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Characterization of the quorum quenching activity of *Streptomyces minutiscleroticus*: A new approach for infection control

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By playing a major role in the virulence of several pathogenic bacteria, N-Acyl homoserine-lactone (AHL) -dependent quorum sensing, is now considered a useful target for antimicrobial therapy. In the present study a total number of 63 *Streptomyces* isolates recovered from different soil samples collected from different localities in Egypt were screened for quorum quenching activity using a fast, reliable, simple screening method and *Chromobacterium violaceum* mutant strain (CV026) as a biosensor. Primary screening against synthetic hexanoyl homoserine lactone (HHL) revealed that 8 isolates showed quorum quenching activity while secondary screening against the extracted naturally produced acyl homoserine lactone (AHL) signals of seven *Pseudomonas aeruginosa* clinical isolates showed a variable profile of activity. Characterization of activity showed that isolate that coded St62, showing highest activity among other *Streptomyces* isolates, could degrade 2 µM HHL in 4 h. The crude enzyme of this isolate was found to have high thermal stability retaining >83% of its activity when pre-incubated at 80°C for 60 min. It also retained >90% of the activity when pre-incubated at pH 8, pH 9 and pH 10 and >80% when pre-incubated at pH 6. All the tested metal ions and EDTA had no effect on activity except for Cu^{++} which caused partial reduction in activity. Enzyme extract was found to have acylase activity equal to 5.2 U/mg total protein. Vmax and Km were found to be equal to 19.92 $nM.min^{-1}$ per mg protein and 23.05 nM, respectively. Maximum catalytic activity was observed at pH 8 over a temperature range of 20-50°C. The enzyme also showed preference in hydrolyzing AHLs with long acyl chains than short acyl chains but yet digested all the tested AHL standards. Finally, identification of the selected isolate using 16S ribosomal RNA gene analysis and phylogenetic analysis showed it to be a *Streptomyces minutiscleroticus* which is, to the best of our knowledge, the first identified quorum quenching bacterial species of its type.

Key words: Quorum sensing, *Streptomyces minutiscleroticus*, Antipathogenic drugs, Acyl homoserine lactones.

INTRODUCTION

The increasing occurrence of multi-resistant pathogenic bacterial strains has gradually rendered the traditional antimicrobial treatment ineffective. Today, a global concern has emerged that we are entering a post-antibiotic era with a reduced capability to combat microbes, and, hence, the development of novel therapeutic approaches for the treatment of bacterial infections constitutes a focal point in modern research (Hentzer and Givskov, 2003).

The term quorum sensing (QS) describes a process in which a signal, known as an autoinducer, accumulates in the environment as the bacterial population grows. At some threshold concentration, the autoinducer affects a

change in the gene expression of the population leading to transcriptional regulation (Fuqua et al., 1994). QS-controlled genes often encode virulence factors and gene products required for bacteria-host interactions (Pirhonen et al., 1993; Parsek and Greenberg, 2000; Pearson et al., 2000). QS was also found to be involved in many other biochemical processes in the bacterial cell (Diggle et al., 2007).

The approach of targeting quorum sensing systems is referred to as 'quorum quenching' and compounds with abilities to stop bacterial virulence through targeting QS system are termed antipathogenic drugs as opposed to antibacterial drug (Hentzer and Givskov, 2003). This approach has the advantage over the traditional treatments which are based on compounds that kill or inhibit bacterial growth and which suffer from problems such as: side effects of chemotherapeutic agents, limited number of newly discovered antibiotics and increasingly emerging resistance to existing antibiotics. There are basically three different targets for QS inhibition which include: the signal generator; the signal receptor and the signal molecule (Rasmussen and Givskov, 2006)

Many Gram negative bacteria, like *Pseudomonas aeruginosa*, have Acyl homoserine lactone (AHL)-dependent quorum sensing systems (Fuqua et al., 2001). Accordingly, inactivation of this signal molecule represents a new effective approach for the control of infection of such pathogenic highly resistant bacteria.

Many different bacteria belonging to various genera have been reported to express quorum quenching through enzymes degrading AHLs. Examples include the degradation of acyl homoserine lactone molecule by lactonases which are produced by some *Bacillus* species (Dong et al., 2001; Lee et al., 2002). O ther example include degradation by *Arthrobacter* sp. (Park et al., 2003), *Variovorax paradoxus* (Zhang et al., 2002; Carlier et al., 2003) and acylases produced by some *Streptomyces* species (Park et al., 2005). A search for bacteria degrading the Autoinducers of QS systems is promising for designing agents to effectively suppress bacterial infections (Khmel and Metlitskaya, 2006)

The goal of the present study was to screen soil for *Streptomyces* with quorum quenching activity against synthetic and naturally produced AHL from clinically isolated *P. aeruginosa* isolates together with the characterization of the activity and the identification of the promising isolate.

MATERIALS AND METHODS

Chemicals

All chemicals were of high quality from available grades purchased from El-Nasr Chemicals (Adwic), Egypt. Acyl homoserine lactone standards (bytanoyl (C4), haxanoyl (C6), heptanoyl (C7), and octanoyl (C8) homoserine lactone) were purchased from Sigma-Aldrich, Germany. Reagents for DNA extraction and PCR were a product of Fermentas, USA.

