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Quorum quenching activity in cell-free lysate of endophytic bacteria isolated from *Pterocarpus santalinus* Linn., and its effect on quorum sensing regulated biofilm in *Pseudomonas aeruginosa* PAO1

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ABSTRACT

Quorum sensing mechanism allows the microorganisms to resist the antibiotic treatment by forming biofilms. Quorum quenching is one of the mechanisms to control the development of drug resistance in microbes. Endophyte bacteria are beneficial to plant growth as they support the immune system against the pathogen attack. The endophytic bacteria present in *Pterocarpus santalinus* were screened for the presence of N-acyl homoserine lactones (AHLs) degrading bacteria using biosensor strains and further confirmed by quantifying the violacein production. Cell-free lysate of endophytic bacteria, *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 exhibited potent AHL degrading ability by inhibiting about 80% violacein production in biosensor strain. Furthermore, when the cell-free lysate was applied to *Pseudomonas aeruginosa* PAO1 and PAO1-JP2 biofilm it resulted in significant ($p < 0.01$) inhibition of biofilm formation. The biofilm inhibition was confirmed by visualization of biofilm slides under fluorescence microscopy, which showed decrease in total biomass formation in treated slides. Isolation and amplification of the gene (*aiiA*) indicated that the presence of AHL lactonase in cell-free lysate and sequence alignment indicated that AiiA contains a "HXHXDH" zinc-binding motif that is being conserved in several groups of metallohydrolases. Therefore, the study shows the potential of AHLs degradation by AHL lactonase present in cell-free lysate of isolated endophytic bacteria and inhibition of quorum sensing regulated biofilm formation in *P. aeruginosa* PAO1.

Introduction

Quorum sensing (QS) is a vital and important mechanism in single celled organisms such as bacteria. Autoinducers like *N*-acylhomoserine lactones (AHLs) are widely conserved signal molecules in quorum sensing systems in Gram negative bacteria (Dong et al. 2000). AHLs are small, diffusible molecules which regulate cell density dependent functions in bacteria, and induce or repress the gene expression when the threshold concentration is reached (Uroz and Heinonsalo 2008). Earlier research has shown the importance of AHLs in the regulation of a range of biological functions in *Vibrio* species (Dong et al. 2002). Some Gram negative food-associated bacteria regulate the biofilm formation, motility and exoprotease production by quorum sensing systems (Medina-Martinez et al. 2007). Quorum sensing actively controls the virulence factors, since treatment with antibiotics make the harsher environment leads to the emergence of multidrug resistance in bacteria which makes more survival of pathogenic bacteria by forming biofilms (Chan et al. 2011). As *Pseudomonas aeruginosa* is capable of developing biofilm, it resists multidrug treatments and emerges as a multidrug resistant strain. Basically, it produces *N*-(butanoyl) homoserine lactone and *N*-(3-oxododecanyl) homoserine lactone as major QS molecules; it enhances the cell growth as well as the formation of biofilm (Flickinger et al. 2011).

Endophytic bacteria colonize internal plant tissues without substantially harming it. Beneficial effects observed in many endophytes includes tolerance to various environmental conditions, the resistance against pathogens and act as effective biocontrol agent (Garafola et al. 2009). Quorum sensing in endophytic *Serratia plymuthica* is controlled by the production of AHLs as signaling molecules, which phenotypically regulate the motility and formation of biofilm (Liu et al. 2011). In ginger (*Zingiber officinale*) rhizosphere *Acinetobacter* and *Burkholderia* exhibited co-existence of quorum sensing and quorum quenching (QQ) activities. These bacteria could degrade a wide range of AHLs, which is referred as collective role in QS-dependent phenotype of a polymicrobial community (Chan et al. 2011).

Quorum sensing inhibitors from natural and synthetic sources are reported to be most effective in preventing quorum sensing regulated gene expression (Morohoshi et al. 2012).

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Quorum quenching (QQ) activity leads to inactivation/degradation of QS molecules (Barrios et al. 2009), as it prevents sufficient accumulation in the bacterial cell thereby disrupting the quorum sensing (Medina-Martinez et al. 2007). Degradation of AHLs molecules is done by major two enzymes, lactonases and acylases (Rashid et al. 2011), as they are specifically acting to break lactone ring and acyl side chain, respectively. In *Bacillus* sp. degradation of AHLs take place in the presence of *aiiA* gene, which encodes the production of AHL-lactonase (Dong et al. 2000). In *Ralstonia* sp., the *aiiD* gene encodes AHL-acylase production (Lin et al. 2003). Therefore, degradation of QS molecules would help in the suppression of virulence related factors in both human and plant pathogens.

Endophytes symbiotically in relation with plant and produce many secondary metabolites including enzymes. *Pterocarpus santalinus* L. (*Fabaceae*), also named "red sandalwood", is a rare tree, commercially used as a substitute for teak. This species is distributed exclusively in Western Ghats of India. *P. santalinus* has been used as a folk remedy for the treatment of inflammation, mental aberrations, ulcers and control of diabetes (Krishnaveni and Rao 2000). The isolation of endophytic bacteria from *P. santalinus* with quorum quenching activity has not so far been reported. Therefore, endophytic bacteria were isolated from *P. santalinus* and cell-free lysate from these bacteria were screened for AHLs-degradation activity by using AHL biosensors, *Chromobacterium violaceum* CV026 and *Escherichia coli* MG4. The AHL-degradation activities were further tested on quorum sensing regulated biofilm formation in *P. aeruginosa* PAO1 wild type.

Methods

Endophytic bacterial strains

The *Pterocarpus santalinus* plant samples collected from Western Ghat forest of Karnataka, India. The plants were identified by consulting taxonomists and the herbarium of the plant was preserved in the Herbaria collection center of Department of Studies in Microbiology (MGMB/002/2011-2012), University of Mysore, Mysore, India. The leaves and bark were detached and surface sterilized as previously reported (Araujo et al. 2002; Rajesh and Rai 2013). Distilled water cleaned samples were soaked in 70% (v/v) ethanol for 2 min. It was then washed several times with sterilized water, dipped in 0.1% mercury chloride for 1 min, again washed with sterilized water 3–5 times and then washed with sterilized distilled water. The samples were then cut into small pieces, each piece put on a plate of Luria-Bertain (LB) agar medium and the plates were cultivated at 30 °C for 2 days to promote endophytic bacterial growth. Individual colonies were selected randomly and inoculated onto another LB plate, and incubated at 30 °C for 24 h. Each bacterial culture was checked for purity and the purified endophytic isolates were numbered, transferred separately to LB slants, and stored at 4 °C.

