Biosynthesis Of Quorum Quenching Cyanobacterial Silver Nanoparticles And Their Prospectives.

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Abstract.
This study aimed to isolate cyanobacterial species and develop silver nanoparticles using Cyanobacterial species. Communication is the crucial rudiment of biotic systems which is regulated by complex networks of biochemical torrents. Microorganisms too have an intricately coordinated communication system known as Quorum Sensing. Quorum sensing stimulates a large array of biological activities like biofilm formation, transfer of antibiotic resistance, virulence, bioluminescence, sporulation and symbiosis within the community. It helps microorganisms to live collectively as a macro community protecting and nourishing its individuals. Quorum sensing stimulates a large array of biological activities like biofilm formation, transfer of antibiotic resistance, virulence, bioluminescence, sporulation and symbiosis within the community. Characterization of cyanobacterial silver nanoparticles using UV Visible spectroscopy was done. Susceptibility of *Pseudomonas aeruginosa* to antibiotics in presence of AgNPs was determined. Cyanobacterial silver nanoparticles found to control the production of pyocyanin in *Pseudomonas aeruginosa* due to quorum quenching activity.

Keywords: Quorum Sensing; Quorum quenching; Silver Nanoparticles; Alkaloids; Flavonoids; antimicrobial activity.

Introduction

Communication is the crucial rudiment of biotic systems which is regulated by complex networks of biochemical torrents. Micro-organisms too have an intricately coordinated communication system known as Quorum Sensing. The intricately coordinated communication in microbes is termed as "Quorum sensing". Any significant fluctuation in microorganism's population density is detected and communicated. This is established by the release of autoinducer signal molecules like acylated homoserine lactone or oligopeptides, into the milieu where other organisms bear receptors for signal reception. The system differs substantially in different bacteria. Gram positive systems involves secreted autoinducing peptides (AIPs), while Gram-negative systems mainly use N-acylated homoserine lactones (AHLs). Also, Gram-negative QS signals are sensed by intracellular molecules, while Gram-positive QS signals usually interacts with the extracellular part of two-component histidine kinase systems that transfer the stimulation by protein phosphorylation inside the cell that activates the response regulator, which is a DNA-binding transcription factor to alter gene expression of QS-controlled target genes. (Miller *et al*., 2001; Whitehead *et al*., 2001). Within a dense population the signaling molecules are not diluted to loss but rigorous uptake takes place by the members within the community. The assimilation of molecules into the cell indicates a populated community. A threshold concentration of these molecules brings about changes in the genetic expression as a response. Quorum sensing stimulates a large array of biological activities like biofilm formation, transfer of antibiotic resistance, virulence, bioluminescence, sporulation and symbiosis within the community. It makes

microorganisms to live collectively as a macro community protecting and nourishing its individuals. (Chauder & Bassler, 2001)

In microorganisms most of the crucial physiological processes take place only after the population reaches a bulk density. After the entry the pathogen firstly increases its population then further it is able to initiate infection by invading the host. When treated by antibiotics some of the organisms escape its action by certain strategies like modification of target, efflux of antibiotics through membrane pumps, or by synthesis of antibiotic degrading enzymes. (Blair et al., 2015). The resistance developed in few organisms can be transmitted to others by horizontal gene transfer. Quorum sensing plays an important role in conjugation between organisms. Also, multidrug-resistant (MDR) and extensive drug resistant (XDR) superbugs have been decades old nuisance to the health care professionals. Microorganisms are known to develop biofilms on a conducive substratum which are difficult to get rid of. Bacteria in biofilm are embedded in self-produced extracellular polymeric matrix produced by the bacteria. Bacteria develop biofilm on submerged surfaces such as natural aquatic systems, water pipes, living tissues, tooth surfaces, indwelling medical devices and implants. (Prakash *et al*., 2003). These biofilms exacerbate the virulence during pathogenesis, invade the implants, catheters causing infection and also lead to biofouling of submerged structures like ship hulls reducing its efficacy. This process had eventually cost millions of lives and money. (Saxena et al., 2019).