Bacterial strains

Chromobacterium violaceum CV026

CV026 is a mutant strain of *C. violaceum* that produces the characteristic purple pigment violacein only in response to the presence of acyl homoserine lactone with C4-C8 side chain and it acts as (AHL) dependent biosensor (McClean et al., 1997). CV026 was subcultured in Luria Bertani (LB) broth for maintenance. For purification, 20 µg/ml kanamycin were added in the growth medium as it is kanamycin resistant (Ravn et al., 2001). It was stored in slant medium or in lyophilized form for long term preservation.

Pseudomonas aeruginosa isolates

Seven *P. aeruginosa* isolates were used in this study. These isolates were previously recovered from clinical specimens, studied for their AHL productivities and had their AHL signals analyzed using thin layer Chromatography (TLC) (Sakr et al., 2014). They were used to study the spectrum of quorum quenching activity of the recovered *Streptomyces* isolates.

Streptomyces isolates

Sixty three *Streptomyces* isolates were recovered onto starch nitrate agar (Waksman, 1959) from 18 soil samples previously collected from different localities in Egypt and pre-enriched with CaCO3 at the Microbiology and Immunology Department, Faculty of pharmacy, Ain shams University. These isolates were routinely maintained onto starch nitrate agar and some selected isolates were subject for long term preservation in slant medium or as spores glycerol stocks (Kieser et al., 2000; Shepherd et al., 2010).

Growth of test isolates for collection of the cell free supernatants

Streptomyces isolates were grown in two steps, first a preculture that was prepared by suspending a fresh slant of 4 days age of *Streptomyces* isolate in 5 ml distilled H_2O from which 1 ml was used to inoculate 5 ml YMG medium (yeast extract 0.4%w/v, malt extract 1%w/v, glucose 0.4%w/v) contained in a test tube. The tube was then incubated for 4 days at 28°C with shaking at 160 rpm. Second, the main culture was done according to (Sun-Yang et al. (2005), with some modifications. From the preculture, 1 ml (about 10^7 cfu/ml) was transferred into 20 ml YMG medium contained in 100 ml conical flask to provide appropriate aeration. Then, the flask was incubated for 7 days at 28°C with shaking at 160 rpm. The *Streptomyces* cells were then removed by centrifugation at 15000 rpm for 15 min and the resulting supernatant was collected. Because AHLs (test substrate) are easily hydrolyzed under alkaline conditions (Yates et al., 2002), the pH of the culture supernatants of the test isolates was adjusted to 7.0 before being tested for AHL degrading activity.

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Primary screening using the synthetic signal molecule (HHL)

Screening was done according to Jafra and his coworkers ((Jafra and van-der-Wolf, 2004), with some modifications. Briefly, *C. violaceum* CV026 strain was grown overnight in 5 ml LB broth at 28°C with shaking at 160 rpm then the optical density (OD_{640}) of the growth produced was adjusted to equal to 0.1-0.2 (about 10^8cfu/ml, Blosser and Gray, 2000). Then, in each well of the microtitre plate, about 50 µl of 0.5 µM hexanoyl homoserine lactone (HHL) were overlaid by 50 µl of collected supernatant, incubated for 4 h at 28°C, then the bacterial cultures were exposed to UV germicidal lamp (working at 254 nm) for 90 min after which, 50 µl of the adjusted count CV026 culture was added to each well. The plates were incubated at 28°C overnight where CV026 produced purple pigment in wells where HHL was present. While, in the wells where HHL was degraded by bacterial isolates, no color was produced. Positive control was prepared by adding 50 µl plain LB broth instead of the collected supernatant.

Secondary screening of the positive isolates using *P. aeruginosa* extracts

Secondary screening were done for the collected supernatants that proved to degrade HHL in the primary screening and was carried out against the extracts of seven *P. aeruginosa* isolates that were previously screened for their abilities to produce acyl homoserine lactone signal molecules and analyzed using TLC. This was performed according to the protocol described by Jafra and coworkers (Jafra and van-der-Wolf, 2004). Briefly, About 0.3 ml aliquots of the *P. aeruginosa* extract was mixed with 1.5 ml M63 buffer ,pH 7.0, prepared according to Heidemarie and coworkers (Heidemarie et al., 1980). About 50 µl of the resulting solution (1.8 ml) was added to each well of the microtitre plate overlaid with 50µl of the supernatants of and the assay was carried out just as described in the primary screening. Positive control for each *P. aeruginosa* extract was done using plain LB broth instead of collected supernatants. Secondary screening was done in triplicates for confirmation of the results.

Testing the activity of tested isolates at different levels of substrate concentration

In an experiment, two dilutions of the collected cell free supernatant of positive *Streptomyces* isolates were tested against different concentrations of synthetic HHL (substrate); 0.5, 2, 5 and 10 µM and this was carried out as described in primary screening.

Measuring the total protein concentration in the crude enzyme extracts of positive isolates

Growth and preparation of cell free supernatants of test isolates were done as previously described. The protein concentrations of the prepared cell free supernatants, termed as crude enzyme extracts, were measured using bovine serum albumin as a standard (Lowry et al., 1951). A standard curve of absorbance at 660 nm as a function of protein concentration of the standard solutions was plot and used to determine the samples' protein concentrations. Then, protein concentration in the crude enzyme was adjusted to 3 mg/ml.

Measuring the activity of the crude enzyme extracts of positive isolates using well diffusion method

This assay was done according to Ravn and his coworkers (Ravn et al., 2001) where 10 ml LB agar was overlaid by 10 ml semis olid LB agar supplemented with HHL at a concentration 100 nM and seeded with the biosensor strain (CV026) . When the overlaid agar had solidified, wells were punched into the agar with a sterile Cork borer of diameter 10 mm. After that, 150 µl of the crude extract of the positive isolates were pipetted into the wells. The plates were then incubated for 24 hours at 28°C. The growth of CV026 showed the purple color in the whole plate except the zones around the wells.

