Malaysian Journal of Microbiology, Vol 10(2) 2014, pp. 80-91
http://dx.doi.org/10.21161/mjm.53613

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Screening and preliminary characterization of quenching activities of soil *Bacillus* isolates against acyl homoserine lactones of clinically isolated *Pseudomonas aeruginosa*

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Received 9 May 2013; Received in revised form 5 September 2013; Accepted 7 October 2013

Aim: Quorum quenching of *Pseudomonas aeruginosa* homoserine lactone signal molecules represents a new approach for control of infection of this pathogenic highly resistant microorganism. The present study aimed at screening of soil for quorum sensing inhibitory bacteria active against clinically isolated *P. aeruginosa* together with the characterization of their activity and finally identification of promising isolates.
Methodology and results: Using a fast, reliable and simple screening method, 161 bacterial isolates collected from soil from different places in Egypt were screened for their quorum quenching activity against synthetic hexanoyl homoserine lactone using *Chromobacterium violaceum* mutant strain (CV026) as a biosensor. All 32 positive isolates were found to belong to *Bacillus* species. Secondary screening against the signals extracted from seven *Pseudomonas* isolates, analyzed by thin layer chromatography, was done. The activity of all the positive isolates was found to be intracellular. Activity against different concentrations of synthetic hexanoyl homoserine lactone showed that some isolates could degrade more than 20 µM even when diluted 100 fold. Selected isolates were found to have broad spectrum activity against other synthetic homoserine lactone standards. Maximum activity for most of the selected isolates was found to occur between 25-60 °C. Crude enzyme extracts of the promising isolates were collected by sonication, protein concentrations of the obtained extracts were measured and their activities were compared by well diffusion method. Finally, the isolates with promising quorum quenching activities were identified using 16S ribosomal RNA sequencing.
Conclusion, significance and impact of study: Having high activity against homoserine lactone autoinducers, the enzyme produced by *Bacillus* isolates represents a new promising antipathogenic drug suppressing *Pseudomonas* virulence.

Keywords: Quorum sensing, acyl homoserine lactones, soil *Bacillus* isolates, quenching activity

INTRODUCTION

Quorum sensing (QS) is a type of gene expression regulation that depends on bacterial population density. This system employs low molecular weight signal molecules, termed autoinducers, that accumulate in the environment as the population grows (Fuqua *et al.*, 1994; Khmel and Metlitskaya, 2006). When a bacterial population increases to a critical level, autoinducers accumulate to a certain threshold concentration after which they affect a change in the gene expression of the population by re-entering the cells and either interacting with a transcription factor or by attaching to a cell-surface receptor causing a signaling cascade that eventually induces differential gene expression (Fuqua *et al.*, 1994; Fuqua *et al.*, 1996; Swift *et al.*, 2001; Cámara *et al.*, 2002; Sifri, 2008). Different functions and phenotypes of bacteria are known to be controlled by quorum sensing. Most importantly, bacterial virulence is now known to be, in many cases, controlled by quorum sensing (Antunes *et al.*, 2009; Galloway *et al.*, 2011). The opportunistic pathogen *P. aeruginosa* possesses two well identified N-acyl homoserine lactone (AHL) quorum-sensing systems that regulate numerous, overlapping sets of genes (Van-Delden and Iglewski, 1998; Schuster and Greenberg, 2006). The QS regulon in *P. aeruginosa*, is believed to encode one third of the virulence factors including elastase, alkaline protease, rhamnolipids, phenazines, lectins, chitinases and other numerous proteins with unknown functions (Hentzer and Givskov, 2003; Schuster *et al.*, 2003; Vasil, 2003; Wagner *et al.*, 2003; Rasmussen *et al.*, 2005; Diggle *et al.*, 2007). Also AHLs have been detected in sputum samples collected from individuals with cystic fibrosis who were infected with *P. aeruginosa* (Singh *et al.*, 2000; Erickson *et al.*, 2002). These studies support the central role for AHL quorum sensing in *P. aeruginosa* disease (Sifri, 2008). In addition to that, genetically engineered quorum sensing system mutants

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

have shown to have reduced virulence in several animal models of infection (Rumbaugh *et al.*, 1999; Pearson *et al.*, 2000; Wu *et al.*, 2001).

The discovery and elucidation of bacterial QS systems has also been paralleled by growing interest in the ability to manipulate signal reception and transduction. Today, one global concern is the control of infection and virulence of highly antibiotic resistant bacteria as *P. aeruginosa*. Thus, the development of novel therapeutic approaches constitutes a major point of modern research. The alternative to antibiotic-mediated bacteria killing or growth inhibition is attenuation of bacterial virulence such that the organism fails to establish successful infection. Compounds with such abilities are termed antipathogenic drugs as opposed to antibacterial drugs (i.e., most traditional antibiotics). Antipathogenic drugs target key regulatory bacterial systems that govern the expression of virulence factors (Hentzer and Givskov, 2003). A search for enzymes degrading the AIs of QS systems is promising for designing such antipathogenic drugs (Khmel and Metlitskaya, 2006). Many different bacteria belonging to various genera have been reported to express activity degrading AHLs where recent studies show that acyl-homoserine lactone (HSL) signaling molecules can be degraded by enzymes produced in several genera of soil bacteria (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000; Flagan *et al.*, 2003; Park *et al*., 2003). Examples include lactonases from *Bacillus* and *Arthrobacter*, acylases from *Streptomyces* and *Variovorax paradoxus* in addition to several other bacteria (Czajkowski and Jafra, 2009; Hong *et al.*, 2012). The aim of the present study was to screen soil for bacteria with high quorum sensing inhibitory activity against both synthetic HSL standards and natural signals produced by seven clinically collected *P. aeruginosa* isolates whose signals were characterized using TLC. Activities of positive isolates were characterized for selection of isolates with potential activity against pathogenic *P. aeruginosa* clinical isolates followed by identification of these isolates to be further studied.

