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Quorum quenching activity of soil isolates against pigment producing *Pseudomonas aeruginosa* SU-8

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ABSTRACT

Quorum Sensing (QS) is a prevalent communication process found in most bacteria. Biofilm formation is believed to be responsible for antibiotic resistance in Pseudomonas aeruginosa. Quorum Quenching (QQ) is an antagonistic mechanism performed by certain bacteria against QS, where they degrade the QS molecule. In this study, pigment producing and biofilm forming P.aeruginosa was isolated from air. This quorum sensing organism was co-cultured with two soil isolates – Brevibacillus brevis and Bacillus subtilis, and their inhibition against quorum sensing organism was studied using crystal violet assay. Brevibacillus brevis was found to show antagonistic activity. Both the organisms were found to inhibit the biofilm formation.

Key words: Introduction Quorum Sensing (QS), Quorum Quenching (QQ), *Pseudomonas aeruginosa*, *Brevibacillus brevis*. *Bacillus subtilis*

INTRODUCTION

Bacteria produce signals among themselves known as quorum sensing (QS). This is responsible for growth, biofilm formation virulence, pigment production, antibiotic resistance etc[1-7]. *Pseudomonas aeruginosa* is a known excellent model for quorum sensing, its pigment production - pyocyanin is maintained by quorum sensing systems[8]. Quorum quenching (QQ) is the process of disruption of quorum sensing (QS) signals[9]. Multi-drug resistant bacteria have become a threat to the world with most of the antimicrobials having lost their capacity due to intercellular signaling i.e., quorum sensing (QS) [10]. In the year 1999, Dong *et al* [11] reported the concept of quorum quenching by isolating soil *Bacillus* sp. 240B1, which had the quality of inhibiting the autoinducer molecules. The gene that was responsible for autoinducer inactivation was aiiA [11]. *Bacillus* sp. 240B1 that was first identified, produced an enzyme called AHL-lactonase that was encoded in the aiiA gene [12]. Another enzyme called AHL-Acylases isolated from *Variovorax paradoxs* (VAI-C) inhibited QS molecules reported by Leadbetter and Greenberg [13]. The gene encoding the acylase enzyme from *V.paradoxus* (VAI-C) is still not determined. Lin et al [14] reported the same AHL-Acylases enzyme from *R. eutropha*, which was encoded by aiiD gene.

Biofilms, which are considered a menace for the environment and healthcare settings were inhibited by isolating quorum quenching bacteria from wastewater treatment plant sludge [15]. Multi drug resistance associated with biofilm formation and pigment production in various microorganisms is emerging as a threat to public health as it complicates treatment of diseases. *Pseudomonas aeruginosa* is one such microorganism that causes Cystic Fibrosis. This study aims at the inhibition of the QS molecules by a degradative mechanism called Quorum Quenching (QQ). Two bacteria isolated from garden soil, believed to contain QQ activity and were used to target the biofilm formation and pigment production of *P.aeruginosa*.

MATERIALS AND METHODS

Isolation

Sterile nutrient agar plates were prepared and kept opened in open air for ten minutes. After exposure the plates were incubated at 37^0C for 24 hours. Green pigmented colony was chosen and transferred to nutrient agar slant. After incubation, slants were stored in refrigerator at 4^0C till further use.

Soil samples were collected in a sterile container and brought to the lab. 1g of soil was serially diluted and pour plate was performed and sterile nutrient agar was added to the plate. The plates were incubated at 37^0C for 72 - 96 hours. The organisms grown were selected on the basis of zone of inhibition around the colonies. The selected organisms were streaked on nutrient agar slant, incubated and stored at 4^0C after growth was seen on slant.

