Int.J.Curr.Microbiol.App.Sci (2017) *6*(3): 2485-2495

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 3 (2017) pp. 2485-2495**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.603.281

Antagonistic Activity of Biogenic TiO_2 Nanoparticles against *Staphylococcus aureus* and *Escherichia coli*

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Keywords

Antibacterial Activity, *Escherichia coli, Staphylococcus aureus*, TiO2 nanoparticles.

Article Info

Accepted:
20 February 2017
Available Online:
10 March 2017

A B S T R A C T

Nanobiotechnology is an emerging field of science that utilizes nanobased systems for various biotechnological and biomedical applications. The synthesis of metal and metal oxide nanoparticles has attracted considerable attention, as they have high surface area and high fraction of atoms which is responsible for their fascinating properties such as antimicrobial, magnetic, electronic and catalytic activity. The antibacterial activities of TiO_2 nanoparticles were studied in *Staphylococcus aureus* and *Escherichia coli*. Treatment of the bacterial cells with TiO_2 NP's resulted in the leakage of reducing sugars, proteins and reduced the activity of the respiratory chain dehydrogenases. In conclusion, the combined results suggested that TiO_2 NP's was found to damage the bacterial cell membrane and depress the activity of some vital enzymes which eventually led to the death of bacterial cells. Thus TiO_2 NP's could be used as an effective antibacterial material in the burgeoning field of Nanomedicine research with tremendous prospects for the improvement of combating human pathogens.

Introduction

Particles having one or more dimensions of the order of 100 nm or less are termed as "Nanoparticles". They have attracted global attention due to their unusual and fascinating properties and applications advantageous over their bulk counterparts (Daniel and Astruc, 2004; Kato, 2011). Nanobiotechnology is an emerging field of science that utilizes nano based-systems for various biotechnological and biomedical applications (Ahmed and Sardar, 2013). Nanoparticles have a high specific surface area and a high fraction of surface atoms and they have been studied extensively because of their unique physicochemical characteristics including catalytic activity, optical properties, electronic properties, antibacterial properties and magnetic properties (Krolikowska *et al*., 2003; Catauro *et al*., 2004). Different types of nanoparticles can be synthesized by a large number of physical, chemical, biological, and hybrid methods (Luechinger *et al*., 2010; Liu *et al*., 2011). Although physical and chemical methods are more popular in the synthesis of nanoparticles, the use of harsh environmental conditions and toxic chemicals greatly limits their biomedical applications (Li *et al*., 2011).

Nanoparticles produced by a biogenic enzymatic process are far superior, in several ways, to those particles produced by chemical methods. The biogenic approach for the synthesis of nanoparticles is thought to be clean, nontoxic and environmentally acceptable "green chemistry" procedure. Nanomedicine is a burgeoning field of research with tremendous prospects for the improvement of the diagnosis and treatment of human diseases (Li *et al*., 2011). Nanotechnology is expected to open new avenues to fight and prevent disease using atomic scale tailoring of materials. Recently it has been demonstrated that metal oxide nanoparticles exhibit excellent biocidal and biostatic action against Gram-positive and Gram-negative bacteria (Lopez Goerne *et al*., 2012). TiO_2 has three crystalline phases: anatase, rutile and brookite. Moreover TiO_2 nanoparticles possess interesting optical, dielectric, antimicrobial, antibacterial, chemical stability and catalytic properties which leads to industrial applications such as pigment, fillers, catalyst supports and photocatalyst (Sundrarajan and Gowri, 2011). Anatase has attracted much attention owing to its application in photovoltaic cells and photocatalysts and for its antimicrobial properties (Ahmed and Sardar, 2013).

TiO_2 nanoparticles have become a new generation of advanced materials due to their novel and interesting optical, dielectric, and photo-catalytic properties from size quantization (Alivisatos, 1996). The present study involves the biogenic approach of TiO_2 synthesis using the culture supernatant of the bacterial strain, *Staphylococcus arlettae* and evaluation of their antibacterial activity against selected bacterial isolates.

Materials and Methods

Biogenic Approach for the Synthesis of Tio_2 Nanoparticle

Chemicals Used

TiO $(OH)_2$ (99.9 %) was procured from Sigma Aldrich Chemicals, Bangalore, India. All other regents used in the reaction were of analytical grade with maximum purity. Deionized water was used throughout the experiment. The glass wares were washed in dilute nitric acid and thoroughly washed with double distilled water and dried in hot air oven.