MATERIALS AND METHODS

Chemicals

All chemicals were of high quality from available grades supplied (unless otherwise indicated) by El-Nasr Chemicals (ADWIC), Egypt. Acyl homoserine lactone standards [butanoyl (C_4), haxanoyl (C_6), heptanoyl (C_7), and octanoyl (C_8) homoserine lactone] were purchased from Sigma-Aldrich, Germany. Reagents for DNA extraction and 16S rRNA identification were a product of Fermentas, USA.

Bacterial isolates and their maintenance

Chromobacterium violaceum CV026

CV026 is a mini-Tn*5* mutant of *Chromobacterium violaceum* and acts as an Acyl homoserine lactone dependent biosensor, producing the characteristic purple pigment violacein in response to the presence of the AHL *N*-hexanoyl homoserine lactone (C6HSL) (McClean *et al.*, 1997). CV026 is kanamycin resistant and so it was subcultured in medium containing 20 µg/mL kanamycin for purification (Ravn *et al.*, 2001). CV026 was subcultured in Luria Bertani (LB) broth for maintenance. It was stored in slant medium and also in lyophilized form for long term preservation.

Pseudomonas aeruginosa isolates

Seven *P. aeruginosa* isolates, screened for their production of acyl homoserine lactone signal molecules, were used in this study. These isolates were recovered from some clinical specimens. These isolates were routinely maintained on LB slants at 4 °C, subcultured every month. For long term preservation, the isolates suspended in phosphate buffered saline or in slant medium were stored at 4 °C and –20 °C, respectively.

Bacterial isolates from soil

A number of 161 isolates were collected from 18 soil samples from different places in Egypt. These isolates were screened for their AHL degrading activity. The isolates were recovered using R_2A agar and maintained on nutrient agar slants.

Extraction of acyl homoserine lactone signal molecules from *Pseudomonas* isolates

This was done according to Ravn and his coworkers (Ravn *et al.*, 2001) where AHL signals were extracted from supernatants of fresh cultures of *P. aeruginosa* using ethyl acetate. The ethyl acetate was evaporated then the extracts were reconstituted in 0.5 mL acidified ethyl acetate, transferred into Eppendorf tubes and stored at –20 °C.

TLC analysis of the extracted HSL signal

To evaluate the profiles of AHLs, TLC was carried out according to the method described by Shaw and his coworkers (Shaw *et al.*, 1997) with some modifications. About 40 µL samples were applied to C18 reversed-phase thin-layer chromatography (TLC) plates (Sigma, Germany). Samples were separated using methanol (60%, v/v) in water as the mobile phase. Once the solvent front migrated up to 2 cm apart from the plate top, plates were air dried. For detection of AHLs, the TLC plate was overlaid with a thin film of 0.8% (w/v) LB agar (100 mL) seeded with 7 mL of an exponentially grown AHL biosensor and was then incubated at 30 °C for 24 h. AHLs were identified by comparing the retention factor of synthetic standard AHLs and test AHL spots. Standard AHLs used were butanoyl (BHL), hexanoyl (HHL), heptanoyl and octanoyl homoserine lactones.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Recovery of bacterial isolates from soil

Eighteen soil samples were collected from different places in Egypt and stored at 4 °C. The samples were taken from a depth of 10 cm below the earth's surface. This precaution was taken to enhance the recovery of bacterial isolates as the surface microbial flora is largely affected by the UV rays of sunlight. Isolates were recovered from the soil samples using the method developed by Bodour *et al.*, 2003 with some modifications. A 5 g amount of each sample was placed into a 250 mL flask containing 50 ml of tap water and incubated at 30 °C in a shaking incubator at 200 rpm for 4 days. Then, a sample from each soil slurry was serially diluted and plated on R_2A agar. The plates were then incubated for 2 days at 37 °C and morphologically different colonies were coded, restreaked on R_2A plates for purification. Pure cultures of the recovered isolates were subjected to Gram stain to classify them according to their Gram reaction and then each isolate was transferred onto nutrient agar slants, incubated at 37 °C for 24 h and then stored at 4 °C until screening for quenching activities against quorum signal molecules was done.

Primary screening using synthetic signal molecule (HHL)

Screening was done according to Jafra and his coworkers (Jafra and van-der-Wolf, 2004), with some modifications as follows: bacterial isolates were cultured in 1 mL aliquots of LB broth and incubated overnight at 28 °C under shaking conditions (160 rpm). CV026 was grown overnight at 28 °C in 5 mL LB broth with shaking at 160 rpm then the count of the overnight culture was adjusted to 10^8 cfu/mL. In each well of a microtitre plate, about 50 µL of 0.5 µM HHL were overlaid by 50 µL of bacterial culture (undiluted and diluted 10 fold), incubated for 4 h at 28 °C before being exposed to UV germicidal lamp (working at 254 nm) for 90 min after which, 50 µL aliquot of CV026 culture of adjusted count was added to each well. The plates were incubated overnight at 28 °C where CV026 produced purple pigment in wells where HSL is present. While, in the wells where HSL was degraded by bacterial isolates, no color was produced. Positive control was made by adding 50 µL plain LB broth instead of bacterial culture.

Secondary screening of bacterial soil isolates with AHL quenching activities using *Pseudomonas* extracts

This was done using 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 also done according to the protocol described by Jafra and van-der-Wolf (2004), as follows: about 0.3 mL aliquots of the *Pseudomonas* extract were mixed with 1.5 mL aliquots of M63 buffer, pH 7.0 prepared according to Krieger-Brauer and his coworkers (Krieger-Brauer *et al.*, 1980). Fifty microliter aliquots of the resulting solution (1.8 mL) were transferred into wells of a microtitre plate, overlaid with 50 µL aliquots of the bacterial culture of test isolates and the assay was completed as described in the primary screening. Positive control for *Pseudomonas* extracts was done using plain LB broth instead of bacterial cultures. Secondary screening was done in three replicates for confirmation of the results.

