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SHORT COMMUNICATION

Production of *N*-acyl homoserine lactones by *Chromobacterium haemolyticum* KM2 isolated from the river water in Malaysia

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Abstract

Quorum sensing (QS) is a term used to describe cell-to-cell communication that enables bacteria to orchestrate group behaviours according to density of bacterial cells. In Gram-negative bacteria, this signalling system is widely known to regulate a variety of different phenotypes such as antibiotic production and biofilm formation. In this study, we report the production of *N*-acyl homoserine lactones produced by *Chromobacterium haemolyticum* strain KM2, a bacterium isolated from a river water of a reserved tropical national park. Preliminary screening of QS activity using biosensor reporter assays indicated that *C. haemolyticum* strain KM2 produces both short- and long-chain AHLs. Analysis with high-resolution liquid chromatography–mass spectrometry (LC–MS/MS) analysis revealed the production of three AHLs by strain KM2: *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-dodecanoyl-L-homoserine lactone (C12-HSL), and *N*-3-oxo-dodecanoyl-L-homoserine lactone (OC12-HSL). This bacterial isolate also exhibited strong β-haemolytic activity. To the best of our knowledge, this is the first documentation of QS activity and multiple AHLs production by *C. haemolyticum* strain KM2.

Keywords *Chromobacterium haemolyticum* · Quorum sensing · Biosensor · Triple quadrupole liquid chromatography–mass spectrometry · *N*-acyl homoserine lactone

Introduction

The term quorum sensing (QS), which was first introduced by Fuqua et al. (1994), describes a phenomenon in which the bacterial cells in a population or niche release small diffusible signalling molecules known as "autoinducers". This is to orchestrate a cell density-dependent behavioural changes or gene expressions. Most Gram-negative bacteria use primarily *N*-acyl homoserine lactone (AHLs) as intercellular signalling molecules. In an AHL-regulated QS system, LuxI is the AHL autoinducer synthase whereas LuxR acts as the AHL signal receptor and activates a downstream signalling cascade (Williams et al. 2007).

The structure of AHLs consist of a homoserine lactone ring that is highly conserved in various bacterial species but differ in the carbon chain length and substitutions in the acyl-side chain. The substitution is either by the presence or absence of a hydroxyl-, or oxo-, at the carbon 3 position (Williams et al. 2007). AHL-based QS has been the subject of extensive investigation in the last decade and has become the paradigm in bacterial cell-to-cell communication. There is mounting data that showed the important roles of AHLs in many bacterial physiological behaviours including expression of virulence factors such as swarming motility, twitching, biofilm formation, antibiotic resistance, and pyocyanin production (Brint and Ohman 1995; Daniels et al. 2004; Kociolek 2009; Shih and Huang 2002).

To date, the genus *Chromobacterium* consists of seven recognised species: *C. violaceum, C. haemolyticum* (Han et al. 2008), *C. subtsugae* (Martin et al. 2007), *C. aquaticum* (Young et al. 2008), *C. piscinae* and *C. pseudoviolaceum* (Kämpfer et al. 2009) *and C. vaccinia* (Okada et al. 2013). Of these species, only *C. violaceum* is extensively studied in terms of its QS machinery (Stauff and Bassler 2011). The

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genus *Chromobacterium* is often isolated from water and soil of tropical and sub-tropical regions (Lima-Bittencourt et al. 2007). Both *C. violaceum* and *C. haemolyticum* are phylogenetically related, making them impossible to be distinguished. Nevertheless, the lack of violacein pigment production, strong haemolytic activity and positive catalase test of *C. haemolyticum* has enabled it to be accurately identified (Okada et al. 2013).