Identification of Microbes

Isolated organisms were identified by performing routine biochemical tests and Gram's staining reaction. DNA was isolated [16] and amplified using universal primers 27F AGAGTTTGATCMTGGCTCAG and 1492R TACGGYTACCTTGTTACGACTT. 1400 - 1500 bp amplified products were subjected for sequencing. Unincorporated primers and dNTPs were removed from PCR products using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers (CCAGCAGCCGCGGTAATACG/ TACCAGGGTATCTAATCC). Sequencing reactions were performed using an ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems)®

Antagonistic activity of Quorum Quenching organisms

Sterile nutrient agar was prepared. 24 hours old *Pseudomonas aeruginosa* was swabbed over the plate. Wells were made using gel puncture kit. 24 hours old *Bacillus subtilis* strain SU17 and *Brevibacillus brevis* strain SU18 were added into well. Sterile nutrient broth was added in one well as control. Plates were incubated for 24 hours. After incubation plates were observed for the antagonistic activity against pigment forming *Pseudomonas aeruginosa*.

Quorum Quenching activity against Biofilms

Crystal violet assay was performed as described by O'Toole [17], with fewer modifications. Inhibition of biofilms by quorum quenching organisms was performed in 96-Well Microtitre plates. 100µl of biofilm forming *Pseudomonas aeruginosa* was grown in nutrient broth and was standardized to OD value of 1.0 at 600nm, was added into microtitre plate. Along with this different volumes (5µl, 10µl, 20µl and 40µl) of quorum quenching organisms grown in nutrient broth (which was also standardized to OD value of 1.0 at 600nm) was added. All the wells were made into 200µl using nutrient broth. Thus the biofilm forming organism i.e. *P.aeruginosa*, was co-cultured with the two different quorum quenching organisms. The plates were incubated at 37^0C for different time intervals - 16h, 24h, 40h, 48h, 64h, 72h, 88h, 96h. For each time interval, a control was also maintained. The nutrient broth was removed from the wells and the cells were washed with sterile saline. Then the cells were stained with 200 µl of 1% crystal violet for 1 minute and then the stain was completely removed by washing the cells with distilled water. The cells were treated with 200 µl of 50% ethanol and then the cell turbidity was measured at 600nm in ELISA reader. A graph was plotted between optical density and time intervals.

Quenching activity against Pigment Production

Pyocyanin producing *P.aeruginosa* was co-cultured with 40µl of two soil isolates- *Bacillus subtilis* and *Brevibacillus brevis.* Pyocyanin pigment production by *P.aeruginosa* was estimated at regular time interval. Pigment extraction was performed following the method of Essar et al[18] and Pyocyanin was quantified by following equation [18,19].

Concentration of pyocyanin (µg/ml) = OD_{520} x 17.072

aii A amplification

Primer sequences in this study used were AiiA1 (forward primer), 5′-ATGACAGTAAARAARCTTTATTTC-3′ and AiiA2 (reverse primer), 5′-TCACTATATATAYTCMGGGAACTC-3′[20]. DNA was isolated [Pitcher et al., 1989]. The full-length *aiiA* gene was amplified with the primer pair AiiA1/AiiA2 in 50 µl reaction volume containing 20 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 0.5 µM of each primer and 2.5 U *Taq* DNA polymerase. PCR was carried out for 30 cycles (at 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min). The PCR product was analyzed on 1% agarose gel [20].

Statistical analysis

All the tests were done in triplicates. Mean value was taken and standard error was calculated. The results were given illustrated as Mean± standard error.