Characterization of the AHL quenching activity of the promising *Bacillus* soil isolates

Detection whether the AHL quenching activity is extracellular or intracellular

Positive isolates were grown in 1 mL LB broth overnight at 28 °C with shaking at 160 rpm then they were centrifuged at 6000 rpm for 15 min. Supernatants were then collected and filtered using sterile membrane filters of pore size 0.22 µM while bacterial cells were reconstituted in 1 mL saline. Both cells and supernatants were prepared in both undiluted and diluted (10 fold) forms and 50 µL of each was applied in the assay that was carried out as described in the primary screening and the activity of both cells and supernatants were examined after 24 h of incubation.

AHL quenching activity at different concentrations of HSL

Here, the assay was done using the positive isolates and the activity was measured at different concentrations of synthetic HHL; 0.5, 2, 5, 10 and 25 µM. The 4 h incubation period of the primary screening assay was applied.

AHL quenching activity at different dilution levels

Different bacterial culture dilutions (undiluted, diluted 10 and 100 fold) were screened against different concentrations of HSL (0.5, 2, 10, 25 µM).

AHL quenching activity at shorter incubation periods

This was done for selected promising isolates where the incubation period of the isolates whole cultures with HSL was reduced once to 2.5 h and 1 h before exposure to UV germicidal lamp.

Effect of incubation temperature on AHL quenching activity of the promising positive isolates

This assay was done as in the primary screening, but the incubation of the bacterial whole cultures and different concentrations of synthetic signal molecule was done at different temperatures, 5, 15, 25, 35, 40, 50, 60, 70 and 80 °C.

Study of AHL quenching activity spectrum

The promising isolates were screened for their activity against standard butanoyl, heptanoyl and octanoyl homoserine lactone. This was carried out as in the

ISSN (print): 1823-8262, ISSN (online): 2231-7538

primary screening using different concentrations of synthetic standards (2, 5, 10, and 25 µM) and different dilutions of the bacterial cultures (undiluted, diluted 10 and 100 fold). Incubation of the bacterial cultures and synthetic signals took place once for 4 h and once for 1 h before exposure to UV germicidal lamp.

Preparation of crude enzyme extract by sonication

Bacillus isolates were grown for 16-18 h in 5 mL LB broth at 28 °C with shaking at 160 rpm. The cells were collected by centrifugation, washed twice with cell washing buffer (Tris-HCl pH 7.5, 25.0 mM), then cells were resuspended in cracking buffer [Tris-HCl pH 7.5, 25.0 mM and Dithiotheritol (DTT) 1.0 mM]. The cells were disrupted by sonication while the tubes containing cell suspensions were immersed in ice bath. Sonication was applied intermittently for 1 min operation and 30 sec stop period for a total operation time of 7 min is applied for 1 min and stopped for 30 sec with total time of 7 min of intermittent sonication. Measuring the absorbance at 600 nm every 1 min was done to validate the efficiency of the sonication process (Fykse *et al.*, 2003). After sonication, Centrifugation was done at 15000 rpm for 15 min to remove the unlysed cells and cell debris (Boyer *et al.*, 2008) and finally the supernatants produced, containing the crude enzyme, were collected.

Measurements of the total protein concentrations of the prepared crude enzyme extracts

Protein concentration was measured by the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Samples and standards were prepared and measured in duplicates and the absorbance values measured were averaged. A calibration curve of absorbance as a function of protein concentration of the standard solutions was plot and used to determine protein concentrations of the samples. For all the tested isolates, the total protein concentration was finally adjusted to 3.5 mg/mL using cracking buffer.

Evaluation of AHL quenching activity of crude enzyme extracts of tested Bacillus isolates using well diffusion method

This assay was done according to (Ravn *et al.*, 2001) where 10 mL LB agar was overlaid by 10 mL semisolid LB agar containing synthetic HSL at concentration 100 nM seeded with the biosensor strain (CV026) . When the overlaid agar had solidified, wells were punched in the agar with a sterile Cork borer (diameter 10 mm). After that, 90 µl of the crude extract of the promising isolates were pipetted into the wells. The plates were then incubated at 28 °C for 24 h. The growth of CV026 showed purple color in the whole plate except the zones around the wells. The experiment was done twice and the diameters of the formed inhibition zones were measured.

Identification of soil *Bacillus* isolates with promising AHL quenching activity

Chromosomal DNA extraction

It was done according to (Pospiech and Neumann, 1995) and the extracted chromosomal DNA was finally dissolved in 500 µL TE buffer containing RNAse with a concentration of 100 µg/mL. It was then placed in eppendorf tubes as aliquots of 20 µL each, stored at –20 °C.

16S rRNA gene analysis

Sequencing of 16S rRNA genes was done at GATC Company, by the use of ABI 3730xl DNA Sequencer. The obtained 16S rRNA gene sequence files of the selected isolates were assembled using Staden package program version 3 (Staden, 1996) and finally the sequences were compared with those in GenBank database using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the identification of the isolates.

