

Full Paper

Characterization of *N*-acyl homoserine lactones (AHLs) producing bacteria isolated from vacuum-packaged refrigerated turbot (*Scophthalmus maximus*) and possible influence of exogenous AHLs on bacterial phenotype

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Quorum sensing (QS) is a cell-to-cell communication mechanism through which microbial cells communicate and regulate their wide variety of biological activities. *N*-acyl homoserine lactones (AHLs) are considered to be the most important QS signaling molecules produced by several Gram-negative bacteria. The present study aimed to screen the AHLs-producing bacteria from spoiled vacuum-packaged refrigerated turbot (*Scophthalmus maximus*) by biosensor assays, and the profiles of AHLs produced by these bacteria were determined using reversed-phase thin-layer chromatography (RP-TLC) and gas chromatography-mass spectrometry (GC-MS). Effects of exogenous AHLs and QS inhibitor (QSI) on the phenotypes (i.e., extracellular proteolytic activity and biofilm formation) of the AHLs-producing bacteria were also evaluated. Our results demonstrated that eight out of twenty-two isolates were found to produce AHLs. Three of the AHLs-producing isolates were identified as *Serratia* sp., and the other five were found to belong to the family of *Aeromonas*. Two isolates (i.e., *S. liquefaciens* A2 and *A. sobria* B1) with higher AHLs-producing activities were selected for further studies. Mainly, RP-TLC and GC-MS analysis revealed three AHLs, i.e., 3-oxo-C6-HSL, C8-HSL and C10-HSL were produced by *S. liquefaciens* A2, while five AHLs, i.e., C4-HSL, C6-HSL, C8-HSL, C10-HSL, and C12-HSL, were produced by *A. sobria* B1. Moreover, production of AHLs in both bacterial strains were

found to be density-dependent, and the AHLs activity reached a maximum level in their middle logarithmic phase and decreased in the stationary phase. The addition of exogenous AHLs and QSI decreased the specific protease activity both of the *Serratia* A2 and *Aeromonas* B1. Exogenous AHLs inhibited the biofilm formation of *Serratia* A2 while it enhanced the biofilm formation in *Aeromonas* B1. QSI inhibited the specific protease activity and biofilm formation in both bacterial strains.

Key Words: biofilm; *N*-acyl homoserine lactones; quorum sensing; protease activity; turbot

Introduction

Quorum sensing (QS) is a cell density dependent mechanism based on the production, secretion and detection of extracellular signaling molecules (Fuqua et al., 1994). QS signaling are small diffusible molecules secreted by bacteria and accumulated in cytoplasm. When the production of these signaling molecules reaches a threshold concentration, bacteria can sense their presence and regulate gene expression, such as exoenzymes production, luminescence and biofilm formation (Gram et al., 2002; Jakobsen et al., 2013). Many Gram-negative bacteria employ *N*-acyl homoserine lactones (AHLs) as signaling molecules that contain a homoserine lactone ring carrying acyl chains of 4 to 18 carbons with or without a modification at the C3 position (Myszka and Czaczyk, 2012). Gram-positive bacteria utilize oligopeptides as QS signals for the cell-to-

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cell communication (Li et al., 2012; Monnet and Gardan, 2015). Another signaling molecule known as autoinducer-2 (AI-2) that was derived from 4,5-dihydroxy-2,3-pentanedione (DPD) is deemed to be the omnipresent chemical as the interspecies communication signal between Gram-negative and Gram-positive bacteria (Ascenso et al., 2011; Galloway et al., 2010).

An AHL-mediated QS system has been reported in a broad range of proteobacteria affiliating with alpha (α), beta (β) and gamma (γ) sub-groups (Chhabra et al., 2005). A well-described example of an AHL-mediated QS system has been reported in the Gram-negative symbiotic bacterium *Vibrio fischeri*, which colonizes the light organ of particular squid and fish and produces bioluminescence at a high cell density (Nealson, 1977). AHL-regulated traits have also been reported in many other bacterial species such as *Vibrio harveyi*, *Erwinia carotovora* and *Pseudomonas aeruginosa* (Bassler et al., 1994; Chatterjee et al., 2010; Williams and Cámara, 2009). Moreover, several reporter systems based on *lux*, *gfp*, *lacZ* reporter gene fusion or pigment induction have been widely employed to screen bacteria for the production of AHL signaling molecules (Shaw et al., 1997). However, these biosensors only provide evidence of the production or tentative identification of AHL signal molecules, and a detailed identification of chemical structures requires advanced technology, i.e., mass-spectrometry or nuclear magnetic resonance spectroscopy (Chhabra et al., 2005).

