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The effect of quorum sensing on anaerobic granular sludge in different pH conditions

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ABSTRACT

The effects of several signal molecules on anaerobic granular sludge at different pHs were investigated. The results showed that pH affected the synthesis and maintaining of the signal molecules. In an alkaline medium (pH 9), sludge bacteria secreted less acyl-homoserine lactone (AHLs), which had an opposite variation with the synthesis of extracellular polymeric substances (EPS), and its effect did not present. Additionally, the synthesis of EPS (mainly loosely bound layer) was majorly promoted by auto-inducers-2 (AI-2), but a lower negative role of diffusible signal factor (DSF). In neutral and weak alkaline media, the combining regulation of increasing AI-2 content and decreasing DSF content enhanced relative hydrophobicity (RH) and granular strength in granular sludge, which made granules to have a greater diameter and a more glossy granular shape. Whereas, in acid medium, the decreasing AI-2 content and increasing DSF content made a decrease in RH and granular strength, resulting in the degeneration of anaerobic granular sludge.

1. Introduction

QS, which is cell–cell communication among bacteria, occurs by an exchange of chemical signal molecules termed AI [1]. When the extracellular signal molecules in their microenvironment reach a threshold concentration, the cell will be activated and begin regulating particular gene expression. Most bacteria have more than one QS system and secrete different classes of signal molecules to jointly regulate the expression of specific genes [2]. These regulations include virulence factor expression, antibiotic and extracellular enzyme production [3], biofilm formation [4], degradation of organic pollutants [5], and plasmid transfer [6] etc. As a special case in biofilm formation, anaerobic granular sludge must be regulated by QS, and this regulation has been demonstrated in the granulating process of aerobic granular sludge [7–10].

QS depends on the bacterial signal molecule density, and our previous studies have shown that there are three types of signal molecules in anaerobic granular sludge, including intraspecies signal molecules (AHLs), interspecies signal molecules (AI-2), and intra- and interspecies signal molecules (DSF) [11]. As signal molecules of Gram-negative bacteria, AHLs contain a range of different length carbon chains and a homoserine lactone ring. They regulate the formation of biofilms through the EPS matrix, including their composition and production [12]. AI-2, the universal signal molecule between Gram-negative and -positive bacteria, plays an important role in guiding bacteria to switch from planktonic growth to attached growth [7–9], which is a prerequisite in biofilm formation, especially in the granulation of aerobic sludge [8,13]. Another type of signal molecule, DSF, plays an adverse role in bacterial cell aggregation [14] and biofilm formation [15–17].

These signal molecules, as effective chemicals in QS, are synthesized in cells and secreted out of the cell, and they will exist in a specific environment for a relatively long time until their concentrations reach a threshold value. The environment included temperature, pH and different ions etc.. Therefore, the external environment, in which the extracellular signal molecules tem-

Abbreviations: QS, quorum sensing; AI, autoinducers; AHLs, acyl-homoserine lactone; AI-2, auto-inducers-2; DSF, diffusible signal factor; EPS, extracellular polymeric substance; AnGS, anaerobic granular sludge; MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; COD, chemical oxygen demand; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry; HPLC, high performance liquid chromatography; RH, relative hydrophobicity; IC, integrity coefficient; LB, loosely bound; TB, tightly bound; PN, protein; PS, polysaccharide; SEM, scanning electron microscopy.

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Table 1
Buffer formula.

pH	Buffer A (mL)	Buffer B (mL)
5	74	26
6	70	30
7	65	35
9	59	41
10	55	45

Buffer solutions with different pH values were obtained using different volume ratios of the two buffers in 100 mL, and the specific ratios are given in Table 1. Buffer A contained phosphoric acid (0.04 M), acetic acid (0.04 M), boric acid (0.04 M), and buffer B was 0.02 M NaOH.

porarily exist, determines their stability and influence the normal transport of signal molecules across cell membrane. The specific mechanism is not clear and needs to be further investigated.

The metabolism of part bacteria in AnGS will be abnormal when the environmental pH deviates from the optimum pH range [18]. For example, the activity of methanogens will be severely inhibited in pH < 6.5 or >8.2, and pH shock will cause a deterioration of granular sludge until granular degeneration [18]. The above process includes the irreversible change of the composition of microflora and internal structure in granules, which is accompanied by the change of the synthesis of signal molecules.

Therefore, this study was conducted to clarify the presence of different signal molecules and to investigate the effective mechanism of QS in AnGS at different pHs. The results here will provide a reference for further research into the granulating mechanism of anaerobic sludge.

2. Materials and methods

2.1. Experimental configuration and operating conditions

The experiment was conducted in a bottle with a total volume of 600 mL and the effective volume of the reactor was 300 mL, and the MLSS was 18.5 g SS/L. The bottle was sealed using a rubber stopper after blowing nitrogen. And the biogas was collected using a plastic pipe. The reactors containing the sludge were incubated at 30 °C and at 130 rpm. Each sample was assayed in triplicate and the mean values reported.