RESULTS AND DISCUSSIONS

TLC analysis of the extracted HSL signal

Extracts of seven clinically isolated *Pseudomonas* isolates that were previously screened for producing AHL were used in this study. The relative quorum sensing signal productivities of the seven *Pseudomonas* isolates were previously studied and shown in (Table 1). Separation of AHLs by TLC coupled with their detection by CV026 biosensor gave an identifying index of the AHL signals produced by the test bacteria. Violacein production in CV026 was proved to be inducible by all the AHL compounds with *N*-acyl side chains from C4 to C8 in length, with varying degrees of sensitivity (McClean *et al.*, 1997). Results of TLC analysis showed that 3 of the tested *P. aeruginosa* isolates (P27, P19 and P14) gave only one purple spot of Rf (retardation factor) value similar to that of standard hexanoyl HSL. While the other 4 isolates (P13, P16, P17 and P18) showed two purple spots-one having Rf value similar to that of standard HHL and the other having Rf similar to that of standard BHL, Figure 1. No spots were found to be parallel to that of heptanoyl or octanoyl homoserine lactone standards. Although it was reported in previous studies that *Pseudomonas* isolates produced a minimum of four detectable signals (Shaw *et al.*, 1997), only two spots could be detected for four of the tested isolates and one spot for the other three in the present study. This indicates that the extracts of 7 isolates contained only 2 types of signals that can be detected by the biosensor used, CV026. In 2011, a study on clinical isolates of *P. aeruginosa* showed that two to six different AHLs were detected in most of the test isolates (Kumar *et al.*, 2011). However, the same study stated that using CV026, only one signal, identified as hexanoyl HSL, was detected. The absence of a spot does not necessarily mean that the *P.*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

aeruginosa isolate does not produce such a signal. Non-detection could be due to the low production of the signal relative to the sensitivity of the biosensor used. From the previous results, identification of spots, based on migration of standards, show that *P. aeruginosa* isolates P14, P19 and P27 produce only one signal detected by CV026, HHL, while P13, P16, P17 and P18 produce 2 signals detected by CV026, identified as BHL and HHL.

Table 1: The relative quorum sensing signal productivities of seven *Pseudomonas* isolates and the signals identified by TLC.

Pseudomonas isolate code no.	Amount of signal produced in terms of HHL of growth supernatant (µM)	No. and identity of signals detected by TLC
P13	0.283	2 (BHL, HHL)
P14	0.086	1 (HHL)
P16	0.006	2 (BHL, HHL)
P17	0.194	2 (BHL, HHL)
P18	0.026	2 (BHL, HHL)
P19	0.095	1 (HHL)
P27	0.395	1 (HHL)

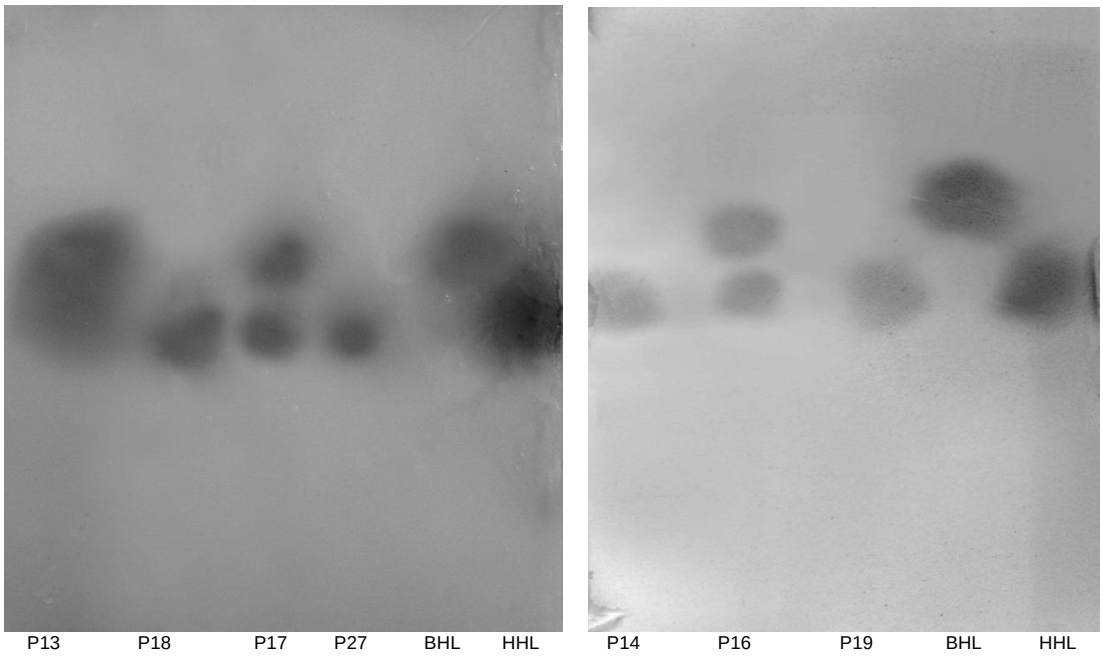

Figure 1: TLC chromatograms of the extracted AHL signals of *P. aeruginosa* isolates (P13, P14, P16, P17, P18, P19 and P27), as detected by CV026 biosensor. HHL, standard hexanoyl homoserine lactone; BHL, standard butanoyl homoserine lactone.

Recovery of bacterial isolates from soil

A total of 161 isolates were recovered from 18 soil samples. All the soil samples had viable count ranging between ($9x10^4$) and ($9x10^7$) cfu/gram. The recovered isolates were classified according to their Gram reaction and their morphology under the microscope. It was found that 66.45% of the isolates were Gram positive bacilli, 14.9% Gram negative rods, 11.18% Gram positive cocci, and 7.45% Gram negative coccobacilli.

