The effect of quorum sensing and extracellular proteins on the microbial attachment of aerobic granular activated sludge

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HIGHLIGHTS
• Vanillin and proteinase K can reduce attachment biomass of AGAS.
• Contents of AHLs and extracellular proteins decrease with vanillin and proteinase K.
• Extracellular proteins can promote microbial attachment of AGAS.
• AHL is involved in microbial attachment by the regulation of extracellular proteins.
• QS and extracellular proteins play important roles in the formation of “AGAS biofilm”.

ABSTRACT
In this study, vanillin, a quorum sensing (QS) blocker, and proteinase K were employed to investigate the effect of QS and extracellular proteins on the microbial attachment of aerobic granular activated sludge (AGAS). Results clearly showed that both vanillin and proteinase K could reduce attachment potential of AGAS, and the combined use of them was more effective in reducing attachment biomass of AGAS. The contents of N-acylhomoserine lactones (AHLs) and extracellular proteins were reduced in the presence of vanillin and proteinase K. Besides, it was found that extracellular proteins could promote microbial attachment of AGAS, and it was also revealed that AHLs-mediated QS might be involved in microbial attachment of AGAS through the regulation of extracellular proteins. This study suggested that both QS and extracellular proteins might play important roles in the development of “AGAS biofilm” from the perspective of the biofilm.

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1. Introduction
Aerobic granular activated sludge (AGAS) is composed of self-immobilized microorganisms, and is cultivated in the sequencing batch reactor (SBR) by regulating various operating parameters such as organic loading rate, dissolved oxygen, hydrodynamic shear force, feeding strategy (Liu and Tay, 2004; Lee et al., 2010). Owing to its excellent settleability, high pollutant degradation capability and strong resistance to toxic substances, AGAS has been widely investigated in laboratory-scale reactors in the last decade (Adav et al., 2008a). Moreover, there are also some pilot-scale applications of AGAS (Liu et al., 2010; Su et al., 2012). In general, AGAS is regard as a promising aerobic biotechnology for wastewater treatment. However, the exact mechanism of granule formation remains unclear till now.

From the microbiological point of view, AGAS can be regarded as a special form of biofilm which is commonly developed by aggregation of a variety of microorganisms (Ren et al., 2010). Therefore, it is believed that the microbial attachment and development of “AGAS biofilm” may play important roles in granule formation. So far, there have been many factors thought to regulate the microbial attachment and biofilm formation (Hall-Stoodley and Stoodley, 2005). One of the important mechanisms is the quorum sensing (QS) (Waters and Bassler, 2005). QS takes place via chemical signal molecules, which are released and detected as a mean to gauge population density. When the population density reaches a threshold, the expression of certain genes is induced by signal molecules. To date, several signal molecules, such as oligopeptides, N-acylhomoserine lactones (AHLs), autoinducer-2 (AI-2) have been identified. Among those, AHL in Gram-negative bacteria is one of important signaling molecules involved in biofilm formation, and has been well characterized. For instance, Lynch et al. (2002) reported that Aeromonas hydrophila of ahyl mutant, could
not produce C4-HSL, and failed to form a mature biofilm. Wang et al. (2012) found that *Pseudomonas* sp. HF-1 biofilm formation was induced by C6-HSL and 3-oxo-C6-HSL. Unfortunately, all studies are almost concerned about the effect of QS on attachment of pure cultures. Ren et al. (2010) revealed that signal chemicals produced by AGAS promoted the attached growth of bacteria obtained from AGAS, but the result was very limited and preliminary, and much effort was needed to explore the relationship between microbial attachment and QS in mixed cultures, especially in AGAS.

Extracellular polymeric substances (EPS), which are secreted by microorganisms, mainly consist of proteins, polysaccharides, extracellular DNA (eDNA), etc. Previous studies demonstrated that EPS were involved in the microbial attachment and biofilm formation (Czaczyk and Myszka, 2007; Yang et al., 2011). Importantly, considering proteins are dominant component of EPS, the effect of extracellular proteins on microbial attachment and biofilm formation has been extensively investigated. Tielen et al. (2010) reported that overexpression of the proteolytic elastase LasB from mucoid *Pseudomonas aeruginosa* had a pronounced effect on biofilms. Over the observation period of 72 h, the borosilicate glass surface was only sparsely and unevenly colonized in the form of films. Over the observation period of 72 h, the borosilicate glass surface was only sparsely and unevenly colonized in the form of films.