Bacterial Strain Used

The bacterial strain used in this study was isolated from sludge and effluents were collected from textile and tannery industries. Based on the morphological, cultural, biochemical characteristics and 16 s rDNA sequencing, the isolate was identified as *Staphylococcus arlettae.* The pure cultures were maintained on nutrient agar slants at 4° C.

Synthesis of TiO_2 Nanoparticles

Staphylococcus arlettae strain IDR-4 cells were allowed to grow as broth culture for 1 week at 37°C in shaking condition at 120 rpm and were treated as source culture. 50 ml of the cultural broth was taken and centrifuged at 8000 rpm for 10 minutes. Following centrifugation, 20 ml of the culture supernatant was mixed with 20 ml of 0.025M $TiO(OH)_2$ to form a ratio of 1:1. The mixture was treated at 80°C for 10–20 min until white deposition starts to appear at the bottom of the flask, indicating the initiation of transformation. The culture solution was cooled and allowed to incubate at room temperature in the laboratory ambience. After

12–48 h, the culture solution was observed to have distinctly markable coalescent white clusters deposited at the bottom of the flask (Kirthi *et al*., 2011; Tharanya *et al*., 2015).

Antibacterial activity of TiO_2 Nanoparticles

The antibacterial effect of TiO_2 nanoparticles were examined by disc diffusion method against gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) gram negative bacteria (*Escherichia coli* and *Serratia marcescens*) collected from lab stock.

Muller Hinton agar was prepared and poured onto the sterile petriplates. After solidification, 2 wells were cut (for test and control) and each culture was swabbed individually on the respective plates. The synthesized TiO_2 nanoparticles were diluted with distilled water (15µg/ml) and placed onto each wells and incubated for 24 hours. Following incubation the zone of inhibition against nanoparticle were observed and measured (Yokeshbabu *et al*., 2013).

Assay the minimum inhibitory concentration of TiO_2 NP's

The minimum inhibitory concentration (MIC) of TiO_2 NP's was determined by using the standard plate count method. The powdered form of TiO_2 NP's was sterilized with UV radiation for 1 h, and the weighed under aseptic conditions. Mueller-Hinton broth containing 10^5 CFU/ml of bacterial cells was used as a starter culture. Various concentrations of TiO_2 NPs (0, 50, 100, 150 and 200 µg/ml) was inoculated onto the above mentioned starter cultures and incubated in a shaking incubator at 37°C for 24 h. Following incubation, 100 µl of the test cultures was spread onto Muller-Hinton agar and incubated at 37° C for 24 h. After incubation, the number of colonies grown on the agar was counted (Wang *et al*., 2006; Kim *et al*., 2011).

Growth curve Determination of bacteria exposed to different concentrations of TiO_2 NP's

To investigate the antibacterial efficacy of TiO_2 NP's, the growth curve of bacterial cells exposed to different concentrations of TiO_2 NP's was taken. Mueller-Hinton broth with different concentrations of TiO_2 NP's powder (0, 50, 100, and 150 µg/ml) was prepared, and the test bacterial culture (10^5 CFU/ml) was inoculated and incubated in a shaking incubator at 37° C for 24 h. Growth curve of bacterial culture were attained through repeated measures of the optical density (O.D) at 600 nm.

Effect of TiO_2 NP's on leakage of reducing sugars and proteins through membrane

To investigate the leakage of reducing sugars and proteins through the host cell membrane, different volumes of Mueller-Hinton medium, TiO_2 NP's and the test bacterial cells were added into 10 ml cultures with final concentration of 100 µg/ml TiO_2 NP's and 10^5 cfu/ml bacterial cells. Control experiments were performed in the absence of TiO_2 NP's. The cultures were incubated at 37°C with shaking at 150 rpm. Following 4 h incubation, 1 ml of the bacterial cultures was sampled and centrifuged at 12,000 rpm, the supernatant liquid was frozen at -30°C immediately and then the concentration of reducing sugars and proteins were determined as soon as possible (Bradford, 1976; Miller, 1959).