Primary screening for AHL quenching activity of recovered bacterial soil isolates using the synthetic signal molecule HHL

Results showed that 27 isolates have the ability to breakdown the HHL, both in the undiluted and 10 fold

ISSN (print): 1823-8262, ISSN (online): 2231-7538

diluted forms, giving no purple color after incubation of treated substrate (HHL) with CV026, while those lacking this ability (negative isolates) showed the purple color of violacein after incubation of the treated substrate (HHL) with CV026. Other five isolates (codes 62, 100, 101, 102 and 114) could completely (no purple color with the biosensor) degrade and 2 other isolates (codes 31 and 144) could partially (faint purple color with the biosensor) degrade the signal molecule only in the undiluted form of the whole culture. The 2 isolates coded 31 and 144 were excluded together with the negative isolates and not subjected to the secondary screening. Although several genus of bacteria isolated from soil were previously reported to have the ability to degrade acyl homoserine lactone signal- like *Arthrobacter* (Park *et al.*, 2003), *Variovorax paradoxus*, *Agrobacterium* (Zhang *et al.*, 2002), *Acinetobacter* (Kang *et al.*, 2004) in the present study results showed that all the positive or even partially positive isolates belong to the genus *Bacillus* where 29.9% of the *Bacillus* isolates gave positive results and 1.86% showed partially positive activity. This indicates the high prevalence of quorum quenching activity among *Bacillus* isolates where approximately one third of the isolates turned to have quorum quenching activity. We believe that even giving a negative result in the screening process does not imply the disability of the other *Bacillus* isolates to produce quorum quenching enzymes as it might be due to the presence of such enzymes in inadequate quantities, insufficient to degrade the concentration of signal used in the assay.

Secondary screening for AHL quenching activity of recovered bacterial soil isolates using *Pseudomonas aeruginosa* extracts

This was done to examine the spectrum of quorum sensing inhibitory activity of the 32 positive *Bacillus* isolates against the extracts of the seven clinically isolated *P. aeruginosa* isolates that were characterized by TLC. Results showed the following; the degrading activity of AHL signals contained in the seven extracts of the tested *P. aeruginosa* isolates was demonstrated by the *Bacillus* isolates (65, 78, 112 and 115), and for six extracts by *Bacillus* isolates (12, 16, 20, 29, 48, 49, 54, 58, 66, 68 and 87) and for five extracts by isolates (47, 50, 70, 97, 100 and 139) when tested in undiluted forms. Other tested *Bacillus* isolates showed activity against equal to or less than four extracts of tested *P. aeruginosa* isolates also when tested in undiluted forms and isolate 114 was found to lack activity on extracts of all *Pseudomonas* isolates. Some *Bacillus* isolates also showed high HHL degrading activity as they still exhibited HHL degrading activity in the 10 fold diluted form. The *Bacillus* genus is reported to exert its quorum quenching activity due to the presence of lactonase enzyme which catalyzes the lactone ring opening in the homoserine moiety of AHLs, without affecting the rest of the signal molecule structure (Dong *et al.*, 2000), so it was expected that activity of positive isolates on *P. aeruginosa* extracts would be the same which was not the case. The failure of some of the isolates which gave positive results in primary screening to degrade AHL signals in the extracts of *P. aeruginosa* may be due to one of the following reasons. The first is that more time was required for the positive isolates to exert their acyl homoserine lactone degrading activity. The second reason may be attributed to the presence of some sort of antagonism between the extracts of *P. aeruginosa* and *Bacillus* cultures. Some studies reported the presence of such antagonism between *Pseudomonas* and *Bacillus* (Simoesa *et al.*, 2008). Some isolates of *P. aeruginosa* are also known to produce lipopeptides which can be extracted using ethyl acetate (Smyth *et al.*, 2010). Studies on lipopeptides have proved that they have antimicrobial activities against some *Bacillus* species (Berry *et al.*, 2012; Govindammal and Parthasarathi, 2013; Lotfabad *et al.*, 2013). Another possibility might be attributed to the presence of an enzyme inhibitor. Despite all these possible explanations, the reason behind the variability of results among *Bacillus* isolates remains unclear. Figure 2 shows the results of secondary screening from which isolates coded 58, 65, 68, 78, 112, and 115 are considered the most promising isolates with reference to their spectrum of activity on *Pseudomonas* signals followed by isolates coded 16, 20, 48, and 66.

Detection whether the AHL quenching activity is extracellular or intracellular

None of the supernatants of the positive isolates showed any positive results, neither in undiluted nor diluted 10 fold forms, while all the cells reconstituted in saline showed activity in both undiluted and diluted forms. This indicates that the quorum sensing inhibitory activity of all *Bacillus* isolates is not extracellular. It is rather intracellular, most probably due to the presence of Lactonase enzyme as stated in literature.

AHL quenching activity at different concentrations of HSL

Results in Figure 3 showed that six isolates, coded (12, 16, 58, 68, 78 and 112), have the highest activity. They have the ability to degrade synthetic hexanoyl homoserine lactone of up to concentration 25 µM in volumes equal to that of the *Bacillus* culture both in undiluted or diluted 10 fold forms. Two isolates, coded 29 and 65 can degrade up to 25 µM of equal volume to the undiluted culture and when diluted 10 fold can degrade up to 10 µM of equal volume. Three other isolates, coded (115, 139 and 48) can degrade up to 10 µM in a volume equal to undiluted culture or 2 µM in diluted 10 fold culture. From these results and the secondary screening results, seven isolates were chosen to be further studied. These were isolates with code no. 16, 58, 65, 68, 78, 112 and 115.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