2.2. Microbial attachment assay

To evaluate the effect of QS and extracellular proteins on the attachment potential of AGAS, vanillin (0.05, 0.15 and 0.3 mg mL⁻¹) (Sangon, China) and proteinase K (0.05, 0.15 and 0.3 mg mL⁻¹) (Merck, Germany) were added to resuspended sludge, respectively. Then, 3 mL of resuspended sludge was dispensed into each well of 12-well polystyrene plates, and six replicate wells were used for each analysis. The plates were statically incubated at 30 °C for 12 and 24 h. After attachment, each well was rinsed with distilled water and was dried at 60 °C for 30 min. Subsequently, each well was stained with 0.2 mL of 0.1% (w/v) crystal violet solution for 20 min. Then the wells were washed with distilled water again, and the remaining crystal violet was dissolved in 1 mL of ethanol. Attached biomass was quantified by measurement of the optical density at 600 nm (OD600). Besides, to determine the effect of the combined use of vanillin and proteinase K on attachment potential of AGAS, four combinations were used as follows: (1) 0.05 mg mL⁻¹ proteinase K + 0.05 mg mL⁻¹ vanillin; (2) 0.05 mg mL⁻¹ proteinase K + 0.15 mg mL⁻¹ vanillin; (3) 0.15 mg mL⁻¹ proteinase K + 0.05 mg mL⁻¹ vanillin; and (4) 0.30 mg mL⁻¹ proteinase K + 0.30 mg mL⁻¹ vanillin.

2.3. Microscopic analysis of microbial attachment

In order to observe the microbial attachment of AGAS with exposure to vanillin and proteinase K, microbial attachment was conducted on glass slides mounted on the bottom of Petri dishes. The sludge concentration and operation conditions were identical to those of microbial attachment assay on 12-well plates except that 40 mL of resuspended sludge was pipetted into Petri dishes. After 12 h and 24 h of incubation, microscopic examination of the attachment microorganisms stained by crystal violet was carried out with transmitted light using an Olympus BX50 system microscope at 200 × magnification.

2.4. Bioassay for AHLs in AGAS

2.4.1. In situ detection of AHLs

The release of AHLs derived from AGAS was estimated based on the QS of reporter strains in the agar plate. To accomplish this, *Agrobacterium tumefaciens* strain NTL4 (pZLR4) was inoculated into Luria–Bertani (LB) broth supplemented by 50 µg mL⁻¹ of gentamicin, and incubated overnight at 28 °C with shaking at 180 rpm. Then, the indicating agar plates were made by diluting overnight cultures of NTL4 into LB agar with the ratio of 1:10. The indicating agar plates were also supplemented by gentamicin and covered with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The AGAS was placed upon a sterilized white filter which was laid on the indicating agar plate, and incubated at 28 °C for at least 4 h. The emergence of blue color was a significant signal indicating the sample produced or contained AHLs.