Assay the effect of TiO_2 NP's on respiratory chain LDH activity in bacterial cells

The dehydrogenase activity was determined according to previous iodonitrotetrazolium chloride method (Kim *et al*., 2009). The bacterial respiratory chain dehydrogenase will reduce colorless INT to a dark red waterinsoluble iodonitrotetrazolium formazan (INF). Different volumes of MH medium, TiO_2 NP's and bacterial cells were added into 10 ml cultures. The bacterial cells were boiled for 20 min to inactivate the enzymes completely as the negative control, while the cells were not boiled, and their enzymes maintained native activity as the positive control. 1 ml culture was sampled and centrifuged at 12,000 rpm, then the supernatants were discarded and the bacteria washed by phosphate-buffered saline (PBS) twice and added 0.9 ml PBS to suspend the bacteria. INT solution (0.1 ml 0.5%) was added, the culture was incubated at 37°C in dark for 2 h, and then 50 µl formaldehyde was added to terminate the reaction. The culture was centrifuged to collect the bacteria and 250 µl solutions of acetone and ethanol 1:1 in volume were used to distill the INF twice. The supernatants were finally combined. The dehydrogenase activity was calculated according to the maximum spectrophotometrical absorbance of INF at 490 nm (Li *et al*., 2010).

Results and Discussion

Nanotechnology is regarded as a key technology which will have economic, social and ecological implication. The field of nanotechnology is one of the most active areas of research in modern materials science. Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. New applications of nanoparticles and nanomaterials are emerging rapidly. Nanotechnology is currently employed as a tool to explore the darkest avenues of antibacterials (Shoba *et al*., 2010).

Biogenic synthesis of TiO_2 nanoparticles using the culture supernatant of IDR-4

The bacterial strain used in this study was isolated from Environmental samples including sludge and effluents were collected from textile and tannery industries located in and around Kanchipuram, Tamil Nadu. The culture supernatant of the bacterial strain possessed the ability to mediate the biosynthesis of TiO_2 nanoparticles, which was apparent by the color change from golden yellow to dark white (precipitated at the bottom of the culture broth) after 24 h of incubation. Similarly titanium oxide nanoparticles were found to be synthesized by using *Planomicrobium* sp. (Malarkodi *et al*., 2013) and *Chromohalobacter salexigens* (Tharanya *et al*., 2015). By 16 S r DNA analysis, the isolate IDR-4 was identified as *Staphylococcus arlettae* strain IDR-4.

Antibacterial activity of TiO_2 nanoparticles

The antibacterial activity of the biogenic TiO_2 nanoparticles were carried out against Gram positive (*Staphylococcus aureus*, *Bacillus subtilis) and* Gram negative (*Escherichia coli Serratia marcescens*) bacterial strains. TiO_2 nanoparticles exhibited maximum antagonistic activity on *E. coli* (16 mm) and *S. aureus* (13 mm).

The formation of zone around the TiO_2 nanoparticles wells clearly proved the antibacterial property of TiO_2 nanoparticles. However, *Bacillus subtilis and Serratia marcescens* showed remarkable resistance against TiO_2. Further studies were carried out with the susceptible isolates - *Escherichia coli* and *Staphylococcus aureus* (Table 1).

The differential sensitivity of Gram-negative and Gram-positive bacteria towards nanoparticles may be depends upon their cell outer layer attribute and their interaction with the charged TiO_2 nanoparticles. It was observed that the negative charge on the cell surface of Gram-negative bacteria was higher than that the Gram-positive bacteria (Roy *et al*., 2010).

Growth curves of bacterial cells treated with different concentrations of TiO_2 NP's

The growth curves of *S. aureus* and *E. coli* cells treated with TiO_2 NP's indicated the suppression of the bacterial growth and reproduction of bacterial cells. In control group (cells not treated with TiO_2 NP's), bacterial growth increased gradually with the increase in incubation time. However, the cells treated with TiO_2 NP's showed gradual decline in their growth curve with increase in the incubation time and increase in the concentration of NPs. When treated in the presence of 150 µg/ml TiO_2 NP's the growth of *S. aureus* and *E. coli* cells were found to be completely inhibited (Fig 1 and 2). Interestingly, upon comparison of the bacterial growth curves of *S. aureus* and *E. coli* cells, TiO_2 NP's exhibited significant growth inhibition of *E. coli* than of *S. aureus*. Similar results were reported by Kim *et al.*, (2011).