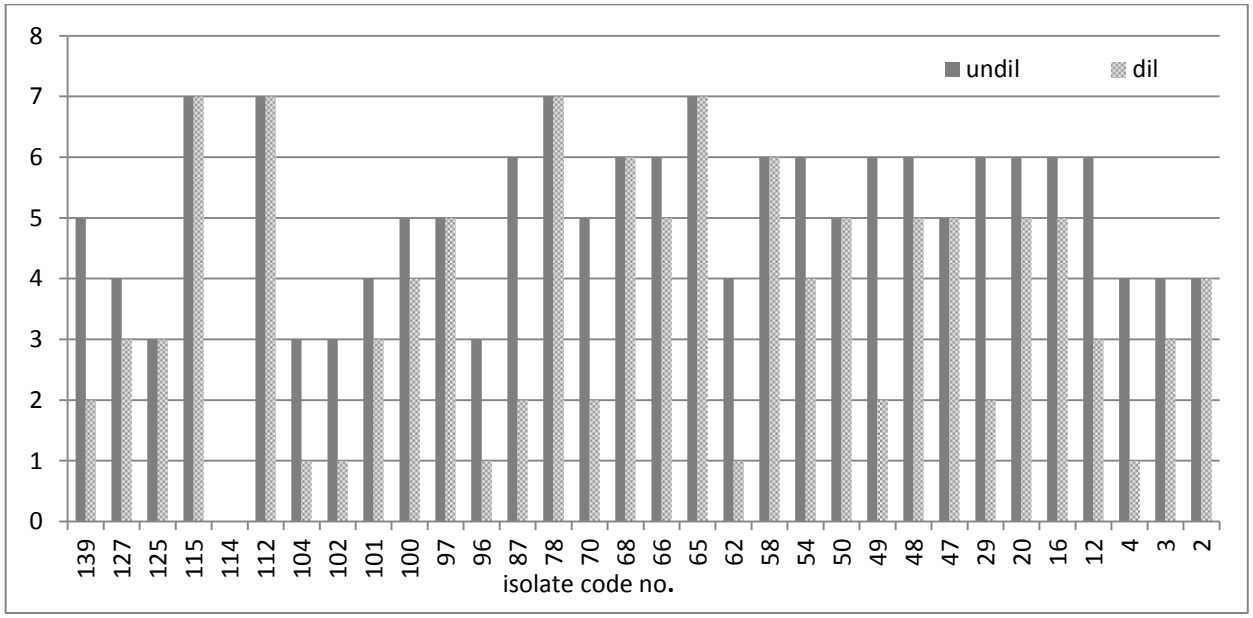

Figure 2: Quenching activity profile of tested *Bacillus* isolates against AHL signals of seven clinically isolated *P. aeruginosa* extracts.

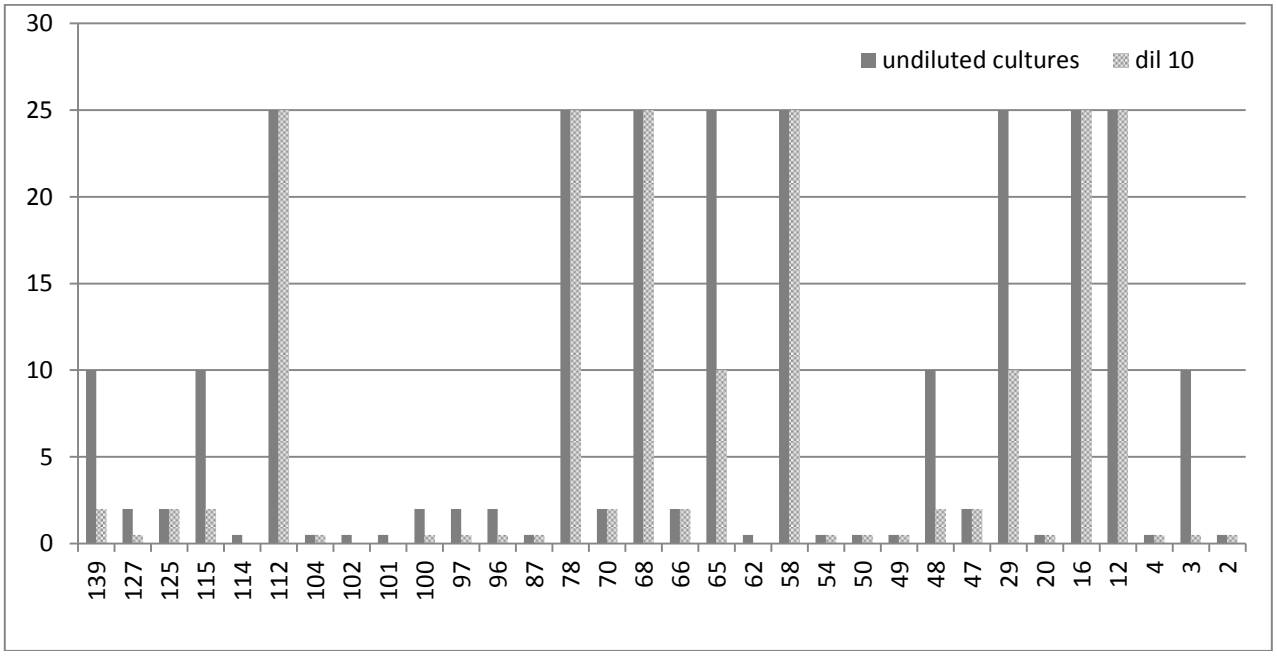

Figure 3: AHL quenching activity at different concentrations of HSL.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

AHL quenching activity at different dilution levels

Results showed that the seven selected isolates have high activity on HHL even when applied in 100 fold dilution form. Activity of isolates coded 16 and 78 was not affected by dilution. Isolate coded 112 could degrade up to 10 µM when diluted 100 fold while three isolates (codes 58, 65, and 68) could degrade HHL of concentration 2 µM and isolate coded 115 could degrade only 0.5 µM when diluted 100 fold.

AHL quenching activity at shorter incubation periods

When the incubation period was reduced to 2.5 h, 5 isolates (coded 58, 65, 68, 78 and 112) could degrade up to 25 µM HHL when used in undiluted form while isolates coded 16 and 115 could degrade up to 10 µM. In 1 h, isolates coded 16, 58, 65, 68 and 78 could degrade up to 10 µM while isolates coded 112 and 115 could degrade up to 2 µM when used in undiluted form. When used in diluted 10 and 100 fold forms, the highest activity was expressed by isolate (78) which could degrade up to 25 µM and 2 µM in 2.5 h when diluted 10 and 100 fold respectively. It could also breakdown a concentration of 2 µM in 1 h even when diluted 100 fold. This assay reflects the rate of degradation of AHLs by selected isolates. Previous studies showed that within 2 h, up to 20 mM 3-oxo-C6 HSL can be completely inactivated by a suspension culture producing lactonase enzyme (Rasmussen and Givskov, 2006).

