu^{2+} or Fe^{2+}, 10 and 80 mg/L K^+, but polysaccharides showed a much greater variation. Hence, it could be concluded that the content of proteins was increased with the addition of selected ions at appropriate concentrations, which was consistent with the previous experiment, which revealed that the metal ions could induce the production of EPS at certain concentrations (Fang et al., 2002). What's more, some studies reported that quorum sensing systems could regulate the expression of proteins of the microorganisms. Chatterjee et al. (2005) showed that In *Erwinia carotovora* subspecies, AHL controls the expression of enzyme/protein production. Bruhn et al. (2004) revealed that *Hafnia alvei* wild-type could produce an extracellular protein of approximately 20 kDa, but this protein was not produced by the AHL-negative mutant and was restored in the mutant when complemented by N-3-oxo-hexanoyl homoserine lactone. In this study, it was shown that more AHLs indeed would be beneficial to the secretion of proteins in both AGAS and FAS; hence, it could be speculated that the AHL-quorum sensing system may regulate the microbial attachment of sludge through playing an important role in the production of proteins. In addition, this study also revealed that EPS in FAS had different changes compared with AGAS. Such distinctions in the characteristics of AGAS and FAS revealed the reason behind their different performance. Actually, some researchers have investigated AGAS by focusing on the role of EPS in determining its physical and microbiological properties, and two quite different exopolysaccharides have been proposed as the gel-forming constituents, with their gel properties clearly different from those of activated sludge EPS (Seviour et al., 2012).

Proteins in the EPS have been considered to have a hydrophobic composition (Dignac et al., 1998) and higher proteins could consequently induce a higher hydrophobicity (Seviour et al., 2009), which played an important function in the microbial attachment of bacteria and the granulation of AGAS. The examination of the relative hydrophobicity of AGAS and FAS with the exposure to metal ions is shown in Fig. 5. Cu^{2+}, Fe^{2+} and K^+ were selected as examples of microbial attachment enhancement or inhibition. It was found that AGAS behaved much more hydrophobically than FAS. Lv et al. (2014) analyzed the differences of bacterial community structure between AGAS and FAS by PCR-DGGE, and found that some hydrophobic bacteria, such as *Flavobacterium*, exclusively existed in AGAS. In addition, the relative hydrophobicity of the sludges exhibited a different profile with the addition of different metal ions. In both AGAS and FAS, the relative hydrophobicity remained nearly unchanged with the addition of K^+, and maintained at the high levels of 45.72%–51.48% and 36.37%–40.86% respectively in all the tested concentrations. In addition, AGAS had a higher relative hydrophobicity than FAS, which was consistent with the biomass attachment measurements. In principle, the high relative hydrophobicity was good for the attachment of biomass on the surface. However, the relative hydrophobicities of the AGAS and FAS were significantly decreased to 33.32% and 27.99%, 22.98% and 21% respectively on exposure to 32 mg/L Cu^{2+} and 32 mg/L Fe^{2+}, despite the fact that there was a slight increase at low concentration. These results demonstrated that the positive effect of ions on microbial attachment would lead to high hydrophobicity, particularly in AGAS, and