Turbot (*Scophthalmus maximus*) is an important commercial marine flatfish in China. It appears to be greatly appreciated by consumers because of its delicious taste, nutritional quality, as well as high economic value (Cai et al., 2014). Turbot are susceptible to spoilage due to their high protein and moisture content. The growth and metabolism of microorganisms are the primary factors associated with food spoilage. During the storage of aquatic product, bacteria always form a microbial derived sessile community biofilm. The formation of a biofilm enhances the resistance for outer pressure and may cause many problems in the food industry. Studies have demonstrated that QS may be involved in the process of exoenzyme activities and biofilm formation: protease production in *Pseudomonas fluorescens* was regulated by AHLs (Liu et al., 2007); the application of furanones (QS inhibitor) prolonged the shelf-life of fermented milk by reducing the motility and exoenzyme activity of *Pseudomonas fluorescens* (Shobharani and Agrawal, 2010); and QS regulated biofilm formation in *Vibrio cholerae* (Antonova and Hammer, 2011). Mainly, the role of AHLs-producing bacteria in the process of refrigerated turbot spoilage suggests a possible link between a QS system and spoilage, which necessitates a new strategy for refrigerated turbot preservation.

In this study, the first objective was to identify AHLs producers from refrigerated turbot packaged under vacuum conditions based on biosensor assays. The types of AHLs were characterized by combining reversed-phase thin-layer chromatography (RP-TLC) with gas chromatography-mass spectrometry (GC-MS) analysis. Then, the possible effects of exogenous AHLs on protease production/biofilm formation were evaluated.

Materials and Methods

Materials and bacterial strains. Live turbot, with a mean weight of 950 ± 50 g, were purchased from Yellow sea farm (Qingdao, China) and transported in oxygenated water to the laboratory. AHLs standards (N-butanoyl-L-homoserine lactone, C4-HSL; N-hexanoyl-L-homoserine lactone, C6-HSL; N-octanoyl-L-homoserine lactone, C8-HSL; N-decanoyl-L-homoserine lactone, C10-HSL; N-dodecanoyl-L-homoserine lactone, C12-HSL; N-tetradecanoyl-L-homoserine lactone, C14-HSL; N-(3-oxohexanoyl)-L-homoserine lactone, 3-oxo-C6-HSL), X-Gal(5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and QS inhibitor(5-bromomethylene-2(5H)-furanone, BMF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used in this study were purchased from sinopharm Chemical Reagent Co., Ltd. (China).

Agrobacterium tumefaciens A136 (pCF218) (pCF372) and *Chromobacterium violaceum* CV026 were kindly donated by Dr. Zhong (Nanjing Agricultural University, Nanjing, China). They were grown in Luria-Bertani (LB) broth at 28°C. The growth medium for A136 was further supplemented with appropriate antibiotics, i.e., spectinomycin ($50 \mu\text{g mL}^{-1}$) and tetracycline ($4.5 \mu\text{g mL}^{-1}$) (McClean et al., 1997; Zhu et al., 2003).

Isolation of bacterial strains. The turbot were killed by a blow on the head, and after being cooled to about 4–6°C, they were individually packed in high-density polyethylene (HDPE) vacuum bags and stored at 4°C for 20 days. Following this, 25 g of turbot sample were aseptically weighed and mixed with 225 mL of sterile physiological saline solution (0.85% sodium chloride). The mixture was homogenized vigorously to produce a suspension, which was subsequently, diluted ten-fold and spread onto plate count agar (PCA) plates. The plates were incubated aerobically for 24–48 h at 28°C. Colonies having different morphologies were streaked onto new PCA agar plates to obtain pure cultures. All the agar plates were incubated at 28°C for 24–36 h.

Identification of AHLs assays.

Extraction of AHLs: Isolated bacterial strains were inoculated separately into fresh LB broth (100 mL) and grown for 24 h at 28°C with shaking (200 rpm). Cell suspensions were then aseptically transferred to sterile centrifuge tubes and were centrifuged for 10 min at 12,000 rpm (4°C). Supernatants were carefully separated and AHLs were extracted with an equal volume of ethyl acetate acidified with formic acid (0.5%). The whole extraction process was repeated three times. The organic phase was collected and evaporated to dryness at 30°C. The dried extracts were further dissolved in 1.0 mL DMSO and stored at –20°C for further use (Zhu et al., 2015).

AHLs bioassay: To detect the presence of AHL in the isolates, well diffusion bioassay was performed using A136 as a reporter strain. LB soft agar (0.7% agar) containing 3–4% A136 was further mixed with X-Gal (final concentration, $50 \mu\text{g mL}^{-1}$) and appropriate antibiotics. Wells were punched after the agar solidified. 30 μL extracts were added into the wells before the plates were incubated at 30°C for 24 h. The appearance of a blue circle indicated

Table 1. Identification of AHLs-producing bacterial strains isolated from spoiled vacuum-packaged refrigerated turbot.

The AHLs-based responses to reporter strain A136 were represented as “++” (strong positive, IDZ > 40) and “+” (positive, IDZ < 40). IDZ is the induction diameter zone expressed in mm.