Quorum quenching is a mechanism to turn- off quorum sensing. Various enzymes, nanoparticles, and biomolecules have been estimated to interrupt this process. Large array of quorum sensing inhibitor ((QSI) molecules has been elucidated from natural sources has been extracted from plants (Koh, 2013), bacteria (Khan et al., 2019) and cyanobacteria (Santhakumari *et a*l., 2016), which possess anti-quorum sensing properties (Asfour *et al*., 2018). Most include phenolics, phenolic acids, quinones, saponins, curcuminoids, FLs, tannins, coumarins, terpenoids, and alkaloids. Cyanobacteria are promising biological source for synthesizing metal nanoparticles. Bio compounds (including alkaloids, phenols, terpenoids, enzymes, co-enzymes, proteins, and sugars) reduce metal salts from positive oxidation state to zero oxidation state. Aquatic cyanobacteria thrive in competitive environment producing bioactive compounds inhibiting other organisms mainly bacteria's propagation. Main strategy adopted is to deter their communication and disrupt their community. Also, cyanobacteria derived nanoparticles can show enhanced quorum quenching activity. (Kumar *et al*., 2008; Gole *et al*., 2001).

Materials & Methods:

Water sample collection from different water bodies

Water sample (250-500 mL) was collected from pond, lake, brackish river and stagnant water bodies in a clean bottle. Samples were stored in dark cool place until arrived at laboratory. 20 mL of each sample was centrifuged at 3000 rpm for 5 minutes.

Enrichment and isolation of aquatic cyanobacterial species

Four samples sediment viz., pond, lake, brackish river and stagnant water bodies was collected. 1gm of each sample sediment was added to sterile BG-11 medium (100mL) supplemented with cycloheximide (50 mg/litre) in sterile Erlenmeyer flask to eliminate contaminating eukaryotes and incubated for 7 days with 16 hours photoperiod at room temperature. Enriched samples were streaked on sterile BG-11 agar plates and incubated for 7 days with 16 hours photoperiod at room temperature (24-27°C). After 24 hrs colonies with different morphological characteristics was selected and characterized morpho-logically.

Microscopic examination of isolated cyanobacteria

Morphologically different colonies were selected and inoculated in sterile BG-11 medium (100ml) and incubated for 7 days with 16 hours photoperiod at room temperature. The cyanobacterial suspension was examined microscopically under oil immersion.

Qualitative biochemical analysis of the cyanobacterial cell extract-

Alkaloids, flavonoids, Glycosides, Quinones, Phenols, Tannins, Saponins, Sugars and Protein content of cyanobacterial extract was determined by standard protocols.

Preparation of silver nanoparticle using cyanobacterial biomass

The Cyanobacterial cells (biomass) were harvested at the beginning of stationary phase by centrifugation, at 5000 rpm for 10 min. To remove traces of medium the pellet was rinsed and washed thrice. One gram of wet weight biomass of each culture was suspended in 20 ml of 1 mM aqueous AgNO3 solution at pH 7 for 24 hours under illumination.

Characterization of nanoparticles using UV Visible spectroscopy

The incubated silver nitrate solution was centrifuged at 3000 rpm for 10 min. The collected sediment was suspended in sterile distilled water and the procedure was repeated twice. Silver nanoparticle synthesis in the test solutions was confirmed by spectral reading of surface plasma resonance at 200-800 nm by UV-Vis spectrophotometry by setting distilled water as blank.