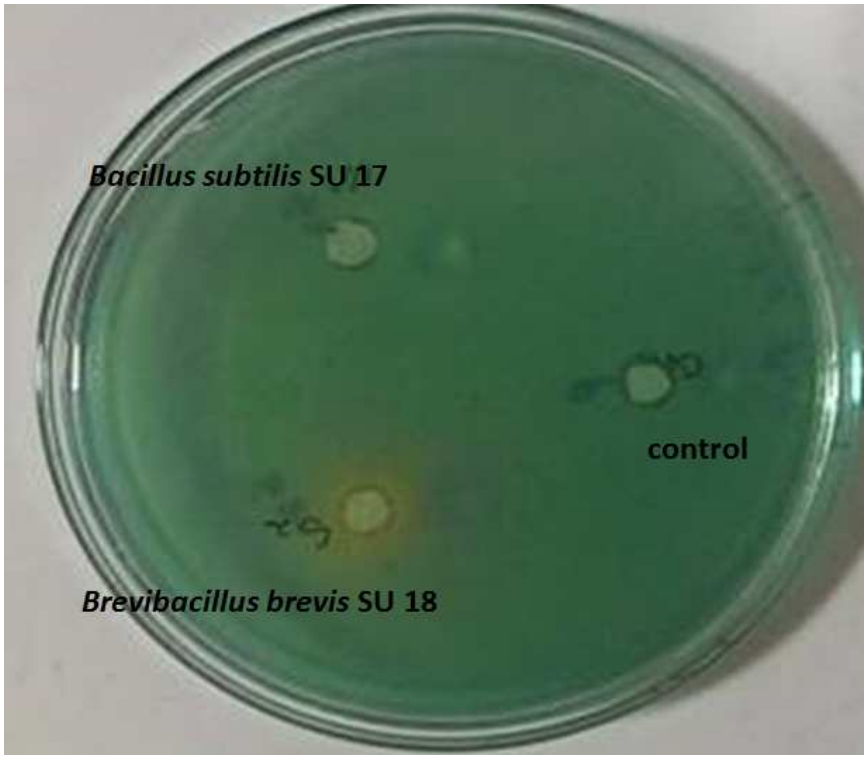

Figure 1. Antagonistic effect of *Brevibacillus brevis* against *P.aeuruginosa*

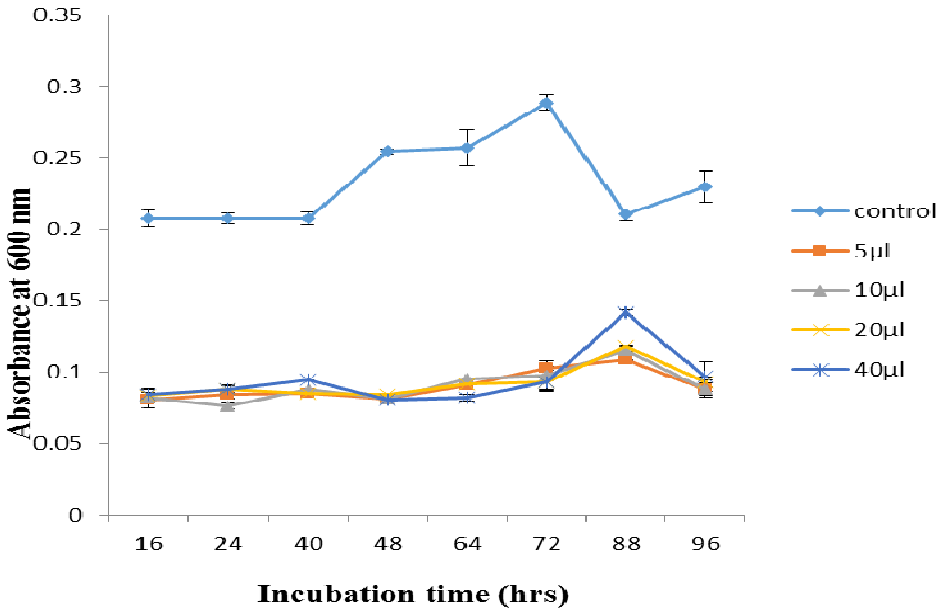

Figure 2. Inhibition of *Pseudomonas aeruginosa* biofilm by *Bacillus subtilis* strain SU-17