Physical parameters that affect the enzyme stability of the selected isolate

Thermal stability

In this assay, the enzyme was incubated at 50, 80 and 90°C for 30 and 60 min. Then, 150 µl of each aliquot was pipetted into the wells of semisolid LB agar prepared as previously described in the well diffusion method (Ravn et al., 2001).The residual activity was then measured by comparing the inhibition zone diameters of the test to that of the control incubated at room temperature. The experiment was done in triplicates and the means and standard deviations were calculated.

Stability at different pH

The assay was carried out according to Cao and his coworkers (Cao et al., 2012) with modifications as follows: 1 ml aliquots of the enzyme extract were transferred to wasserman tubes and the pH of each was changed to the required pH using either McIlvaine buffer (prepared by mixing 19.6 ml 0.1M citric acid and 0.4 ml 0.2M Na_2HPO_4) or Glycine-NaOH buffer (0.1 M glycine-NaOH adjusted to pH 12). The tubes were incubated at room temperature for 30 min and then neutralized to pH 7 using the counter buffer. Afterwards, the volumes of all the aliquots were adjusted equally. Then, 150 µl of each aliquot was pipetted into the wells and the activity was measured using the well diffusion method as previously described. The residual activity was then measured by comparing the inhibition zone diameters at different pH to that of pH 7. The experiment was done in triplicates and the means and standard deviations were calculated. The stability of the enzyme was studied over a pH range of 4 to 10.

Effect of some divalent metals and EDTA on activity

Concentrations of 1 and 10 mM of Ca^{++}, Fe^{++}, Mg^{++} and EDTA were added to the enzyme extract and incubated for 30 min. For Cu^{++} and Zn^{++}, a concentration of 10mM was found to have an inhibitory effect on the growth of CV026, so they were used in concentrations 1 and 2 mM. A sample without an additive served as a control. Then, 150 µl of the tested aliquots and the control were pipetted into the wells of semisolid LB agar, as previously described, and the inhibition zone diameters compared to that of the control. The experiment was done in triplicates and the means and standard deviations were calculated.

Catalytic parameters of crude enzyme of the selected isolate

Catalytic activity rate

This was done according to Cao and his coworkers (Cao et al., 2012) with modifications as follows: aliquots of the enzyme extract (0.5 ml) were incubated with 10 µM HHL at 28°C with shaking at 160 rpm and the reaction was terminated using 2% SDS after 4, 8, 12, 16 and 20 h. The control was done using plain medium of pH 7

containing HHL of 10 µM final concentration incubated under the same conditions, for the same time period and also treated with SDS. Afterwards, the residual amount of HHL was measured by well diffusion assay as follows: 10 ml LB agar were overlaid by 10 ml semisolid agar seeded with CV026 and the plates were left to solidify. Wells were punctured into the agar and 60 µl of the tested samples were transferred into them. The plates were then incubated for 24 hours at 28°C for color development. The HHL in the wells form zones of purple color around the well, the diameter of which was proportional to the HHL concentration. Measured zones were then compared to standard curve showing the diameters of the purple zones as a function of the different HHL concentrations (with 2% SDS) pipetted into the wells. One unit of AHL degrading enzyme activity was defined as: the amount of enzyme that hydrolyzed 1nM HHL in 1 min and was determined after 8 h reaction time.

Effect of temperature on catalytic activity

This was done as described for the determination of the catalytic activity rate of the enzyme but with the incubation temperature of the reaction mixture adjusted at 5, 20, 40, 50 or 70°C. A control was prepared and incubated at 28°C. The rate of reaction was determined for both the control and the test after 8 h and compared.

Effect of pH on catalytic activity

Aliquots (1 ml each) of the enzyme had their pH values adjusted to 6, 7, 8, 9 and 10 using McIlvaine or glycine-NaOH buffer. Then, they were incubated with HHL (10 µM) at 28°c. After 8 h the reaction was terminated using 2% SDS and the procedure was completed as previously described. The reaction rates after 8 h were determined and activity at different pH was compared to activity at pH 7.

Determination of kinetic constants; Vmax and Km

Aliquots of the enzyme extract (0.5 ml) were incubated with different concentrations of HHL; 5, 10, 15, and 20 µM at 28°C with shaking at 160 rpm. Then, the reaction was terminated after 8 h by 2% SDS and the residual amount of HHL measured by well diffusion assay as previously described. The initial reaction velocity was calculated from the following equation:

$$\text{Rate of HHL degradation} = \frac{-\,d[A]}{d[t]}$$

Where A is the concentration of HHL and t is the time in min. The data were plot as a Lineweaver-Burk curve from which kinetic constants, Vmax (the maximum velocity of reaction) and Km (Michaelis–Menten constant) were calculated.

Enzyme substrate specificity

The ability of the enzyme to hydrolyze AHLs was assessed using standard signals, butanoyl (C4), heptanoyl (C7) and octanoyl (C8) homoserine lactones. This was done by measuring the catalytic activity rate of the enzyme as described above for hexanoyl homoserine lactone. The reaction was carried out for 8 h at 28°C and pH7. Units of enzyme activity to total protein concentration was determined in terms of C4, C7 and C8 HSL and compared to that previously measured for HHL standard.