Investigation of enhanced susceptibility of EPS producing organisms to antibiotics in presence of silver nanoparticles

Mueller-Hinton agar plates were inoculated with *P. aeruginosa, S. aureus* and *K. pneumoniae*. One sterile disk loaded with 20 µL of silver nanoparticles, one disk with antibiotics and one disk with antibiotic supplemented with 20 µL of with AgNP was placed on inoculated media. The plates were incubated aerobically at 37°C for 24 h. Zones of inhibition for AgNPs, antibiotics, and antibiotics + nanoparticles were measured and expressed radii in millimetres

Qualitative estimation of antibiofilm activity by Congo red agar

Sterile Congo red agar plates was prepared. 5mL *S. aureus* suspension was incubated with non-inhibitory concentration of NP (1mL) for 30 minutes and streaked on test Congo red agar plate. *S. aureus* suspension was streaked as control on Congo red agar plate. The colour of colonies was observed biofilm producers form black colonies with a dry crystalline consistency and non- biofilm producers forms pink coloured colonies.

Qualitative estimation of antibiofilm activity by Tube Method

St. Nutrient broth were inoculated with loopful of *P. aeruginosa* and 100 µL silver nanoparticle solution and control was maintained without nanoparticle incubated at 37° C for 24hrs. Tubes were decanted & washed with phosphate buffer (PH=7.3) and dried. Dried tubes were stained with crystal violet (0.1%), stain was removed & washed with deionized water and dried and compared.

Estimation of pyocyanin repression activity

P. aeruginosa was inoculated in 10 ml Nutrient broth with 100 µL silver nanoparticle solution and control was maintained without nanoparticle and incubated at 37° C for 24hrs. Broth culture was centrifuged at 5000 rpm for 10 minutes. The culture supernatants were transferred into new tubes and extracted with chloroform (1:2), and vortex for 1 min. The bottom organic phase was collected and re-extracted with 1 ml of 0.2 N HCl until colour change is observed. Absorbance of the aqueous phase was measured and compared at 520 nm in UV Vis spectrophotometer.

Estimation of swarming inhibition activity

2 µl of *P. aeruginosa* control and treated with silver nanoparticle was placed at the centre of LB swarming agar plates (agar 0.6%) and incubated at room temperature for 24 h. Swarming motility was compared by measuring the radius of the swarmed area in both control and treated cells.

Results and Discussion:

Enrichment and isolation of aquatic cyanobacterial species-

300-500 ml water was collected from rice farmlands, brackish water (Kalundre river), lake (Balleshwar lake) and home water tank in a cleaned plastic bottle and was immediately transported to laboratory for inoculation in BG-11 broth for enrichment.

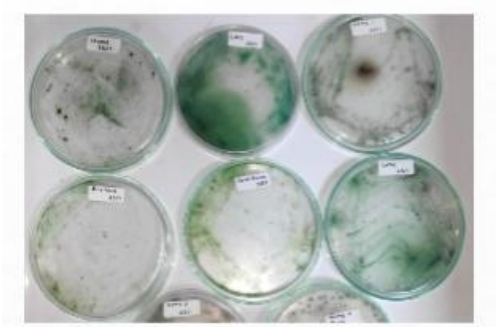


Fig 1. Isolation of Cyanobacteria on BG-11 agar plates

Fig 2. Mass culturing of Cyanobacteria

Mass sub-culturing and microscopic examination of isolated cyanobacteria

Five cyanobacterial colonies were selected for screening nanoparticle synthesizing ability. Genus of Cyanobacteria were identified based on their colony characteristics and microscopic examination.

Colonies growing on the plates were distinguished by colour. Biochemical characterisation of cyanobacteria was done by Stenholz *et al*. (2001) and Rippka *et al.* (1979). Isolates A, B, C, D and E were assigned to genus *Pseudanabaena*, *Aphanocapsa*, *Geitlerinema*, *Aphanothece* and O*scillatoria* respectively based on colony morphology and microscopic examination.