Chromobacterium haemolyticum is a Gram-negative beta-proteobacterium and non-pigmented haemolytic bacterium. Earlier studies have indicated the pathogenicity of this species causing severe bacteremia and leading to necrotizing fasciitis in a young male adult after an injury and exposure to river water in Japan. In addition, *C. haemolyticum* isolated from a male adult was also found to be extremely resistant to beta-lactam antibiotics (Han et al. 2008; Okada et al. 2013). However, to date, no QS activities on *C. haemolyticum* has been elucidated and the regulation of QS system on pathogenicity of the bacterium remains elusive. In this study, we aim to highlight on the QS activities of *C. haemolyticum* isolated from a reserved national park in Malaysia. This is the first documentation on the isolation of AHLs from *C. haemolyticum*. With the identified AHL profile, this facilitates the characterisation of *luxI* homologue in strain KM2 and a further understanding of the QS mechanism in this bacterial isolate.

Materials and methods

Sample collection

Water sample collection was performed at Kuala Marong (02°30.832′N, 103°21.270 E) of the Endau Rompin National Park, Malaysia on 16 April 2013. The water samples were collected using Van Dorn water sampler (Wildco, Yulee, FL) from a depth of 1 m. Then, the samples were transferred into sterile bottles and stored on ice during transportation to the laboratory.

Isolation and culturing of bacterial strains

The water samples were immediately processed upon arrival to the laboratory. One hundred microlitre of the water samples were pipetted in duplicate onto the surface of Luria–Bertani (LB) agar (Scharlau, Barcelona, Spain). All plates were incubated at 28 °C for up to 72 h. Bacteria with observable differences in morphology were isolated for several passages to form pure culture. Stock cultures were preserved in LB broth and 20% glycerol at − 80 °C. The biosensor reporter strains, *Chromobacterium violaceum* CVO26, *Erwinia carotovora* Attn, and *Erwinia carotovora* A20, were routinely cultured in LB media with shaking (220 rpm) at 28 °C. On the other hand, *E. coli lux*-based biosensor strains (pSB401 and pSB1075) were cultured at 37 °C in LB broth supplemented with 20 µg/mL tetracycline (Winson et al. 1998). All the strains were cultured in the conditions mentioned unless otherwise stated.

Screening for AHL production

The screening for AHL-producing bacterial isolates was performed by cross-streaking the isolates with the biosensor strain, *C. violaceum* CVO26 (How et al. 2015; Ngeow et al. 2013). The production of purple violacein pigmentation by CVO26 occurs in the presence of short-chain AHLs ranging from 4 to 8 carbons of the acyl group (McLean et al. 1997). *E. carotovora* Attn, and *E. carotovora* A20 served as the positive and negative controls, respectively.

AHL extraction and detection of short- and long-chain AHLs in spent cultures

Bacterial isolates that showed positive QS properties from initial screening were cultured for 18 h in 100 mL of LB broth buffered with 50 mM of 3-(*N*-morpholino) propanesulfonic acid (MOPS) to pH 6.5 at 28 °C and with agitation at 220 rpm. The spent culture supernatants were then extracted thrice with equal volume of acidified ethyl acetate (0.1% v/v glacial acetic acid). The extracted AHLs were evaporated to dryness in fume hood and stored at − 20 °C for further analyses.

Biosensor strains *E. coli*, pSB401 and pSB1075 were used to detect short- and long-chain AHLs, respectively. The bioluminescence assay were performed as previously described with minor modifications (Han-Jen et al. 2013; Lau et al. 2013). Overnight cultures of the biosensors were diluted to OD_{600} of 0.1 and were used to resuspend the extracted AHL. A volume of 200 µL of this bacterial suspension was added into each well of the 96-well plate. The luminescence and bacterial growth (OD_{495}) were measured every 30 min for 24 h using Tecan Infinite M200 Luminometer (Infinite M200, Mannerdorf, Switzerland). The bioluminescence produced over a period of 24 h was expressed as relative light units (RLU) per OD_{495}.

