

Enzymatic hydrolysis of molecules associated with bacterial quorum sensing using an acyl homoserine lactonase from a novel *Bacillus thuringiensis* strain

Carmen Julia Pedroza · Alvaro Mauricio Flórez · Orlando S. Ruiz · Sergio Orduz

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Abstract *N*-acyl homoserine lactones are key components of quorum sensing, the bacterial communication system. This communication mechanism regulates the expression of genes, including those involved in virulence and biofilm formation. This system can be interrupted by the action of enzymes that hydrolyze the signaling molecules. In this work, we studied the enzymatic properties of a recombinant AHL-lactonase from *Bacillus thuringiensis* strain 147-11516, using substrates with acyl chains of different length (C4-HSL, C6-HSL, C7-HSL, C8-HSL and C10-HSL), we also investigated the effect of pH (5.0–9.0), temperature (20–70 °C), concentration of monovalent, divalent and trivalent metals ions (0.2 and 2.0 mM) and EDTA. The results showed that the recombinant AHL-lactonase had biological activity in alkaline pH conditions (8.0) and high temperature (47 % of hydrolyzed substrate at 60 °C). The recombinant AHL-lactonase has activity on substrates with different acyl chain length. However, the activity of the recombinant enzyme was decreased in the two

concentrations of all metal ions evaluated but was not inhibited by EDTA. The affinity of the enzyme for all substrates tested and its performance, in the evaluated conditions, suggest that the AHL-lactonase from *B. thuringiensis* strain 147-11516 could be used as a strategy for disruption of the Gram-negative bacteria communication system under normal and challenging conditions.

Keywords Quorum sensing · Quorum quenching · AHL-lactonase · *N*-acyl homoserine lactone

Introduction

Gram-negative bacteria use quorum sensing system (QS) to communicate with each other to regulate gene expression. This system that was discovered in the marine bacterium *Vibrio fischeri*, allows the regulation of the expression of bioluminescence (Nealson et al. 1970) and involves the production, releasing, detection and response of small signaling molecules whereas is increasing population density. The bacteria may respond individually and collectively to environmental changes by inducing gene expression involved in conjugation, virulence factors production and biofilm formation, development of genetic competence and differentiation of spores as well as antibiotics production etc. (Lazazzera and Grossman 1998; Lazazzera 2000; Miller and Bassler 2001; Lin et al.

C. J. Pedroza · O. S. Ruiz · S. Orduz
Facultad de Ciencias, Universidad Nacional de Colombia,
Medellín, Colombia

A. M. Flórez (✉)
Laboratorio de Biología Molecular y Biotecnología,
Escuela de Medicina de la Facultad de Ciencias de la
Salud, Universidad de Santander, UDES, Bloque
Arahuaco, 201, Bucaramanga, Colombia
e-mail: amflores@udes.edu.co

2003; Qin et al. 2007). There is a wide range of QS signals, but only two are the most widely studied: *N*-acyl-homoserine lactones (AHLs) produced by more than 70 species of Gram negative bacteria and peptide based QS system in Gram positive bacteria. In the Gram negatives, the AHLs signals diffuse across the cell membrane and bind to regulatory proteins and in the Gram positives such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, the QS signal is detected by a two component response regulator system associate to cell membrane by bound to histidine kinases receptor (Amara et al. 2011; Waters and Bassler 2005).

The QS system allows bacteria to compete and dominate the bacterial community in which they develop strategies to increase opportunities for co-exist. In this scenario, the competition operates through quenching of QS system mediated by wide range of compounds (Kalia 2013). Among the different inhibitors, the enzymatic degradation by AHL-lactonases and AHL-acylases has been studied in order to find applications in a diverse range of fields (Galloway et al. 2012). Lactonases encoded by the gene *aiiA* hydrolyze the lactone ring of AHL of Gram-negative pathogens, and are found in numerous *Bacillus* sp. (Dong et al. 2001, 2002; Lee et al. 2002).

With the discovery of antibiotics and vaccine development in the past 70 years many diseases were controlled, but as in most cases its use impose a selection pressure on microorganisms, inducing resistance development to one or more antibiotics. It had been suggested that quorum quenching (QQ) of bacterial cell–cell communication, could be a promising strategy that can be exploited to prevent food spoilage, bacterial infections and bioremediation (Kalia et al. 2011). In medicine, disrupting QS rather than using antibiotics is a highly attractive concept since it is less likely to induce bacterial resistance (Camara et al. 2002; Hentzar and Givskov 2003).

Previous work by Dong et al. (2000, 2004) showed that the *aiiA* gene product of *Bacillus thuringiensis* (*Bt*) cause inactivation of the AHL autoinducer, inhibiting the virulence of the phytopathogen *Pectobacterium carotovorum* (*Erwinia carotovora*). This mechanism of QQ has also been recently discovered in *Bacillus marcorestinum* sp. nov. (Han et al. 2010). Latest studies revealed that many organisms possess conserved domains for AHLs lactonases and acylases including those such as *Deinococcus radiodurans* R1,

Hyphonomas neptunium ATCC 1544 and *Photorhabdus luminicens* subsp. *laumondii* TT01 that contains genes for both lactonase and acylase (Kalia et al. 2011). The presence of another AHL-lactonase in various species of the genus *Microbacterium* (Wang et al. 2012) and efficient control and attenuation of *Aeromonas hydrophila* virulence have been also reported when the enzyme was used as diet supplement in Zebra fish (Cao et al. 2012). Furthermore, bioassay results of AHL-lactonase activity produced by a Colombian *Bt* strain 147-11516 isolated from bean leaves against plant pathogens and that is also toxic to lepidopteran insects, indicate the importance of the complete biochemical characterization of this enzyme. In this study the activity of the recombinant AHL-lactonase encoded by the *aiiA* gene of *Bt* strain 147-11516 in various conditions of pH, temperature, presence of monovalent, divalent and trivalent ions, in addition to the determination of the specificity and kinetics of the enzyme is described.