Bacterial strains and chemicals

AHL biosensor *C. violaceum* CV026 (a mutant, which responds to short chain of AHLs) was maintained in Luria-Bertani (LB) broth at 30 °C in rotary shaker incubator (Labline Industries, Cochin, India) for 24 h (McClean et al. 1997) and *E. coli* MG4 (pKDT17) which responds to long chain of AHLs was maintained in LB broth with 100 μg/ml ampicillin at 37 °C (Kumar et al. 2011). The authentic culture samples used in the study were procured from MTCC, IMTECH, Chandigarh, India. *Bacillus cereus* (MTCC 1272) used as positive control strain for AHL-degrading activity (Mani et al. 2012), *Pseudomonas aeruginosa* PAO1 (wild type) and PAO1-JP2 (*lasI* and *rhlI* mutant type was kindly provided by Kalai Mathee, Florida International University, USA) was used for biofilm study. *N*-Butanyl-L-homoserine lactone (C_4-HSL, gift from Dr. Sang Sun Yoon, Korea) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL, Sigma–Aldrich, Bangalore, India) were used as substrate for *C. violaceum* CV026 and *E. coli* MG4, respectively.

AHL degradation assay

Preparation of cell-free lysate

The endophytic bacterial isolates were grown in the minimal medium containing (per liter) NaCl, 1 g; KCl, 0.5 g; $MgCl_2$, 0.4 g; $CaCl_2$, 0.1 g; Na_2SO_4, 0.15 g; KH_2PO_4, 2 g; Na_2HPO_4, 2.25 g and trace elements were added to final concentration containing 1 mg $FeCl_3$, 0.1 g $MnCl_2$ and 46 mg $ZnCl_2$ per liter (Christiaen et al. 2011), incubated at 30 °C with shaking for 48 h followed by harvesting by centrifugation at 12,000 rpm for 10 min. The cell pellets were collected and resuspended in 10 ml of potassium phosphate buffer (PPB, 100 mM; pH 7.0) and ground to get cell-free lysate and centrifuged; supernatant was filtered through 0.45 μm filter and the filtrate was collected as "cell-free lysate" which was stored at −20 °C until use. All assays were performed with assay control (PPB) and positive control (cell-free lysate of *B. cereus* MTCC 1272).

AHLs degradation by agar overlay assay

Degradation of AHLs was detected by previously described method (Rashid et al. 2011) with some modifications. LB broth was taken in microcentrifuge tubes loaded with 50 μl of cell-free lysate supplemented with 400 μM of C_4-HSL to get 1.5 ml final concentration. Then tubes were incubated at 30 °C for 2 h. After incubation, it was heated at 95 °C for 10 min. The LB agar plates (10 ml of LB, 1.5% agar) were overlaid with *C. violaceum* CV026 biosensor (0.8% agar) and the wells were loaded with 0.45 μm filter sterilized 50 μl of the reaction mixture and incubated at 30 °C for 24 h along with positive and assay controls. The presence of clear zone (inhibition of violacein production) around the wells is the indication for AHLs degradation. The above assay was repeated using *E. coli* MG4 biosensor for 3-oxo-C_{12}-HSL signaling molecule supplemented with 40 μg/ml X-gal (5-bromo-3-indolyl-β-D-galactopyranoside, Himedia, India). To confirm the enzymatic activity, the cell-free lysate was heat treated at 95 °C for 10 min and the above stated agar overlay method was repeated with heat treated cell-free lysate by using *C. violaceum* CV026 and *E. coli* MG4 biosensors.

Quantification of AHLs degradation by microtiter plate assay

The degradation of AHLs was quantified as per the reported methods (Zhu et al. 2011) with few modifications. Briefly, *C. violaceum* CV026 was incubated for 18 h in Erlenmeyer flasks containing LB. It was inoculated to LB medium supplemented with C_4-HSL along with cell-free lysate from each endophytic bacterial isolate in 100 ml conical flasks. The flasks were incubated at 30 °C for 24 h in shaker incubator (120 rpm). Then 1 ml of culture from each flask was taken in microcentrifuge tube and 1 ml of DMSO (dimethyl sulphoxide) was added, then it was centrifuged at 8000 rpm for 5 min to solubilize violacein and to remove the cells. Two hundred microlitres of violacein containing supernatants were added to 96-well microplates (Tarsons, U bottom) and the absorbance was read with a microplate reader (Themo Scientific, USA) at a wavelength of 585 nm. The harvested bacterial cells were resuspended in 1 ml sterile water to evaluate cell growth by measuring the optical density at 600 nm with a microplate reader (200 μl). The results were compared with respective assay control (PPB), positive control results.

Quorum sensing inhibitors and activators

The cell-free lysate were extracted with equal amount of ethyl acetae and the organic phase was concentrated using Rotary evaporator (Buchi India Pvt. Ltd., Mumbai, India), then the concentrated extract was dissolved in 0.9% of sterile DMSO. The overnight grown culture of *C. violaceum* CV026 was inoculated into LB medium supplemented with C_4-HSL and the extract (100 μg/ml). After incubation, the violacein was extracted with DMSO and 200 μl were loaded into 96-well round bottomed microplates and the absorbance was read with a microplate reader at a wavelength of 585 nm. The presence of quorum sensing inhibitors (QSI) was indicated by a decrease in production of violacein whereas presence of quorum sensing activators (QSA) was indicated by an increase in production of violacein (Zhu et al. 2011).