Strain No.	Closest relative	Similarity (%)	GeneBank No.	A136	IDZ
A1	<i>Serratia</i> sp.	97	KJ601753.1	++	40.5 ± 0.35
A2	<i>Serratia</i>	98	CP006253.1	++	42.5 ± 0.35
A3	<i>Serratia</i> sp.	99	KJ601747.1	++	41.0 ± 0
B1	<i>Aeromonas sobria</i>	100	KJ777693.1	+	30.5 ± 0.35
B2	<i>Aeromonas sobria</i>	100	KC951914.1	+	19.0 ± 0.70
B3	<i>Aeromonas</i> sp.	100	HF678886.1	+	22.0 ± 0
B4	<i>Aeromonas</i> sp.	100	KF317749.1	+	29.5 ± 0.35
B5	<i>Aeromonas hydrophila</i>	100	CP006579.1	+	15.0 ± 0.70

the presence of AHLs. C6-HSL (final concentration, 100 μ M) was used as a positive control.

RP-TLC assay: RP-TLC analysis was performed as described by Shaw et al. (1997). 2–5 μ L of ethyl acetate extracts and AHL standards were loaded onto TLC plates (20 cm × 20 cm, RP-18 F254S, Merck, Germany), and developed with methanol-millipore water (60:40, v/v) (Chu et al., 2011; Ravn et al., 2001). Subsequently, the plates were air dried in a fume hood and overlaid with a thin layer of LB soft agar (0.7% agar) containing A136 or CV026 bacterial biosensor. X-Gal and antibiotics were supplemented for the agar medium with A136 biosensor. Then the TLC plates were incubated at 28°C for 12–18 h. The standards applied on the TLC plates were C4-HSL (1 mM), C6-HSL (1 mM) and 3-oxo-C6-HSL (1 μ M).

GC-MS analysis: AHLs were further evaluated by GC system 6890 N connected to a mass selective detector Agilent-5973 (Agilent Technologies) with the NIST library. The identification of AHLs was performed using a HP-5 MS capillary column (30 m × 0.25 mm ID and 0.25 μ m film thicknesses). Pure helium (He) was used as the carrier gas at a flow rate of 0.8 mL/min. The GC oven temperature was increased from 150°C (held for 3 min) to 280°C (held for 3 min) at a rate of 25°C/min. The GC injector temperature was 270°C and the transfer line was adjusted to 280°C. Mass spectrometry conditions were adjusted to be: electron ionization energy 0.7 kV; emission current 500 μ A; ion source temperature 200°C; and the MS source temperature was 230°C. 1 μ L of AHL standards or extracts was injected in a splitless mode. The mass spectrometer was run at a single ion monitoring (SIM) mode at m/z 143. AHLs in bacterial extracts were identified by comparing the mass spectra and the retention times with AHL standards (Cataldi et al., 2004, 2007).

Identification of bacterial strains. AHLs-producing bacteria were identified by 16S rRNA gene sequencing analysis. Bacterial DNA was extracted and purified using a bacterial genomic DNA extraction kit (Tiangen Biotech, Beijing) as described (Ee et al., 2014). The 16S rRNA gene was amplified from the resulting DNA using universal primers B8F (5'-AGAGTTTGATCCTGGCTCAG-3') and B1510 (5'-GGTTACCTTGTTACGATT-3') through a polymerase chain reaction (PCR) technique under standard conditions (Chan et al., 2011). PCR products were sent for sequencing. The 16S rRNA gene sequences were

Table 2. AHLs detected by RP-TLC and GC-MS, respectively.

Strain	RP-TLC	GC-MS
<i>Serratia</i> A2	3-O-C6-HSL	C8-HSL C10-HSL
<i>Aeromonas</i> B1	C4-HSL C6-HSL	C4-HSL C6-HSL C8-HSL C10-HSL

blasted with those in the Genbank database using the NCBI (<http://www.ncbi.nlm.nih.gov/Blast.cgi>) alignment search tool.

β -galactosidase activity analysis. The bacterial strains *Serratia* A2 and *Aeromonas* B1 were grown in LB broth. Sterile supernatants containing AHLs signaling molecules were obtained by centrifuging (8000 rpm, 5 min) the broth culture every 4 h for 24 h. Supernatants were obtained by passing through a 0.22 μ m syringe filter. AHLs activity was evaluated using the β -galactosidase method, as reported by Miller, using A136 as a reporter strain. The Miller Units were defined as the required amount of β -galactosidase to decompose 1 nmol ONPG in 1 min (Miller, 1972).