Identification of bacterial strain by 16 s rDNA amplification

The genomic DNA of strain KM2 was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA). The quality of the extracted DNA was assessed with Nanodrop Spectrophotometer (Thermo Scientific, USA) while DNA quantification was performed with a Qubit® 2.0 Fluorometer (dsDNA High-Sensitivity Assay Kit, Invitrogen, USA). The bacterial 16S

rRNA genes were amplified as previously described using universal primers, 27F forward primer and 1525R reverse primer (Chong et al. 2012). The polymerase chain reaction was performed with the following parameters: initialization step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1.5 min, and final extension at 72 °C for 7 min. Sequence verification of the amplicon was performed by automated Sanger DNA sequencing. The 16 s rDNA sequence of strain KM2 was compared to sequences in GenBank database using BLASTn programme. Closely related hits were aligned and a phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) version 6.06 (Tamura et al. 2013).

Haemolysis on blood agar

To determine the haemolytic ability of strain KM2, the bacterium was cultured on Columbia agar with 5% v/v sheep blood (ISOLAC®, Isolab, MY) (Chang et al. 2012). The colonies were observed for the presence beta-haemolysis (complete haemolysis) by examining the blood agar plates on transmitted light to look for halo zone.

AHLs profiling using triple quadrupole liquid chromatography–mass spectrometry (LC–MS) system

The extracted AHLs of strain KM2 were reconstituted with 1 mL of acetonitrile, mixed well, and centrifuged. A volume of 100 µL of reconstituted AHLs was pipetted from the top layer and dispensed into sample vials for triple quadrupole liquid chromatography–mass spectrometry (LC–MS/MS) analysis. The instrumental settings and parameters of the LC system and mass spectrometry were followed as described by How et al. (2015). However, five different collision-induced dissociation (CID) or collision energies were used (5, 9, 15, and 20 eV) to enable complete fragmentation of product ions. The mass spectra with the best fragmentation of ions were chosen for analysis. Synthetic AHL standards at concentration of 10 ppm (Cayman Chemicals, Ann Arbor, MI, USA) were used for comparison in the LC analyses.

Results and discussion

Molecular identification of strain KM2

The 16S rDNA phylogenetic analysis of strain KM2 showed a 99% similarity to *C. haemolyticum* strain MDA0585. Strain MDA0585 was previously isolated from a sputum culture (Han et al. 2008). The closest hits with similarity more than 95% were downloaded from GenBank database and were clustered together in a phylogenetic tree with bootstrap test of 1000 replicates (Felsenstein 1985). All base positions containing gaps and missing data were eliminated prior to phylogenetic tree construction. The evolutionary analysis was inferred using neighbour-joining algorithm (Saitou and Nei 1987; Tamura et al. 2004). The 16S rDNA sequence for strain KM2 has been deposited into GenBank database with the accession number KJ845679.

Detection of AHLs production by strain KM2 using biosensors

The screening of short-chain AHLs produced by strain KM2 was performed using *C. violaceum* CVO26. This biosensor is a double Tn*5* mutant that responds by producing the purple pigment violacein in the presence of exogenous short-chain AHLs (McLean et al. 1997). This pigmentation was observed when strain KM2 was cross-streaked against CVO26, indicating that the former is a short-chain AHL producer (Fig. 1). The production of AHLs by strain KM2 was further verified with bioluminescence biosensors as CVO26 is limited to the detection of short-chain AHLs. An increase in bioluminescence activity in both *E. coli* pSB401 and *E. coli* pSB1075 biosensor assays indicated that strain KM2 produced both short- and long-chain AHLs (Fig. 2). Following this, further analyses using LC–MS/MS were performed to obtain the AHL profile of strain KM2 (Figs. 3, 4).