Extraction of total DNA, PCR analysis and sequencing

To identify the bacterial species, only AHLs degrading endophytic bacteria were selected and DNA was extracted according to the method of Araujo et al. (2002). Amplification of 16s rRNA gene of endophytic bacteria was performed with a universal set of forward and reverse primers (27F-5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R-5′ACGGCTACCTTGTTACGCTT-3′, Bangalore Genie, India), respectively. The 25 μl of reaction mixture contained 1 μl of each primer, 1 μl of template DNA and 22 μl of one fold diluted master mix (Bangalore Genie, India). The thermocycling conditions (Eppendroff Mastercycler, Germany) maintained were initial denaturation at 94 °C for 4 min, 35 amplification cycles of 94 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min and final polymerization step of 94 °C for 8 min. The final PCR product was resolved in 1.2% agarose gel and purified using the GenElute gel elution kit (Sigma–Aldrich, USA). DNA sequencing was performed and sequence was subjected to BLAST analysis and Nucleotide sequence similarities were determined by NCBI (National Center for Biotechnology Information databases). The identified isolates sequences were deposited to GenBank database and assigned with accession numbers.

Effect of quorum quenching activity on P. aeruginosa PAO1 biofilm

The effect on biofilm by cell-free lysate of endophytic bacteria was tested using static microtiter plate assay of Cady et al. (2012) with some modification. *P. aeruginosa* PAO1 was grown in tryptic soy broth(TSB, Himedia, India) for 18 h at 37 °C with rotary shaker at 150 rpm. The culture was resuspended in M9 minimal media supplemented with 0.4% (w/v) glucose to get approximately 1.5×10^8 cfu/ml (determined by optical density). Then 190 μl of freshly inoculated culture along with 0.45 μm filter sterilized 10 μl cell-free lysate were dispensed into 96-well microtiter plate. The assay controls were maintained without enzyme treatment (PPB). The plate was incubated at 37 °C for 24 h without agitation. The planktonic cells were removed, read at 600 nm and biofilms formed were detected by staining with 10 μl of crystal violet [0.1% (w/v) in water], incubated for 15 min at room temperature and then washed thoroughly with sterile distilled water. Ethanol (95%) was used to remove crystal violet from the biofilm and absorbance was measured at 590 nm.

To confirm the effect of quorum quenching on biofilm formation by the cell-free lysate, the *P. aeruginosa* PAO1-JP2 was grown in TSB for 18 h at 37 °C with rotary shaker at 150 rpm. The culture was resuspended in M9 minimal growth media supplemented with 0.4% (w/v) glucose to get approximately 1.5×10^8 cfu/ml. Aliquots of cells (190 μl) were then added to individual wells of a sterile 96 well microtiter plate. Autoinducers (10 μl of 10 μM 3-oxo-C_{12} HSL or C_4-HSL) were added to each well, followed by cell-free lysate at 1% of the final concentration. Control wells contained only M9 media, cells without autoinducers, cells with autoinducers but without cell-free lysate, cells with cell-free lysate without autoinducers, cells with autoinducers and PPB. The initial reading was taken at 600 nm and the loaded microtiter plate was incubated at 37 °C for 24 h without agitation. The planktonic cells were removed, read at 600 nm and biofilm formed was detected by staining with 10 μl of crystal violet.

Microscopy

Visualization of *P. aeruginosa* PAO1 biofilm formation and effect of cell-free lysate were performed as reported earlier (Vallet et al. 2004) with minor modifications. Briefly, *P. aeruginosa* PAO1 was grown in TSB for 18 h at 37 °C with rotary shaker operating at 150 rpm. The culture was resuspended in M9 minimal growth media supplemented with 0.4% (w/v) glucose to get 1.5×10^8 cfu/ml (measured by optical density). Biofilm was grown in 50 mm glass slide taken in 90 mm petridish along with cell-free lysate from isolated bacteria to get final concentration of 1% and control samples were taken without cell-free lysate treatment. The petridish was incubated at 37 °C for 24 h. The slides were rinsed with sterile distilled water, air dried and stained with 0.1% of acridine orange (Lobo Chemie Indoaustranal Co., Mumbai, India) for 2.5 min. The biofilm cell attachment was observed under fluorescence microscope (excitation at 490 nm; emission at 525 nm) and inhibition of biofilm formation was compared visually with respective of cell-free lysate treated and control samples.

PCR amplification of aiiA homologue gene

The bacteria which were positive for AHLs degradation bioassay were confirmed for AHL-lactonase activity by amplification of *aiiA* homologue gene by previously reported (Dong et al. 2002) with some modifications. Briefly, genomic DNA was amplified using forward and reverse primer *aiiAF2* (5′-CGGAATTCATGACAGTAAAGAAGCTTTA-3′) and *aiiAR2* (5′-CGCTCGAGTATATATTCAGGGAACACTT-3′) (Nusrat et al. 2011). The thermal cycling conditions were maintained as initial denaturation at 94 °C for 5 min, 5 cycles of 94 °C (45 s), 44 °C (45 s), 72 °C (1 min); 30 cycles of 94 °C (45 s), 53 °C (45 s), 72 °C (1 min), followed by primer extension at 72 °C for 8 min. The PCR amplicons were gel purified and sequenced. Sequence obtained was analyzed and the open reading frame (ORF) was detected using Geneious 6.1; annotated sequences were submitted to GenBank database.

Statistical analysis

Statistical significance of variance for collected data was determined by ANOVA. The quantification of AHL degradation by microtiter plate assay and inhibition of biofilm formation by cell-free lysates data were analyzed by one way ANOVA followed by Tukeys test. Statistics were performed using Graphpad Prism 5.03 software (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Isolation and identification of endophytic bacteria

Totally 39 endophytic bacteria were isolated from the bark and leaves of *P. santalinus*. These were screened for AHL degradation and among these positive isolates were identified by 16s rRNA gene sequencing. The BLAST search was used to compare with 16s rRNA reference gene sequence available in the GenBank database. It was observed that two AHLs degrading strains were closely related to the genera *Bacillus* and *Enterobacter* (Table 1). The phylogenetic tree was generated taking 16s rRNA sequence closest BLAST hits of the query sequence, to test the clustering of our isolates among endophytes, pathogens and environmental samples (Fig. 1). But, phylogram showed a unique subclade of *Bacillus firmus* PT18 and