Protease activity assay. Proteolytic activity was determined using azocasein as the substrate according to the method described by Gram et al. (2002) with some modifications. Briefly, bacterial strains were inoculated into 100 mL LB broth in the absence or presence of exogenous AHLs (C4-HSL, C6-HSL, 3-oxo-C6-HSL, final concentration of 10 μ M) (Kastbjerg et al., 2007). QS inhibitor (BMF), final concentration of 5.4 mg/L, that have no effect on the growth of the two bacterial strains were also used. The flasks were incubated at 28°C for 24 h (200 rpm). At 4 h intervals, growth was measured at OD₆₀₀ and the protease activity was determined as follows. 0.1 mL culture supernatant and 0.1 mL 3% azocasein solution were dissolved in 50 mM phosphate buffer (pH 7.5). The mixture was incubated at 30°C for 1 h, and reactions were stopped by adding 0.5 mL 20 % (w/v) trichloroacetic acid (TCA). A blank control was then performed, and only azocasein and phosphate buffer were added and incubated 1 h at 30°C. The reaction was stopped with TCA before the addition of 0.1 mL of culture supernatant. The mixture was then centrifuged at 12000 rpm for 10 min, and the absorbance of the supernatant obtained was determined at 366 nm. One unit of proteolysis was defined as an in-

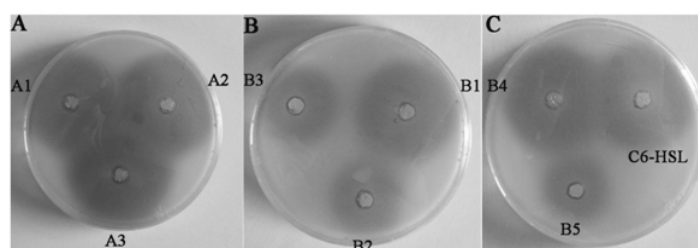


Fig. 1. AHL produced by the isolated bacterial strains in a well diffusion bioassay using *A. tumefaciens* A136 as a biosensor.

Plate A shows the presence of AHLs in bacterial extracts of A1, A2 and A3. Plate B indicates the presence of AHLs in extracts of B1, B2 and B3, while Plate C shows the AHLs in extracts of B4 and B5, C6-HSL (100 μ M) represents positive control.

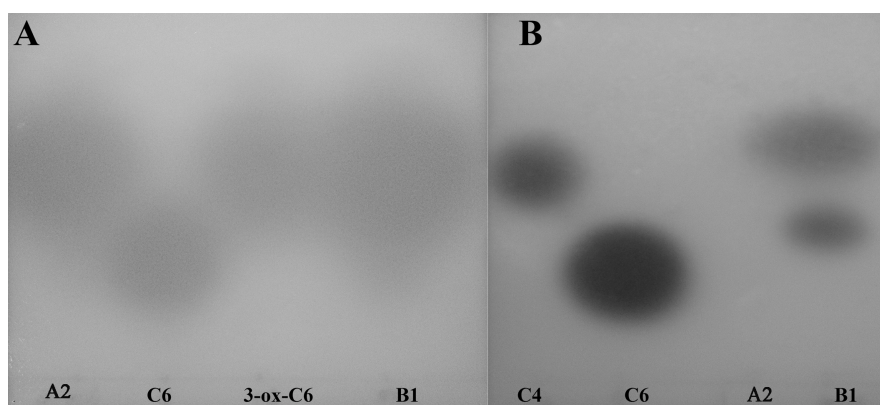


Fig. 2. Separation of AHL molecules from the culture extract of the bacterial strain *Serratia* A2 and *Aeromonas* B1 by RP-TLC.

A. tumefaciens A136 (A) and *C. violaceum* CV026 (B) were used as reporter strains.

crease of one absorbance unit at 366 nm h^{-1} per cell (bacterial density was measured at 600 nm).

Biofilm assay. The biofilm biomass was assessed by the crystal violet (CV) method as described by Wang et al. (2013) with modifications. Briefly, 10 μ L of bacterial strain ($OD_{600} = 0.1$) were added to 1 mL of LB medium and cultivated in the absence or presence of AHLs (C4-HSL, C6-HSL, 3-oxo-C6-HSL, final concentration 10 μ M; BMF, 5.4 mg/L) in a 48-well plate for 12, 24 and 36 h, respectively. An equal volume of DMSO was added as a control. After incubation, the plates were washed three times with sterile deionized water to remove loosely attached cells. The washed wells were air-dried and stained with 1 mL of 0.1% CV solution for 15 min. Stained wells were washed three times with deionized water to remove excess stain. The CV in the stained cells was solubilized with 1 mL of 95% ethanol and the absorbance was measured at 590 nm using a UV-visible spectrophotometer.

Result

Identification of AHLs-producing bacteria

Approximately twenty-two bacterial strains were isolated from spoiled refrigerated turbot packaged under vacuum conditions. All of these bacterial strains were screened for the production of AHLs in well diffusion bioassay using A136 as a reporter strain. AHLs detection

using bacterial biosensors has been proven to be a suitable, rapid and efficient technique. A preliminary well diffusion bioassay revealed that eight of the twenty-two isolates were capable of producing AHLs. Moreover, 16S rRNA gene sequencing analysis indicated that three of the eight AHLs-producing bacterial strains belong to *Serratia* sp.; while the other five were in the family of *Aeromonas* (Table 1). Two of these bacterial strains such as *S. liquefaciens* A2 and *A. sobria* B1 that exhibited higher AHLs-producing ability by stimulating A136 and hydrolyzing X-Gal into blue color were studied further (Fig. 1).

