Synthesis and Evaluation of Quorum Quenching activity of Ag, TiO2 and ZnO metal nanoparticles against wild type and mutant strains of *Chromobacterium violaceum*

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Abstract

The conventional strategies to combat pathogenic microbes seem to be inefficient to curb the menace of pathogens. The bacterial biofilm is a product of the quorum sensing (QS) process mediated by acyl-homoserine lactones (AHL) in many pathogens. The QS inhibitors or quorum quenching (QQ) molecules that disrupt bacterial communication offer an alternative promising strategy to lower antibiotic resistance in pathogens. The nanoparticles can exhibit QQ potential that interrupts cell-to-cell communication. In the present study, laboratory synthesized metal-based nanoparticles such as silver (Ag), titanium oxide(TiO_2) and zinc oxide (ZnO) were characterized using powder X-Ray diffractometer, UV-Visible spectroscopy, EDS, Cyclic voltammetry and TEM imaging technique. Ag and TiO_2 nanoparticles were synthesized by the green synthesis method in the form of quantum dots, which was evidenced from TEM images on a less than 7nm scale. Silver quantum dots with size variation from 2.4nm to 4.2nm and TiO_2 with size ranging from 4.9nm to 8.5nm were detected.

The synthesized nanoparticles were elucidated for their potential as QQ by employing both wild and mutant strains of Chromobacterium violaceum. Nanoparticles affected violacein pigment production in both the strains of C. violaceum. Lower concentrations of NPs did not show any considerable effect on the AHL-dependent Quorum sensing system, however, at moderate to higher concentrations, metal NPs exhibit Quorum Q activity. Higher concentrations of AgNPs and ZnONPs (i.e. 250 ppm and above) show cell inhibitory activity. In the percent pigment(violacein) inhibition assay, maximum pigment inhibition was detected at a concentration range of 100-125ppm in both the strains of C. violaceum without inhibiting cell growth. In the case of TiO_2NPs, maximum pigment inhibition was detected at 500ppm without affecting cell growth in both the strains of C. violaceum. The percent pigment inhibition observed in all nanoparticles was nearly similar in both strains of C. violaceum indicating that the mechanism of action of pigment inhibition is due to interference at the reception of the AHL signaling molecule.

Keywords: Quorum sensing, Quorum quenching, *Chromobacterium violaceum*, Zinc oxide nanoparticles, Titanium dioxide nanoparticles and Silver nanoparticles.

Introduction

These days significant increase in multidrug resistance pathogens has been observed because of the overuse of antibiotics. Cytotoxic approaches like the use of antibiotics either kill or inhibit bacterial growth by interfering with cellular housekeeping functions such as DNA, RNA and protein synthesis. Hence these antibiotics lead to the emergence of multidrug-resistant microbial pathogens[9,11]. Noncytotoxic approaches to control infections such as hindrances in bacterial communication can prevent antibiotic resistance. In the field of antibiofilm agents, a study of quorum sensing (QS) inhibitors has become one of the most promising areas of research.

Antimicrobial agents involved in inhibiting or interfering with QS have emerged as novel tools[5,9]. These antimicrobial agents target virulence mechanisms in many biofilm-forming pathogens. Targeting virulence instead of killing pathogens is a very promising strategy to control disease without creating life-death pressure on pathogens[22]. Thus, quorum quenchers act as anti-virulent agents which have the potential to interfere with virulence, reducing the emergence of resistance against QQ molecules[6].

QS is the cell-to-cell communication system by which bacteria monitor their population density by generating and detecting signaling molecules. When the bacterial population when reaches the required density, synchronization of expression of a different set of genes occurs and all cells in quorum respond simultaneously[22]. QS occurs in both gram positive and gram negative bacterial species[23]. Different types of signaling molecules used for QS are identified such as acyl-homoserine lactones (AHL) in gram negative bacteria, auto-inducing peptides (AIPs) in gram positive bacteria and AI-2 s molecules produced by both gram positive and gram negative bacteria[3].