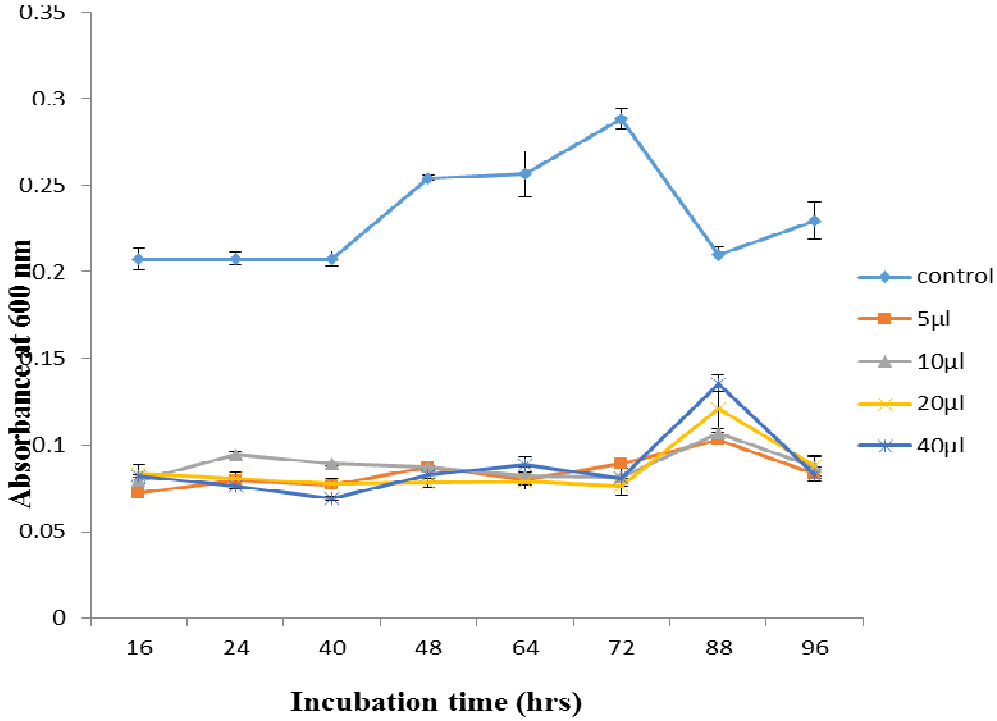

Figure 3. Inhibition of *Pseudomonas aeruginosa* biofilm by *Brevibacillus brevis* strain SU-17