2.4.2. Determination of relative AHLs content

AGAS (equivalent to 10 mg dry biomass) was centrifuged at 3600 g for 5 min. The pellets were washed with deionized water and resuspended in 5 mL of deionized water. Then the sample was sonicated for 30 min at 20 kHz. After that, suspension was filtered through 0.22 µm membranes to obtain cell free culture fluids. The determination of relative AHLs content was carried out based on the method by Singh and Greenstein (2006) with some modifications. The reporter strain NTL4 was cultivated to late exponential phase in a rotary shaker (180 rpm, 28 °C) in minimal medium (2 g L⁻¹ glucose, 10.5 g L⁻¹ KH₂PO₄, 4.5 g L⁻¹ KH₂PO₄, 1.5285 mg; KH₂PO₄, 80.00 mg; CaCl₂, 16.00 mg (All analytical reagents were purchased from Xilong Chemical Co., Ltd. (Guangdong, China)). The SBR was fed with synthetic wastewater with the following compositions (per liter): CH₃COONa, 819.20 mg; NH₄Cl, 152.85 mg; KH₂PO₄, 70.21 mg; NaCl, 160.00 mg; MgSO₄·7H₂O, 80.00 mg; CaCl₂, 16.00 mg (All analytical reagents were purchased from Xilong Chemical Co., Ltd. (Guangdong, China)).
2 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.2 g L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O, 15 mg L\(^{-1}\) CaCl\(_2\)·2H\(_2\)O, 10 mg L\(^{-1}\) FeSO\(_4\)·7H\(_2\)O and 3 mg L\(^{-1}\) MnSO\(_4\)·H\(_2\)O; adjust pH to 6.8) supplemented with 50 μg mL\(^{-1}\) of gentamicin. This bacterial culture was diluted in fresh minimal medium containing no antibiotics to an OD\(_{600}\) of 0.100 and added 50 μL per well which contained 50 μL of cell free sample fluids. The deionized water was used as sample control. The plates were incubated for 18 h at 28 °C with shaking at 180 rpm. Afterwards, an equal volume of X-gal (40 mg mL\(^{-1}\)) was added, and the plates were kept at ambient temperature with shaking for 120 min. Then the absorbance of suspension was determined at 635 nm. The relative AHLs content was quantified as the fold of the absorbance of sample over the suspension was determined at 635 nm. The relative AHLs content was quantified as the fold of the absorbance of sample over the absorbance of control. All assays were repeated three times.

2.5. Extraction and analysis of EPS

The EPS was extracted by the procedure of Yang and Li (2009) with some modifications. Briefly, sludge suspension was first dewatered by centrifugation at 6000 g for 5 min. The pellet was washed with deionized water and centrifuged at 6000 g for 5 min. Next, the washed pellet was diluted with deionized water to 40 mL. Then the mixture solution was heated to 80 °C for 30 min in a water bath, and was centrifuged at 6000 g for 10 min. The supernatant was filtered through 0.45 μm acetate cellulose membranes. Proteins (PN) were measured by the coomassie brilliant blue method (Bradford, 1976) using BSA as the standard. Polysaccharides (PS) were measured by the Anthrone method (Gaudy, 1962) with glucose as the standard.

2.6. Statistical analysis

Attached biomass was expressed as the mean ± standard deviation. Pearson correlation analysis was conducted by SPSS software (version 16.0), and there was a significant relationship when \(p < 0.05\).

3. Results

3.1. Effect of vanillin and proteinase K on attachment potential of AGAS

Table 1 showed the attachment potential of AGAS exposed to various concentrations of vanillin and proteinase K. As shown in Table 1, when AGAS was exposed to different concentrations of vanillin, attachment biomass on polystyrene interface was reduced after 24 h of incubation. Compared to the control, attachment biomass was reduced by 7.56%, 20.93%, and 29.07% after exposure to 0.05, 0.15, and 0.3 mg mL\(^{-1}\) vanillin for 24 h. Similar phenomenon was also observed when AGAS was exposed to different concentrations of proteinase K. There were 32.95%, 39.88%, and 76.88% reduction of attachment biomass after exposure to 0.05, 0.15, and 0.3 mg mL\(^{-1}\) proteinase K for 24 h, respectively (Table 1). The behaviors of attachment potential of AGAS with exposure to vanillin and proteinase K for 12 h were similar to those for 24 h, and detailed results were shown in Table S1. From Table 1 and Table S1, it was noted that attachment biomass of AGAS increased with culture time, regardless of AGAS with and without treatment by vanillin and proteinase K. Additionally, Fig. S1 clearly showed that there was a strong negative correlation between attachment biomass of AGAS and concentration of vanillin and proteinase K, especially after 24 h of incubation \(p < 0.05\). In other words, attachment biomass of AGAS was gradually decreased with the increase of concentration of vanillin and proteinase K. In general, both vanillin and proteinase K could effectively reduce attachment potential of AGAS, and higher concentration of vanillin and proteinase K led to more reduction of attachment biomass.