Minimum inhibitory concentration of TiO_2 NP's

The minimum inhibitory concentration (MIC) was evaluated to determine the lowest concentration of the TiO_2 NP's that could completely inhibit the viability of the *S. aureus* and *E. coli* cells. The viability of bacterial cells gradually decreased with the increase in the concentration of TiO_2 NPs. The MIC of TiO_2 NP's against *S. aureus* and *E. coli* was found to be 150 µg/ml, at which the growth of both the bacterial strains was completely inhibited. The antibacterial activities of the TiO_2 NP's against the Gram-positive *S. aureus* and Gram negative *E. coli* were almost identical (Fig 3 and 4). Similarly, TiO_2 nanoparticles biosynthesized by using the culture supernatant of *Planomicrobium sp.* exhibited remarkable antagonistic activity against *Bacillus subtilis* and *Klebsiella planticola* respectively (Malarkodi *et al.*, 2013).

Table.1 Antibacterial activity of biogenic TiO_2 NP's against the selected bacterial isolates

S. No	Bacterial strains	Zone of Inhibition
1.	*Staphylococcus aureus*	13 ± 0.5 mm
2.	*Bacillus subtilis*	6 ± 0.4 mm
3.	*Serratia marcescens*	7 ± 0.6 mm
4.	*Escherichia coli*	16 ± 0.8 mm

Fig.1 Growth curve of *Staphylococcus aureus* in the presence of TiO_2 nanoparticles