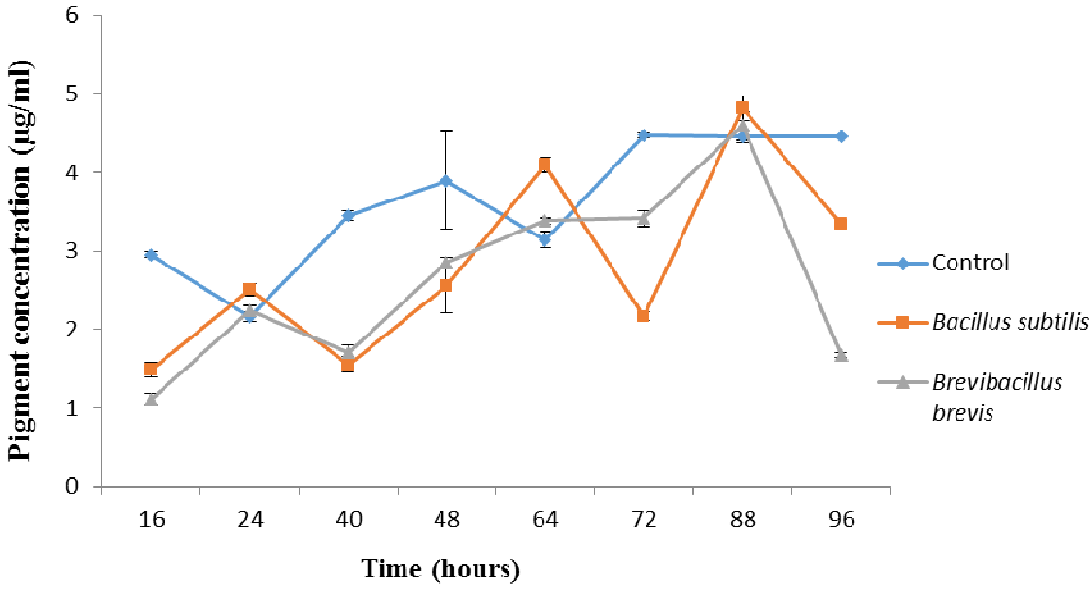

Figure 4. Quenching of Pigment Production by quorum quenching organisms

RESULTS AND DISCUSSION

Isolation and Identification of Organism

The organism found to produce bluish-green pigment on cetrimide agar and nutrient agar was isolated and their 16srRNA was sequenced. The organism was identified as *Pseudomonas aeruginosa* SU-3. The 16srRNA sequence was submitted and GENBANK accession number is GU395986. Two organisms, isolated from garden soil produced distinct morphological characteristics on nutrient agar. Both the organisms were sequenced and confirmed to be *Bacillus subtilis* and *Brevibacillus brevis* using NCBI Nucleotide BLAST. The sequence of *Bacillus subtilis* strain SU17 and *Brevibacillus brevis* strain SU18 were submitted in GENBANK and their accession number are KT119833 and KT119834 respectively

Antagonistic activity of Quorum Quenching organisms

Zone of inhibition and no pigment production was observed around the *Brevibacillus brevis* strain SU18. Thus *Brevibacillus brevis* strain SU18 was found to have antagonistic activity against *Pseudomonas aeruginosa* SU-3 (Figure 7). This might be the ability of *Brevibacillus brevis* strain SU18 to produce antibiotic and also quorum quenching enzymes. *Brevibacillus brevis* is known to exhibit antibacterial activity against *P. aeruginosa* [21].

Quorum Quenching of Biofilms

Crystal violet assay was performed for *Pseudomonas aeruginosa* with two soil isolates with different concentration (5µl, 10µl, 20µl and 40µl). *Bacillus subtilis* strain SU17 and *Brevibacillus brevis* strain SU18 were observed to inhibit biofilm formation (Figure 8 and 9). In all the time points, of the 2 organisms, the latter was found to be affecting the biofilm formation more than the former. *Brevibacillus brevis* strain SU18 has shown its maximum inhibitory activity in 40th hour. *Pseudomonas* biofilm is more resistant (>1000 times) than planktonic cells [1] because of reduced penetration of the antibiotic [22]. Even some antibiotic azithromycin are successful against biofilm formation but fails to inhibit *P. aeruginosa* growth [23].

Kim et al [15] found *Afipia* sp. and *Acinetobacter* sp. to produce intracellular QQ enzyme, whereas *Pseudomonas* sp. and *Micrococcus* sp. produced the extracellular QQ enzyme. Kim et al [15] also found *Microbacterium* sp. and *Rhodococcus* sp. to inhibit AHL activity and biofilm formation in a whole-cell assay. Quorum-quenching activity of cell free extract of *Bacillus licheniformis* DAHB1 against *Vibrio* sp biofilm formation has been studied by Vinoj et a [24]. Further study will give us either an antibiotic or antibiofilm compound. Wang et al [25] have shown in vitro that enzymes which degrade AHLs can reduce biofilm and virulence factor production, but this also remains to be demonstrated in vivo.

Quenching of Pigment Production

The pyocyanin pigment produced by *P.aeruginosa* was inhibited using the two soil isolates- *Bacillus subtilis* strain SU17 and *Brevibacillus brevis* strain SU18 (Figure 12). The inhibition of pigment was visible when compared to control for the different time intervals. At 16th and 40th hour, maximum inhibition of pigment was seen. This inhibition may be because of the enzymes like AHLase or competition exhibited by the organisms or may be any product of the organisms.

aii A amplification

No amplified product for the aii A gene was seen, thus the organisms might be having other way of antagonistic activity.

CONCLUSION

Two soil bacteria *Brevibacillus brevis* strain SU17 and *Bacillus subtilis* strain SU18 were isolated and were co-cultured with pigment producing *Pseudomonas aeruginosa* SU-3. Both the organisms were showing influence against biofilm formation and pigment production of *Pseudomonas aeruginosa* SU-3.

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