Identification of the selected *Streptomyces* isolate

This was done by 16S ribosomal RNA gene analysis where DNA extraction, PCR and PCR clean up took place at Sigma Scientific Services Company, Egypt. Sequencing was done at GATC Company, by the use of ABI 3730xl DNA Sequencer. Afterwards, the obtained 16S ribosomal RNA gene sequence files of the selected isolate were assembled using Staden package program version 3 (Staden, 1996) and the obtained sequence was compared with those in GenBank database using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the identification of the selected isolate and phylogenetic tree was constructed.

RESULTS

Primary screening using the synthetic signal molecule (HHL)

Results show that out of the 63 screened isolates, only eight isolates (12.69% of the total *Streptomyces* isolates) were found to have quorum sensing inhibitory activity while the rest of isolates (55 isolates) showed negative results and failed to degrade HHL. The eight positive isolates with HHL degrading activity resulting from primary screening (St11, St13, St14, St35, St38, St57, St61 and St62) were subjected to secondary screening.

Secondary screening of the positive isolates using *P. aeruginosa* extracts

From the results of Table 1, the degradation of AHL signals contained in 6 of the tested *P. aeruginosa* extracts was demonstrated by the supernatant of *Streptomyces* isolate St62 and for 4 extracts by supernatants of *Streptomyces* isolates St11, St14, St35, St38 and St61 and for 2 extracts by supernatants of isolates, St13 and St57. It was also found that none of the isolates could completely degrade AHL signals of the extract of *P. aeruginosa* isolate P13.

Testing the activity of tested isolates at different levels of substrate concentration

Results from Figure 1, shows that only supernatants of *Streptomyces* isolates St11, St61 and St62 could degrade 2 µM HHL while the rest of isolates supernatants showed no ability to degrade HHL in concentrations higher than 0.5 µM. The supernatants of two isolates, St13 and St38 showed faint purple color at concentration 2 µM of the HHL but it was considered and represented as a negative result.

Measuring the activity of the crude enzyme extracts of positive isolates using well diffusion method

The extracts of five tested isolates (St11, St13, St38, St61 and St62) exhibited degrading activity against HHL,

Table 1. Degrading activity profile of tested *Streptomyces* isolates' supernatants against AHL signals contained in the extracts of seven *P. aeruginosa* isolates.

Tested *Streptomyces* supernatants code no.	Degrading activity of AHL signals contained in tested extracts of *P. aeruginosa* isolates							Total no. of *P. aeruginosa* extracts showed degradation*
	P13	P14	P16	P17	P18	P19	P27	
St11	-	++	+	+	++	++	++	4
St13	-	++	-	-	+	++	-	2
St14	-	++	+	-	++	++	++	4
St35	-	++	-	-	++	++	++	4
St38	-	++	+	+	++	++	++	4
St57	-	++	+	+	+	++	+	2
St61	+	++	+	+	++	++	++	4
St62	+	++	++	++	++	++	++	6

++, Complete degradation of target AHL signals; +, Partial degradation of target AHL signals. -, No degradation of target AHL signals; Shaded row refers to the isolate selected for further studies; *refers to degradation in their signal contents.

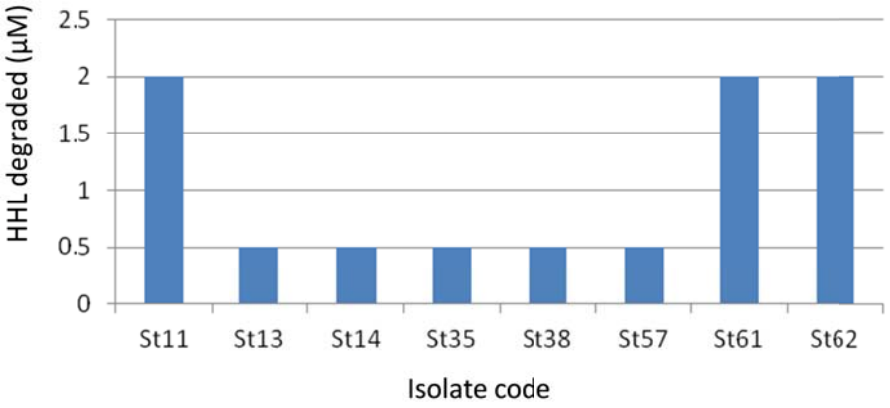

Figure 1. AHL degrading activity of tested *Streptomyces* isolates at different HHL concentrations.

demonstrated by the development of colorless zones by the biosensor around extracts containing wells. On the other hand, the crude enzyme extracts of the other three tested isolates (coded St14, St35 and St57) failed to degrade AHL around the wells evidenced by the absence of inhibition zones. The diameters of colorless zones developed are recorded in Table 2.

Comparing the HHL degrading activity of the tested *Streptomyces* isolates and their activity spectrum against *P. aeruginosa* extracts, isolate coded St62 which showed activity against signals contained in six *P. aeruginosa* extracts, was selected for further studies.

Physical parameters that affect the enzyme stability of the isolate St62

Thermal stability

When incubated at 50°C for either 30 or 60 min, the enzyme of isolate St62 retained more than 98% of its activity. When incubated at 80°C for 30 min, it retained about 92% of the activity and when incubated for 60 min, it retained about 83% of the activity. When the enzyme was incubated at 90°C for 30 min the activity was lost completely evidenced by the absence of inhibition zone in comparison to the control (Figure 2).