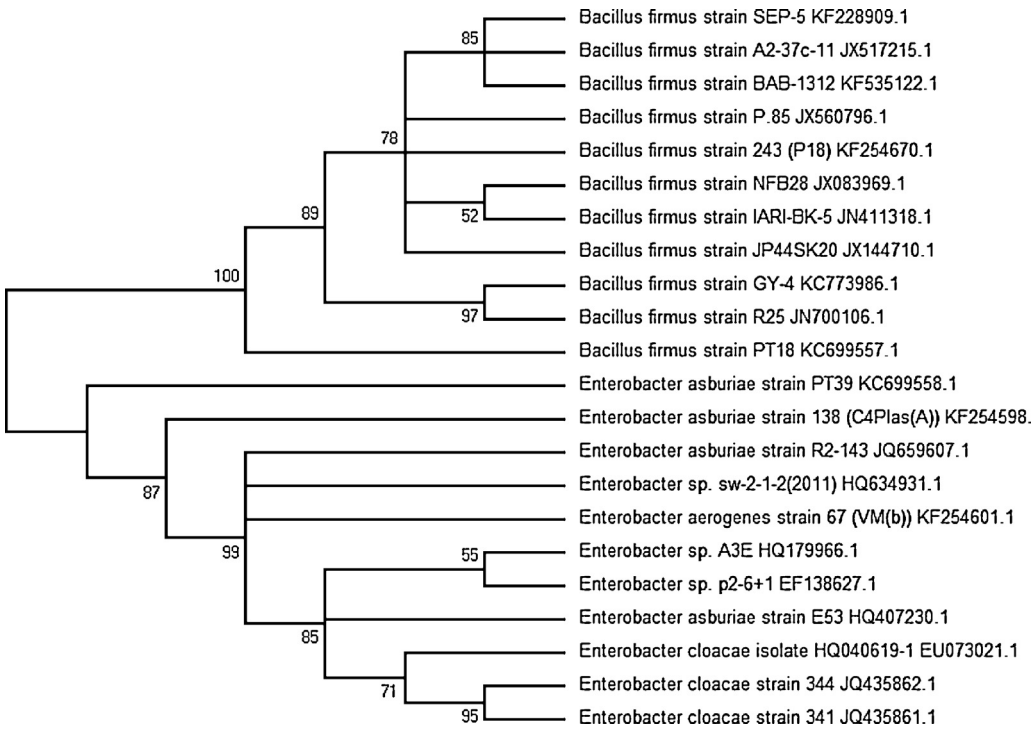

Fig. 1. Unrooted phylogenetic tree deriving from neighbor-joining showing the evolutionary relationship of endophytic *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 with its closest BLAST hits. The neighbor-joining tree was maintained by 1000 boot straps using the MEGA-5 software.

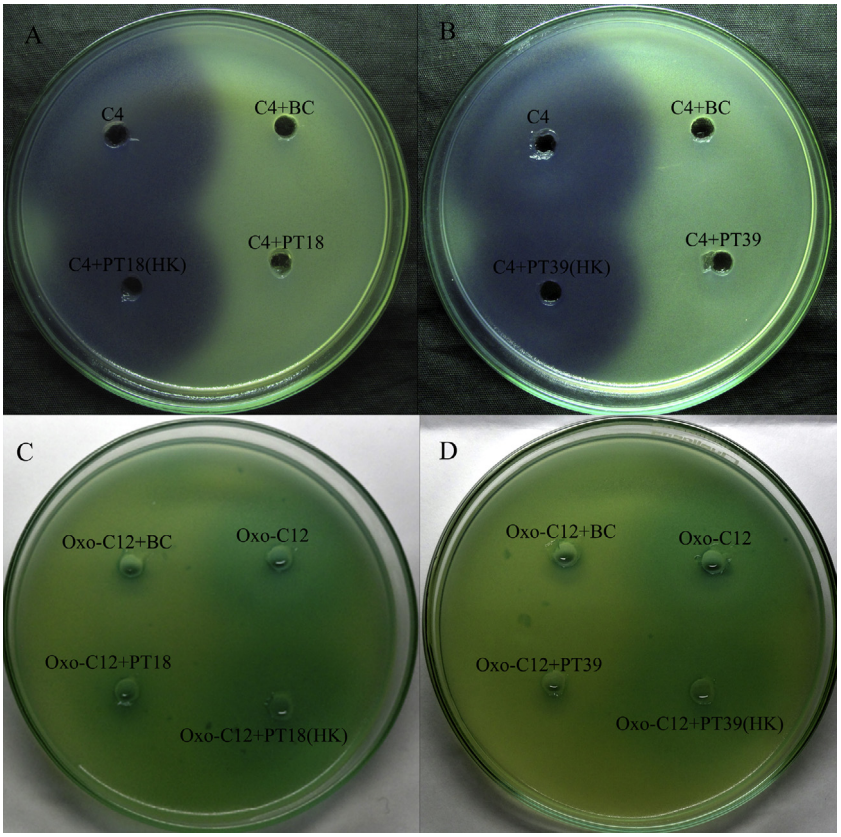

Fig. 2. Agar overlay assay to detect AHLs degradation using biosensor strains. Degradation of synthetic C_4-HSL was analyzed by suppression of violacein production in *C. violaceum* CV026 treated with cell-free lysate and heat killed (HK) controls (A and B). Degradation of synthetic 3-oxo-C_{12}-HSL was analyzed by suppression of blue color production in *Escherichia coli* MG4 treated with cell-free lysate and heat killed (HK) controls (C and D).

Table 1
The AHL degradation activity of endophytic bacterial isolates of *Pterocarpus santalinus*.

Endophytic bacterial isolates codes (sample)	Identified endophytic bacteria (Genbank accession number)	C_4-HSL degradation	3-oxo-C_{12}-HSL degradation	GenBank accession number of *aiiA* gene
PTB18 (Bark)	*Bacillus firmus* PT18 (KC699557)	+	+	KF699528
PTL 39 (Leaves)	*Enterobacter asburiae* PT39 (KC699558)	+	+	KF709655
Bacillus cereus	ND	+	+	ND
Assay control	ND	–	–	ND

ND, not done; +, positive; –, negative.