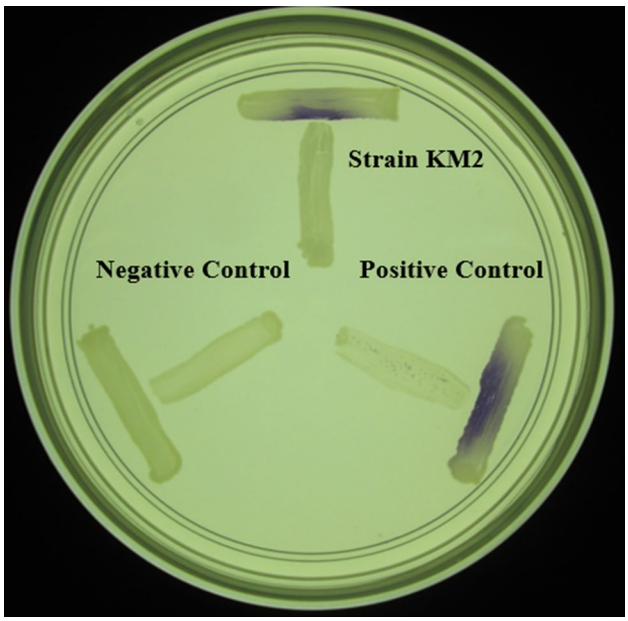

Fig. 1 Preliminary screening for detection of short-chain AHLs using CVO26 biosensor. *E. carotovora* Attn and *E. carotovora* A20 served as positive and negative controls, respectively. Strain KM2 triggered purple violacein production in CVO26 after 18 h of incubation

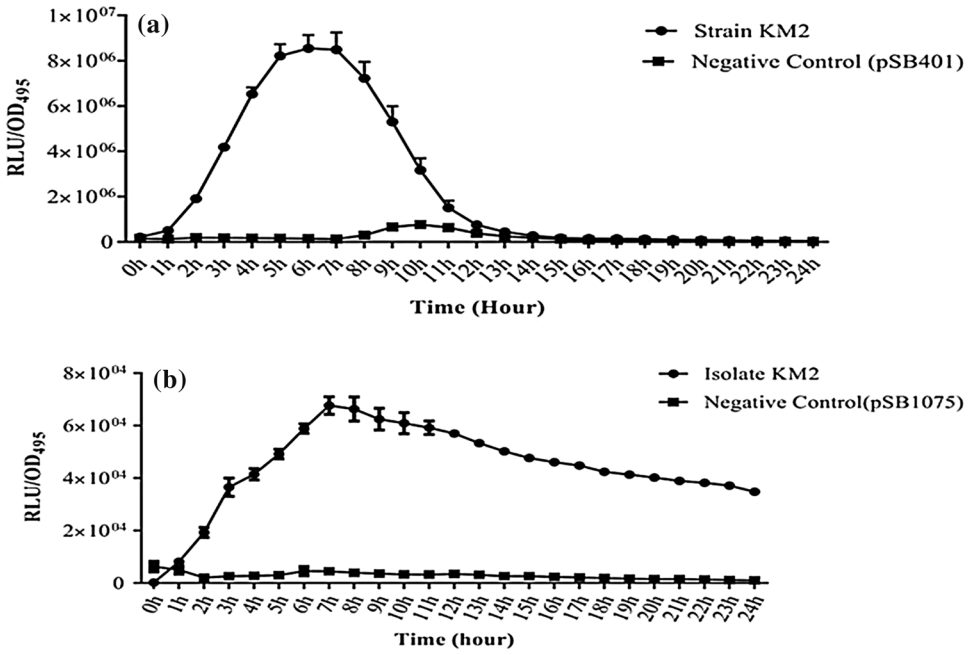

Fig. 2 Spent culture supernatant of strain KM2 induced bioluminescence activity of both short and long AHL biosensors. **a** *E. coli* (pSB401) and **b** *E.coli* (pSB107) after 24 h of incubation. Both biosensors that were grown without the presence of AHLs served as negative controls

