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Nanotoxicological study of Cu-doped TiO2 nanoparticles on Gram positive bacteria *Bacillus amyloliquificans*

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ABSTRACT
Titanium dioxide is being one of technologically important material in the field of nanotechnology. Titanium dioxide doped with copper nanoparticles are widely used because of its thermodynamic stability, anticorrosion, high photo catalytic activity, wide band gap, high transmittance in visible and infrared spectral range. In the present study, TiO_2 doped with copper nanoparticles was synthesized from Titanium isopropoxide as a precursor using by hydrothermal method and sol-gel technique. Cu doped TiO_2 nanoparticles were characterized by Fourier-transform infrared spectroscopy (FTIR), UV-Visible spectroscopy, and scanning electron microscopy with Energy Dispersive X –ray Spectroscopy (SEM / EDX). The Cu doped TiO_2 nanoparticles were found to be spherical, ellipsoidal and irregular in shape. Individual nanoparticles as well as a few aggregates are found having the size of 5-20 nm. The biocompatibility of the Cu doped TiO_2 nanoparticles with their photo catalytic activity make them future candidate for the development of sustainable environmental remediation technologies. To assess bioremoval of the cu doped nanoparticles on the microorganisms, this study was undertaken. In this study growth of Bacillus amyloliqufcans was checked against various concentration of nanoparticles prepared by the both methods (2, 3, 4 and 5w/v %). It was seen that the microorganism has ability to grow in presence of nanoparticles with increase in the total protein content. The 5% concentration of Cu doped TiO_2 enhanced the cell mass protein of Bacillus amyloliqufcans by 3.63 times.
Keywords: *Bacillus amyloliqufcans, Cu doped TiO_2, Sol Gel, and Bioremediation*

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INTRODUCTION:

Due to the enormous applications of nanotechnology, the environmental and ecological effects ofnanomaterials have to be considered. Changes of nanomaterials will not only help ensure thesafety of Nano technological applications, but also help design functional materials that haveminimal adverse effects [3]. Titanium dioxide (TiO2) has been widely used in many fields [2]. Toenhance the functional properties and applicability of titanium dioxide, doped versions of TiO2are benign synthesized to enhance catalytic activity for light harvesting applications [5]. Many researchers have conducted studies toevaluate if nano-scale titanium dioxide would have biological impacts [1]. TiO2 NPs has been reported to have antimicrobial activities due to the reactive oxygen species formation. On theother hand, copper NPs appear to have higher cytotoxicity than copper ions because they maypenetrate the cell membrane and release copper ions inside the cell [15]. However, it is still not clear whether there is synergistic effect when TiO2 NPs are doped with CuO. Also, very few studies have examined the natural remediation of toxic metal NPs from the environment [17],which can be another important consideration of NPs; ecological impact. This study employed a model bacterial species: *Bacillus amyloliquefaciens* a Gram-positive bacterium and a model strain for the study of Nano toxicology. The objectives ofthis study are: 1) to determine the toxicity of Cu-doped TiO2 NPs; and 2) to investigate bacterial responses to NPs.

MATERIAL AND METHODS

Synthesis of Cu doped Nanoparticals:

Cu doped NP s are Synthesized by two methods i) Hydrothermal Method ii) Sol gel Method

Hydrothermal Method:

The term hydrothermal process is define as performing chemical reaction in solvent contained in sealed vessels in which the temperature of solvent can be brought to around their critical points via heating concurrently with autogenous process[13]. Hydrolyzation7.45 ml of Titanium (IV) Isopropoxide (TTIP) was performed with 100ml distilled water. White precipitate of Titanium hydrous oxide was formed instantly; this mixture was stirred for10min for complete hydrolysis process and allowed for undisturbed settling of precipitate. The precipitate was washed with distilled water for complete removal of alcohol. Precipitate was kept in ice bath for maintaining condition. The mixture was added slowly with 30ml of aqueous hydrogen peroxide (30%).This step leads to formation of transparent solution. During this process the complex get converted to the orange colour. After complete dissolution of precipitated solution was diluted to 100ml using distilled water.5% Copper Nitrate solution was added to Titanium peroxide solution with continuous stirring for 30min. The mixture get converted to viscous gel. In this way copper doped TiO_2 gel is dried at appropriate temperature. In further process this gel converted to fine powder. Add with 50ml of Milli-Q water and10ml alcohol 2gm of copper doped Titanium peroxide gel powder was this mixture is transferred into sealed Teflon container with a SS caving and heated in an oven for temperature (120^0 c)