Characterization of AHLs in *Serratia* A2 and *Aeromonas* B1

RP-TLC plates overlaid with a thin layer of LB-soft agar containing A136 or CV026 as biosensor strains have been extensively used to characterize AHLs (Shaw et al., 1997). AHLs produced by *Serratia* A2 and *Aeromonas* B1 bacterial strains were characterized by the RP-TLC method. TLC separation of the bacterial extracts with an A136 biosensor indicated different AHL profiles and recorded a single spot of an AHL molecule produced by *Serratia* A2, which was equivalent in size and *R_f*-value to 3-oxo-C6-HSL. Whilst an ethyl acetate extract of the bacterial strain *Aeromonas* B1 induced two spots on TLC plate, whose size and *R_f*-value were in accordance with 3-oxo-C6-HSL

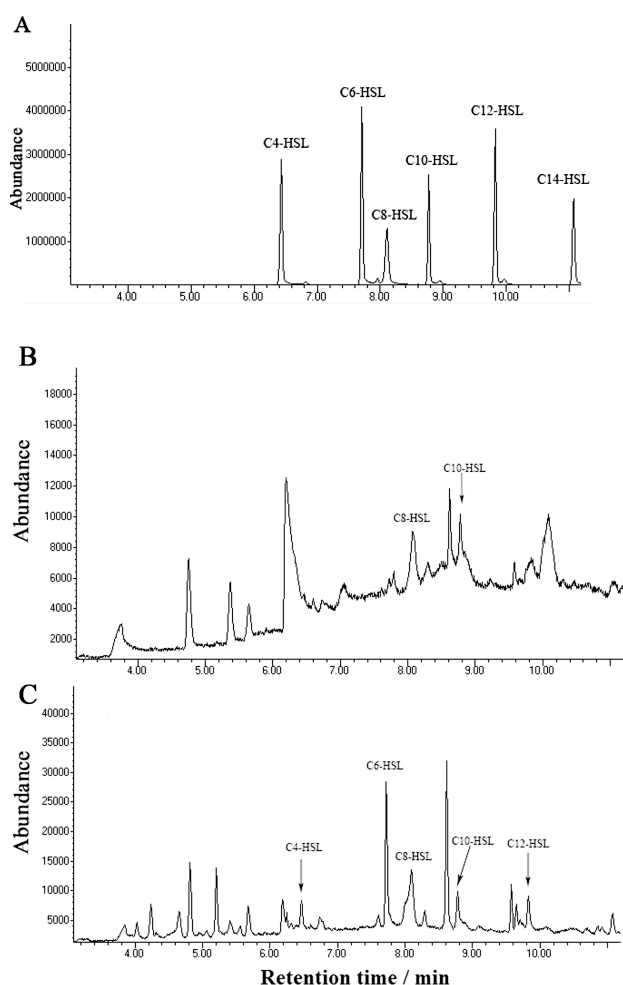


Fig. 3. GC-MS chromatogram in SIM mode at m/z 143 of a mixture of six AHL standards (C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL and C14-HSL) (A), ethyl acetate extracts of the bacterial strain *Serratia* A2 (B) and *Aeromonas* B1 (C).

and C6-HSL, respectively. Moreover, TLC analysis with a CV026 biosensor did not reveal any such spots produced by *Serratia* A2. However, *Aeromonas* B1 showed two spots having a purple color, which were similar in R_f -value with C4-HSL and C6-HSL (Fig. 2). As has been reported, CV026 cannot be activated by 3-oxo-C6-HSL to produce a purple color, so the AHLs produced by *Aeromonas* B1 were C4-HSL and C6-HSL, not 3-oxo-C6-HSL.

In the present study, identification of AHLs signaling molecules was further evaluated by the GC-MS system which has been reported as a non-target screening technique with better separation power, sensitivity and good structural information (Cataldi et al., 2004, 2007).

A common fragmentation was determined with the maximum ion at m/z 143, with other secondary peaks at m/z 101, 57, and 43. Mainly, the fragment ion at m/z 143 is due to a McLafferty rearrangement with the usual carbonyl groups carrying a hydrogen atom in the γ -position. A chromatogram carrying a mixture of six AHL standards (C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL and C14-HSL) indicated clearly resolved peaks under optimized GC-MS conditions. Moreover, GC-MS analysis of the bacterial extracts identified two AHLs (C8-HSL and C10-HSL) produced by *S. liquefaciens* A2, and five