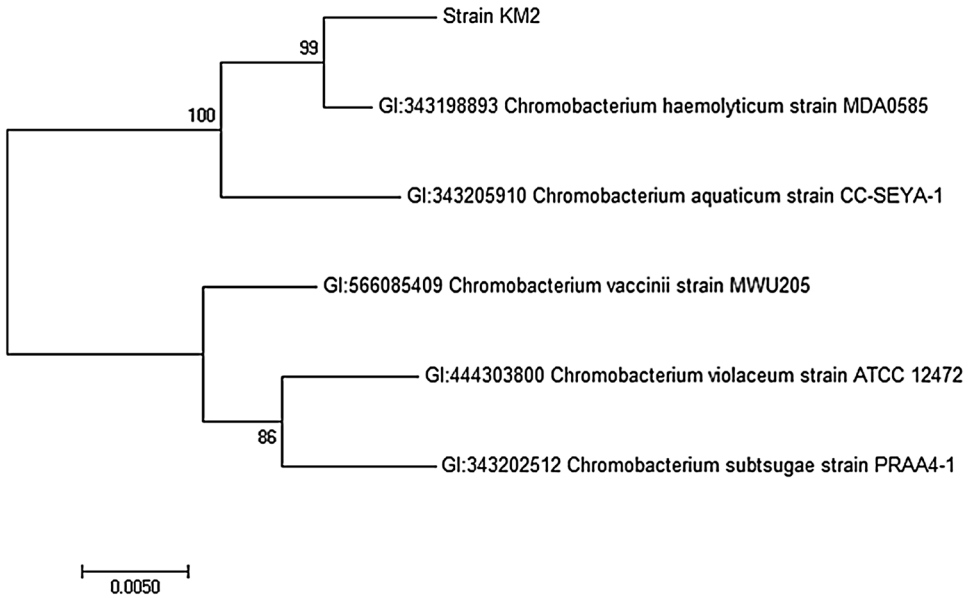

Fig. 3 Phylogenetic analysis, generated using neighbour-joining algorithm, shows that strain KM2 is very closely related to *C. haemolyticum* strain MDA0585. Bootstrap values based on 1000 replications are listed at the branching points as percentages. The horizontal bar at the bottom represents evolutionary distance as 0.005 change per nucleotide position GenBank accession numbers are stated next to the bacterial species

AHL profile of strain KM2

LC–MS/MS analyses were performed to verify the identity of the AHLs produced by strain KM2. The mass spectra of the spent culture supernatant of strain KM2 were compared to the synthetic AHL standards at their respective retention time. The analyses revealed that strain KM2 produced three types of AHLs, namely *N*-octanoyl-L-homoserine lactone (C8-HSL, Fig. 5), *N*-dodecanoyl-L-homoserine lactone (C12-HSL, Fig. 6), and *N*-3-oxo-dodecanoyl-L-homoserine lactone (OC12-HSL, Fig. 7). In all the mass spectra shown, the molecular mass of *m*/*z* 102 refers to the presence of lactone moiety of AHL. The *m*/*z* ratio of the synthetic AHLs reported by Ortori et al. (2011) were used as a reference to identify the peaks produced by the AHLs standards and spent culture supernatant of strain KM2. The *m*/*z* values obtained from the culture supernatant were very close to the theoretical value of the synthetic standards.

To our acknowledgement, this is the first documentation of QS activity in *C. haemolyticum*. Other members of the *Chromobacterium* genus, *C. aquaticum* and *C. subtsugae*, have been found to produce *N*-octanoyl homoserine lactone (C8-HSL) and *N*-hexanoyl homoserine lactone (C6-HSL), respectively (Rekha et al. 2011). They are short-chain

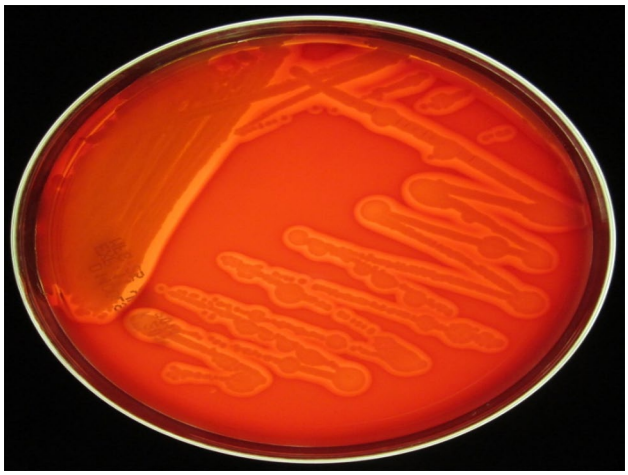

Fig. 4 β-Haemolytic activity of strain KM2 after 24-h incubation. Approximately, 3-mm clear zones formed around the growth areas of the bacteria

AHL-producers. In this study, strain KM2 is both short- and long-chain AHL producer. Such unique finding may indicate a possible different language code used for intra- and inter-cellular interaction among bacteria.