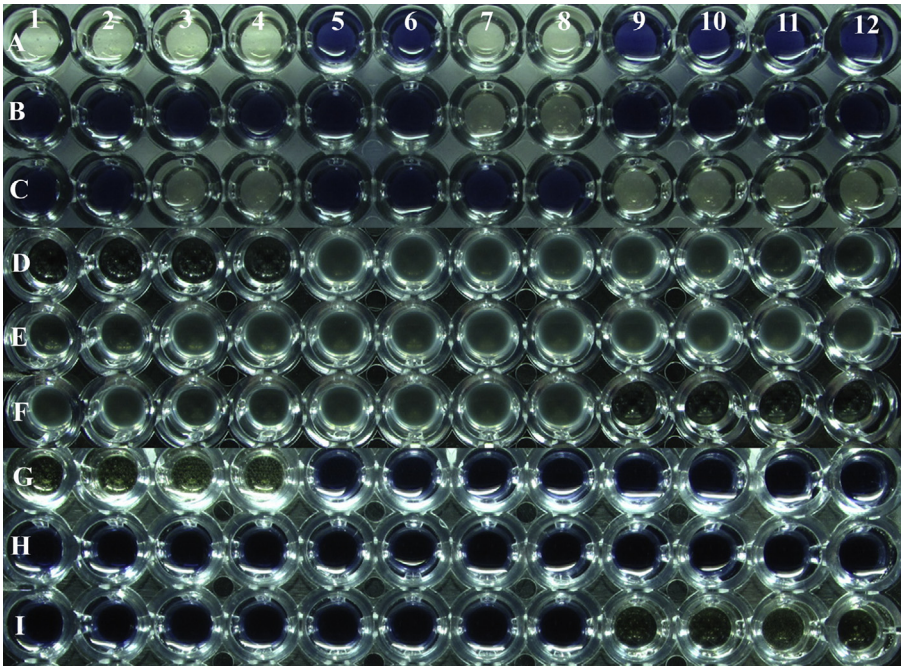

Fig. 3. AHLs degradation assay by microtiter plate method. AHLs degradation was analyzed by measuring the intensity of violacein production extracted from *C. violaceum* CV026 (row number A_1–A_4 sterile control, A_7 and A_8 treated with *B. cereus*, B_7 and B_8 treated with *Bacillus firmus* PT18 and C_3 and C_4 treated with *Enterobacter asburiae* PT39). Column number D–F indicates the growth measurement at 600 nm and column number G–I indicates the absence of QSI and QSA.

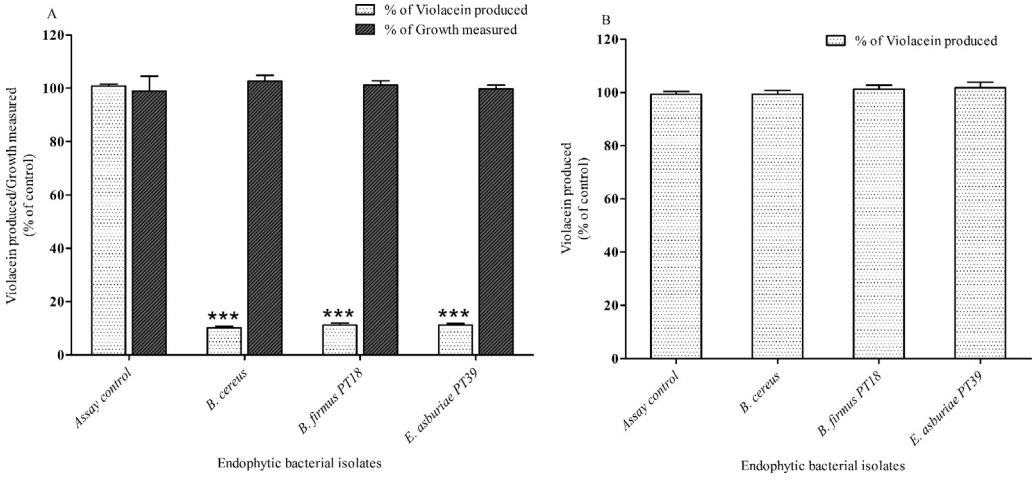

Fig. 4. Quantification of violacein production and measurement of growth under assay condition by microtiter plate method. (A) Quantification of violacein produced in percentage, more than 80% inhibition of violacein production was observed by *Bacillus firmus* PT18, *Enterobacter asburiae* PT39 and positive control ($p < 0.0001$), and absence of growth inhibition. (B) Absence of QSI and QSA, measured by amount of violacein production compared to control. The assay control value was set 100% and the error bar represents standard deviations from reproduced results in three repeated experiments.