Materials and methods

Bacterial strains and culture media

Reporter strains *Agrobacterium tumefaciens* NTL4 and *Chromobacterium violaceum* CV026 were obtained from Dr. J. Fuqua at Indiana University, USA and the Institute of Infection Immunity and Inflammation, University of Nottingham, England respectively. The first strain carries a *lacZ* fusion with *traI*, which produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) in response to AHLs. The *C. violaceum* strain CV026 regulates production or inhibition of a pigment in the presence of AHLs (Ravn et al. 2001). These strains were grown in Luria Bertani (LB) (1 % peptone, 0.5 % yeast extract, 1 % NaCl) with 1.0 % agar, supplemented with appropriate antibiotics tetracycline 4.5 μ g/ml and spectinomycin 50 μ g/ml for *A. tumefaciens* and kanamycin 20 μ g/ml for *C. violaceum*.

To study the activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆, the following substrates were used, *N*-decanoyl, octanoyl, heptanoyl, butanoyl and hexanoyl-DL-homoserine lactones (Fluka). To evaluate the effect of ions on the AHL-lactonase activity eight salt solutions were used MgCl₂, CaCl₂, MnCl₂, Pb(NO₃)₂, CuCl₂, AlCl₃, FeCl₂ and KCl (Merck).

Escherichia coli ArticExpress (DE3) cells were transformed with plasmid *aiiA*-pCold IV containing the *aiiA* gene from *Bt* strain 147-11516 and six histidine residues at the N-terminus were added to target gene and were preserved after purification. The gene was sub-cloned into the vector pCold IV (Takara- BIO-INC, Shiga, Japan) using *NdeI* and *XbaI* and the correct sequence of the *aiiA* gene inserted in plasmid pCold IV was verified by DNA sequencing on both strands.

Expression and purification of the AHL-lactonase₁₄₇₋₁₁₅₁₆

Recombinant *E. coli* cells were cultured for 4 h in 1 L of LB medium containing 50 µg/ml of ampicillin, subsequently gene expression was induced by adding 1 mM IPTG and incubated again at 15 °C overnight. Cells were harvested by centrifugation at 5,000 rpm for 10 min and the pellet was resuspended in 30 ml of lysis buffer (20 mM Tris-HCl containing 500 mM NaCl, 20 mM imidazole, 1 mM PMSF and bacterial protease inhibitors, pH 8.0), then bacteria were lysed by sonication in an ice bath. Bacteria lysate was centrifuged at 11,000 rpm for 20 min and the supernatant was collected. Proteins were separated by affinity chromatography using a Ni-NTA column equilibrated with buffer A (20 mM Tris-HCl, 500 mM NaCl and 20 mM imidazole pH 8.0). Proteins were eluted with buffer B (20 mM Tris HCl, 500 mM NaCl and 250 mM imidazole at pH 8.0). The fractions collected were analyzed by 12 % SDS-PAGE and proteins stained with Coomassie brilliant blue. Several fractions containing the AiiA protein were mixed and dialyzed against 1× PBS. Finally the protein AIIA AiiA was lyophilized and stored at -70 °C for later use.

Detection of AHL-lactonase₁₄₇₋₁₁₅₁₆ activity with reporter strains

The biological activity of the purified AHL recombinant lactonase₁₄₇₋₁₁₅₁₆, was detected using 35.6 mM of enzyme mixed with 20 µM of *N*-hexanoyl-DL-homoserine lactone (C6-HSL) or *N*-octanoyl-DL-homoserine lactone (C8-HSL) dissolved in 1× PBS (pH 7.4), after incubation periods of 30, 60 and 120 min at 30 °C with gentle agitation, the hydrolysis products were analyzed with the reporter strains CV026 and *A. tumefaciens* NTL4.

The effect of temperature on AHL-lactonase₁₄₇₋₁₁₅₁₆ activity, was established using 20 µM of C6-HSL in a solution of 100 mM Tris HCl (pH 7.4) and incubated at different temperatures (0, 15, 26, 30, 40, 50, 60 and 70 °C) for 30 min; after this time, the AHL-lactonase₁₄₇₋₁₁₅₁₆ was added to a final concentration of 100 µM. After 120 min, the reactions were stopped with heat (100 °C for 90 s) and the hydrolysis products were analyzed using the reporter strain *C. violaceum* CV026.

Thermal stability of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ was determined taken 1.97 µl of the enzyme to a final concentration of 100 µM in 100 mM Tris HCl (pH 7.4) and incubated at different temperatures (30, 40, 50, 60 and 70 °C). After various times, 2, 3, 4 and 5 h, 28 µl of the substrate (C6-HSL) were added to a final concentration of 20 µM and the reactions were incubated at the same temperature for additional 120 min with gentle shaking. To determine the effect of pH on the AHL-lactonase₁₄₇₋₁₁₅₁₆ activity, 0.93 µl of the enzyme were mixed to a final concentration of 110 µM with C6-HSL to a final concentration of 20 µM in a 100 mM Tris HCl solution at different pH values (4, 5, 6, 7, 8 and 9). The reaction mixtures were incubated at 32 °C for 60 and 120 min with gentle agitation and the hydrolysis products were analyzed with the reporter strain *C. violaceum* CV026.

To determine the effect of ions on the AHL-lactonase₁₄₇₋₁₁₅₁₆ activity, Mg²⁺, Ca²⁺, Mn²⁺, Pb²⁺, Cu²⁺, Al³⁺, Fe²⁺ and K⁺ were evaluated in two concentrations, 0.2 and 2.0 mM. The reactions were incubated during 60 and 120 min at 32 °C. AHL-lactonase₁₄₇₋₁₁₅₁₆ activity was evaluated at enzyme and C6-HSL concentrations of 120 and 20 µM, respectively, in 1× PBS, pH 7.4. Ion solutions containing Mn²⁺, Pb²⁺, Cu²⁺, Al³⁺ and Fe²⁺, were mixed with EDTA at a final concentration of 10 mM to avoid precipitation. They were analyzed using the reporter strain *C. violaceum* CV026.

The effect of metal ions on the enzyme activity was determined using EDTA at concentrations of 10, 25 and 50 mM, under the same conditions described above, but without the ion solutions. The hydrolysis products were analyzed using the reporter strain *C. violaceum* CV026. To evaluate the effect of EDTA in the reporter strain, in this assays two negative controls were set, one containing buffer, EDTA and substrate while the other control had only buffer and substrate. The evaluation of the AHL-lactonase₁₄₇₋₁₁₅₁₆ with the reporter strains was

performed by triplicate including the respective controls.