3.2. Effect of the combined use of vanillin and proteinase K on attachment potential of AGAS

The effect of the combined use of vanillin and proteinase K on attachment potential of AGAS was analyzed at the present study. After 24 h of incubation, there were 31.22%, 49.74%, 75.66%, and 82.01% reduction with exposure to 0.05 mg mL\(^{-1}\) proteinase K + 0.05 mg mL\(^{-1}\) vanillin, 0.05 mg mL\(^{-1}\) proteinase K + 0.15 mg mL\(^{-1}\) vanillin, 0.15 mg mL\(^{-1}\) proteinase K + 0.05 mg mL\(^{-1}\) vanillin, and 0.3 mg mL\(^{-1}\) vanillin, respectively (Table 1). The similar profiles were also found after 12 h of incubation (Table S1). It was interesting to note that the combined use of vanillin and proteinase K was more effective in reducing attachment biomass of AGAS. For instance, the attachment biomass was reduced by 7.56% and 39.88% with exposure to 0.05 mg mL\(^{-1}\) vanillin and 0.15 mg mL\(^{-1}\) proteinase K for 24 h. But there was 75.66% reduction with exposure to 0.05 mg mL\(^{-1}\) vanillin and 0.15 mg mL\(^{-1}\) proteinase K. Microscopic images showing in Fig. S2 also indicated that both vanillin and proteinase K reduced the attachment potential of AGAS, compared to the control.

3.3. In situ bioassay and relative AHLs content of AGAS treated by vanillin and proteinase K

AHLs were signaling molecules that existed in many Gram-negative bacteria, and known to regulate many biological functions including biofilm formation. Fig. 1 showed that the relative AHLs content of AGAS with exposure to different treatments was lower

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### Table 1

<table>
<thead>
<tr>
<th>Treats</th>
<th>Concentration (mg mL(^{-1}))</th>
<th>Attachment biomass (OD(_{600}))</th>
<th>Percentage of reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.72 ± 0.04</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.59 ± 0.06</td>
<td>7.56</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>1.36 ± 0.04</td>
<td>20.93</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>1.22 ± 0.05</td>
<td>29.07</td>
<td></td>
</tr>
<tr>
<td>Proteinase K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.73 ± 0.11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.16 ± 0.12</td>
<td>32.95</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>1.04 ± 0.07</td>
<td>39.88</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.40 ± 0.05</td>
<td>76.88</td>
<td></td>
</tr>
<tr>
<td>Combination of proteinase K and vanillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>1.89 ± 0.03</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.05 + 0.05</td>
<td>1.30 ± 0.07</td>
<td>31.22</td>
<td></td>
</tr>
<tr>
<td>0.05 + 0.15</td>
<td>0.95 ± 0.08</td>
<td>49.74</td>
<td></td>
</tr>
<tr>
<td>0.15 + 0.05</td>
<td>0.46 ± 0.02</td>
<td>75.66</td>
<td></td>
</tr>
<tr>
<td>0.30 + 0.30</td>
<td>0.34 ± 0.03</td>
<td>82.01</td>
<td></td>
</tr>
</tbody>
</table>
that the content of AGAS was reduced owing to treatment with vanillin and proteinase K. Compared to the control, the relative AHLs content of AGAS treated by 0.3 mg mL\(^{-1}\) proteinase K was slightly decreased. These results were also confirmed by in situ bioassay for AHLs after 24 h culture (Fig. S4), which indicated that the blue color was stronger on the bioassay agar plate of AGAS without treatment and with treatment by 0.3 mg mL\(^{-1}\) proteinase K, compared with AGAS exposed to 0.3 mg mL\(^{-1}\) vanillin or the combination of 0.3 mg mL\(^{-1}\) vanillin and 0.3 mg mL\(^{-1}\) proteinase K.