Effect of incubation temperature on AHL quenching activity of the promising positive isolates

Most of the isolates showed maximum activity between 25 and 60 °C having their activities almost unchanged in undiluted or even diluted forms. Activity decreased at 70 °C except for isolates 16 and 115 and for the undiluted culture of isolate coded 78 while at 80 °C the positive control did not show purple color at concentration 0.5 µM of the signal molecule so the results could not be assessed. Isolate 115 showed a maximum activity between 15 and 70 °C while the activity of isolate coded 112 and diluted culture of isolate coded 65 slightly decreased at 60 °C. All the isolates showed considerable activities at 5 and 15 °C where undiluted cultures of 5 isolates could degrade up to 10 µM at 5 °C and the other two isolates, codded 65 and 115, could degrade 2 and 0.5 µM respectively. The effect of incubation temperature on the activity of isolate coded 78 is represented in Figure 4. Retaining maximum activity within such a wide range of temperature is considered very positive as it reflects the relative stability of the enzyme produced by these isolates.

Study of AHL quenching activity spectrum

Like their activity on HHL, the seven selected isolates showed high activity on butanoyl, heptanoyl and octanoyl homoserine lactone standards. Results in Figure 5 and Figure 6 show that in 4 h, they could degrade up to 25 µM and up to 10 µM in 1 h except for isolate 115 which could degrade only 10 µM and 2 µM of the signal in 4 h and 1 h respectively. This indicates that these isolates have a broad spectrum of activity against acyl homoserine lactones. Several studies support that lactonase enzyme has a non-specific substrate activity against homoserine lactones. For example, in 2004, Wang and coworkers carried out a study on a lactonase enzyme that displayed strong enzyme activity towards all tested AHLs (Wang *et al.*, 2004). Also in 2012, another study on the hydrolytic activity of an enzyme, $AiiA_{A196}$ from a *Bacillus* species proved that it had a broad spectrum including C4-HSL, C6-HSL, C7-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3-oxo-C8-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, 3-hydroxy-C8-HSL, and 3-hydroxy-C14-HSL (Cao *et al.*, 2012).

Evaluation of AHL quenching activity of crude enzyme extracts of tested *Bacillus* isolates using well diffusion method

Using the crude enzyme extracted by sonication, the activity of the seven isolates was compared by well diffusion method. Results showed that purple color was observed in the whole plate except around the wells were the activity of the crude enzyme of the *Bacillus* isolates degraded the signal molecule around the wells thus inhibiting the development of purple color. The recorded inhibition zone diameters of the crude enzyme extracts of the tested isolates ranged between 21-23 mm (Table 2).

Identification of soil *Bacillus* isolates with promising AHL quenching activity

The sequences of 16S RNA when compared with GenBank database using BLAST were found to belong to the following *Bacillus* species, isolates 58, 68, 78, 112 and 115 were identified as *Bacillus cereus*. Isolate 16 was identified as *Bacillus thuringiensis* while isolate 65 was identified as *Bacillus weihenstephanensis.*

Table 2: AHLa quenching activity of the selected *Bacillus* isolates as determined by well diffusion method.

Bacillus isolate code no.	AHL quenching activity expressed as inhibition zone diameter[b] (mm)
16	23
58	21.5
65	21
68	22.5
78	23
112	22
115	23

[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.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

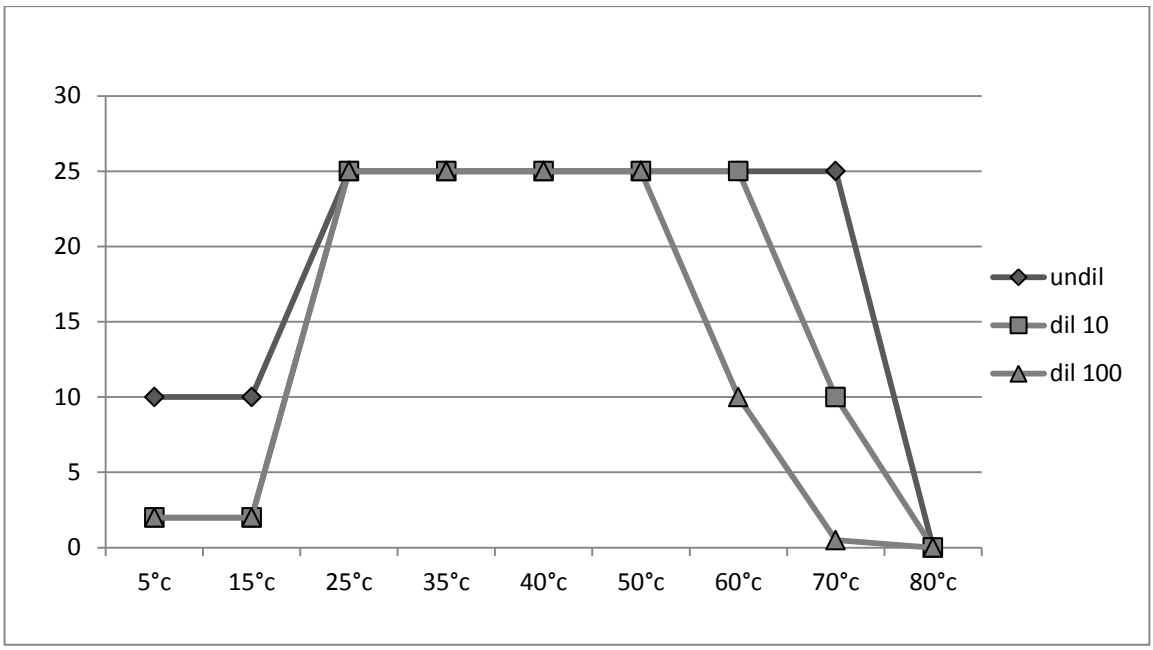

Figure 4: Effect of incubation temperature on AHL quenching activity of the isolate coded 78.