Evaluation of the AHL hydrolysis by the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆

A reaction mixture was prepared by adding 3.37 μl (120 μM) of the purified AHL-lactonase₁₄₇₋₁₁₅₁₆ and 1.99 μl (0.25 mM) of C6-HSL to a 100 mM Tris HCl buffer (pH 7.4) with a final volume of 1 ml. The reaction was incubated at 32 °C for 10 min at 250 rpm and stopped by heating the solution at 100 °C for 90 s. After the enzymatic hydrolysis, the resulting solution was extracted three times with ethyl acetate (Carlo Erba) in a 1:1 ratio; each organic phase was separated by centrifugation (11,000 rpm for 2 min), then the mixture of the extracts were dried in an eppendorf vacufuge concentrator at 30 °C. Dried residues were dissolved in methanol–water (50:50, v/v) and hydrolysis products were analyzed and quantified by HPLC–MS. AHL-lactonase activity was defined as the amount of micromoles of AHLs hydrolyzed per minute per milligram of AHL-lactonase.

Quantitative determination of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ activity by HPLC–ESI–MS

The activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ was quantified by liquid chromatography using an Agilent 1200

series HPLC. Two microliters of the reaction mixtures in methanol–water were injected on a C18 reverse phase column (Zorbax XDB, 4.6 \times 150 mm, particle size 5 microns). Elution was performed with methanol–water (50:50, v/v) with ammonium acetate and trifluoroacetic acid (TFA) (5 mM and 0.05 %, respectively) with an isocratic profile at a 0.8 ml/min flow using a micro-divider valve (Upchurch Scientific, USA). The separated components were injected for detection and quantified by quadrupole mass spectrometer (ESI–MS) (model V1, Agilent) under positive ion and nitrogen was used for nebulization (1 psi = 6,894.76 Pa). For quantification of non-hydrolyzed substrate, calibration curves were prepared for each substrate. The conditions for mass spectrometry using electrospray ionization (ESI–MS) are specified in Table 1.

Activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ under different temperature and pH condition

Thermal stability of the AHL lactonase₁₄₇₋₁₁₅₁₆ was determined by treating 120 μM solutions of the enzyme at different temperatures (20, 30, 40, 50, 60 and 70 °C) in 1 ml of 100 mM Tris HCl, pH 7.4, for 2 h, then C6-HSL was added to a final concentration of 0.25 mM and the mixture was incubated for 10 more minutes at each temperature and the reactions were stopped at 100 °C for 90 s. The non-hydrolyzed substrate was measured as described above.

Table 1 Optimization conditions of ESI–MS for quantification of *N*-acyl homoserine lactones (A). Ions selected to quantify the residual activity of the recombinant enzyme AHL-lactonase₁₄₇₋₁₁₅₁₆ on *N*-acyl homoserine lactones (B)

| Parameters in (ESI–MS) | <i>N</i> -acyl homoserine lactones | | | |
|--------------------------------|--|----------------------|---|---------------------|
| | C ₄ -HSL | C ₆ -HSL | C ₇ -HSL | C ₈ -HSL |
| A | | | | |
| Capillary voltage (V) | 2,500 | 2,700 | 2,700 | 4,000 |
| Fragmentation voltage (V) | 70 | 90 | 90 | 110 |
| Drying gas flow (l/min) | 12.0 | 12.0 | 12.0 | 12.0 |
| Nebulization pressure (p.s.i) | 60 | 60 | 60 | 60 |
| Drying gas temperature | 350 °C | 350 °C | 350 °C | 350 °C |
| Detection range (<i>m/z</i>) | 100–200 | 100–220 | 100–240 | 100–250 |
| N-HSL | Ions <i>m/z</i> | | | |
| | [C ₄ H ₇ NO ₂ + H] ⁺ | [M + H] ⁺ | [M + H – C ₄ H ₇ NO ₂] ⁺ | |
| B | | | | |
| C4-HSL | 102 | 172.2 | | |
| C6-HSL | 102 | 200.2 | | |
| C7-HSL | 102 | 214.3 | 113.3 | |
| C8-HSL | 102 | 228.3 | 127.3 | |

To determine the effect of pH on the activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ the same procedure was followed as described above, except that the enzyme substrate mixture was incubated at 32 °C in 100 mM Tris HCl buffer under different pH conditions (5, 6, 7, 8 and 9). The concentration of the non-hydrolyzed substrate was quantified by HPLC–MS.

Effect of metal ions and EDTA on the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ activity

A reaction mixture composed of 1.7 µl (120 mM) of the AHL-lactonase₁₄₇₋₁₁₅₁₆ and 1.0 µl (0.25 mM) of C6-HSL in 1× PBS (pH 7.4) with different ions (Mg²⁺, Ca²⁺, Mn²⁺, Pb²⁺, Cu²⁺, Al³⁺, Fe²⁺ and K⁺) were set to obtain two concentrations of each ion, 0.2 and 2.0 mM. Solutions containing Mn²⁺, Pb²⁺, Cu²⁺, Al³⁺ and Fe²⁺ were prepared containing 10 mM EDTA (pH 8.0) at pH 7.4. The resulting solutions, with final volumes of 500 µl, were incubated for 30 min at 32 °C. The reactions were stopped by heating the solutions, as mentioned before.

As control, EDTA 10 mM was used without ion solution in the same assay conditions and concentrations of enzyme and substrate as described above. Residual lactones from the enzymatic hydrolysis were quantified by HPLC–MS with their respective controls by triplicate.

Kinetics and specificity of the enzyme AHL-lactonase₁₄₇₋₁₁₅₁₆

To determine the AHL-lactonase₁₄₇₋₁₁₅₁₆ kinetics, the enzyme was mixed to a final concentration of 120 µM in a Tris HCl, pH 7.4, 100 mM with the substrates *N*-decanoyl, octanoyl, heptanoyl, hexanoyl and butanoyl-DL-homoserine lactone at concentrations of 0.16, 0.31, 0.625, 1.25, 2.5, 5, 10 and 20 mM in a final volume of 500 µl. The reactions were incubated at 30 °C and stopped by treating the reactions at 100 °C for 90 s, before 10 % of each substrate was consumed. For the specificity tests, the same substrates were evaluated at a final concentration of 3 mM with 120 mM of the enzyme; the reactions were incubated at 32 °C and after 10 min were stopped as mentioned before. Residual lactone concentrations after the enzymatic hydrolysis were quantified by HPLC–MS. All assays were performed by triplicate with their

controls. The negative controls were substrate, buffer without enzyme.