Compounds that inhibit or interfere with bacterial cell to cell communication systems and biofilm formation are called quorum quenchers (QQ). QQ target the cell signaling molecules, by affecting the virulence of bacteria without inhibiting its growth. QQ molecules do not impose selective pressure on pathogens, thus reduce the chances of the development of resistance. Recently, various QQ biomolecules have been reported from a wide range of

sources that control pathogenic infection without modifying them to an antibiotic-resistant pathogen[4].

Clinical trials of QQ biomolecules are under investigation, however, a limited success rate has been observed due to their inefficient stability, biocompatibility, bioavailability and delivery to their target site[8]. Nanotechnology has revolutionized many fields of human endeavor. To overcome this problem, recently researchers are focusing on nanomaterials as QS inhibitors as they possess numerous advantageous properties such as effective biofilm penetration, efficient delivery at the target site, stability, biocompatibility and lesser toxicity to the host system.

Nanoparticles are preferred for various therapeutic applications because of their extensive reactivity which is contributed by their large surface area to volume ratio[17.] Metal nanoparticles (NPs) with QQ potential have shown promising strategy to control QS dependent virulence. Although literature regarding the QQ potential of NPs is scarce, yet some studies have mentioned the direct involvement of NPs as QS inhibitors or their role as carriers of QQ[20].

Most of the studies on the screening of QQ molecules employed *Chromobacterium violaceum* as a biosensor organism. *C. violaceum*is a gram negative bacterium, commonly found in soil and water. In this bacterium, the production of violet colored pigment violacein is controlled by QS regulated process. Violacein pigment can be easily detected and quantified. Therefore, this organism is commonly used in the detection of QQ molecules. Along with *the C. violaceum* wild-type strain, the mutant strain *C. violaceum* CV026 has been also employed in QQ studies. *C. violaceum* CV026 is deficient in the synthesis of homoserine lactone molecule and subsequently pigment production[13,18].

The differential properties of *C. violaceum* strains can be applied to reveal QQ potential of nanoparticles[10]. Current work focuses on the elucidation of the effect of nanoparticles on bacterial QS systems by employing both wild and mutant strains of *C. violaceum.* Laboratory synthesized metal based nanoparticles such as silver (Ag), zinc oxide (ZnO) and titanium oxide (TiO_2) were used to understand the mechanism of inhibition of QS system to find new strategies to control virulence in biofilm forming pathogens.

Material and Methods:

Synthesis and characterization of nanoparticles: Dried fruits of *Embillicaofficinalis, Terminaliabelerica* and *Terminaliachebula* were taken from Ayurved Rasashala, Pune. Fruits were washed with distilled water, dried and ground in a mixer to obtain a fine powder. The powdered mixture was added to 100 ml deionized water and boiled for 30 minutes in a water bath. The clear filtered mixture extract was used for the synthesis of AgNPs and TiO_2NPs from 0.02M $AgNO_3$ and 0.01M $TiCl_3$ solutions respectively. The mixtures were sonicated in a sonicator at room temperature for 10 to 15 minutes[18]. ZnONPs were chemically synthesized from zinc acetate dehydrate [$Zn(CH_3COO)_2.2H_2O$] and sodium hydroxide (NaOH) as per the method prescribed.

Zinc acetate dihydrate and sodium hydroxide were dissolved in deionized water to form the liquid media of the desired concentrations of 0.05M and 0.1M respectively. ZnONPs were synthesized by mixing two solutions in a 1:1 ratio of the concentrations [$Zn(CH_3COO)_2.2H_2O$: NaOH][15]. Nanoparticles were characterized using powder X-Ray diffractometer (Bruker Advance D8), UV spectrometry, EDS (Perkin Elmer), Cyclic voltammetry and TEM techniques.