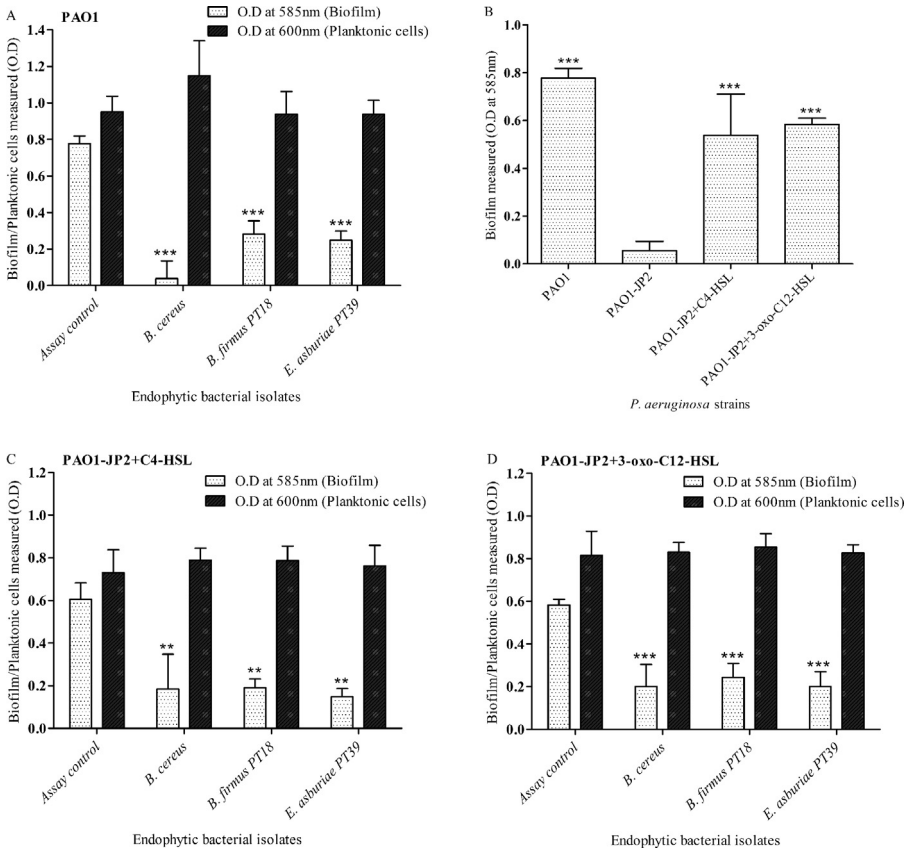

Fig. 5. Effect of cell-free lysate on quorum sensing regulated biofilm formation in *P. aeruginosa* PAO1 and PAO1-JP2. (A) Percentage of biofilm inhibition formed by *P. aeruginosa* PAO1 represented by crystal violet staining for biomass. Significant biofilm inhibition was observed ($p < 0.0001$) in treated cells. (B) Percentage of biofilm measured in PAO1, PAO1-JP2, PAO1-JP2 with 10 μM C_4-HSL and PAO1-JP2 with 10 μM 3-oxo-C_{12} HSL. (C) Effect of cell-free lysate treatment on PAO1-JP2 with 10 μM C_4-HSL biofilm formation ($p < 0.01$). (D) Effect of cell-free lysate treatment on PAO1-JP2 with 10 μM 3-oxo-C_{12} HSL biofilm formation ($p < 0.001$). The error bar represents standard deviations from reproduced results in three repeated experiments.

Enterobacter asburiae PT39 related to an uncharacterized genomic system and it may suggest novel strains as endophytes.

Screening of endophytic bacteria for AHLs-degradation

The AHLs degradation activity was screened by agar overlay method. Degradation of AHLs was indicated by absence of violacein around the loaded wells. In assay control (only QS molecule) violacein production was evident around the loaded wells and in *B. cereus* (positive control) and cell-free lysate of endophytic bacteria treatment exhibited degradation of AHLs molecules (Table 1). *B. firmus* PT18 and *E. asburiae* PT39 degraded AHLs molecules and heat treated cell-free lysate of AHLs degrading endophytic bacteria were unable to inhibit production of violacein in the agar overlay assay (Fig. 2). The degradation was further confirmed and quantified by microtiter plate assay (Fig. 3). The cell-free lysate of *B. firmus* PT18 and *E. asburiae* PT39inhibited more than 80% of violacein (Fig. 4A) along with positive control *B. cereus* ($p < 0.0001$) as observed by microtiter plate assay. The quantification of biomass shows that there was no inhibition of cell biomass in quorum quenching positive isolates (Fig. 4A). The QSI and QSA quantifications show the absence of these (QSI and QSA) molecules (Fig. 4B). Therefore, absence of QSI and inactivation of AHLs degradation upon heat treatment are the indication of the possible enzymatic activity in cell-free lysate of endophytic bacteria.

Effect of cell-free lysate on P. aeruginosa PAO1 biofilm

The cell-free lysate was subjected to effect of activity on biofilm formation in *P. aeruginosa* PAO1 by static microtiter plate method. The biofilm was stained with crystal violet to understand the total intact biomass in microtiter plate under experimental conditions. The results showed more than 80% inhibition of biofilm formation cell-free lysate of positive control and more than 60% inhibition by *B. firmus* PT18 and *E. asburiae* PT39 ($p < 0.0001$) (Fig. 5A). The quantification of biofilm and planktonic cells indicates the inhibition of biofilm formation and the planktonic cells are not influenced by cell-free lysate treatment. In quorum sensing induced study on PAO1-JP2 with quorum sensing molecule C_4-HSL or 3-oxo-C_{12} HSL showed more than 70% regeneration of biofilm when compared to mutant without QS molecule treatment (Fig. 5B). To confirm the effect of AHLs degradation on biofilm by cell-free lysate treatment mutant strain PAO1-JP2 was supplemented with QS molecules and treated along with cell-free lysate. When PAO1-JP2 was supplemented with C_4-HSL molecules and treated with cell-free lysate, it exhibited more than 60% inhibition of biofilm formation compared to control ($p < 0.01$, Fig. 5C). Similarly, when PAO1-JP2 was supplemented with 3-oxo-C_{12} HSL molecules and treated with cell-free lysate it exhibited more than 50% inhibition of biofilm formation compared to control ($p < 0.001$, Fig. 5D). Therefore, regeneration of biofilm formation capacity in PAO1-JP2 by the addition of major QS molecules shows the importance of quorum sensing in biofilm

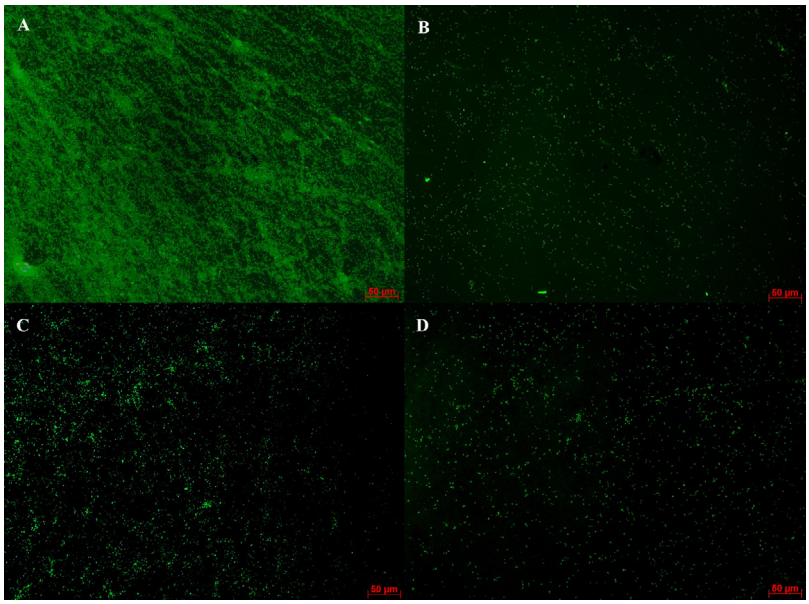

Fig. 6. Fluorescence microscopy for visualization of biofilm formation by *P. aeruginosa* PAO1 stained with acridine orange. (A) Assay Control (without treatment), (B) treated with *B. cereus*, (C) treated with *Bacillus firmus* PT18 and (D) treated with *Enterobacter asburiae* PT39. The decrease in total biomass represents the inhibition of biofilm formation in treated when compared to control in 24 h incubation.

formation which could be controlled by inhibition of quorum sensing by using cell-free lysate of endophytic bacteria.