Statistical analysis

The resulting data from the assays to determine the effect of ions and EDTA on the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ activity were analyzed by two ways analysis of variance and Tukey's test for multiple comparisons using the statistical program SigmaStat (Systat Software Inc). Specificity assays were analyzed with the same statistical package by one way analysis of variance and Tukey's test. Data from the enzyme activity under different pH and temperature was calculated as the mean and standard deviation for all data taken by triplicate.

Results

Activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ determined with the reporter strains

The recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ encoded by the gene *aiiA* of *Bt* strain 147-11516 was expressed in *E. coli* and purified in a Ni–NTA column. SDS-PAGE analysis of the AHL-recombinant lactonase showed a purity of 85 %, with a molecular weight of approximately 28 kDa (Fig. 1). The AHL-lactonase₁₄₇₋₁₁₅₁₆ contains 250 aminoacids (Fig. 2) and its sequence has been deposited in GenBank under the accession number ABN51242. This enzyme showed biological

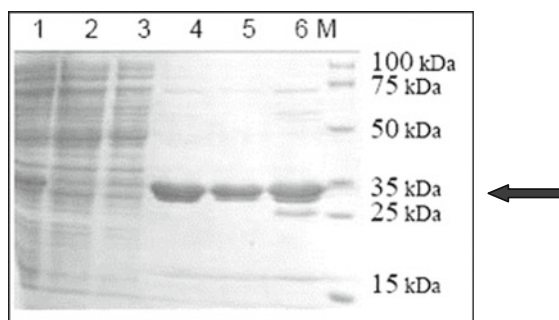


Fig. 1 The recombinant protein AIIA from *Bacillus thuringiensis* strain 147-11516 was purified on a Ni column and fractions were analyzed by SDS-PAGE stained with Coomassie brilliant blue. Lane M, low molecular weight marker. Lane 1, supernatant after sonication of the recombinant cells. Lane 2, column flow. Lane 3, column wash. Lanes 4–6, AIIA protein fractions

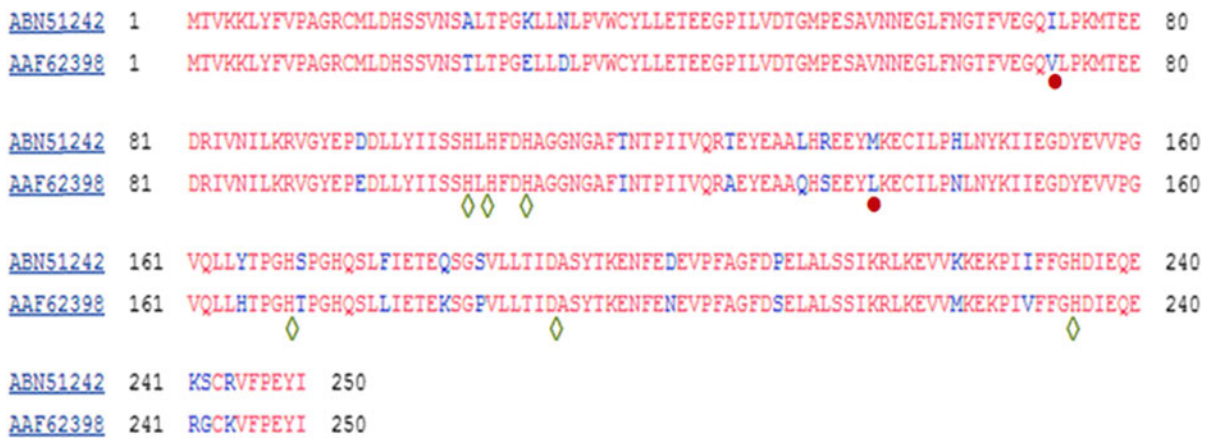


Fig. 2 Sequence alignments of AHL-lactonase₁₄₇₋₁₁₅₁₆ (ABN51242) and AHL-lactonase_{240B1} (AAF62398). Residues involved in Zinc coordination and denoted as *rhombi*. *Red*

circles indicate aminoacid residues involved in the hydrophobic channel. Aminoacid differences in the sequence are indicated by *blue letters*

activity with C6-HSL and C8-HSL as evidenced by absence of pigmentation in the CV026 reporter strain and a decrease in color of NTL4. Indirect measurement of AHL-lactonase activity with the reporter strains showed a possible preference for C6-HSL, for this reason and to facilitate the selection of conditions to evaluate and quantify the enzymatic activity by HPLC–MS, all subsequent trials were performed with strain CV026. It was determined that the minimum concentration of enzyme capable of hydrolyzing C6-HSL and to show detectable activity with the reporter strains was between 100 and 120 μ M. Similarly, the buffer Tris HCl 100 mM was chosen to evaluate the hydrolysis of the lactones because phosphate buffers significantly inhibited the AHL-lactonase₁₄₇₋₁₁₅₁₆ activity.

Low temperatures (0 and 15 °C) inhibited the activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ when it was evaluated with strain CV026, but enzyme activity increased as temperature increased. In the range between 30 and 70 °C, the recombinant enzyme hydrolyzed all the substrate C6-HSL; result that was supported by thermal stability tests where the enzyme showed 100 % activity when it was incubated at 60 °C for up to 5 h. Negative controls showed a slight hydrolysis of the substrate at 60 °C and even at 70 °C there was only 13 % hydrolysis (Table 2; Fig. 3a). EDTA did not alter the enzyme in any of the three concentrations tested, but in the negative control, growth of strain CV026 was slightly inhibited at 50 mM (Fig. 3b).