Cultivation and Maintenance of bacterial strains: In this study, two bacterial strains *Chromobacterium violaceum 2656* and *C.violaceum* CV026 were used as biosensor strains to monitor Acyl Homoserine Lactone N-hexanoyl-L – Homoserine lactone (C6-HSL) inactivation activity[16]. *C. violaceum* CV026 is a mini tn5 mutant of wild type *C.violaceum*. *C. violaceum 2656* was obtained from MTCC, IMTECH, Chandigarh, India and *C.violaceum* CV026 is obtained from NCMR-NCCS, Pune, Maharashtra. *C.violaceum 2656* was cultivated and maintained in nutrient broth tubes and incubated at 30ºC. Similarly, *C. violaceum* CV026 was also cultivated and maintained at 30ºC in nutrient broth tubes supplemented with 25µg ml^{-1} Kanamycin (Gibco BRL).

Effect of nanoparticles on viability *C. violaceum* strains: Well, diffusion assay of AgNPs, ZnONPs and TiO_2NPs was carried to know the effect of nanoparticles on the viability of cells. *C.violaceum 2656* was inoculated in 5 ml nutrient broth tubes. Similarly, *C.violaceum* CVO26 was also inoculated in 5 ml nutrient broth tubes supplemented with 25µg ml^{-1} Kanamycinand at 12.5mM concentration. Both cultures were incubated for 18-20 hours at 30ºC. O.D of both samples was adjusted to 1×10^8 CFU /ml and 100 µl of each culture was spread on nutrient agar plates. Differential concentrations of the above-mentioned nanoparticles (5-500ppm) were loaded into wells formed in the plates. These plates were incubated at 30ºC for 24 hrs. Appropriate controls were kept to compare. After incubation, zones of growth inhibition were observed.

Violacein pigment inhibition assay: Inhibition of violacein production by Ag, ZnO and TiO_2NPs was performed by using a previously reported method by Khan et al[12] with slight modifications. In this method, all NPs were diluted to appropriate concentrations (5-500ppm) with nutrient broth. Further, all the concentrations of NPs were tested for their QQ activity in pigment inhibition assay which was performed in triplicate for each sample separately. In this assay, each concentration of NPs was inoculated separately in 2 ml of $1/100^{th}$ dilution of freshly grown biosensor cultures of *C.violaceum 2656* and *C.violaceum* CV026 in nutrient broth. In the experiments conducted with

C.violaceum CV026, 12.5mM of N-hexanoyl-L-homoserine lactone (C6-HSL) was added to induce the production of violacein pigment.

Control sets were kept for all the concentrations of NPs. Cultures were incubated at 30º C for 24hrs with shaking at 120 rpm. After incubation, tubes were centrifuged at 10,000 g for 10 min. To the resulting cell pellet, 1ml of DMSO was added. The mixture was vortexed until the violacein pigment completely solubilizes and further, centrifuged at 10000g for 10 min. 200µl of the supernatant containing solubilized violacein pigment was placed in 96 wells flat-bottom microtiter plate. The absorbance of extracted pigment was measured at 590nm wavelength using the microtiter reader (Thermo scientific Multiscan Go).

Violacein production by *C. violaceum* strains without being treated with nanoparticles was also measured at 590nm and which was considered as 100% violacein production. Furthermore, the absorbance of nutrient broth medium and nanoparticles dispersed in nutrient broth was also measured to monitor interferences of nanoparticles in absorbance measurement. Reduction in violacein pigment production in presence of NPs was measured in terms of percentage inhibition as [(O.D of control)-(O.D of test)/OD of control] X 100.

Results and Discussion

Synthesis and Characterization of nanoparticles: Nanoparticles were synthesized using green synthesis and chemical synthesis approaches. Green synthesis methods employed for the synthesis of AgNPs and TiO_2NPs were reported earlier by several researchers using dried fruits of *Embillica officinalis*, *Terminali abelerica* and *Terminalia chebula*[2,18,19]. Fig. 1a shows the visible color change of $AgNO_3$ from colorless to dark brown in presence of dried fruit extract indicating possible synthesis of AgNPs.