3.4. EPS content of AGAS treated by vanillin and proteinase K

EPS was mainly composed of proteins and polysaccharides, and believed to induce the microbial attachment. The polysaccharides and proteins contents of AGAS treated by vanillin and proteinase K were summarized in Fig. 2. It was shown that the proteins content of AGAS was reduced owing to treatment with vanillin and proteinase K, while the polysaccharides content almost remained constant. Exactly, after 24 h culture, the proteins content of the control was 27.36 mg g\(^{-1}\) MLSS, while the proteins content of AGAS treated by vanillin, proteinase K and the combination was 21.96, 21.28, and 11.67 mg g\(^{-1}\) MLSS, respectively. The polysaccharides content of sludge was stabilized at around 11.24 and 12.37 mg g\(^{-1}\) MLSS. The profiles of proteins and polysaccharides contents with exposure to vanillin and proteinase K for 12 h were similar to those for 24 h, and detailed results were shown in Fig. S5. In addition, it should be noted that the proteins content was increased with culture time, regardless of AGAS with and without treatment by vanillin and proteinase K. However, the polysaccharides content was stable during the culture time.

3.5. The correlations among attachment biomass, relative AHLs content and extracellular proteins

As shown in Fig. 3a, there was a significant positive relationship between attachment biomass and extracellular proteins of AGAS with and without exposure to both vanillin and proteinase K (\(p < 0.05\)). In other words, extracellular proteins could effectively promote microbial attachment of AGAS. Besides, there was also a significant positive relationship between extracellular proteins and relative AHLs content with and without exposure to vanillin (\(p < 0.05\)), which indicated that AHLs might regulate extracellular proteins synthesis (Fig. 3b). It appeared from Fig. 3a and Fig. 3b that AHLs-mediated QS might be involved in microbial attachment of AGAS through the regulation of extracellular proteins. The result was also confirmed by Fig. 3c, showing that there was a significant positive relationship between attachment biomass and relative AHLs content with and without exposure to vanillin (\(p < 0.05\)).

4. Discussion

At the present study, it is the first comprehensive investigation on the effect of vanillin and proteinase K on microbial attachment of AGAS. Table 1 showed that microbial attachment of AGAS on polystyrene interface was reduced by vanillin and proteinase K. To date, no related information was available for functions of vanillin and proteinase K in controlling microbial attachment of AGAS. Izano et al. (2009) showed that the biofilm formation of nontypeable Haemophilus influenza in polystyrene tube was significantly inhibited by 1 mg mL\(^{-1}\) proteinase K. Moreover, the biofilm development of Streplococcus pneumoniae was almost inhibited at the concentration of 25 \(\mu\)g mL\(^{-1}\) proteinase K (Moscoso et al., 2006). Ponnusamy et al. (2009) reported that the maximum A. hydrophila biofilm inhibition of 46.3% was observed at the high concentration of 0.25 mg mL\(^{-1}\) vanillin on polystyrene surface after 6 h of incubation. All these studies indicated that both vanillin and proteinase K could inhibit the biofilm development of pure cultures, which was confirmed again in our mixed culture of AGAS. More importantly, as shown in Table 1, it was interesting to find that the combined use of vanillin and proteinase K was more effective in reducing attachment biomass of AGAS, which was similar to the result showing the combination treatment of phages and chlorine was more effective in controlling and removing P. aeruginosa biofilms than single treatment (Zhang and Hu, 2013). It was speculated that the presence of proteinase K was likely to be beneficial to digest extracellular proteins, leading to the vanillin penetrates into cells easily.

Fig. 1 showed that the relative AHLs content of AGAS was significantly decreased after treatment by 0.3 mg mL\(^{-1}\) vanillin and the combination of vanillin and proteinase K. Ponnusamy et al. (2009) determined the effect of vanillin on QS property of bio-indicator strains C. violaceum CV026 and A. tumifaciens NTL-4 supplied with exogenous signaling molecules. It was found that 0.25 mg mL\(^{-1}\) vanillin showed the inhibition for short-chain (C\(_{12}\)-HSL (69%) and 3-Oxo-C\(_{12}\)-HSL (59.8%)) and long-chain AHL molecules (C\(_{14}\)-HSL (13.5%) and C\(_{16}\)-HSL (12%)). Similarly, Yeon et al. (2009) used another QS blocker, acylase, to investigate the role of QS in membrane fouling, and found that biocake in the acylase-added reactor showed much weaker AHLs activity than in the con-
control membrane bioreactor (MBR). These results suggested that blocking QS would inhibit the production of AHLs, which was also found in our experiment. Additionally, as shown in Fig. 2, the proteins content of AGAS was reduced after exposure to vanillin and proteinase K. Proteinase K was an endolytic protease, which could cleave peptide bonds at the carboxylic sides of aliphatic, aromatic, proteinase K. Proteinase K digested most extracellular proteins of AGAS. Although no report showed that the presence of vanillin could inhibit the production of extracellular proteins, another QS blocker acylase indeed reduced the extracellular proteins concentration per unit mass of biocake in a MBR system (Yeon et al., 2009). Considering the vanillin also blocked the QS circuit, it was inferred that vanillin could affect the extracellular proteins production as shown in our results.