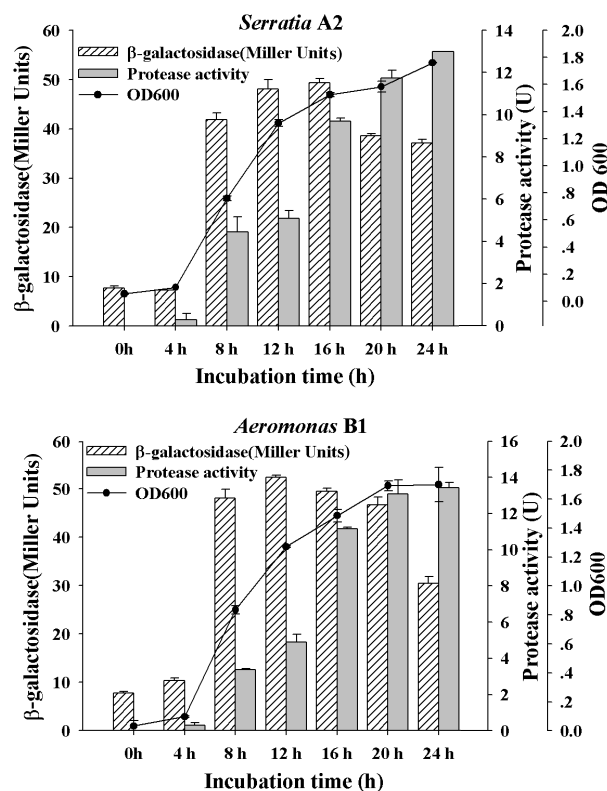


Fig. 4. The protease activity and AHLs activity of *Serratia* A2 and *Aeromonas* B1 were detected during 24 h incubation in LB broth.

Data are expressed as the mean of three replicates.

AHLs (C4-HSL, C6-HSL, C8-HSL, C10-HSL and C12-HSL) produced by *A. sobria* B1 (Fig. 3).

Effect of AHLs on protease activity of *Serratia* A2 and *Aeromonas* B1

As the results in Fig. 4 demonstrated, where AHLs activities were determined using the β -galactosidase method, AHLs in both bacteria were hardly detected at a low population density ($OD_{600} < 0.2$) and significantly greater amounts of AHLs were produced at 8 h when the cell densities of OD_{600} reached 0.6. Also, AHLs activity decreased in *Serratia* A2 at the stationary phase (20 h). *Aeromonas* B1 showed a reduced AHLs activity at 24 h. These results indicated that the AHLs productions in *Serratia* A2 and *Aeromonas* B1 were density dependent.

The protease activity produced by both *Serratia* A2 and *Aeromonas* B1 increased when the bacterial strains entered into the mid-phase ($OD_{600} > 0.6$) and reached a maximum at the beginning of the stationary phase (Fig. 4). Meanwhile, the effect of different exogenous AHLs and QSI on specific protease activity (protease activity divided by bacterial density, A_{366}/OD_{600}) of the two bacterial strains was evaluated. Mainly, the addition of exogenous AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) influenced the protease activity in both of the bacterial strains. Of the three AHLs, C6-HSL and 3-oxo-C6-HSL decreased the specific protease activity at a low level in *Serratia* A2 after 24 h. While, the addition of the three exogenous AHLs (C4-HSL, C6-HSL, 3-oxo-C6-HSL) decreased the specific protease activity in *Aeromonas* B1 (Fig. 5). Moreover, the

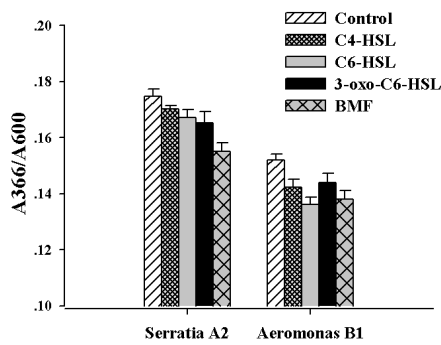


Fig. 5. The effect of exogenous AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) and QSI (BMF) on the specific protease activity of *Serratia A2* and *Aeromonas B1* bacterial strains detected after 24 h incubation.

Data are expressed as the mean of three replicates.

addition of QSI clearly inhibited the protease activity in both of bacterial strains (Fig. 5).

Effect of AHLs on biofilm formation of *Serratia A2* and *Aeromonas B1*

Biofilm is a structure enabling a microbial community to survive a deteriorated environment. As showed in Fig. 6, the addition of exogenous AHLs influenced differently the process of biofilm formation of the two bacteria. After 12 h incubation, exogenous AHLs had no effect on biofilm formation either in strain A2 or B1, and then biofilm mass increased and reached a maximum at 24 h. In *Serratia A2*, biofilm formation was lightly inhibited at the stationary phase (24 h) with the addition of 3-oxo-C6-HSL, while C4-HSL and C6-HSL had no significant effect. Nevertheless, exogenous AHLs promoted the biofilm formation of *Aeromonas B1* at 24 h; especially C4-HSL, which resulted in a greater than 40% increase. Subsequently, the biofilm mass decreased when the microbial communities entered into a decline phase (36 h). At 36 h, all three AHLs exhibited a significant inhibition on the biofilm formation of *Serratia A2* and the inhibition ratio reached nearly 50% by the addition of 3-oxo-C6-HSL, while having no significant effect on the biofilm mass in *Aeromonas B1* after 36 h incubation. For both two bacterial strains, the addition of QSI had no significant effect on biofilm formation at 12 h. While, biofilm formation of both bacterial strains QSI were dramatically inhibited after 24 h and 36 h incubation.