Similarly, TiO_2 solution showed a color change from colorless to yellowish-orange in presence of the dried extract of three herbs. Plant material contains diverse biomolecules and metabolites like proteins, vitamins, coenzymes based intermediates, phenols, flavonoids and carbohydrates having hydroxyl, carbonyl and amine functional groups that react with metal ions and reduce their size into the nano range[14]. These biomolecules reduce the size of the nanoparticles and also play an important role in the capping of the NPs which is important for stability and biocompatibility of nanoparticles[14].

The UV/Visible spectra of AgNPs(Fig.1a) showed a clear indication of synthesis of AgNPs with color change from colorless to dark brown. This is supported with a UV-Visible peak at 500nm which is the surface plasmon resonance peak for AgNP. TEM data ((Fig. 1c and 1d) explains crystalline nature and polydisperse silver quantum dots with size variation from 2.4nm to 4.2nm. Energy dispersive spectra (fig. 1e) indicate evidence of silver concentration in mapping. The figure 1(f) explains the redox behavior of silver nano quantum dots indicating on the surface of silver quantum dots that many reactions were taking place which was not in uniform redox couple. Fig. 1g indicates XRD 2θ data for silver nanoparticles as per JCPDS file 04-0783 in which FCC structure is with planes 111, 200, 220, 311 and 222.

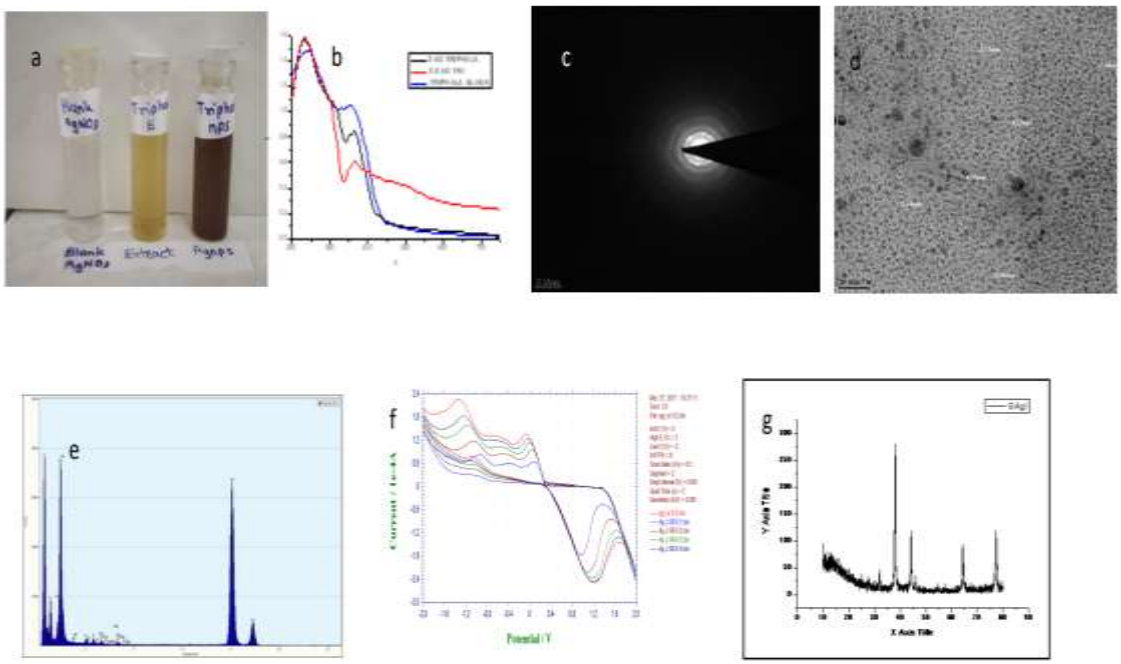

Figure 1: Characterization of silver nanoparticles (AgNPs): (a) color change during synthesis, (b) UV-Visible plots for AgNP, (c,d) TEM images of AgNP, (e) EDS mapping for AgNP, (f) Cyclic voltammogram for AgNP, and (g) XRD pattern of AgNP