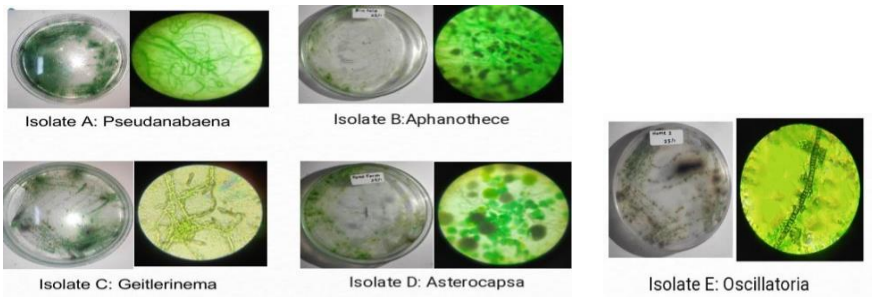
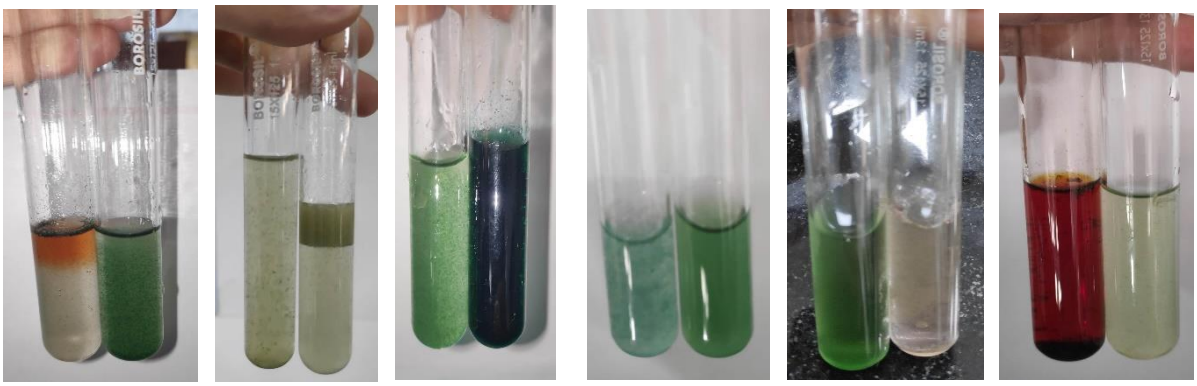


Fig 3. Microscopic and colony characteristics of isolates

Qualitative biochemical analysis of the cyanobacterial cell extract

The isolates were tested qualitatively for biochemicals viz, Alkaloids, Flavanoids, Quinones, Phenol, Tannin, Saponin and Proteins which have been theorized to act as reducing agents for nanoparticle synthesis as shown in Figure 5.



Glycosides +ve **Quinones +ve** **Tannins +ve** **Protein +ve** **Phenol +ve** **Alkaloids +ve**

Fig 4. Biochemical results

Key: +ve: Positive results, -ve: negative results

Preparation of silver nanoparticle using cyanobacterial biomass

One gram of wet weight biomass of each isolate was suspended in 20 ml of 1 mM aqueous AgNO3 solution at pH 7 for 24 hours under illumination. The Isolate A and D

solution, which was initially colourless, turned green and reddish-brown indicating the biotransformation of ionic silver to the reduced silver, and the subsequent formation of NPs in an aqueous medium. Reddish -brown colour of AgNPs was observed in aqueous solutions due to the excitation of surface plasmon resonance (SPR) of AgNPs. According to the obtained results, the experimented biomass of two isolated cyanobacterial cultures were able to reduce silver ion to AgNPs as verified by visual observation.

The nanoparticle solution was centrifuged at 3000 rpm for 10 min and sediment was suspended in sterile distilled water. The process was repeated twice to remove traces of silver nitrate. At the end 0.03 g of sediment was collected and added to 3 ml of sterile distilled water making concentration of 10 mg/ml. This nanoparticle solution was screened for quorum quenching activity.