The pHs were controlled using different pH buffer solutions. In this study we used the Britton–Robinson buffer, and it contained two kinds of buffer, buffer A and buffer B (Table 1). Buffer solutions with different pH values were obtained using different volume ratios of the two buffers in 100 mL. The composition and specific ratios of two buffer solutions are given in Table 1. The synthetic wastewater consisted of the buffer solution with nutrients (cane sugar, NH_4Cl, KP_2PO_4) and trace elements, and it also considered the impact of carbon source from buffer solution. The wastewater was replaced every 24 h and the experiments lasted 6 days.

2.2. Signal molecules add-back studies

C4-HSL, AI-2 and DSF, three types of signal molecules in AnGS, were used as exogenous additions for AnGS. For example, the standards of C4-HSL and DSF (Sigma, USA) were added into reactors with synthetic wastewater and the final concentrations were 100 nM and 4.7 μM, respectively. And ultra-pure water was added as the control (CK1) of AHLs and DSF. As for AI-2, the centrifugal supernatant of BB170 culture (OD_{600} ≈1.0) was collected and replaced the added standards. The culture containing AI-2 was used as the solvent of synthetic wastewater. And flesh culture was included as its medium control (CK2). The wastewater was replaced every 48 h and the study lasted 6 days at 30 °C and at 130 rpm.

2.3. Seeding sludge

The AnGS used in this experiment was obtained from papermaking factory and cultivated using cane sugar as a carbon source with a COD = 3000 ± 100 mg/L. The COD removal efficiency was above 90%. The buffer system had an alkalinity of 2000 mg $CaCO_3$/L. The MLVSS/MLSS ratio of the seeding sludge was 77.5%. And its granular diameter ranged from 0.5 mm to 4 mm.

2.4. Extraction of signal molecules

The mixture of sludge and water was used to extract and measure AHLs and DSF [11], specific pretreatment methods were described as follows: 100 mL of the mixture was divided into sludge and water, respectively. The water was used to resuspend the sludge after crushing. The mixture after pretreatment was extracted with an equivalent volume of ethyl acetate, and evaporated to dryness and re-dissolved in 2 mL of 50% acetonitrile for subsequent analysis [19] The efficiency of the above extraction processes is over 80%. As for AI-2, 1 mL of supernatant from the mixture was collected, and filtered through a 0.22 μm syringe filter and stored at -20 °C for the AI-2 assay.

2.5. Analytical methods

2.5.1. UPLC-MS/MS determination of signal molecules

AHLs were analysed using a Waters UPLC-MS/MS (Xevo TQD, Waters, USA) system with a waters ACQUITY UPLC BEH C-18 column (1.7 μm d, 2.1 × 50 mm, Waters, Ireland). The nebulizer gas was N_2; the flow rate of the dryer gas was set at 800 L/h. The heated capillary and voltage were maintained at 500 °C and 0.5 kV, respectively [20]. The samples were then passed through a 0.22-μm filter and eluted with ammonium acetate buffer (0.05 M) and acetonitrile. The RSD of AHLs is less than 4%. Because of its different molecular weight, the detection limit of AHLs ranging from C4-HSL to 3-oxo-C8-HSL is 0.1–1.0 pM.

2.5.2. HPLC determination of signal molecules

DSF was analysed using a Waters HPLC system with an X Bridge C-18 column (5 μm d, 4.6 × 250 mm). The samples were then passed through a 0.22-μm filter for HPLC analysis. More precisely, the DSF was eluted with 80% acetonitrile in water at a flow rate of 0.7 mL/min with a separation injection volume of 10 μL. The wavelength was set to 210 nm [11]. Besides, RSD in DSF detection is less than 5% and its detection limit is 2 μM.

2.5.3. AI-2 determination

The AI-2 activity was measured by an indicator strain, *Vibrio harveyi* BB170, which produces light in the presence of AI-2 and was measured by a multifunctional microplate reader (SpectraMax M5, USA) [11]. The relative AI-2 content calculated as the intensity of the sample at 490 nm dived by the intensity of the media control, and its RSD is less than 2%.

2.5.4. Other methods

COD was measured using a DR2800 spectrophotometer (Hach Company, Loveland, CO, USA) and the granular diameter was measured by wet sieving method. Volatile fatty acids were measured by withdrawing a 2-μL sample from the headspace using a gas-tight syringe followed by gas chromatography with a chromatograph (GC 7890 II, Shanghai Tianmei Science Instrument Co., Ltd., Shanghai, China) equipped with a flame ionization detector. The operating parameters are given in Huang et al. [21]. RH was measured by bacterial adhesion to hydrocarbons [22].

A two-step heating EPS extraction procedure [23] was used to extract the easily extractable EPS, including LB-EPS and TB-EPS. PS