Haemolytic activity of strain KM2

Strong haemolytic activity of strain KM2 was observed following 24-h incubation at 28 °C in Columbia Agar supplemented with 5% (v/v) sheep blood. Clear zones on the surface of the blood agar indicated the presence of β-haemolysin activity of the bacterium (Ariyanti et al. 2011; Han et al. 2008). Haemolysin is a cytolytic toxin well documented as the potential pathogenicity of *Chromobacterium*. Previous study also reported that *C. haemolyticum* strain MDA0585^T showed strong haemolytic activity as well as causing severe bacteremia in a healthy young patient following trauma and exposure to river water (Okada et al. 2013). Other species of the same genus such as *C. subtsugae* and *C. aquaticum* have also been reported to exhibit β-haemolytic activity (Martin et al. 2007; Young et al. 2008). However, these bacteria were never reported to have caused severe bacteremia or necrotizing fasciitis. *C. haemolyticum* thus have a stronger haemolytic activity as well as pathogenicity to cause such a severe infection. From the phylogenetic analysis, strain KM2 was demonstrated to have the closest relationship with *C. haemolyticum* strain MDA0585^T. The latter strain also possesses a remarkable haemolytic activity (Han et al. 2008). However, it has been reported that haemolytic activity of genus *Chromobacterium* is not regulated or controlled by AHL-mediated QS system (Sivendra and Tan 1977). Thus, we believe the AHL production by *C. haemolyticum* strain KM2 may serve as a regulator for different pathogenic traits or even genes crucial for its survival in the environment. However, more studies needs to be done to elucidate the functions of the QS system in this species.

On the other hand, AHLs are found to be fundamental regulatory agents in haemolytic activities of certain bacteria (De Kievit and Iglewski 2000). *Pseudomonas aeruginosa* PAO1, for instance, employs Rhl QS system to regulate the