Stability at different pH

As shown from the results in Figure 3, the enzyme of isolate St62 retained more than 90% of the activity when pre-incubated at pH 8, pH 9 and pH 10 while the activity was lost completely after pre-incubation at pH 4 and pH 5. When pre-incubated at pH 6 for 30 min, the enzyme retained more than 80% of the activity relative to the control.

Effect of some divalent metals and EDTA on activity

At the two test concentrations of Ca^{++}, Mg^{++}, Zn^{++}, Fe^{++}

Table 2. AHL[(a)] degrading activity of the tested *Streptomyces* isolates as determined by well diffusion method.

Streptomyces isolate code	AHL degrading activity expressed as inhibition zone diameter[(b)] (mm)
St11	22
St13	18
St38	14
St61	19
St62	21

[a]HHL was used as a substrate; [b] The listed values were the average of replicate readings and inhibition zone refers to zone showing no purple color development by the grown biosensor cells.

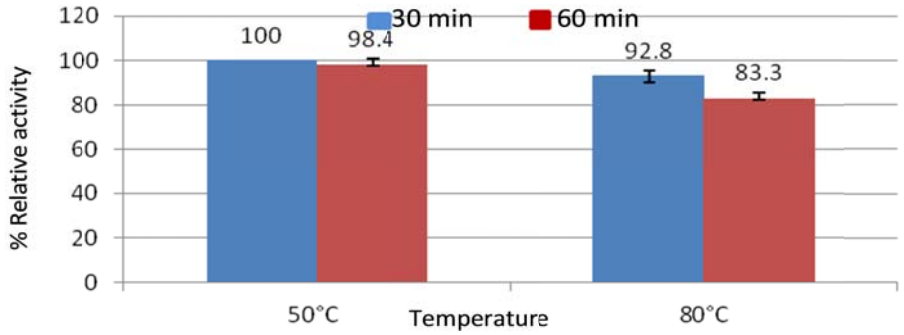

Figure 2. Residual activity after pre-incubation at different temperatures. % relative activity was calculated with respect to control (sample kept at room temperature).

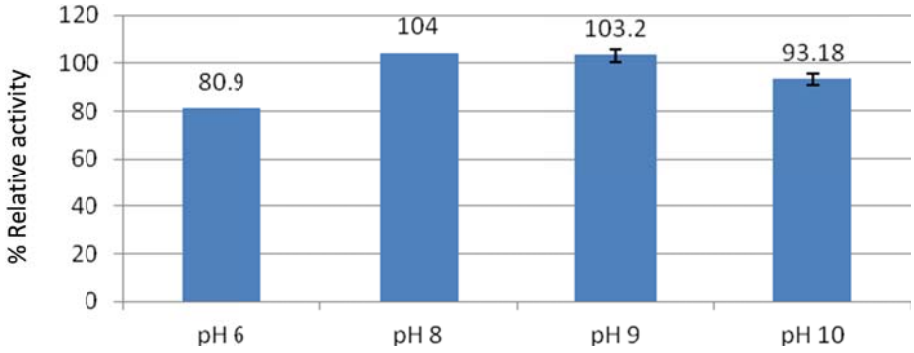

Figure 3. Residual activity after pre-incubation at different pH. % relative activity was calculated with respect to control (sample at pH 7).

and EDTA, the recorded enzyme activity was ~100%. Activity was neither stimulated nor inhibited. However, a concentration of 1 mM Cu^{++} reduced the activity to 90.5% and a concentration of 2 mM Cu^{++} to 81.8% (Figure 4).

Catalytic parameters of crude enzyme of isolate St62

Catalytic activity rate

The time course of HHL degradation by the enzyme of the tested *Streptomyces* isolate St62 is shown in Figure 5. The catalytic activity rate after 8 h was 7.8 $nM.min^{-1}$ then it decreased to 6.04 $nM.min^{-1}$ after 12 h. The activity in terms of acylase activity units to total protein concentrations was found to be equal to 5.2 U/mg total protein. One acylase activity unit is defined as the amount of enzyme that hydrolyzed 1 nM of the substrate in 1 min. amount of enzyme that hydrolyzed 1 nM of the substrate in 1 min.

Effect of temperature on catalytic activity

The activity was almost not affected between 20 and

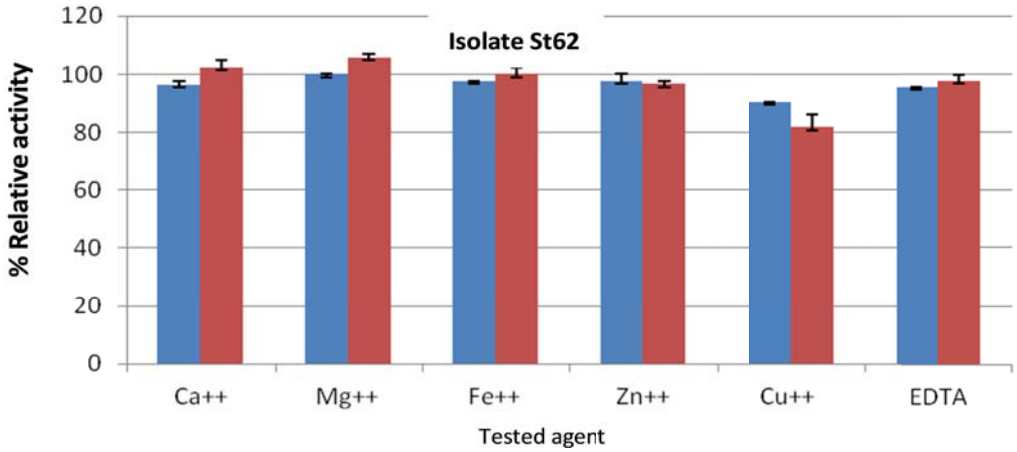

Figure 4. Effect of some divalent metals and EDTA on activity of isolate St62. % relative activity was calculated with respect to control (sample without an additive). Blue: 1 mM concentration, red: 10 mM concentration except for Cu^{++} and Zn^{++} (2 mM).