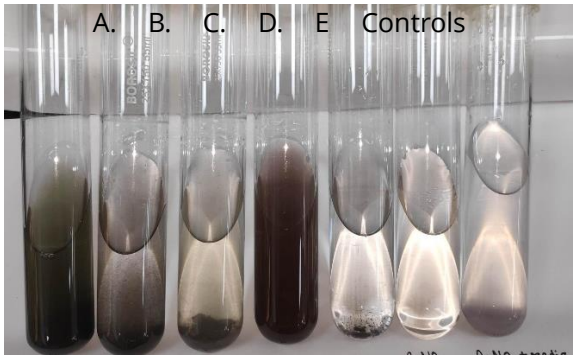
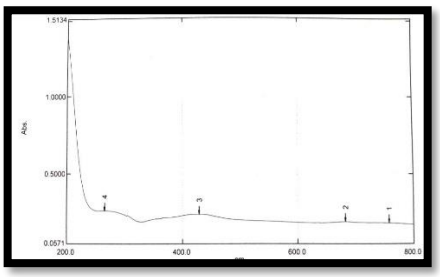


Fig 5. Silver nanoparticles production

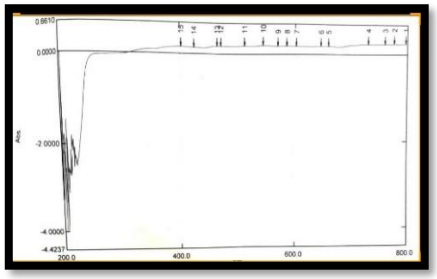
Characterization of nanoparticles using UV Visible spectroscopy

Nanoparticles have unique optical properties which makes UV-Vis a valuable tool for identifying, characterizing, and studying nanomaterials. figure 3 shown the absorption peak of Nanoparticles of various isolates. The surface plasmon resonance bands indicated the production of AgNPs.

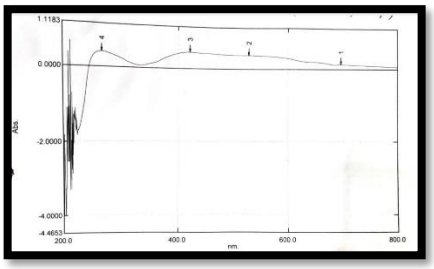
Other isolates did not exhibited the characteristic absorption peak around 200-400 nm indicating no biosynthesis of nanoparticles.



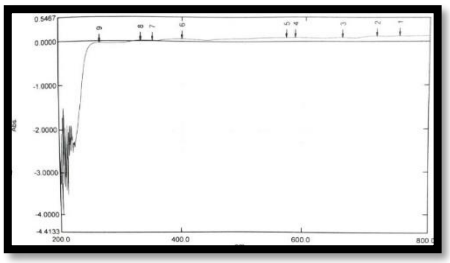
A-AgNP



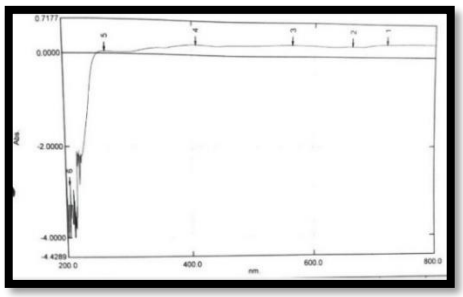
B- AgNP



C- AgNP



D- AgNP



E -AgNP

Fig 6. UV-vis spectrum of nanoparticles

Investigation of enhanced susceptibility to antibiotics in presence of AgNPs

Mueller-Hinton agar Plates were inoculated with *P. aeruginosa*, *S. aureus* and *K. pneumoniae*.

A sterile disk loaded with 20 µL of silver nanoparticles, one disk with antibiotics and one disk with antibiotic supplemented with 20 µL of with AgNP was placed on inoculated media .The plates were incubated aerobically at 37ºC for 24 h. Zones of inhibition for AgNPs, antibiotics, and antibiotics + nanoparticles were measured and expressed radii in millimetres. Antibiotic sensitivity test was done as per Kirby-bauer disc diffusion method as shown in figure 8. The combined use of an antibiotic and AgNP generally resulted in enhanced killing as compared to killing by an antibiotic alone. This data suggest that AgNPs may act synergistically with chemotherapeutics to increase the success of antibiotic treatment by enhancing the susceptibility of bacterial biofilms by interfering with quorum sensing and reducing their virulence.