Visualization by fluorescence microscopy

In inhibition of biofilm formation in *P. aeruginosa* PAO1 by treating with cell-free lysate can be understood better by visualization of biofilm under fluorescence microscopy. The result exhibited the suppression of total biomass in treated slides when compared to control (Fig. 6). In control slide a thick biofilm in 24 h of incubation was observed, but not in treated slides with *B. firmus* PT18 and *E. asburiae* PT39, which showed decrease in biofilm biomass of *P. aeruginosa* PAO1. Therefore, the results support the inhibition of biofilm formation by *P. aeruginosa* PAO1 as indicated by crystal violet staining.

PCR amplification of aiiA homologue gene

Using the extracted genomic DNA, an PCR amplicons of approximately 800 bp was detected by gel electrophoresis (Fig. 7). PCR amplicon obtained was sequenced and compared with those found in online data search of NCBI (http://www.ncbi.nlm.nih.gov). The BLAST search exhibited high similarity with coding sequence of the closely related aiiA gene (responsible for AHLs degradation, called as AHL lactonase). The contig sequence of our enzymes was obtained by the CAP3 sequence assembly program and amino acid sequence alignment of our enzymes with AHL lactonases from other species was done using NCBI database and Clustral W (Fig. 8). Multiple alignment of AHL-lactonase sequence releaved the presence of the motif "HXHXDH" and presence of metal ligands for the dinuclear zinc form.

Discussion

The endophytic microorganisms live symbiotically in plants and support the plant immune system to counter the attack by the plant pathogens. These microorganisms are soil borne bacteria which can also penetrate plant roots, and some strains may move to aerial parts like xylem, bark, leaves *etc.*, but with decreasing bacterial density in comparison with root colonizing populations (Compant et al. 2010). Most of the Gram negative pathogens which cause diseases in plant as well as in humans maintain their threshold cell growth by the quorum sensing mechanism by producing AHLs as major signaling molecules. Any degradation of these signaling molecules will result in the suppression of virulence of pathogens and lowering of disease severity (Dong et al. 2000).

In the present study, AHLs degrading bacteria *B. firmus* PT18 and *E. asburiae* PT39 which were isolated from among the endophytic bacteria were inhabiting *P. santalinus*. *B. firmus* was previously isolated as endophyte from rice seeds (Kaga et al. 2009) and *E. asburiae* from peanut as endophytic plant growth promoting

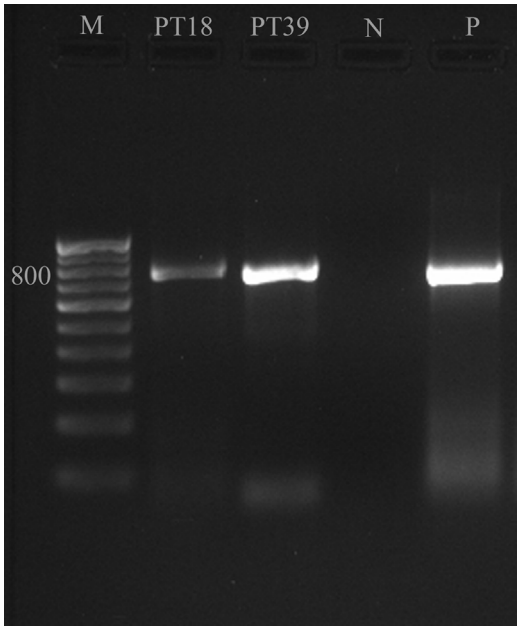

Fig. 7. PCR amplification of the *aiiA* gene (about 800 bp) from AHLs degrading endophytic bacteria using specific primers *aiiAF2* and *aiiAR2*. Lanes: M – 100 bp DNA ladder; PT18 – *Bacillus firmus* PT18; PT39 – *Enterobacter asburiae* PT39; N – negative control (without DNA template); P – *B. cereus*.

