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Determination of quorum-sensing signal substances in water and solid phases of activated sludge systems using liquid chromatography–mass spectrometry

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ABSTRACT

The detection of acyl homoserine lactones (AHLs) in activated sludge is essential for clarifying their function in wastewater treatment processes. An LC–MS/MS method was developed for the detection of AHLs in both the aqueous and solid phases of activated sludge. In addition, the effects of proteases and extracellular polymeric substances (EPS) on the detection of AHLs were evaluated by adding protease inhibitors and extracting EPS, respectively. Recoveries of each AHL were improved by adding 50 μL of protease inhibitor, and recoveries were also improved from 0 to 56.9% to 24.2%–105.8% by EPS extraction. Applying the developed method to determine the type and concentration of AHLs showed that C_4-HSL, C_6-HSL, C_8-HSL and 3-oxo-C_8-HSL were widely detected in a suspended activated sludge system. The dominant AHL was C_8-HSL, with a highest concentration of 304.3 ng/L. C_4-HSL was mainly distributed in the aqueous phase, whereas C_6-HSL, C_8-HSL and 3-oxo-C_8-HSL were preferentially distributed in the sludge phase.

Introduction

Quorum sensing (QS) is a communication mechanism among microorganisms within a species or between inter-species (Bassler, 2002). QS was found in the marine bacterium *Vibrio fischeri* that is responsible for bioluminescence. The bioluminescence of *V. fischeri* occurs after secretion of sufficient signal substances that serve as autoinducers. Once a threshold concentration of signal substances is reached, activation of target genes will be triggered, inducing the detection of the luciferase (Nealson and Hastings, 1979). The regulation of virulence gene expression, biofilm formation, swarming and sporulation by QS has been confirmed (Bassler, 2002; Nealson and Hastings, 1979). During regulation, signal substances corresponding to inter- and intra-species communication play a vital role in QS. The N-acyl homoserine lactones (AHLs) are well-characterized bacterial communication languages in Gram-negative bacteria that vary in the length and saturation degree of the acyl chain. In contrast, communication among Gram-positive bacteria usually uses oligopeptides as signal substances. Unlike AHLs and oligopeptide autoinducers, AI-2, a novel furanosyl borate diester with no similarity to other autoinducers, is a universal signal that functions in interspecies cell-to-cell communication (Waters and Bassler, 2005).

Recently, the function of QS in wastewater treatment processes has received considerable attention. Studies have shown that AHL-mediated QS is strongly linked to nitrification, biofilm formation, granular sludge formation and membrane

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biofouling (Jiang and Liu, 2012; Huang et al., 2016; Tan et al., 2014, 2015; Gao et al., 2014; Yeon et al., 2008). The types and concentrations of AHLs varied at different stages of granulation, with increased amounts of N-[(RS)-3-Hydroxybutyryl]-L-HSL (C_4-HSL), N-octanoyl-L-homoserine lactone (C_8-HSL) and N-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL) during granulation and decreased amounts of N-hexanoyl-L-homoserine lactone (C_6-HSL) and C_8-HSL during granule disruption (Tan et al., 2014). Similar results were observed in biofilm systems. C_6-HSL, C_8-HSL, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and N-tetradecanoyl-L-homoserine lactone (C_{14}-HSL) were regularly and positively correlated to biofilm formation by affecting the production of extracellular polymeric substances (EPS), with varied AHLs concentrations of 0–100 ng/L (Hu et al., 2016a). The addition of AHLs (5 nmol/L AHLs, C_6-HSL:C_8-HSL:C_{14}-HSL:3-oxo-C_{12}-HSL of 1:1:1:1) affected the ratio of ammonia-oxidizing bacteria to total bacteria in a biofilm system (Hu et al., 2016b). In addition, C_{14}-HSL, N-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C_{14}-HSL) and C_4-HSL were closely correlated to nitrification and denitrification of *Pseudomonas aeruginosa* (Gao et al., 2014; Toyofuku et al., 2007). In membrane bioreactors, C_6-HSL, C_8-HSL and 3-oxo-C_8-HSL were shown to be the three main signal substances causing membrane biofouling (Yeon et al., 2009). Therefore, AHLs are pivotal substances that can clarify the quorum sensing mechanism involved in wastewater treatment processes.