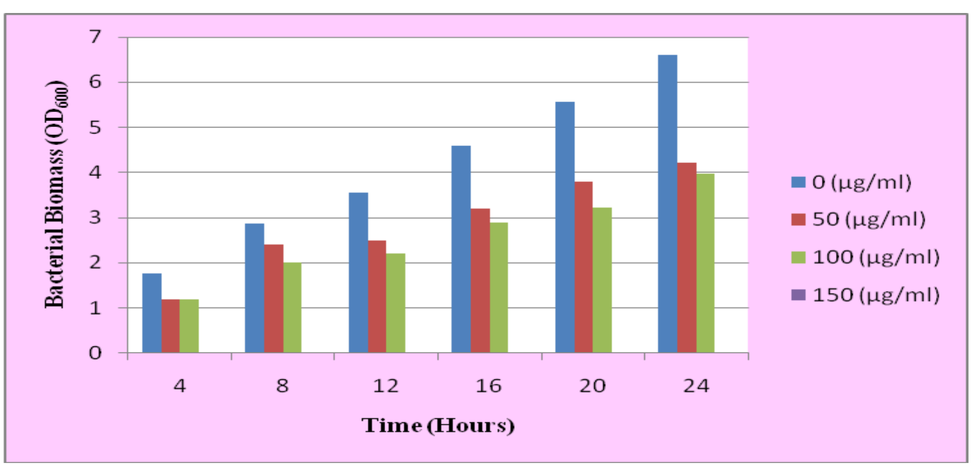


Fig.2 Growth curve of *Escherichia coli* in the presence of TiO_2 nanoparticles

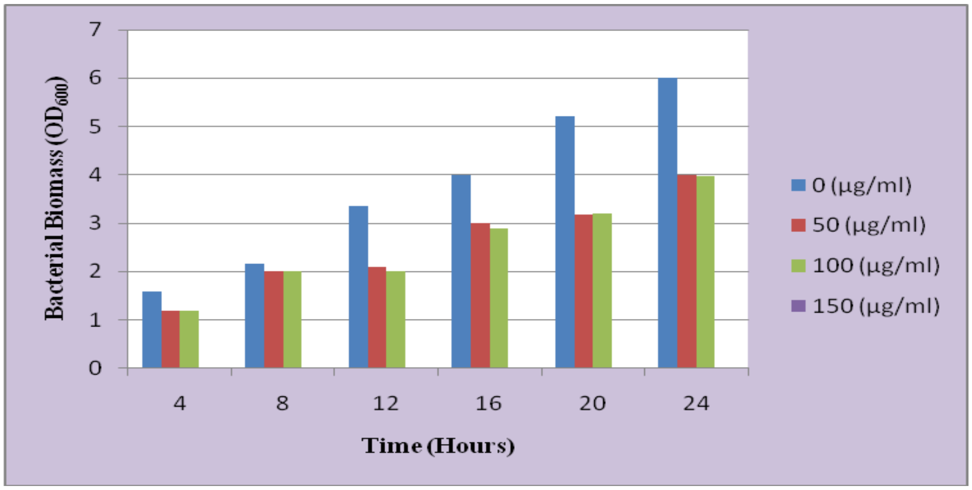


Fig.3 Minimum Inhibitory Concentration of TiO_2 NP's on *Staphylococcus aureus*

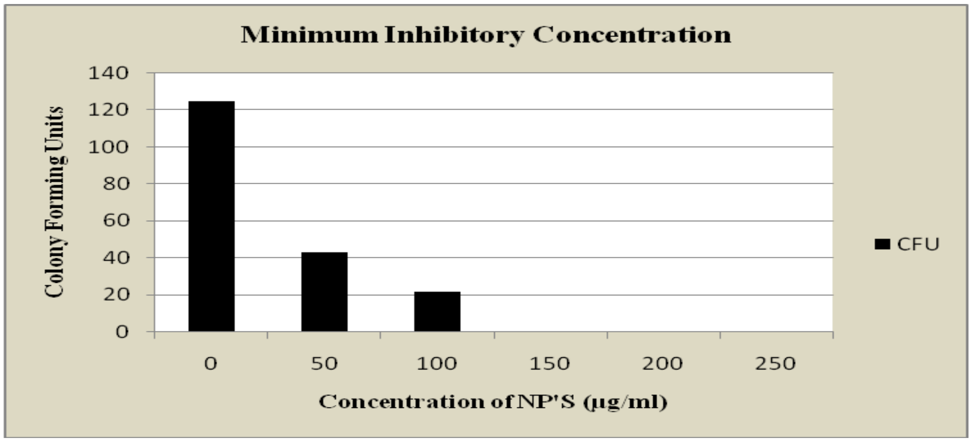


Fig.4 Minimum Inhibitory Concentration of TiO_2 NP's on *Escherichia coli*

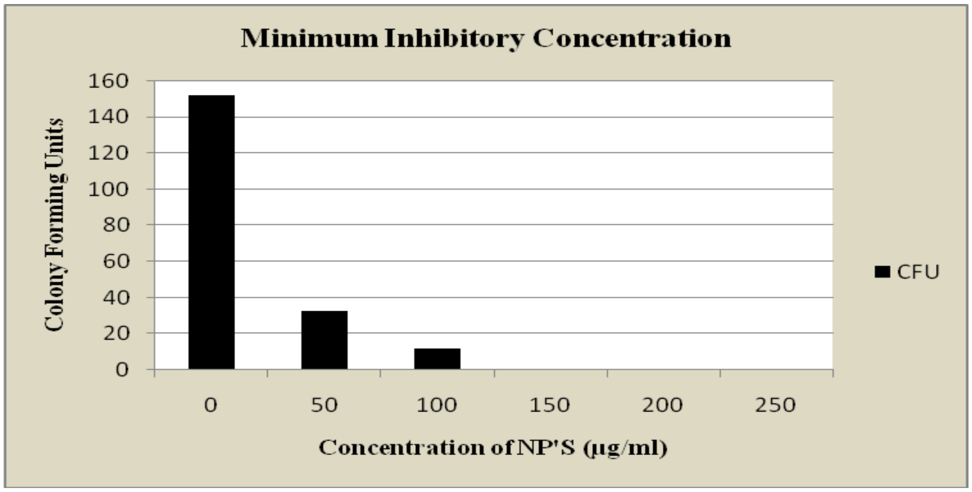