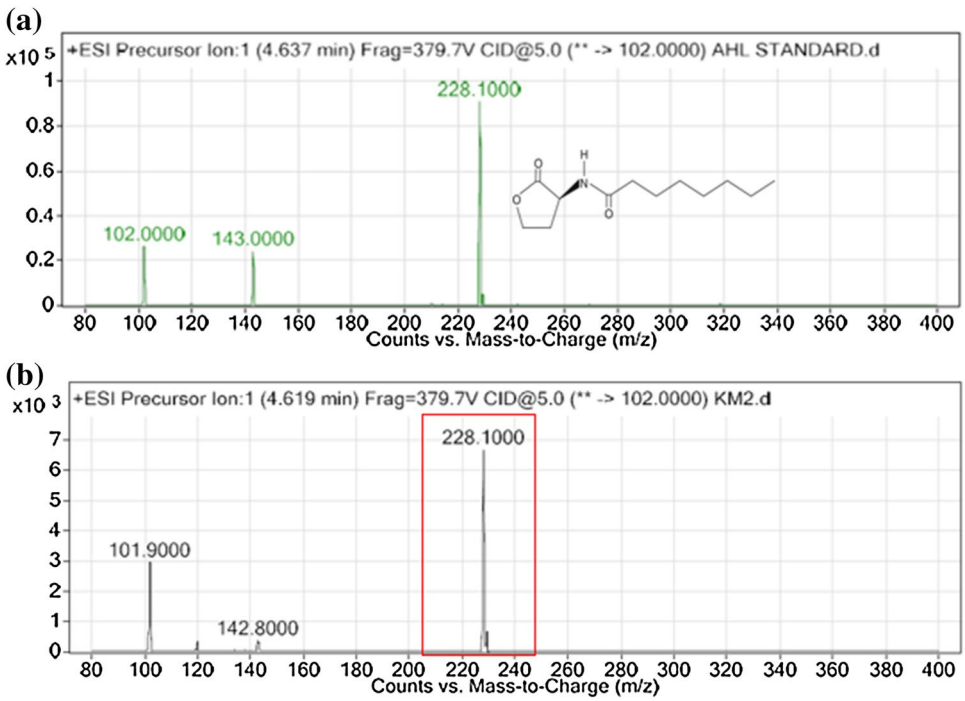

Fig. 5 The mass spectra showing the presence of C8-HSL produced by strain KM2. Mass spectra of (**a**) synthetic C8-HSL standard with *m/z* value of 228.1 with ion abundance of 91096.93 (100% base peak abundance), and **b** C8-HSL produced by strain KM2 with *m/z* value of 228.1 with ion abundance of 6696.74 (100% abundance). The retention times for the peaks of C8-HSL standard and C8-HSL produced by strain KM2 were similar, at 4.6 min. The chemical structure of C8-HSL is shown in **a**

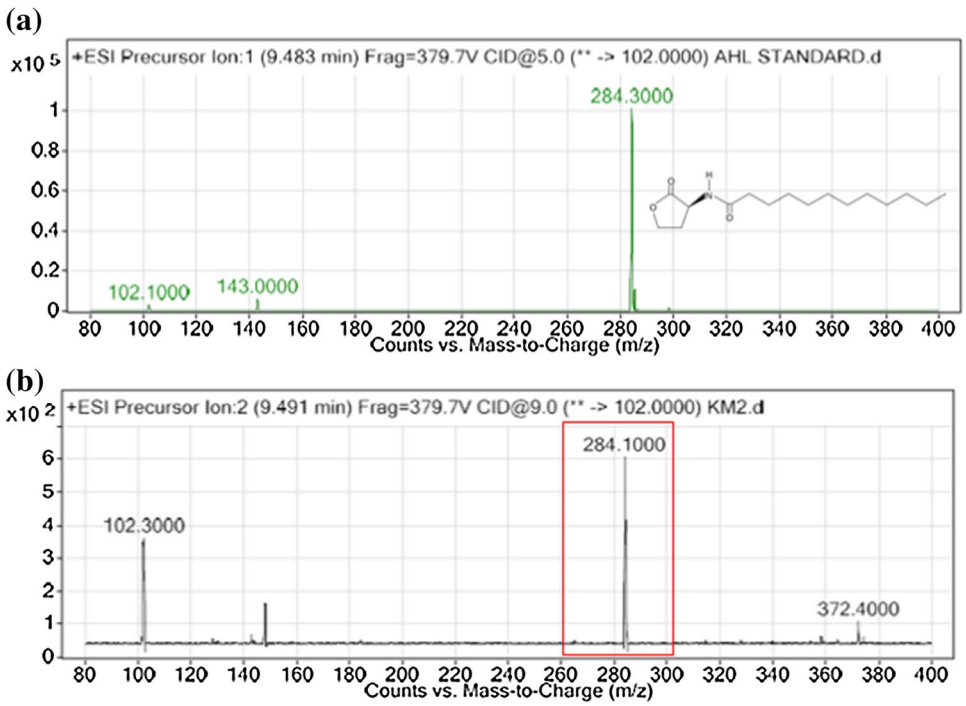

Fig. 6 The mass spectra showing the presence of C12-HSL produced by strain KM2. Mass spectrometry analysis of (**a**) synthetic C12-HSL standard with *m*/*z* value of 284.3 and abundance of 101385.52 (100% base peak abundance) and **b** C12-HSL produced by strain KM2 (*m*/*z* value of 284.1) with an abundance of 657.08 (100% abundance). C12-HSL produced by strain KM2 showed the same retention time (9.4 min) as the standard. The chemical structure of C12-HSL is shown in **a**