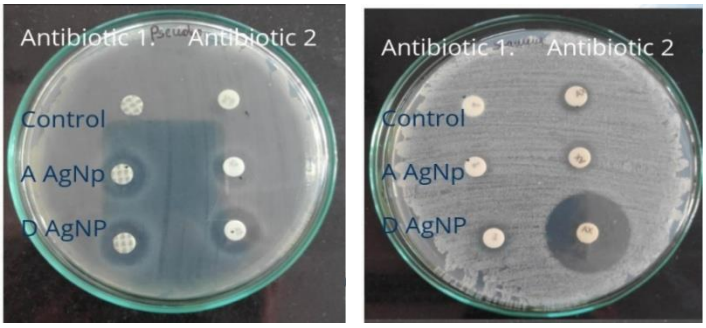


Fig 7. Antibiotic Susceptibility test in presence of AgNP

Table 1. Antibiotic Susceptibility results for *Klebsiella pneumoniae*

	K. pneumoniae + 'A'AgNP	'A' AgNP	*K. pneumoniae* + 'D'AgNP	'D' AgNP	*K. pneumoniae* control
Piperacillin	10	8	11	9	3
Novobiocin	9	8	8	9	6
Nalidixic acid	6	8	6	9	6
Gentamycin	9	8	11	9	6
Cefuroxime	6	8	6	9	6
Cefoperazone	9	8	11	9	6
Cephotax	6	8	6	9	6

Table 2. Antibiotic Susceptibility results for *S. aureus*

	S. aureus +'A'A gNP	'A' AgNP	*S. aureus* + ' D' AgNP	'D' AgNP	*S. aureus* control
Clindamycin	36	8	38	8	36
Oxacillin	9	8	11	8	6
Amoxycillin	10	8	25	8	9
Co-trimoxazole	32	8	32	8	32
Methicillin	8	8	10	8	6
Vancomycin	21	8	22	8	20

Table 3. Antibiotic Susceptibility results for *P. aeruginosa*

	P. aeruginosa+ 'A'AgNP	'A'AgNP	*P.aeruginosa*+ 'D' AgNP	'D' AgNP	*P.aeruginosa* control
Piperacillin	12	8	15	8	6
Novobiocin	9	8	8	8	6
Nalidixic acid	14	8	13	8	13
Gentamycin	18	8	19	8	18
Cefuroxime	12	8	12	8	6
Cefoperazone	11	8	11	8	6
Cephotax	10	8	9	8	6

Qualitative estimation of antibiofilm activity

Freeman *et.al* have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. 5mL *S. aureus* suspension was incubated with non-inhibitory concentration of NP (1mL) for 30 minutes and streaked on test Congo red agar plate and *S. aureus* suspension was streaked as control on CRA plate and incubated at 37ºC for 24 h aerobically. Biofilm producers form black colonies with a dry crystalline consistency and non biofilm producers forms pink coloured colonies on CRA.

In presence of AgNPs *S. aureus* formed pink colonies and exhibited slight blackening whereas control plate had colonies with dry crystalline consistency indicating biofilm production.

Semi-quantitative estimation of antibiofilm activity by Tube method

St. Nutrient broth were inoculated with loopful of *P. aeruginosa* and 100 µL silver nanoparticle solution and control was maintained without nanoparticle incubated at 37° C for 24hrs. After incubation, tubes were decanted and washed with phosphate buffer saline and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water and dried in inverted position. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. In presence of AgNPs *Pseudomonas* exhibited lower biofilm formation as compared to the control tube as shown in figure 9.