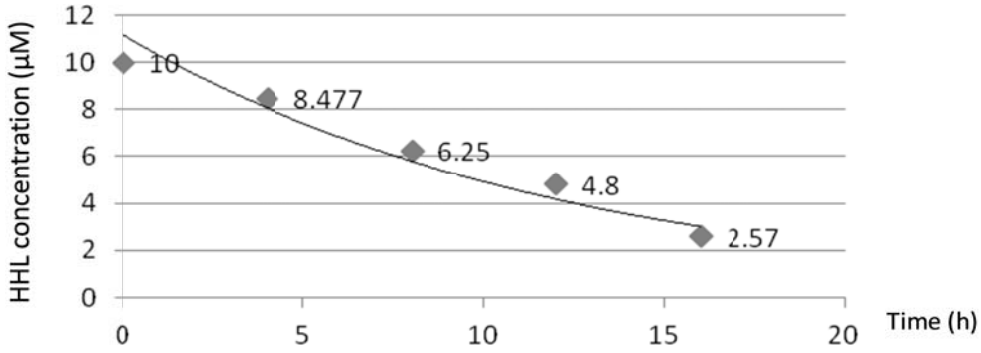

Figure 5. Time course of HHL degradation by the enzyme of *Streptomyces* isolate St62

Table 3. Effect of temperature on catalytic activity of crude enzyme of isolate St62.

Temperature (°C)	% relative activity
5	84.40
20	98.5
40	111
50	100
70	ND*

*ND= not determined

Table 4. Effect of pH on catalytic activity of crude enzyme of isolate St62.

pH	% Relative activity
pH 6	84.85
pH 8	126
pH 9	ND*
pH 10	ND*

*ND= not determined.

50°C. At 5°C, relative activity was reduced to 84.4% while at 70°C the HHL was partially degraded so results could not be assessed (Table 3).

Effect of pH on catalytic activity

At pH 6, relative activity decreased to 84.85% while it increased at pH 8 to 126% (Table 4). However, at pH 9 and 10, activity could not be measured accurately due to the partial degradation of the synthetic signal used.

Determination of Kinetic constants; Vmax and Km

From Lineweaver-Burk plot (Figure 6) Vmax and Km were calculated and they were found to be equal to 19.92 nM.min^{-}1 per mg protein and 23.05 nM, respectively.

Enzyme substrate specificity

Results from Table 5, show that the tested enzyme has a

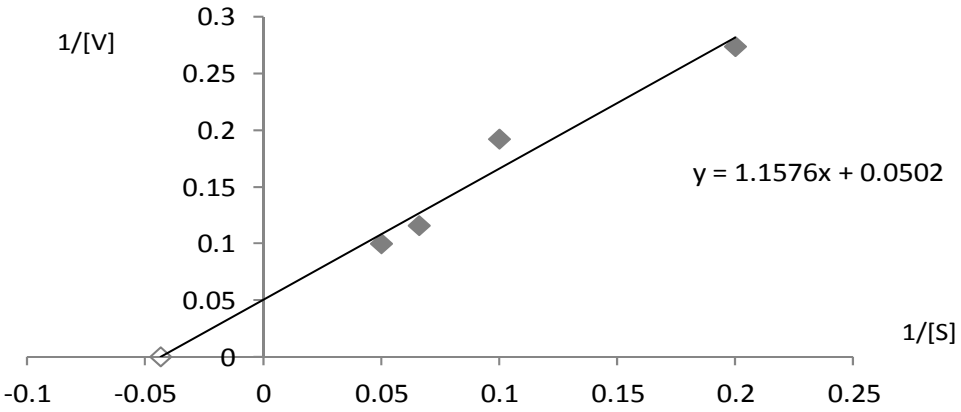

Figure 6. Lineweaver-Burk plot for AHL degrading enzyme of *Streptomyces* isolate St62.

broad substrate spectrum evidenced by its ability to degrade the 4 tested AHL compounds used in the study. Maximum activity was observed against C7-HSL and C8-HSL with very slight difference between them while least degrading activity was observed against C4-HSL (almost one third the activity against C7-HSL).

Table 5. Enzyme substrate specificity of the AHL degrading enzyme of *Streptomyces* isolate St62 against different AHL standards expressed as activity units per mg total protein.

Substrate	Activity (U/mg)
C4-HSL	2.5
C6-HSL	5.2
C7-HSL	7.2
C8-HSL	7.08

Identification of the selected *Streptomyces* isolate St62

According to the 16S ribosomal RNA sequencing, the selected *Streptomyces* isolate St62 was found to have 97% similarity with *Streptomyces minutiscleroticus* strain NRRL B-12202 (accession code=NR_044149.1) and was identified as *Streptomyces minutiscleroticus.* It is worth noting that although other *Streptomyces* isolates were reported to exert quorum quenching activity, this is a newly identified quorum quenching *Streptomyces* species. Figure 7 shows the phylogenetic tree for this isolate.