Furthermore, as shown in Fig. 3a, it was revealed that high content of extracellular proteins was beneficial to the attachment of AGAS. Heilmann et al. (1996) found that a Tn917 insertion mutant of Staphylococcus epidermidis lacked four cell surface proteins, was less hydrophobic, and was affected in primary attachment to polystyrene surface. Dignac et al. (1998) indicated that the proteins and amino acids were the hydrophobic components of the EPS, while carbohydrates were hydrophilic. Moreover, some studies showed that lectin proteins existed in EPS of activated sludge were responsible for activated sludge bioflocculation (Higgins and Novak, 1997; Park and Novak, 2009). All these studies suggested that the hydrophobicity and functionality of extracellular proteins might be beneficial to attachment ability of microorganisms. Besides, it was found that extracellular proteins synthesis was regulated by AHLs (Fig. 3b). Nouwens et al. (2003) reported that P. aeruginosa PA01 mutants deficient in one or more of the lasRI, rhlRI and vfr genes significantly released lower amounts of protein than the wild-type. Morohoshi et al. (2004) revealed that the expression of the 55 kDa protein in fish pathogen Edwardsiella tarda, which was reported as a virulent-strain-specific protein, was controlled by AHLs. Nevertheless, there were some other reports indicating that exopolysaccharide was regulated by AHLs-mediated QS (Marketon et al., 2003; Quiñones et al., 2005), which was not consistent with our results showing exopolysaccharide seemed to be stable with and without treatment. In this study, proteins were more abundant than polysaccharides in AGAS, which was similar to McSwain et al. (2005) and Zhang et al. (2007) indicating that extracellular proteins were the predominant EPS component of AGAS. Therefore, it was believed that extracellular proteins were more susceptible to be regulated by AHLs in AGAS. From the results described above, it was speculated that AHLs-mediated QS might be involved in microbial attachment of AGAS through the regulation of extracellular proteins. The result was consistent with that in Fig. 3c, showing that AHLs promoted microbial attachment of AGAS. So far, the effect of QS mediated by AHLs on microbial attachment of pure cultures has been well studied. For instance, Labbate et al. (2004) reported that a mutant of Serratia liquefaciens MG1 that was incapable of synthesizing extracellular signal formed a thin and nonmature biofilm lacking cell aggregates and differentiated cell chains. However, signal-based complementation of this mutant resulted in a biofilm with the wild-type architecture. Wang et al. (2012) reported exogenous addition of C6-HSL and 3-oxo-C12-HSL induced biofilm formation in Pseudomonas sp. HF-1 when compared with the control algal group. Importantly, Ren et al. (2010) reported the effect of QS on bacterial adhesion of AGAS. It was showed that the signal chemicals produced by AGAS induced the attachment growth of bacteria with a high adhesion capability, leading to initial cell attachment and subsequent biofilm growth on flow-cell cover slide. In general, all results above fully supported the conclusion, QS mediated by AHLs contributed to the microbial attachment of AGAS.

![Fig. 3. The relationship between attachment biomass and extracellular proteins (a), extracellular proteins and relative AHLs content (b), attachment biomass and relative AHLs content (c).](image-url)
5. Conclusion

In this study, it was found that microbial attachment of AGAS could be inhibited by both vanillin and proteinase K. The contents of AHLs and extracellular proteins were decreased with vanillin and proteinase K. Besides, it was found that extracellular proteins were in favor of microbial attachment of AGAS, and AHLs-mediated QS might be involved in microbial attachment of AGAS through the regulation of extracellular proteins. These results clearly indicated that both QS and extracellular proteins played important roles in the development of “AGAS biofilm”.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.10.097.

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