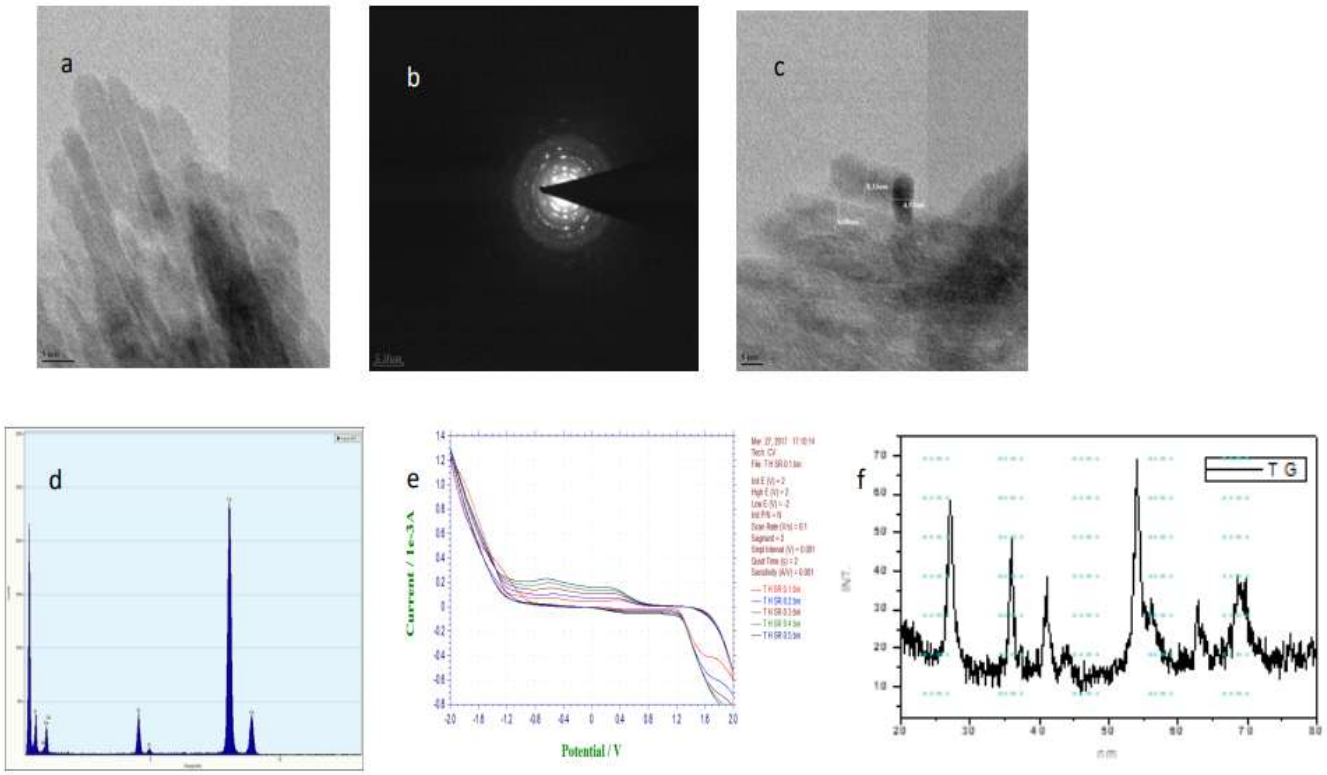

Figure 2: Characterization of Titanium nanoparticles (TiO_2NPs): (a, b, c, d) TEM images of TiO_2NPs with EDS mapping, (e) Cyclic voltammogram of TiO_2NPs, (f) X-ray Diffraction pattern of TiO_2NPs.

Fig. 2 (a, b, c) shows that TiO_2 NPs are also quantum dots as per TEM data with a polydisperse rod-like structure with a size ranging from 4.9nm to 8.5nm. EDS plot (fig. 2d) confirms the formation of TiO_2 NPs. XRD data (Fig. 2e) shows the anatase structure of TiO_2 which matches with JCPD file no. 21-1272. Cyclic voltammogram data (Fig. 2f) shows a redox couple with electron transfer at the surface. As already reported method for synthesis of ZnO NPs was followed, hence its characterization was not performed.