DISCUSSION

In this study, we screened soil *Streptomyces* for quorum quenching activity and we studied the activity of enzyme of the isolate St62. Screening showed that out of the 63 recovered isolates, only eight isolates were found to have quorum quenching activity with a prevalence of quorum quenching enzymes of 12.69% in the recovered *Streptomyces* isolates. Secondary screening results showed the extract of only one *Streptomyces* isolate-St62- could degrade the signal molecules contained in 6 of the *P. aeruginosa* extracts. It was also found that none of the isolates could completely degrade AHL signals of the extract of *P. aeruginosa* isolate P13. The explanation behind this weak activity of *Streptomyces* supernatants on the naturally produced *P. aeruginosa* signals might lie in the substrate specificity of the acylase enzymes believed to be responsible for quorum quenching activity. In 2005, Park and his coworkers stated that the acylase enzyme in their study exhibited a relatively low activity of short acyl side chain AHLs, C6-HSL and 3-oxo-C6-HSL and did not degrade detectable amounts of C4- HSL (Park et al., 2005). It is also observed here that the *P. aeruginosa* isolate P13 whose signals were not degraded by any of the *Streptomyces* supernatants produces two signals detected by CV026. These two signals are namely C4-HSL and C6-HSL. This isolate also displays high AHL productivity (0.283 µM). This directs us to believe that the reason behind the inability of the *Streptomyces* supernatants to degrade the signals of this *P. aeruginosa* isolate is due to its production of high amounts of C4-HSL which exceed the low capability of the acylase of *Streptomyces* isolates to degrade C4-HSL. The same reasons could also be used to explain why only one *Streptomyces* isolate could degrade the signals of *P. aeruginosa* isolates P16 and P17, both are also BHL producers. Signals of isolate P18, degraded by 6 out of the 8 *Streptomyces* isolates, seem to contain a concen-tration of C4-HSL less than those produced by other *P. aeruginosa* isolates.

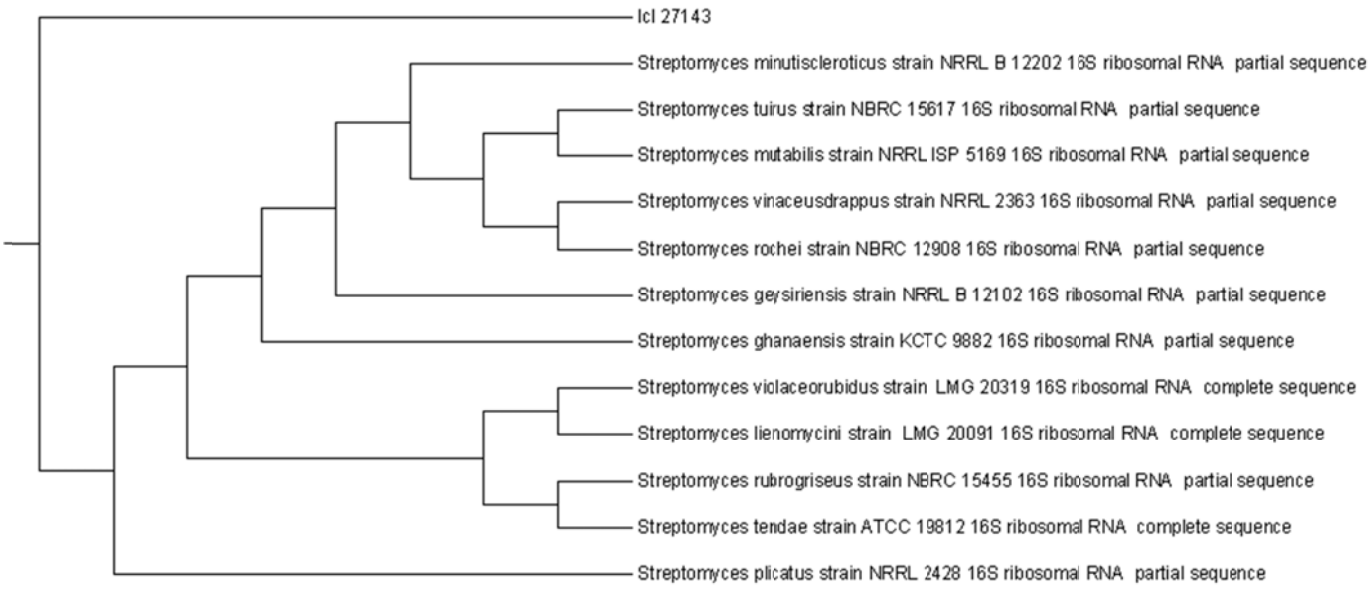

Figure 7. The phylogenetic tree for *Streptomyces minutiscleroticus*

Signals of isolates P14 and P19 were degraded by the supernatants of the eight tested *Streptomyces* isolates. This may be attributed to their production of C6-HSL only as displayed using TLC (Sakr et al., 2014). This was not the case for *P. aeruginosa* isolate P27 although it was proved by TLC to produce C6-HSL only. This may be due to the increased production of C6-HSL signal by this isolate (with AHL productivity of 0.395 µM).