Fig.5 Effect of TiO_2 NP's on protein leakage from *Staphylococcus aureus* cells

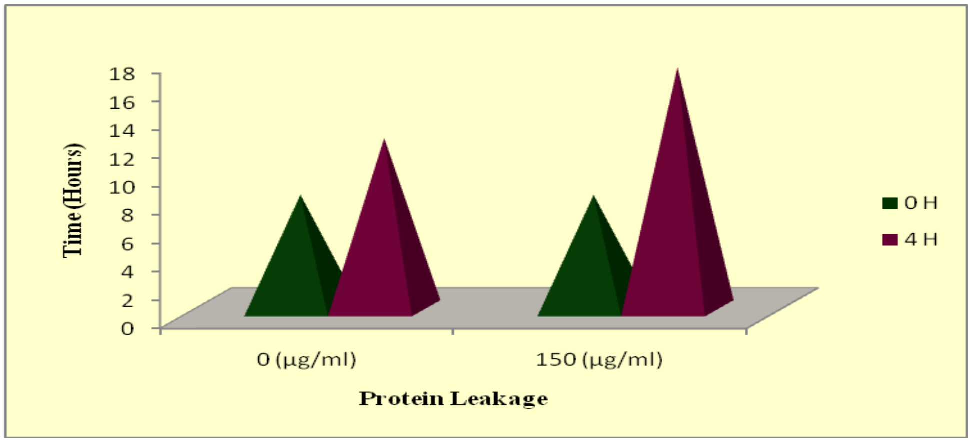


Fig.6 Effect of TiO_2 NP's on protein leakage from *Escherichia coli* cells

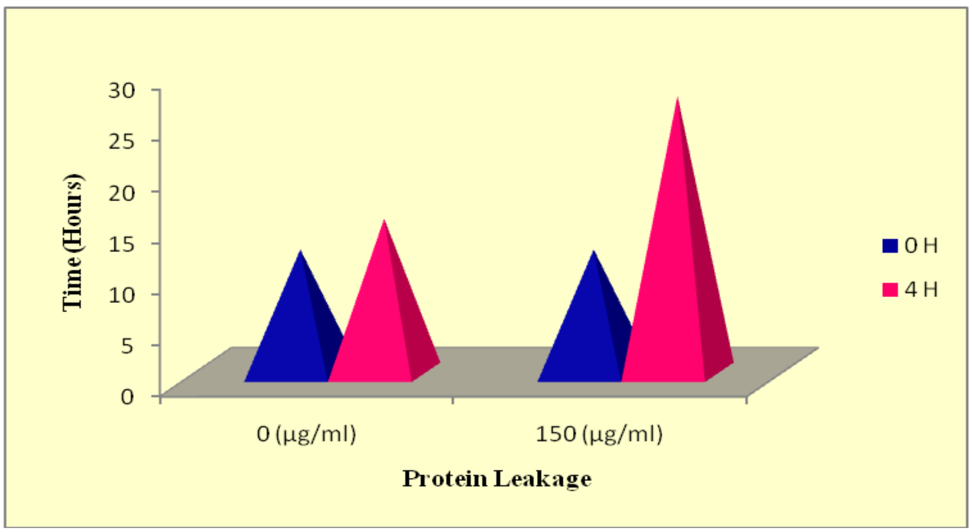


Fig.7 Effect of TiO_2 NP's on leakage of reducing sugars from *Staphylococcus aureus* cells

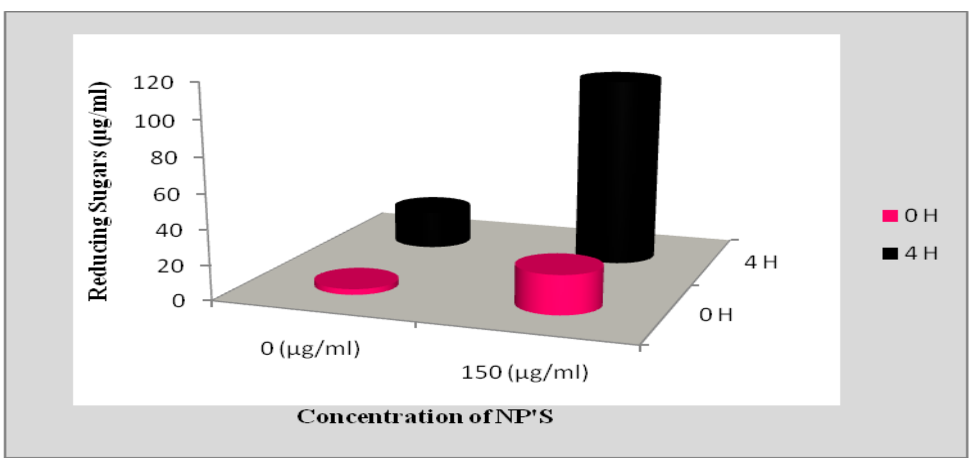


Fig.8 Effect of TiO_2 NP's on leakage of reducing sugars from *Escherichia coli* cells