Effect of nanoparticles on viability *C. violaceum* strains: *C. violaceum* strains are being used as biosensors to study the quorum quenching potential of various molecules due to their ability to produce violet colored pigment through an acyl-homoserine lactone regulated QS process. The inactivation of cell-to-cell communication can occur at three different levels: 1)Inhibition of synthesis of a signaling molecule,2)Inhibition of accumulation, exchange and transport of QS signaling molecule and 3) Inhibition of binding of a signaling molecule with the receptor[7,20]. The significant reduction in violacein production in *C. violaceum* 2656 compared to *C. violaceum* CV026 suggests the interference in the biosynthesis of quorum sensing signaling molecule by the studied metal nanoparticle.

Alternatively, the decrease in violacein production in both strains of *C. violaceum* indicates interference of nanoparticles in the binding of a signaling molecule with the receptor. QS regulated process depends on cell population density. Therefore, it is necessary to evaluate the effect of NPs on *C. violaceum* viability as it will directly alter the production of violacein. So along with violacein pigment inhibition assay, the effect of NPS on cell growth was detected by well diffusion assay.

Zinc oxide nanoparticles(ZnO): ZnONPs affected violacein production in both the strains i.e. *C.violaceum 2656* and *C. violaceum* CV026. In CV026 maximum violacein, pigment inhibition was 43% at 125 ppm concentration of nanoparticle without inhibiting cell growth. However, pigment inhibition was not observed at lower concentrations i.e. 5ppm and 25 ppm. Also in *C.violaceum 2656,*a maximum of 41% pigment inhibition was observed at 100ppm without inhibiting cell growth. Percent pigment inhibition was similar at 250 ppm and 500ppm concentrations of ZnONPs for both the strains (fig. 3and 4).

The pigment production has been reduced up to 45% when *C.violaceum 2656* was treated with 250 ppm and 500ppm concentration of ZnONPs. Also when *C.violaceum CV026* was treated at 250 ppm and 500ppm concentration respectively, 42% and 44% pigment inhibition were detected. However, for both the strains of *C. violaceum*, inhibition of growth was detected at concentrations 250ppm and 500 ppm of ZnONPs.

Thus from the above findings, it can be concluded that at concentrations 50ppm,100ppm and 125ppm, ZnONPs act as quorum quenching molecules. However, higher concentrations of ZnONPs show antibacterial activity.