Comparing the activity of the positive isolate by well diffusion method showed that isolates St11 and St62 showed the largest inhibition zones, three isolates, that coded St14, St 35 and St 57 did not show any inhibition zones. This might be attributed to the slow reaction rate of degrading enzymes and their low yield by the test isolates which might have been slower than the ability of the biosensor, CV026, to sense HHL in the area around the wells. In other words, CV026 sensed HHL and started synthesizing violacein before the enzyme started to degrade HHL in considerable amounts. Having the broadest spectrum of activity on signals in the clinically isolated *P. aeruginosa* extracts and a relatively high activity on different HHL concentrations (2 µM), isolate St62 was chosen for conducting further characterization of its enzyme.

Studying the physical stability of the enzyme of isolate St62 showed that the enzyme has high thermal stability. *Streptomyces* isolates are believed to exhibit AHL degrading activity through acylase enzyme (Park et al., 2005), accordingly, results in this study are consistent with two previous studies on an amino-acylase from a Streptomyces mobaraensis isolate (Koreishi et al., 2005), and a penicillin acylase (Torres-Bacete et al., 2007). In their study, Torres-Bacete and his coworkers stated that the enzyme was found to have a thermostable structure, showing a midpoint transition temperature of 81.5°C where unfolding was observed at high temperatures (Torres-Bacete et al., 2007).

The enzyme also displayed high stability over a wide pH range where it retained more than 90% of the activity when pre-incubated at pH 8, 9 10 and when pre-incubated at pH 6 for 30 min, the enzyme retained more than 80% of its activity. In a previous study on an amino-acylase, Koreishi and his coworkers stated that the enzyme was stable between pH 5.5-8 when pre-incubated for 1h at 37°C while at pH 10, the enzyme retained about 45% of its activity (Koreishi et al., 2005).

As for the effect of some metals and EDTA on activity, the enzyme in the present study proved to maintain its activity in the presence of all tested metal ions and EDTA except for partial reduction in activity by Cu++. In their study, Koreishi and his coworkers stated that a concentration of 0.1 mM Cu++decreased the activity of amino-acylase to about 20% (Koreishi et al., 2005). The same study reported a reduction in the relative activity of the enzyme by the addition of 0.1 mM Mg++, Fe++ and Ca++ to a range of 25-56% and an increase in activity when 0.1 mM Zn++ was added to 142% which wasn't the case in the present study. According to the same study, EDTA did not have an effect on activity as was the case here. The study also stated that amino-acylase is a metalloenzyme evidenced by the reduction in activity achieved by phenanthroline(Koreishi et al., 2005). On the other hand, in their study on a Penicillin acylase, Torres-Bacete and coworkers stated that the enzyme does not require any external cofactor, metal ion, or reducing agent for maximal activity (Torres-Bacete et al., 2007).

Studying the catalytic activity, the crude enzyme of isolate St62 with total protein concentration of 3 mg/ml

could effectively degrade HHL with a rate of 7.8 $nM.min^{-1}$. The specific activity in terms of AHL acylase activity units per mg total protein was found to be equal to 5.2 U/mg.

Catalytic activity was almost not affected between 20 and 50°C with maximum activity displayed at 40 °C which is different from the penicillin acylase in the study Torres-Bacete and coworkers where the highest hydrolytic activity was achieved at 75°C (Torres-Bacete et al., 2007). Maximum catalytic activity was observed at pH 8 where it reached 126%.

Results also show that the studied enzyme has a broad substrate spectrum evidenced by its ability to degrade the 4 tested AHL compounds in its crude form. However, a notable difference was observed in the catalytic spectrum of the enzyme towards tested AHLs. Maximum activity was observed against C7-HSL and C8-HSL and was almost three times the activity against C4-HSL. Activity against C6-HSL was almost double the activity against C4-HSL. The findings in this study are consistent with several other studies on different acylases that reported high deacylation activity against AHLs with long acyl chains and weak activity on short chained AHLs with sometimes no activity at all detected with C4- HSL (Huang et al., 2003; Park et al., 2005; Romero et al., 2008). An acylase from a *Ralostonia* isolate however, has been reported to rapidly degrade C4-HSL and 3-oxo-C12-HSL with equal efficiencies while showing a significantly low activity against 3-oxo-C6-HSL (Lin et al., 2003).

By conducting the 16S ribosomal RNA gene sequence analysis, alignment of the obtained sequence against the respective nucleotides sequences of the 16S rRNA genes present in the database could be carried out. Displaying an identity percentage of 97%, the selected *Streptomyces* isolate St62 was identified as *Streptomyces minutiscleroticus.* Some previously studied *Streptomyces* isolates were reported to have enzymes with quorum quenching abilities like the study carried out by Park and coworkers (Park et al., 2005)and *Streptomyces mobaraensis* in the study of Koreishi and coworkers (Koreishi et al., 2005), however, the isolate in this study appears to be the first identified quorum quenching *Streptomyces* of this type.

Conclusion

AHL degrading enzyme of *S. minutiscleroticus* isolate in this study has proved to establish high thermal stability and high stability over a wide pH range. The enzyme also proved to be resistant to the effect of heavy metals and metal chelating reagent, EDTA, and retained maximum activity in the presence of all the tested metal ions and EDTA except for partial reduction in activity when Cu^{++} was added. Maximum catalytic activity was maintained over a wide range of temperature of 20-50°C and maximum catalytic activity was observed at pH 8. The enzyme displayed preference in hydrolyzing AHLs with long acyl chains. In conclusion, AHL degrading enzyme of *S. minutiscleroticus* appears to be a good candidate for further development into an antipathogenic drug.

Conflict of interests

The authors did not declare any conflict of interest.

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