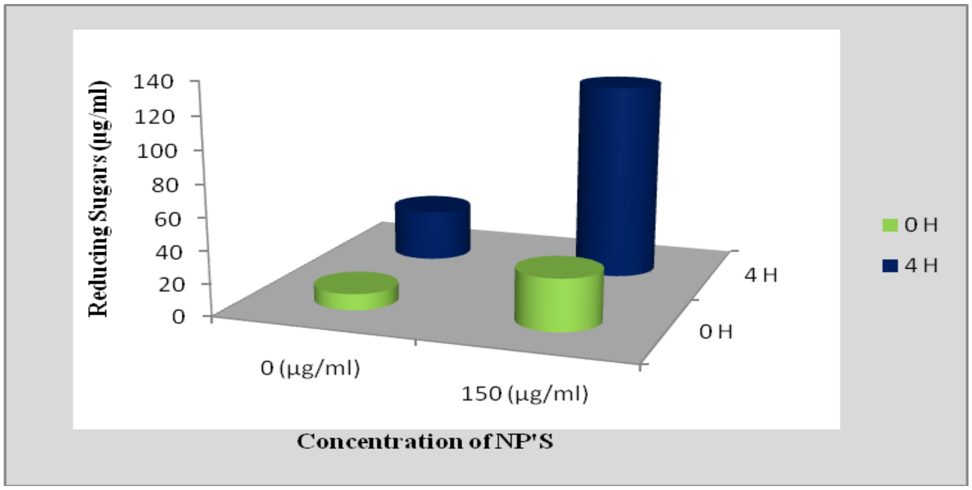


Fig.9 Effect of TiO_2 NP's on the activity of Respiratory Chain Dehydrogenases in *Staphylococcus aureus* cells

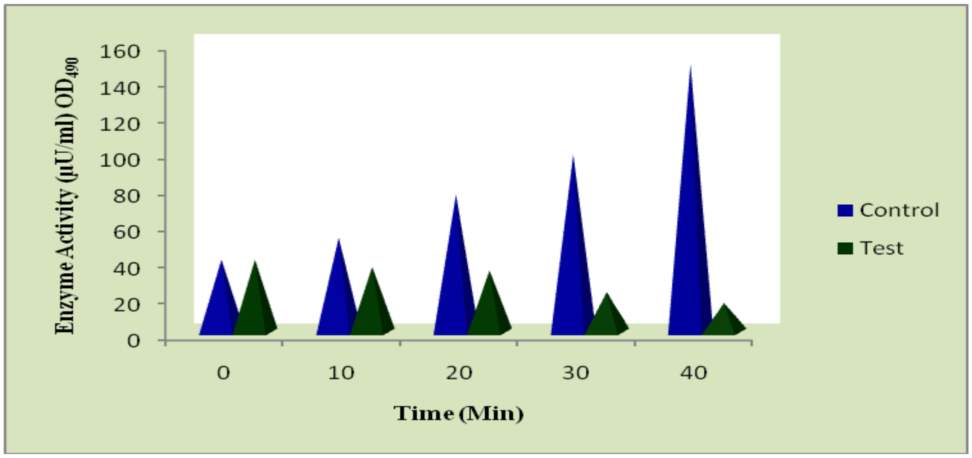
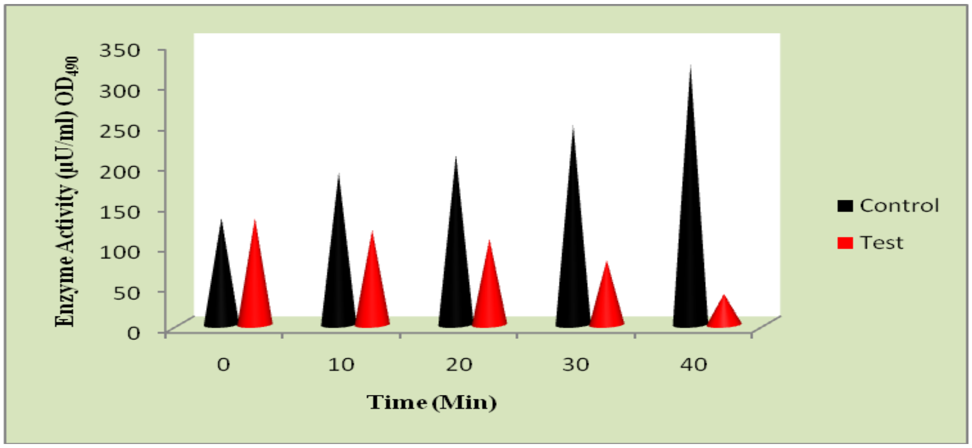