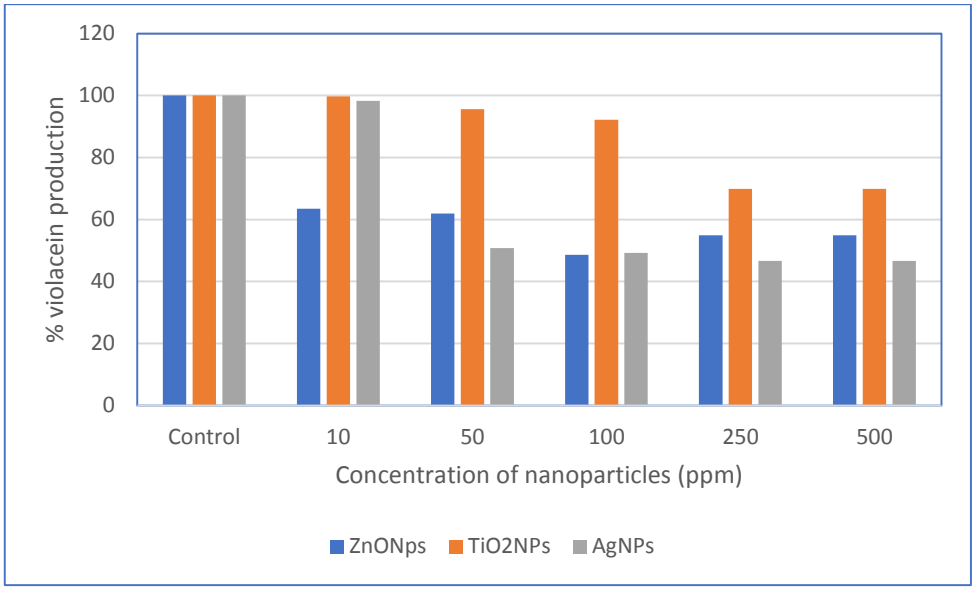

Fig. 3: Effect of metal nanoparticles (Ag, ZnO and TiO_2) on violacein production in *C. violaceum 2656* wild type.

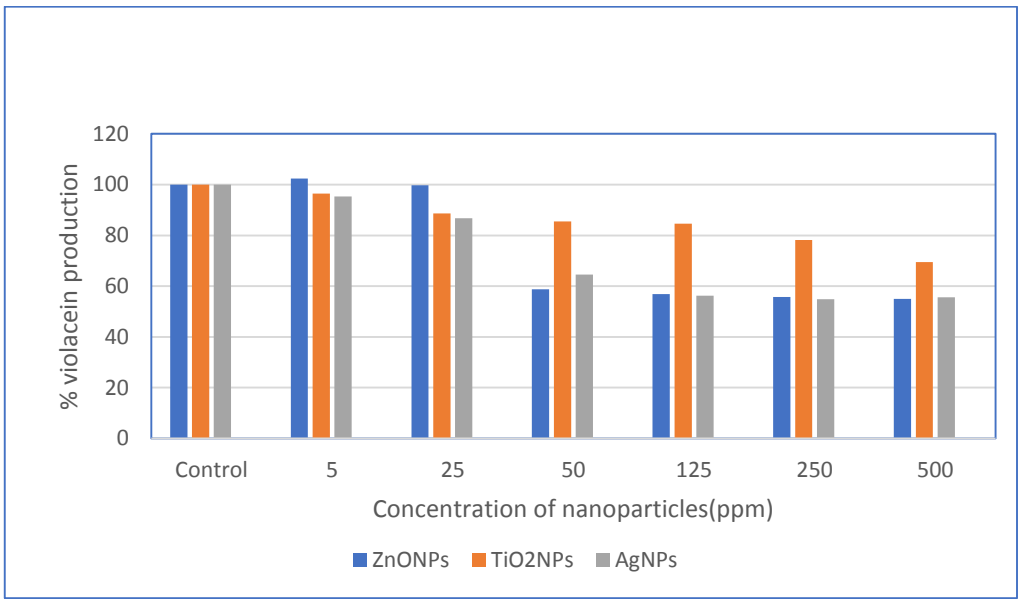

Fig. 4: Effect of metal nanoparticles (Ag, ZnO and TiO_2) on violacein production in *C. violaceum* CV026.