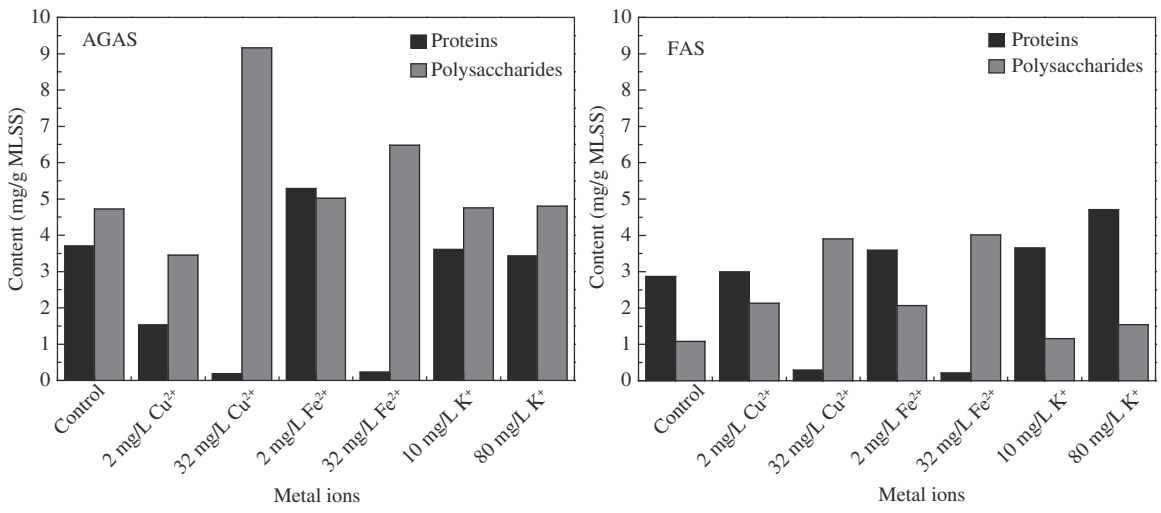

Fig. 4 – The proteins and polysaccharides of AGAS and FAS with different metal ions.

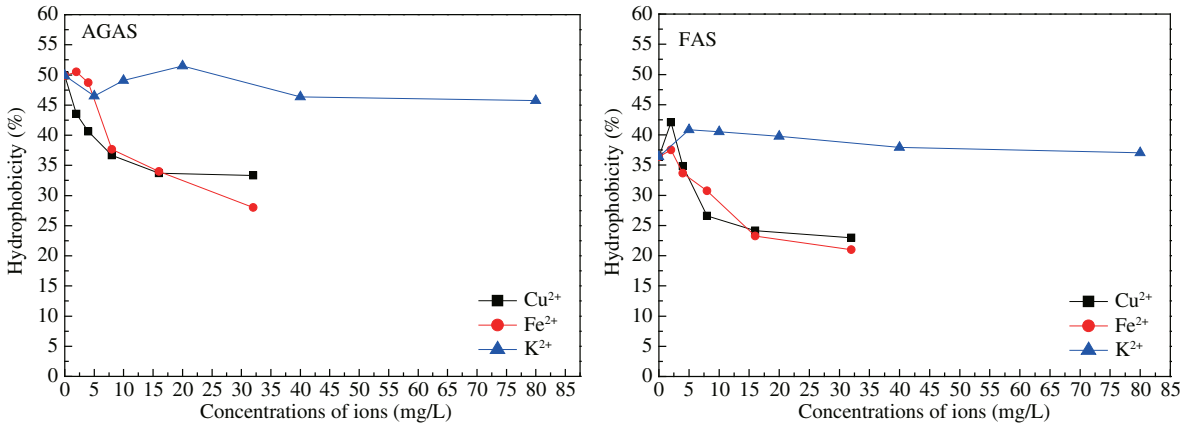

Fig. 5 – The hydrophobicity of AGAS and FAS with different metal ions.

negative ions would produce a low hydrophobicity value. Also, it was confirmed that the relative hydrophobicity of sludges had a close relationship with microbial attachment, and the profile of relative hydrophobicity of sludge conformed well with the behavior of biomass attachment. Similar experimental results were reported in the studies of biofilm formation and AGAS granulation (Branda et al., 2006; Zhang et al., 2007).

3. Conclusions

Microbial attachment ability was very important for the granulation of AGAS, in which there are many factors that could play a role, and the presence of metal ions in the wastewater was one of the most important factors. Metal ions like Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and K^{+} affected the microbial attachment of AGAS and FAS through inducing changes in the quorum sensing system, EPS, and relative hydrophobicity of the sludges. Different ions had varying effects on the microbial attachment of AGAS and FAS, and AGAS and FAS reacted differently to the metal ions as well. At appropriate concentrations, Ca^{2+}, Mg^{2+}, and K^{+} could enhance the microbial attachment ability of both AGAS and FAS, and Cu^{2+}, Fe^{2+}, and Zn^{2+} could improve the microbial attachment of FAS at lower concentrations; but even at low concentrations, Cu^{2+}, Fe^{2+}, and Zn^{2+} would greatly inhibit the attachment process of AGAS. In general, AGAS was more sensitive to the presence of metal ions. Hence, selection of appropriate metal ions at appropriate concentrations would be important for the rapid cultivation of AGAS.

Acknowledgments

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