Fig.10 Effect of TiO_2 NP's on the activity of Respiratory Chain Dehydrogenases in *Escherichia coli* cells



Effect of TiO_2 NP's on protein leakage from bacterial cell membranes

It was found that TiO_2 NPs could enhance the leakage of protein by elevating the membrane permeabilities of the susceptible bacterial cells. Initially, protein leakage from the membranes of control *S. aureus* cells (without TiO_2 NP's treatment) and test *S. aureus* cells (treated with TiO_2 NP's) remained almost the same (10.24 and 12.12 µg/mg respectively). After 4 h incubation, protein leakage from *S. aureus* cells treated with TiO_2 NP's considerably increased (18.52 µg/mg); however, the protein leakage from cells in the control group was found to be 12.22 µg/mg (Fig 5). Similarly, TiO_2 NP's also increased the leakage of proteins through the membrane of *E. coli*. At start time (0 h), the leakage of proteins from cells in control experiment was 12.22 µg/mg, while leakage of proteins from cells treated with TiO_2 NPs was 14.08 µg/mg. The leakage of proteins in *E. coli* treated with TiO_2 NP's for 4 h was found to be 19.06 µg/mg, in contrast the protein liberation from control experiment was found to be 12.24 µg/mg (Fig 6).

Effect of TiO_2 NP's on the membrane leakage of reducing sugars

Fig 7 and 8 revealed that TiO_2 NP's could elevate the leakage of reducing sugars from the bacterial cell membranes. At start point (0 h), only traceable amount of reducing sugars was found be leaked from *S. aureus* cells in control experiment, while the leakage amount of reducing sugars from *S. aureus* cells treated with TiO_2 NP's reached 22.06 µg per bacterial dry weight of 1 mg (µg/mg). After treatment with TiO_2 NP's for 4 h, the leakage amount of reducing sugars was found to be 108.72 µg per mg, but the leakage was only 26.36 µg/mg in control cells. At start point (0 h), only traceable amount of reducing sugars was found be leaked from *E. coli* cells in control experiment, while the leakage amount of reducing sugars from *E. coli* cells treated with TiO_2 NP's reached 32.12 µg per bacterial dry weight of 1 mg (µg/mg). After treatment with TiO_2 NP's for 4 h, the leakage amount of reducing sugars was found to be 122.60 µg per mg, but the leakage was found to be 32.12 µg/mg in case of control cells.

Effect of TiO_2 NP's on Respiratory Chain Dehydrogenases

In case of *S. aureus* control cells, the enzyme activity was found to be in increased with the increase in incubation time reaching the maximum of 148 µU/ml after 40 min of incubation. Interestingly, enzymatic activity of *S. aureus* cells treated with TiO_2 NP's was found to be inversely proportional to the increase in incubation time (Fig 9). In case of *E. coli* control cells, the enzyme activity was found to be in increased with the increase in incubation time reaching the maximum of 322 µU/ml after 40 min of incubation. Interestingly, enzymatic activity of *E. coli* cells treated with TiO_2 NP's was found to be inversely proportional to the increase in incubation time (i.e.) the initial enzyme activity at start time (40 µU/ml) was drastically reduced to 16 µU/ml after 40 min of incubation (Fig 10). According to Ahearn *et al*. (1995), nanoparticles can lead to enzyme inactivation via formatting complexes with electron donors containing sulfur, oxygen or nitrogen (thiols, carboxylates, phosphates, hydroxyl, amines, imidazoles, indoles). Nanoparticles may displace native metal cations from their usual binding sites in enzymes (Ghandour *et al*., 1988).

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How to cite this article:

Durairasu, M., V. Indra, N. Arunagirinathan, J. Hemapriya and Vijayanand, S. 2017. Antagonistic Activity of Biogenic TiO2 Nanoparticles against *Staphylococcus aureus* and *Escherichia coli*. *Int.J.Curr.Microbiol.App.Sci.* 6(3): 2485-2495.
doi: https:// doi.org/10.20546/ijcmas.2017.603.281