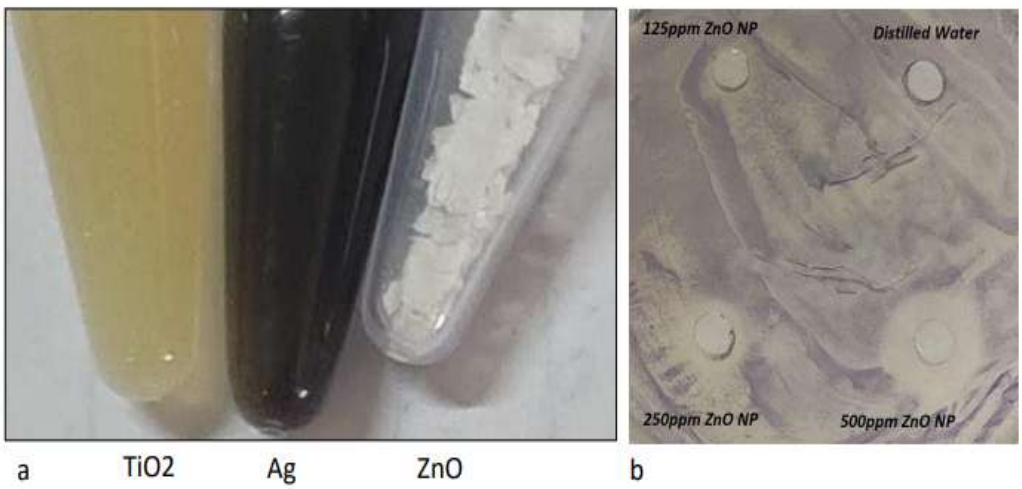

Fig. 5: a) Nanoparticles used in the study, b) Zone of inhibition of growth indicating cell inhibitory effect of ZnONPs against *C. violaceum 2656* at 250ppm and 500ppm

Titanium Oxide Nanoparticles (TiO_2NPs): As shown in fig. 3 and fig. 4, lower concentrations of TiO_2NPs did not affect the synthesis of violacein pigment in both strains of *C. violaceum*. In *C. violaceum* CV026, 31% and 22% violacein pigment inhibition were detected at 500 ppm and 250 ppm respectively. Also when *C. violaceum 2656* is treated with TiO_2NPs, almost 22% pigment inhibition was detected at 250 ppm and 500 ppm. In both the strains, maximum pigment inhibition (30%) was detected at 500ppm without affecting cell growth. Along with this cell growth, inhibition was not detected in TiO_2NPs which indicates its quorum quenching potential. Violacein pigment inhibition in TiO_2NPs is not because of the inhibition of cells of *C. violaceum* strains, but it is due to TiO_2NPs arresting the QS mechanism of these strains. Previously, Gomez et al[7] also reported the quorum quenching potential of TiO_2NPs.

Silver Nanoparticles (AgNPs): Further AgNPs were also evaluated for quorum quenching properties against *C. violaceum* strains. Considerable reduction in pigment production was detected when *C. violaceum* CV026 was cultured in presence of AgNPs. When *C. violaceum* CV026 was cultured with AgNps violacein pigment percent reduction was detected up to 36% at 50ppm concentration, 44% at 125 ppm and almost 45% at 250ppm and 500ppm concentrations. Whereas, when *C. violaceum* 2656 was cultured with AgNPs, comparatively more violacein pigment inhibition was detected. More than 49% pigment was inhibited above 50 ppm concentration of AgNPs. Along with this, as shown in fig. 3, at concentrations 250 ppm and 500 ppm, cell inhibitory activity against the both strains of *C. violaceum* strains was detected, indicating that at low concentrations, AgNPs have QQ potential. Maximum violacein pigment inhibition (50.79%) was detected without cell inhibition when *C. violaceum* 2656was treated with a 100 ppm concentration of AgNPs. Wagh et al[22] also reported the QQ activity of silver-based nanoparticles.

Conclusion

The results from the current study demonstrate that metal nanoparticles affect AHL-dependent QS systems. The action of metal nanoparticles depends upon the concentration of nanoparticles. The lower concentrations of NPs do not show any considerable effect on AHL dependent QS system, however, moderate to higher concentrations behave as QQ molecules. At higher concentrations i.e. 250 ppm and above, both AgNPs and ZnONPs show cell inhibitory activity. In all of these nanoparticles detected, pigment inhibition was approximately similar for both *C. violaceum* strains indicating, the action of NPs at the level of reception of AHL signaling molecule.

In the case of TiO_2NPs, cell inhibitory activity was not detected at higher concentrations but QQ activity has been detected. Also, comparatively equal percent violacein pigment inhibition was detected in both the strains of *C. violaceum* indicating that the mechanism of the action of pigment inhibition is interference at the reception of AHL signaling molecule. These findings suggest that TiO_2NPs have the potential to be used as QQ molecule. Thus further studies on the optimization of the concentration of these NPs may reveal new insights into the strategies to be applied to control QS-regulated virulence in pathogens. Also, studies on the application of these NPs along with the antibiotics may reduce the dose of antibiotics which may further control the emergence of multidrug resistance in pathogens.

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