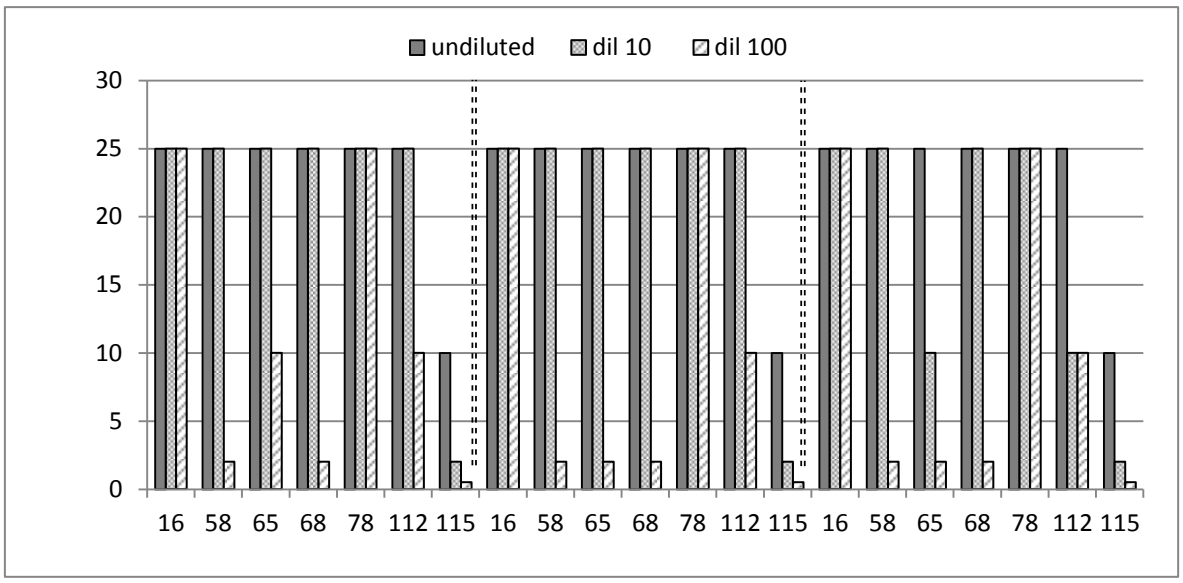

Figure 5: AHL quenching activity spectrum of the seven selected isolates against synthetic HSL standards; C4, C7 and C8 HSL after 4 h.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

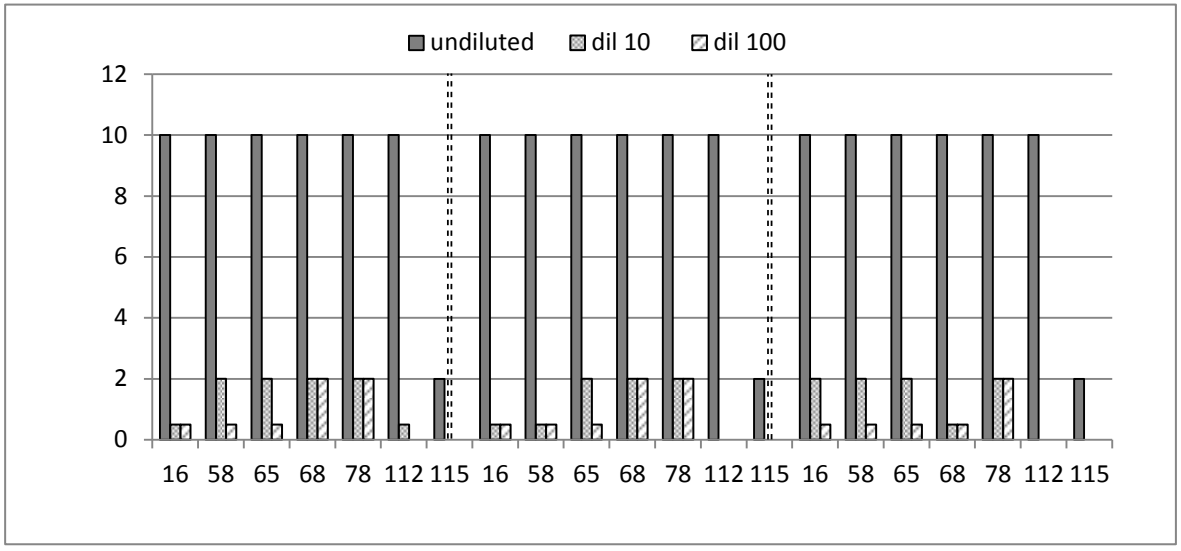

Figure 6: AHL quenching activity spectrum of the seven selected isolates against synthetic HSL standards; C4, C7 and C8 HSL after 1 h.

CONCLUSION

In this study, screening of soil for AHL quenching activity bacteria revealed that seven *Bacillus* isolates have promising activity against acyl homoserine lactones. These isolates showed activity against high concentrations of both synthetic and naturally occurring signals that were separated and identified from clinically isolated *P. aeruginosa*. Besides, they proved to maintain maximum activity over a wide range of temperature and possess a broad spectrum when tested against different synthetic signal molecules. The crude enzyme extracts of the tested *Bacillus* isolates with AHL degrading activities were prepared by sonication and characterized. Identification using 16S rRNA was done and revealed that five out of the seven tested isolates were identified as *Bacillus cereus* and the other two isolates were identified as *Bacillus thuringiensis* and *Bacillus weihenstephanensis,* respectively. The study outcome contributes to the development of an alternative therapeutic approach for bacterial infections that be used with or without antimicrobial agents.

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