Control S. Aureus **S. Aureus + 'A' –AgNP** **S. Aureus + 'D' –AgNP**

Fig 8. Congo red agar results

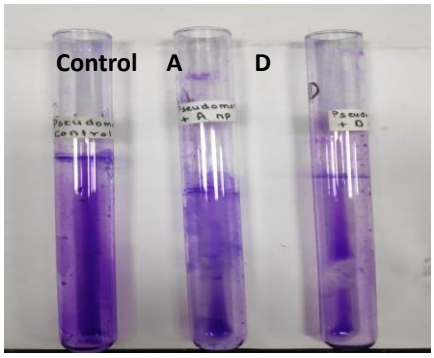


Fig 9. Tube method results

Estimation of pyocyanin inhibition

Pyocyanin is an extracellular redox-active

phenazine that contains a peculiar blue-green pigment. This compound can be isolated from cellular contaminants by isolating it in an aqueous suspension formed by the addition of chloroform. Because of the unique pigmentation this compound can then quantified with a spectrophotometer.

To determine the pyocyanin production *P. aeruginosa* was inoculated in 10 ml Nutrient broth with 100 µL silver nanoparticle solution and control was maintained without nanoparticle and incubated at 37° for 24hrs. Broth culture was centrifuged at 5000 rpm for 10 minutes. The culture supernatants were transferred into new tubes and extracted with chloroform (1:2), and vortex for 1 min. The bottom organic phase was collected and re-extracted with 1 ml of 0.2 N HCl until colour change is observed.
Absorbance of the aqueous phase was measured and compared at 520 nm in UV Vis spectrophotometer.

Pseudomonas with A- AgNPs exhibited 56.25% reduction in pyocyanin production as compared to the control whereas with D-AgNPs it exhibited 31.20% reduction. This indicates that silver nanoparticles have downregulated the production of pyocyanin due to quorum quenching activity.

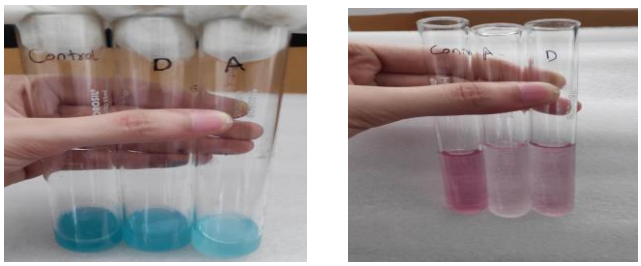


Fig 10. Pyocyanin extraction in chloroform

Table 4. Pyocyanin production results

	P. aeruginosa+ 'A' AgNP	P. aeruginosa+ 'D' AgNP	P. aeruginosa control
O.D at 520nm	0.07	0.11	0.16
Concentration (µg/ml) O.D ×17.072	1.19504	1.87792	2.73152
% Reduction	56.25%	31.20%	-

Estimation of swarming inhibition

Bacterial invasion is a necessity for biofilm formation. The effect of AgNPs was studied on the motility of biofilm forming *P. aeruginosa* bacterial cells. Figure 11 indicates that nanoparticles A and D inhibit the swarming motility of *P. aeruginosa* strains in the plate assay 76.66% and 70% respectively. It can be concluded from the results that silver nanoparticles reduced flagellum driven motility of *P. aeruginosa* in the treated sample compared to the control exhibiting quorum quenching activity. The AgNPs might impact motility by disturbing the Quorum sensing system.

Fig 11. Swarming inhibition results

4. Conclusion:

The present study concludes that silver nan

oparticles of cyanobacteria isolates A & D s howed good anti-bacterial activity. The com bined effect of an antibiotic and AgNP generally resulted in enhanced killing as compared to antibiotic alone. In presence of AgNPs, *Pseudomonas* exhibited lower biofilm formation. Also, *Pseudomonas* with Isolate A Cyanobacteria AgNPs exhibited 56.25% reduction in pyocyanin production as compared to the control whereas with D isolate Silver Nanoparticles exhibited 31.2% reduction. This indicates that silver nanoparticles have down regulated the production of pyocyanin due to quorum quenching activity. It was observed that silver nanoparticles of cyanobacterial isolates A and D inhibited the swarming motility of *P. aeruginosa* in the plate assay 76.66% and 70% respectively.

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