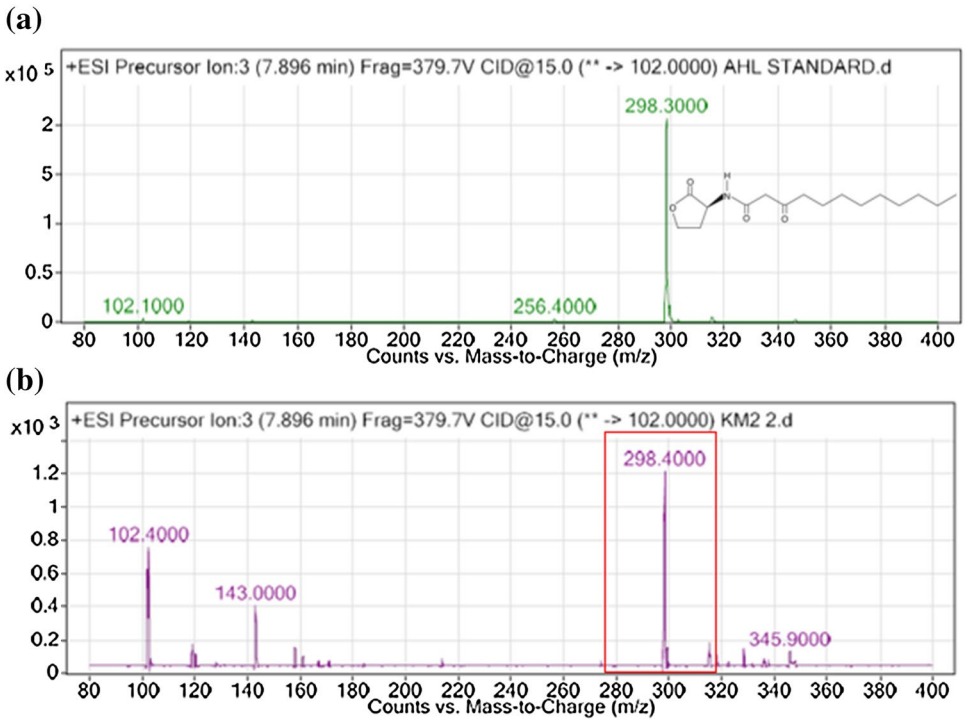

Fig. 7 The mass spectra showing the presence of OC12-HSL produced by strain KM2. Mass spectra of **a** synthetic OC12-HSL standard with m/z value of 298.3 and ion abundance of 207021.78 (100% base peak abundance), and **b** OC12-HSL produced by strain KM2 with m/z value of 298.4 and ion abundance of 1333.92 (100% abundance). The retention time for both OC12-HSL peaks were 7.896 minutes. The chemical structure of OC12-HSL is shown in (**a**)

production of haemolysin (Brint and Ohman 1995). Likewise, *Enterobacter faecalis* produces cytolysin, a major virulence factor that possesses haemolytic activity that is lethal to many eukaryotic cells. This cytolysin serves as autoinducer for QS induction of the cytolysin operon (Reading and Sperandio 2005). However, there are some strains such as *Pseudomonas* sp. W3 that could produce haemolysin in the absence of QS circuit (Chang et al. 2012). Interestingly, Saroj et al. (2017) reported that haemolytic activity and viability of *Streptococcus pyogenes* M6 S165 was inhibited by external long-chain AHLs, namely oxo-C12-HSL or oxo-C14-HSL. This shows that AHLs may play different

regulatory roles in haemolytic activity of different bacteria. In our study, further validation is needed to investigate the roles of the AHLs in haemolytic activity of strain KM2.

Conclusions

In this study, we report for the first time, the acyl-homoserine lactone profile of *C. haemolyticum*. Three AHLs, C8-HSL, C12-HSL, and OC12-HSL, were detected in the spent supernatant cultures of *C. haemolyticum* strain KM2 using LC–MS/MS. Efforts are now being focused on the whole genome sequencing of strain KM2 to study the possible association of QS-genes and pathogenic factors.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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