Discussion

Microbiological activities are the main cause of aquatic food product spoilage. QS has been reported to be involved in food spoilage, i.e., some specific spoilage organisms may produce an autoinducer to regulate their collective behavior leading to food spoilage, or bacterial species may sense the autoinducer secreted by others to monitor the density change (Zhu et al., 2015). AHLs are the most common autoinducers utilized by Gram-negative bacteria to regulate various biological functions. The preservation of refrigerated turbot by vacuum packing is extensively applied in China. Hence, understanding the production of

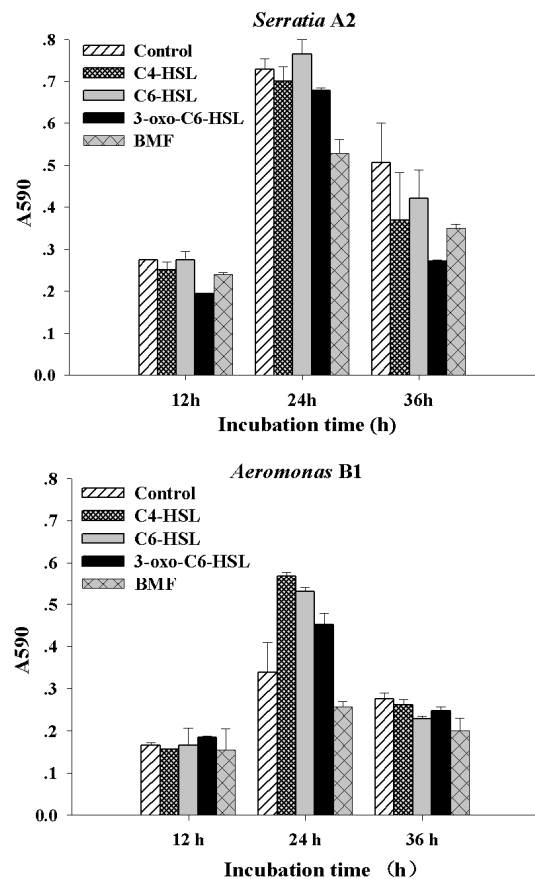


Fig. 6. The determination of exogenous AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) and QSI (BMF) on biofilm formation of *Serratia A2* and *Aeromonas B1* incubated for 36 h.

Data are expressed as the mean of three replicates.

QS signaling molecules and exploring its influence on microbiological activities is of importance.

In this study, AHLs-producing bacteria were screened using bacterial biosensors. The AHLs producers (*Serratia* sp. and *Aeromonas* sp.) were identified by 16S rDNA and Blast with the NCBI database. The genus *Serratia* is a Gram-negative, facultative anaerobic and rod-shaped member of the family *Enterobacteriaceae*. Bacterial strains belong to *Aeromonas* sp. are Gram-negative, straight cells. These bacterial species are food-borne health-hazard opportunistic pathogens leading to pneumonia, intravenous catheter-associated infections, and osteomyelitis (Chan et al., 2013). Both of the bacterial strains widely exist in vacuum-packed refrigerated or iced raw food materials and have been reported to be involved in the spoilage of various aquatic food products, i.e., they have been isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) and spoiled raw salmon (*Salmo salar*) fillets stored under modified atmosphere packaging (Macé et al., 2012, 2013).

Serratia sp. and *Aeromonas* sp. have been reported to be capable of producing AHLs based on the *luxI* homogene system. Both of these bacterial species mainly secrete short chain AHLs (i.e., C4-HSL, C6-HSL, 3-oxo-C6-HSL) and some hydroxyl-AHLs (i.e., 3-OH-C6-HSL) detected using the TLC method with combined reporter strain. Several reporter strains with different specificities have al-