The AHL-lactonase₁₄₇₋₁₁₅₁₆ hydrolyzed C6-HSL in the treatments containing neutral and basic pH, but had no detectable activity with the reporter strain CV026 in the treatments containing acid pH (Table 2). In the negative control treatments containing pH solutions of 4, 5, 6, 7 and 8 there were not hydrolysis of the lactone ring; however, in the negative control of pH 9.0, 50 % hydrolysis of C6-HSL was observed, interfering with the measurement of the enzyme activity under that condition.

Al³⁺ and Cu²⁺ dissolved in 1× PBS (pH 7.4) completely inhibited the enzyme activity in the two concentrations tested (0.2 and 2.0 mM). Increasing the concentration of Fe²⁺, Mn²⁺, Ca²⁺ and Mg²⁺ to 2.0 mM, partially affected the performance of the AHL-lactonase, while at 0.2 and 2.0 mM, Pb²⁺ and K⁺ did not affect the enzyme activity.

Assessment of recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ activity by HPLC–MS

The activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ with C6-HSL was 0.8 μ mol/min/mg in the first 10 min of the reaction, when 10 % of the substrate was consumed. The substrate concentration in the negative control was 98 %. Quantification of AHLs not hydrolyzed by the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ was performed using HPLC–ESI–MS, after selecting the ions for each substrate found in higher abundance (Table 1), but C10-HSL was precipitated, demonstrating low solubility in the mobile phase. To quantify the

Table 2 Effect of temperature and thermal stability of the AHL-lactonase₁₄₇₋₁₁₅₁₆ in the presence of C6-HSL and the reporter strain CV026 (A). Effect of pH on the enzymatic activity of AHL-lactonase₁₄₇₋₁₁₅₁₆ with C6-HSL and the reporter strain CV026 (B)

| Effect of temperatures in the enzymatic activity | | Thermal stability of the enzyme | |
|--|----------|---------------------------------|----------|
| Temperature (°C) | Activity | Temperature/ time (°C/h) | Activity |
| A | | | |
| 0 | – | 30/5 | +++ |
| 15 | – | 40/5 | +++ |
| 26 | ++ | 50/5 | +++ |
| 30 | +++ | 60/5 | +++ |
| 40 | +++ | 70/2 | ++ |
| 50 | +++ | 70/3 | + |
| 60 | +++ | 70/4 | + |
| 70 | +++ | 70/5 | + |

Effect of pH in the enzymatic activity

| pH | Activity 60 min | Activity 120 min |
|----------|-----------------|------------------|
| B | | |
| 4 | – | – |
| 5 | – | – |
| 6 | + | + |
| 7 | +++ | +++ |
| 8 | +++ | +++ |
| 9 | +++ | +++ |

Activity evaluation scale of AHL-lactonase₁₄₇₋₁₁₅₁₆ according to the coloured area around the well

Diameters of 0–1.9 mm (Activity +++), 2–3.5 mm (Activity ++), 3.6–5.0 mm (Activity +) y > 5.1 mm (Activity –)

AHLs, calibration curves were performed with known concentrations of each substrate with correlation coefficients ≥ 0.995 , which increased as the injection volume was reduced. Subsequently, with the selected ions and the optimized ESI–MS conditions (Table 1), all substrates were separated and the retention times for each one were identified (C4-HSL: 2.3 min; C6-HSL: 4.8 min, C7-HSL: 8.4 min and C8-HSL: 16 min). In order to verify the presence of the product of the enzymatic hydrolysis, the detection range (m/z) was increased and all the product ions were found (C4-HSL: 190.2; C6-HSL: 218.2, C7-HSL: 232.3 and C8-HSL: 246.3 m/z), thereby confirming the incorporation of a water molecule to the homoserine lactone ring, as seen in the chromatogram and in the mass

spectrum MS ESI-C6-HSL substrate used to characterize the enzyme (Fig. 4).

After 2 h of incubation under controlled conditions and at different temperatures, the AHL-lactonase₁₄₇₋₁₁₅₁₆ showed the greatest stability and activity on the substrate (1.5541 $\mu\text{mol}/\text{min}/\text{mg}$ defined as 100 %) at 30 °C, however, at 40 and 50 °C maintained 75 and 60 % of its biological activity, whereas at 20 and 70 °C the AHL-lactonase hydrolyzed 45 and 33 % of the C6-HSL (Fig. 5a). Hydrolysis of the substrate was not observed in the negative controls, except at 70 °C where 20 % degradation of the substrate was detected. In the other hand, enzyme activity increased with increasing pH and reached its maximum activity at pH 8.0, then declined at pH 9.0. Under acidic conditions, the activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ was reduced to 28 % (Fig. 5b). Negative controls showed a marked lactonolysis at pH 9.0, confirming the results found with the reporter strain CV026.

The activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ showed statistically significant differences compared to the control (no EDTA added), when evaluated in monovalent, divalent and trivalent ion solutions. The results showed that in the enzymatic reactions containing ions at concentrations of 0.2 mM, the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ activity was between 0 and 82 %, being completely abolished by Cu^{2+} ; in the same way, when the concentration of ions was increased to 2.0 mM, the enzyme activity decreased in all treatments and was completely abolished in solutions containing Cu^{2+} and Mn^{2+} (Fig. 5c). The recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ in the presence of 10 mM EDTA displayed a 95.7 % of activity compared to control and showed no statistically significant differences, indicating no negative effects on the enzyme.

The kinetic parameters of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ were determined using the Michaelis–Menten model and the graphical representation of Lineweaver–Burk. The recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ showed hydrolytic activity in AHLs with acyl chains of different lengths, with K_m values between 0.5 and 8.84 mM, indicating high affinity of the enzyme for the substrates; however, the enzyme showed low catalytic and efficacy constants (Table 3). The C8-HSL hydrolysis values could be considered as an estimate because it showed low solubility at concentrations of 2.5 mM in the Tris HCl 100 mM buffer (pH 7.4) used for these tests.