The concentration of QS signal substances ranges from ng/L (ng/g) to μg/L (μg/g) (Tan et al., 2014; Hu et al., 2016b). Therefore, suitable pretreatment and accurate quantitative methods are required in the study of QS in activated sludge systems. Initially, signal substances were detected by strain reporters such as *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026 with the production of β-Galactosidase and violet pigment in response to AHLs, respectively (Fuqua and Winans, 1996; Stickler et al., 1998; McClean et al., 1997). Based on reports, the quantitation of AHLs was achieved by estimating β-Galactosidase activity (Zhu et al., 2003). However, this method provided only preliminary quantitation, and the number of detectable signal substances was limited. To further improve the accuracy and precision of the AHLs assay, methods based on high-performance liquid chromatography (HPLC) (Lépine and Déziel, 2011; Tang et al., 2015), gas chromatography–mass spectrometry (Cataldi et al., 2007), and high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Ortori et al., 2011; Li et al., 2006) were developed for the quantitation of signal substances. Specifically, most AHLs could be detected by the LC–MS/MS method (Ortori et al., 2011). However, previous studies mainly detected AHLs in the culture supernatants using a liquid–liquid extraction method, including AHLs produced by *P. aeruginosa* (Ortori et al., 2011; Wang et al., 2012) and *Burkholderia cepacia* LA3 grown on the LB medium (Li et al., 2006). Only limited studies have attempted to determine AHLs in activated sludge and supernatant (Feng et al., 2014). According to Feng et al. (2014), although the detection of AHLs in anaerobic activated sludge is divided into an aqueous phase, a water wash phase and a sludge phase, and AHLs were estimated based on the reported strains of QS and HPLC, with the detected AHLs limited to C_4-HSL and 3-oxo-C_8-HSL. Additionally, the effect of complex physicochemical characteristics of activated sludge was not taken into account in the detection of AHLs in previous studies. The detection of AHLs in activated sludge may be affected by many factors, such as EPS components of polysaccharides and proteins (Song et al., 2014), enzyme activity (Lade et al., 2014), pH and temperature. Even though the effects of these factors on the detection of AHLs are not well understood, some indirect evidences have suggested that these factors are connected with AHL-mediated processes in activated sludge systems. First, AHLs were likely to react with some components of the EPS, thereby slowing diffusion of AHLs (Song et al., 2014). Moreover, some specific regions of polysaccharides and proteins can adsorb organic matter, including AHLs (Tan et al., 2014). In addition, the degradation of AHLs through structural changes catalyzed by quorum-quenching (QQ) enzymes has been confirmed (Lade et al., 2014). The chemical stability of AHLs under alkaline and acidic conditions was investigated by Wang et al. (2012), who showed that an acidic environment was beneficial for the persistence of AHLs. Because it is difficult to detect AHLs in activated sludge, a comprehensive method for the detection of AHLs in both activated sludge and the supernatant should be developed; this method would be useful for clarifying their function in wastewater treatment processes.

In this study, a comprehensive method for QS signal-substance detection from water and the solid phases of activated sludge systems was developed by LC–MS/MS. In addition, factors affecting QS signal-substance extraction and detection were examined intensively for further optimization of the developed method. Finally, the developed method was applied to detect both the type and concentration of AHLs in an activated sludge system.

1. Materials and methods

1.1. Reagents

Standard AHLs *viz.* N-(β-ketocaproyl)-L-Homoserine lactone (3-oxo-C_6-HSL) was purchased from the Cayman Chemical Company (Ann Arbor, USA). C_4-HSL, C_6-HSL, C_8-HSL, 3-oxo-C_8-HSL, N-decanoyl-L-homoserine lactone (C_{10}-HSL), N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C_{10}-HSL), N-dodecanoyl-L-homoserine lactone (C_{12}-HSL), 3-oxo-C_{12}-HSL, C_{14}-HSL and 3-oxo-C_{14}-HSL were purchased from Sigma-Aldrich (Shanghai, China) and stored at −20°C. Protease inhibitor P (Phenylmethylsulfonyl fluoride) was purchased from Roche (Basel, Switzerland). Protease inhibitor C (Cocktail, EDTA-Free, 100× in DMSO) was purchased from Biotool Company (Houston, USA). HPLC-grade formic acid and acetonitrile were purchased from J. T. Baker Chemicals Company (Wisconsin, USA). HPLC-grade methanol was purchased from Merck Company (Darmstadt, Germany).

1.2. Tested activated sludge

The activated sludge used in this experiment was obtained from a lab-scale sequencing batch reactor (SBR) operated in a multiple anoxic and aerobic mode. The SBR was operated on a 6-hr cycle, and each cycle consisted of a 120-min anaerobic phase (including 10 min filling), a 120-min intermittent

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