ways been applied to detect AHLs. In this study, two reporter strains, i.e., A136 and CV026, were also applied to detect AHLs. A136 is a Gram-negative soil bacterium which carries a *lacZ* fusion to the *traI* and cannot produce its own AHLs, but can induce *traI:lacZ* reporter in the presence of exogenous AHLs. A136 detects a broad range of AHLs with high sensitivity to these compounds (Zhu et al., 2003). CV026 is a double mini-Tn5 mutant bacterial strain which regulates the violacein production via the CviI/R AHL QS system in response to exogenous AHLs (McClellan et al., 1997). CV026 mainly detects the AHLs with short-length side chains, i.e., C4 to C8 with varying degrees of sensitivity. However, these bacterial reporter strains cannot identify the chemical structures of the AHL molecules and the bioassay with TLC does not provide accurate structural information, and thus their structural identification by chemical methods requires a more sensitive and reliable technique. In the present study, further identification of the AHLs was confirmed by GC-MS analysis, which revealed the presence of long-chain AHLs in *Serratia* A2 and *Aeromonas* B1. This result was consistent with Cataldi et al. (2004, 2007) that some long-chain AHLs can be detected by GC-MS, but not using reporter strains. Moreover, 3-oxo-C6-HSL in *Serratia* A2 was not detected using GC-MS because of its heat instability. LC-MS is maybe a good choice for the detection of oxo-substitutions AHLs. Chan et al. (2011) has detected some long-chain AHLs in *A. sobria* isolated from patients in Malaysia using the TLC method, but did not speculate which species. Consistent with our result, they may have been C10-HSL or C12-HSL. The superiority of GC-MS compared with RP-TLC has also been exhibited in recent studies (Cataldi et al., 2007; Chan et al., 2011, 2013). In summary, short-chain AHLs were the predominate signaling in *Serratia* sp. and *Aeromonas* sp., the concentration of long-chain AHLs was low and their detection requires a more sensitive method.

The spoilage of aquatic products mainly occurs due to the degradation of protein, and both of the isolated bacterial species exhibited protease activity. Our results have revealed that the production of AHLs in *Serratia* A2 and *Aeromonas* B1 were density-dependent. The protease activity in these two bacterial strains was induced from the mid-logarithmic phase reaching a maximum at the stationary phase. Exoenzyme production has been reported to be regulated by a QS system in several Gram-negative bacteria. Christensen et al. (2003) have described that the *lipB* gene required for the production of proteolytic and lipolytic activities in *Serratia proteamaculans* B5 α was regulated by 3-oxo-C6-HSL. Jatt et al. (2015) have indicated that exogenous AHLs enhance the production of alkaline-phosphatase in *Pantoea ananatis* B9 isolated from marine snow; nevertheless, the addition of AHLs did not affect the exoenzyme activities of amylase, protease, and lipase. Whether the phenotype of bacteria regulated by QS was always studied by constructing AHLs synthase mutant strain, then the possible effect of QS signaling compounds on bacteria was known through the phenotype difference between the wild strain and mutant strain. However, in the food matrix, AHLs-producing communities coexist, so it is useful to understand what happens when

AHLs are in excess. The present study has shown that exogenous AHLs have inhibited the specific protease production in both bacteria. Morohoshi et al. (2007) reported that an SpnR protein belonging to the LuxR family functioned as a negative regulator in *Serratia marcescens*. Liu et al. (2011) have indicated that the production exoenzyme was positively regulated but indol-3-acetic acid was negatively regulated by QS in *Serratia plymuthica* G3 strain. Protease production regulated by C4-HSL and glucose in *Aeromonas* spp. has also been demonstrated (Kirke et al., 2004).

Biofilm, as a complex differentiated community, is produced by bacteria to enhance the resistance to outer pressure. The process of biofilm formation involves attachment, development and maturation, and is influenced by a combination of environmental factors and cell-to-cell communication mechanisms (Wang et al., 2013). The present study have shown that the addition of exogenous AHLs has a different influence on biofilm formation in both of the isolated bacteria. Exogenous AHLs negatively regulated the biofilm formation of *Serratia* A2 after 36 h incubation, while had no significant effect at 12 h and 24 h. Interestingly, the addition of three exogenous AHLs promoted the biofilm formation of *Aeromonas* B1 at 24 h and had no significant influence at 12 h and 36 h. Kjelleberg and Molin (2002) have indicated that the effect of QS on gene expression may be dependent on the growth phase. The utilization passway of AHLs may strain dependent. However, QS regulated the gene expression of *Serratia* A2 only in a poor nutrient environment. The fact that microbial cells show a different response to AHLs is interesting, and investigation of this mechanism is of importance.

The screening of AHLs-producing bacteria in the present study is mainly based on a culture-dependent method. A limitation is that cultural microorganisms represent only a small part of microbial diversity. Maybe some other microorganisms are capable of producing AHLs during the process of turbot spoilage which needs to be studied further, although our study has provided evidence that *Serratia* sp. and *Aeromonas* sp. were the dominant AHL producers during the process of turbot spoilage, irrespective of uncultured microorganisms. Three AHLs (3-oxo-C6-HSL, C8-HSL and C10-HSL) were produced by *S. liquefaciens* A2 and five different AHLs (C4-HSL, C6-HSL, C8-HSL, C10-HSL and C12-HSL) were produced by *A. sobria* B1. Aquatic product spoilage is the consequence of microbiological activities, such as protease secretion and biofilm formation, which have been reported to be regulated by a QS system. Meanwhile, the addition of exogenous AHLs decreased the specific protease production in both the AHLs-producing bacteria investigated here. Biofilm formation was inhibited in *Serratia* A2, while being promoted in *Aeromonas* B1 by exogenous AHLs. The present study provides a new food preservation strategy for prolonging the shelf-life of vacuum-packed refrigerated turbot.

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