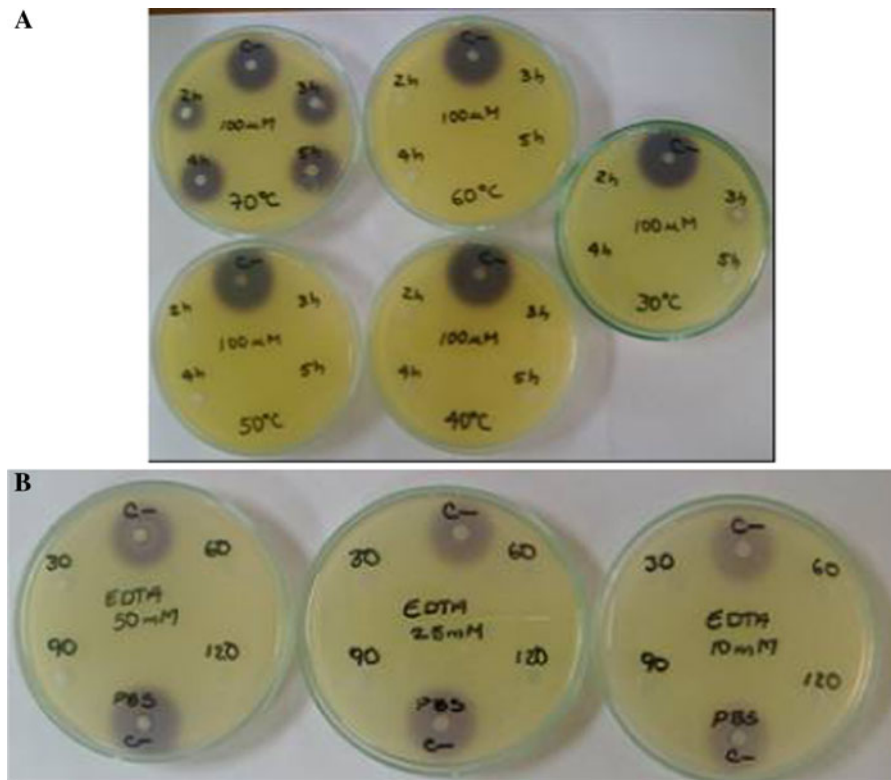


Fig. 3 Thermal stability and activity in EDTA solutions of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆. **a** Activity was evaluated at 20 and 100 μ M of C6-HSL and enzyme, respectively, at 30, 40, 50, 60 and 70 $^{\circ}$ C and incubation times up to 5 h. C– negative control contained only 100 mM Tris HCl buffer

In order to determine the specificity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆, *N*-decanoyl, octanoyl, heptanoyl, hexanoyl and butanoyl-DL-homoserine lactones were used, which have different acyl chains lengths without substitutions (Table 3). The results indicate that the AHL-lactonase₁₄₇₋₁₁₅₁₆ prefers AHLs with shorter acyl chains and the higher activity was observed with C4-HSL. It was not possible to determine the enzyme activity on C10-HSL and the activity of C8-HSL could be considered an estimate due to the poor solubility of these substrates in the buffer used for the enzymatic reaction.

Discussion

There are several reports showing the potential of the AHL-lactonase of *Bt* as a strategy for controlling diseases caused by pathogens, but little information is available on the biochemical characterization of these

(pH 7.4) and C6-HSL. **b** EDTA was evaluated at concentrations of 50, 25 and 10 mM for 30, 60, 90 and 120 min. C–, 1 \times PBS solution with EDTA and C6-HSL. (C-PBS), 1 \times PBS solution with C6-HSL without EDTA

enzymes which are inhibitors of QS. The results presented here indicate that the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ effectively hydrolyzed several AHL substrates of different acyl chain length at alkaline pH and high temperatures. The recombinant enzyme also displayed activity in the presence of high concentrations of EDTA, despite containing the Zn motif in its sequence.

The AHL-lactonase₁₄₇₋₁₁₅₁₆ (GenBank ABN51242) is a protein of 250 amino acids containing the motif “His¹⁰⁶-Asp¹⁰⁸-His¹⁰⁹-X⁵⁹X²¹-His¹⁶⁹-Asp¹⁹¹” and displays a 91 % of identity with the AHL-lactonase_{240B1} (GenBank AAF62398) characterized by Wang et al. (2004), as indicated in Fig. 2. These two enzymes contain 250 aminoacids and share the Zinc motif of the metalloenzymes. However, they differ in 23 aminoacids with special reference to Ile-73 and Met-138, replaced by Val and Leu in the AHL-lactonase_{240B1}. These amino acids are part of the hydrophobic channel contacting the substrate (Kim et al. 2005; Liu et al. 2005). These two

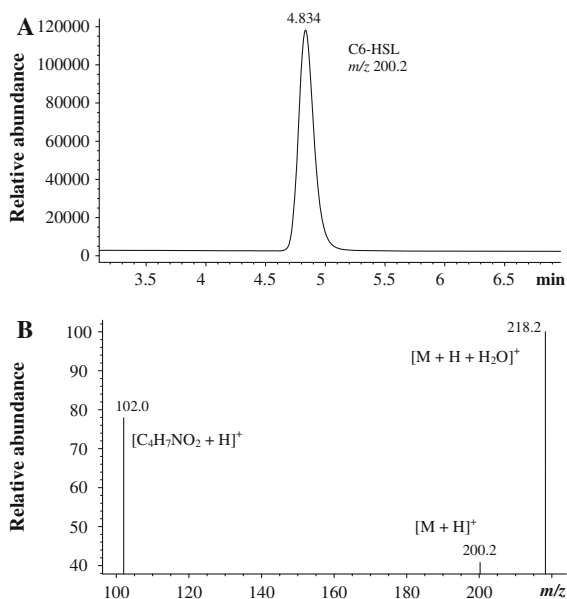


Fig. 4 Mass spectrum chromatogram and ESI-MS of C6-HSL and degradation products obtained after treatment with the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆. **a** C6-HSL had a retention time of 4.8 min. **b** The ion with a *m/z* of 218.2 corresponds to the hydrolysis product of C6-HSL. The selected chromatographic conditions and ions are described in the text and in Table 1, respectively

enzymes also differ in residue 170, close to His-169, amino acid of importance in Zn coordination a common feature of the metallohydrolases. There are differences between the publications of Kim et al. (2005) and Wang et al. (2004) regarding whether the AHL-lactonase is a metalloenzyme despite having binding sites for two Zn atoms.