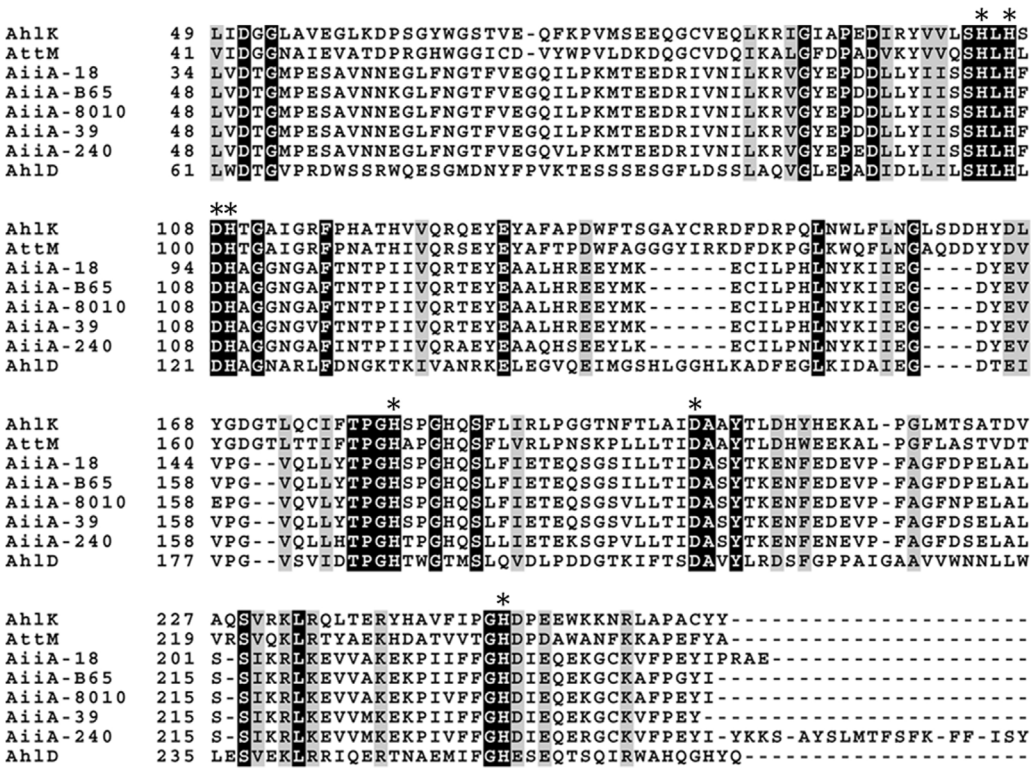

Fig. 8. Multiple alignment of amino acid sequence of lactonase from *Bacillus firmus* PT18 (AiiA 18) and *Enterobacter asburiae* PT39 (AiiA 39) with other known sequences. AHL lactonase of AiiA 18 and AiiA 39 was aligned with *Klebsiella pneumoniae* (AhlK, accession number: AY222324.1) *Bacillus* sp. 240B1 (AiiA 240, accession number: AF196486.1), *Arthrobacter* sp. IBN110 (AhlD, accession number: AF525800.1), *Bacillus thuringiensis serovar kurstaki* strain 8010 (AiiA 8010. accession number: AY943832.1), *Bacillus weihenstephanensis* strain B65 (AiiA B65, accession number: KC823046.1), *Agrobacterium tumefaciens* (AttM, accession number: U59485.2). ClustralW2 was used for alignment of sequences and BoxShade server was used to shade identical residue with black and conserved residues with gray. Metal ligands in the dinuclear zinc form of AHL lactonase was designated with an asterisk.

bacteria (Wang et al. 2013). The cell-free lysate of endophytic bacteria was screened for AHLs degradation activity and confirmed the inhibition of quorum sensing. The absence of QSI and heat denatured assay indicates the possible enzyme activity in cell-free lysate of *B. firmus* PT18 and *E. asburiae* PT39. In endophytic actinomycetes HSL-acylase activity was reported with C_{10}-HSL (*N*-decanoyl-L-homoserine lactone) as most favorable substrate. Similarly, the quorum quenching activity of isolates from endophytic actinomycetes exhibited highest activity when compared to isolates of rhizosphere soil (Chankhamhaengdecha et al. 2013).

The quorum quenching activity in cell-free lysates was applied to *in vitro* inhibition of biofilm formation in *P. aeruginosa* PAO1. In *P. aeruginosa*, biofilm formation is one of the virulence factors which causes complicated biofilm associated infections and stimulates drug resistance (Vallet et al. 2004). The QSI and quorum quenching enzymes have the ability of suppressing the biofilm formation in *Pseudomonas* sp. In order to measure the extent of quorum quenching of cell-free lysate in inhibition of *P. aeruginosa* biofilm, we studied biofilm formation in PAO1 and PAO1-JP2 as model organisms. In wild type PAO1 >80% biofilm inhibition was observed by cell-free lysate treatment, similarly treatment with cell-free lysate upon PAO1-JP2 in presence of 10 μM 3-oxo-C_{12} HSL or C_4-HSL exhibited >50% inhibition of biofilm formation. It indicates the role of quorum sensing in *P. aeruginosa* biofilm formation by the influence of major two quorum sensing molecules. Favre-Bonte et al. (2003) demonstrated the restoration of biofilm formation capacity by the addition of an exogenous C_4-HSL molecule in *rhlI* mutant and treatment with azithromycin, which is known to reduce the production of 3-oxo-C_{12} HSL and C_4-HSL with 45% of biofilm inhibition in wild type *P. aeruginosa* PT5. Shih and Huang (2002) reported that the biofilm formed by PAO1 is much thicker than double mutant PAO1-JP2 and biofilm formation reached a plateau phase in 24 h in PAO1 but it took 36–48 h of lags in PAO1-JP2 before entering the maximum accumulation phase. The secretion of Homoserine lactones (HSLs) from a biofilm enhances the growth of neighboring cells, which makes possible for fewer planktonic cells to transit to life on a surface (Flickinger et al. 2011).

The fluorescence microscopy study showed decrease in development of total biomass in treated cells when compared to control. It indicates that the ability of cell-free lysate with removal of biofilm adhesion capacity in *P. aeruginosa* PAO1. Recent reports shows that inhibition of *P. aeruginosa* biofilm formation by small synthetic cationic peptides. The peptides effectively prevented more than 50% of biofilm formation by reducing swimming and swarming motilities, but they failed to inhibit planktonic cells as do antimicrobial agents (Fuente-Nunez et al. 2012). Similarly, α-helical peptides were able to reduce the biofilm formation by *P. aeruginosa* without planktonic cells inhibition. Inhibition of biofilm formation due to high affinity to extracellular DNA which plays a major role in biofilm formation might facilitate the detachment or disruption of stable biofilms (Pompilio et al. 2012). Interference with cell signaling leads to inhibition of biofilm formation in *las* quorum sensing dependent systems. This type of mechanism observed in QSI molecule furanone activity against *P. aeruginosa.* The furanone is capable of penetrating the biofilm matrix and interfere with quorum sensing gene expression, as a consequence inhibition of biofilm maturation results (Hentzer et al. 2002).

The presence of planktonic cells indicates that cell-free lysates in biofilm inhibition assay target the AHLs degradation instead of biomass inhibition. Also, cell-free lysate avoids the creation of harsh environment as evidenced by the treatment of antibiotics and mutations in response to changes in environmental conditions.

Therefore, the reports suggest the role of quorum sensing in biofilm formation of *P. aeruginosa*. The quorum quenching strategy demonstrates the blocking of cell to cell communication, thereby reducing the biofilm formation. This method could be useful in the treatment of infection by drug resistant *P. aeruginosa*.

Amplification and sequencing of AHL lactonase from genomic DNA were done for the identification of AHL degrading protein present in cell-free lysate of endophytic bacteria. Sequence analysis of our AHL lactonase revealed the presence of several highly conserved domains shared with lactonases from other species. The presence of motif "HXHXDH" believed to play a role in metal binding and is conserved in almost all metaloenzymes (Thomas et al. 2005).

In conclusion, the ability of endophytic bacteria to degrade AHLs as major signaling molecules in Gram negative bacteria suggests the novel source for AHL lactonase. The study suggests that *in vitro* inhibition of biofilm formation in *P. aeruginosa* PAO1 by AHL lactonase present in cell-free lysate of endophytic bacteria could be applicable in clinical situations also.

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