The AHL-lactonase₁₄₇₋₁₁₅₁₆ showed an optimum pH of 8.0, but its catalytic activity was reduced when the pH decreases, although at pH 5.0 had better activity than the AHL-lactonase_{240B1}. Unlike the results shown by Wang et al. (2004), negative controls of pH 9.0 showed a remarkable lactonolysis that possibly interfered with the activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆, consistent with what was observed in the bioassays with the reporter strain CV026. The alkaline hydrolysis of AHL has been reported and there are observations that plants attacked by *Pectobacterium carotovorum*, increase the pH in the infected areas (Byers et al. 2002); however, the lactonolysis can be reversed at acid pH conditions (Camara et al. 2002). The low catalytic activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ at low pH is consistent with the results of circular

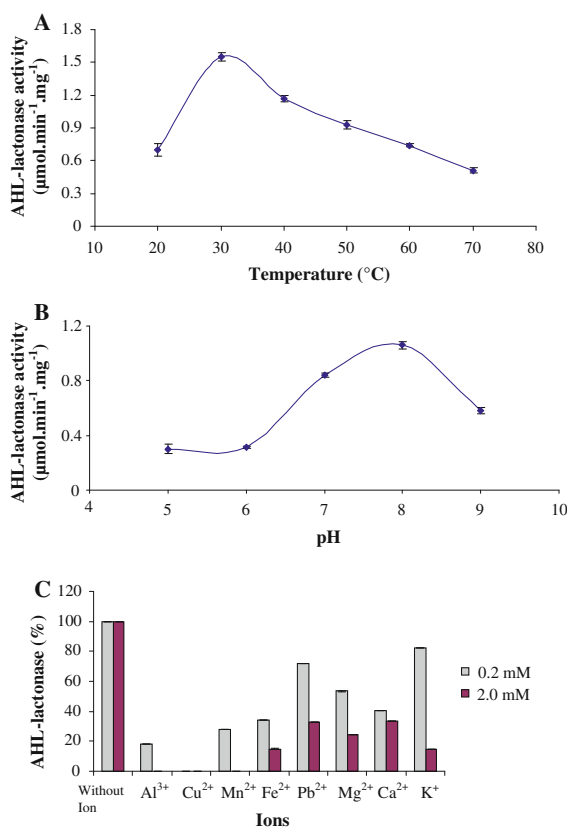
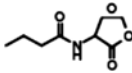
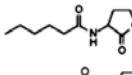
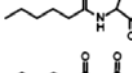

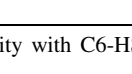


Fig. 5 Thermal stability evaluation (a), effect of pH (b) and ion concentration (c) in the activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ tested with C6-HSL

dichroism obtained by Wang et al. (2004), indicating a drastic change in the conformational structure of the AHL-lactonase_{240B1} at low pH, which also suggested that the ionization state of the side chains and hydrogen bonds has an important role in maintaining the conformational structure of the protein, similarly indicate that the pH range of 5.0 to 6.0, is the pH that allows ionization of the histidine side chain present in the active site, which is essential for enzyme activity (Wang et al. 2004).

The AHL-lactonase₁₄₇₋₁₁₅₁₆ showed biological activity at 40 and 50 °C with C6-HSL, producing degradation of 75 and 60 % of the substrate respectively, and under these conditions, the enzyme significantly increased the reaction rate (Fig. 5a). The activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ at high temperatures is superior to the activity shown by the AHL-lactonase_{240B1} which was inactive when it was incubated at 45 °C (Wang et al. 2004). The AHL-lactonase₁₄₇₋₁₁₅₁₆ activity decreased to 47 % at

Table 3 Kinetic parameters of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ using different acyl chain lengths (A). Specificity of the enzyme by the substrate (B)

| AHLs | K _m (mM) | k _{cat} (s ⁻¹) | k _{cat} /K _m (mM ⁻¹ /s ⁻¹) |
|----------|---|-------------------------------------|---|
| A | | | |
| C4-HSL | 0.50 | 5.49 × 10 ⁻³ | 1.09 × 10 ⁻² |
| C6-HSL | 0.86 | 1.02 × 10 ⁻² | 1.19 × 10 ⁻² |
| C7-HSL | 8.84 | 2.35 × 10 ⁻² | 2.66 × 10 ⁻³ |
| C8-HSL | 3.81 | 4.46 × 10 ⁻² | 1.17 × 10 ⁻² |
| AHLs | Structure | Activity (%) | |
| B | | | |
| C4-HSL |  | 190.3 (0.767) ^a | |
| C6-HSL |  | 100 (0.374) | |
| C7-HSL |  | 73.7 (1.748) | |
| C8-HSL |  | 457.6 (0.984) | |
| C10-HSL |  | ND | |

The specific activity with C6-HSL (6.554 μmol/min/mg) was defined as 100 %

Experiments performed by triplicate

ND not determined due to low solubility of the substrate

^a Numbers in brackets indicate standard deviation of mean values

60 °C, whereas at 70 °C the enzyme activity was only 33 %, possibly due to denaturation of the enzyme; however, Cao et al. (2012) reported thermal stability of AiiA_{AI96} at 70 °C. The AHL-lactonase₁₄₇₋₁₁₅₁₆ showed the higher activity at 30 °C, which is the optimum growth temperature of *Bt*, and accordingly with the results obtained with the reporter strain *C. violaceum* CV026. In preliminary tests performed with strain CV026 it was observed 100 % activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ with C6-HSL, in the reaction mixtures incubated at 60 °C, this hydrolysis may be produced by the prolonged incubation time of the enzyme with the substrate (120 min).

The activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆ significantly decreased in the presence of all metal ions used and in the two concentrations tested, but Cu²⁺ completely abolished the activity of the enzyme on C6-HSL, possibly due to interaction with the sulfhydryl groups of the enzyme. Unlike the AHL-

lactonase_{240B1}, which was not inhibited by divalent ions such as Mg²⁺, Ca²⁺ and Mn²⁺ at concentrations of 0.2 and 2 mM, the AHL-lactonase₁₄₇₋₁₁₅₁₆ was inhibited by several of them as shown in Fig. 5c, and at concentrations of 2.0 mM of Mn²⁺ the enzymatic activity completely disappeared, although in bioassays using the reporter strains and Mg²⁺ a faint shadow around the well was detected, indicating good biological activity. It is possible that this inconsistency was due to the difference in the incubation time used, 2 h for the reporter strains, while in the enzyme reactions, the incubation time was 30 min. The inhibitory effect of Ca²⁺ and Mn²⁺ are consistent with the results obtained with the reporter strain CV026, although there is no literature to explain the effect of Mn²⁺ in the activity of the AHL-lactonase, there is information regarding loss of α helical content of Gyrase B caused by Mn²⁺ (Sissi et al. 2005). Treatment of the AHL-lactonase₁₄₇₋₁₁₅₁₆ with Fe²⁺, Pb²⁺ and Cu²⁺ showed the same activity as in the case of AHL-lactonase_{240B1} and CV026 trials (Wang et al. 2004). It is possible that Fe²⁺ may have the same effect as in the case of urease, which accelerates oxidation of thiol groups, and therefore decreases the enzyme activity (Tirrell and Middleman 1978). We have not found reports of the effect of K⁺ and Al³⁺ in AHL-lactonase activity; however, recent studies on paraoxonases (PON1 and PON3) indicate that amino acids like histidine, aspartic acid and phenylalanine, have the ability to bind metal ions and these amino acids have been reported as essential for catalytic activity of the AHL-lactonases; for instance, aspartic acid at positions 50 and 108 and histidine at positions 104, 106, 109, 169, and 235 are involved in the interaction with the HSL ring, in addition to coordination and stabilization of Zn. Similarly, F¹⁰⁷ is involved in the acyl chain binding in the hydrophobic active site channel (Kim et al. 2005; Pla et al. 2007).

In the other hand, EDTA did not affected the activity of the AHL-lactonase₁₄₇₋₁₁₅₁₆, even at the higher concentration tested according to the results with the biosensor strain CV026, as well as in the quantitative assays, results that are consistent with those of the AHL-lactonase_{240B1}. The activity of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ on C6-HSL in the presence of high concentrations of EDTA is interesting, because despite having the same catalytic motif of metalloenzymes, ¹⁰⁶XDH¹⁰⁹ ~ H¹⁶⁹ (Copeland 2000; Bebrone 2007), the AHL-lactonase₁₄₇₋₁₁₅₁₆

was not negatively affected by EDTA. The same observation and results have been reported by Cao et al. (2012) regarding the enzyme AiiA_{AI196} and by Wang et al. (2004) with the AHL-lactonase_{240B1}.

The AHL-lactonase₁₄₇₋₁₁₅₁₆ showed higher affinity for the substrates than the AHL-lactonase_{240B1}, but the catalytic and efficacy constants were lower. When the kinetic parameters K_M , k_{cat}/K_M , k_{cat} of the enzymes were compared reacting with the substrates C4-HSL, C6-HSL, C7-HSL and C8-HSL, the K_M values were superior for the AHL-lactonase_{240B1}, but the k_{cat}/K_M (specificity index) and k_{cat} , were lower in the AHL-lactonase₁₄₇₋₁₁₅₁₆ (Table 3). The K_M values obtained with the AHL-lactonase₁₄₇₋₁₁₅₁₆, show higher affinity for the substrates but the k_{cat} and k_{cat}/K_M suggest that higher amount of energy is required to break the ES[#] complex (Enzyme-Substrate transition state); therefore, lower specificity index (k_{cat}/K_M) values are associated to relative changes of k_{cat} and K_M ; condition found when reduction of k_{cat} is higher than reduction of K_M (Bauer et al. 2001). This situation could be associated to changes in structural factors which could affect the catalytic activity of the enzyme and probably due to differences in the amino acid sequences of the proteins.

The results of the specificity assays are consistent with the kinetic parameters. Similarly, the saturation condition used by Wang et al. (2004), 1:6,000 was superior to ours, but the specific activity for C4-HSL (12.47 $\mu\text{mol}/\text{min}/\text{mg}$) exceeded the activity of 10.04 $\mu\text{mol}/\text{min}/\text{mg}$ achieved by the AHL-lactonase_{240B1}. Likewise, the AHL-lactonase₁₄₇₋₁₁₅₁₆ had an activity of 6.554 $\mu\text{mol}/\text{min}/\text{mg}$ on C6-HSL, which represents a little more than half of the activity of the AHL-lactonase_{240B1} with this same substrate. An assay performed under the same saturation conditions of Wang et al. (2004), in a 1:6,000 ratio with C6-HSL showed that the AHL-lactonase₁₄₇₋₁₁₅₁₆ activity was 31 fold higher than that determined under lower saturation conditions and significantly exceed the reported for the AHL-lactonase_{240B1} with the same substrate. Hydrolysis of C7-HSL by the AHL-lactonase₁₄₇₋₁₁₅₁₆ was similar to the hydrolysis of C6-HSL, possibly due to the similarity of the acyl chain length, but so far there are no reports indicating that this substrate is produced in nature (Morin et al. 2003); Draganov et al. (2005) have reported the activity of paraoxonases on C7-HSL, indicating that this substrate is susceptible to enzymatic hydrolysis. The high hydrolytic activity (29.99 $\mu\text{mol}/\text{min}/\text{mg}$) of the AHL-lactonase₁₄₇₋₁₁₅₁₆ with C8-HSL is

uncertain since this substrate showed very low solubility at the concentration tested and therefore the quantification can be concealed by precipitation. Furthermore, this result agrees with the data observed with the reporter strain NTL4, which detected residual C8-HSL, while under the same experimental conditions, but with C6-HSL, the CV026 biosensor indicated no residual substrate.

The experimental results of the recombinant AHL-lactonase₁₄₇₋₁₁₅₁₆ show that this enzyme is stable at high temperature and alkaline pH conditions and has affinity for different communication molecules of gram negative bacteria; therefore, this enzyme could be a tool applicable in crops affected by pathogens that use AHLs as their bacterial communication system. There are many opportunities for future applications in other fields of microbiology such as the development of pharmaceutical innovations, since these enzymes inhibit cell communication of several bacterial species affecting vertebrates (Chun et al. 2004; Yang et al. 2005; Cao et al. 2012) and could be an alternative to control diseases caused by pathogens in humans and animals.

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Conflict of interest The authors declare that they have no conflict of interest.

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