Sol gel Method:

The sol-gel process is a more chemical method (wet chemical method) for the synthesis of various nanostructures, especially metal oxide nanoparticles[13]. Molecular precursor titanium isopropoxide was dissolved in water and then the solution was converted to the gel by heating and stirring by hydrolysis/alcoholysis. In some cases, this term is also used to describe processes conducted at ambient conditions. For the clarification, "Solvothermal process" was used. Hydrolyzed 7.45 ml of Titanium (IV) Isopropoxide (TTIP) was with 100ml distilled water. White precipitate of Titanium hydrous oxide was formed instantly; this mixture was stirred for10min for complete hydrolysis process and allowed for undisturbed settling of precipitate. The precipitate was washed with distilled water for complete removal of alcohol. Precipitate was kept in ice bath for maintaining condition. The mixture was added slowly with 30ml of aqueous hydrogen peroxide (30%).This step leads to formation of transparent solution. During this process the complex get converted to the orange colour. After complete dissolution of precipitated solution was diluted to 100ml using distilled water. 5% Copper Nitrate solution was added to Titanium peroxide solution with continuous stirring for 30min. The mixture get converted to viscous gel. In this way copper doped TiO_2 gel is dried at appropriate temperature. In further process this gel converted to fine powder. Add with 50ml of Milli-Q water and10ml alcohol 2gm of copper doped Titanium peroxide gel powder was this mixture is transferred into sealed Teflon container with a SS caving and heated in an oven for temperature (400^0 c)[19]

Characterization of Nanoparticles

FTIR:

When infrared radiation is bombarded on a sample, it absorbs the light and creates various vibration modes. This absorption relates precisely to the nature of bonds in the molecule [14]. The frequency ranges are measured as wavenumbers typically over the range of 4000-600 cm . TheFTIR spectrum is measured as wavenumber because wave number is directly related to the energy and frequency, thus providing an easy. Way for interpreting the spectrum. Prior to the sample analysis, the back ground is recorded, to avoid air and water vapour contamination peaks. The proportion of the background and the sample spectrum are directly related to the absorption spectrum of the sample. The absorption spectrum indicating various vibrations of the bonds presents in the sample molecule. Several modes arise due to the various bond vibrations. For the purpose of FTIR it is very necessary to prepare the sample. The sample should be as small as 10microns. Tiny sample size allowed good effective identification of residual particles' analysis also measures levels of oxidation along with degrees of cure of some polymer.

Contaminants and additives also give peak so the sample should be properly process for purity[9]. Results are interpreted in the form of graph as shown in (Table.1; Fig1)

UV Spectroscopy:

The absorption curves of Cu-doped TiO2 nanoparticles are shown in Fig. 2. Pure TiO2 exhibits an absorption peak at around330nm whereas Cu doped TiO2 nanoparticles exhibit peak at 230 nm.

Scanning electron microscopy. (SEM)

FE SEM was conducted to determine the morphology and elemental composition of Cu-dopedTiO2 nanoparticles, shown inFig.3. Cu doped TiO2 nanoparticles found to have size in the range of 13.16 nm and 51.54 nm.

Energy Dispersive X –ray Spectroscopy (EDS)

Energy-dispersive X-ray spectroscopy (EDS, EDX, EDXS or XEDS),), is an analytical technique used for the elemental analysis of nanoparticles [20,6]. Based on the EDX data, it can be confirmed that Cu doped nanoparticles were formed by Sol gel method (Fig.4). Elemental composition of Cu doped TiO2 nanoparticles is given in the table 2.

X-ray Diffraction (XRD)

For the size and plane confirmation, XRD of Cu-doped TiO2 nanoparticles was done. As per the report, the peaks positioned at 2Θ values of 25, 38, 49, 55,62,70, and 75 are indexed as (101), (004), (200), (105), and (213) reflections of crystalline anatase phase with average crystalline size of3.465nm.(Fig 5)[47]

3)Nano toxicological Study

In the nutrient broth Cu doped TiO2 nanoparticles were spiked in the concentrations of 2 to 5 %. Tubes were incubated at room temperature for 48 hours. Cell mass was further processed for the determination of total protein content by Folin Lowry method. With increase in the concentration of Cu doped TiO2 nanoparticles, total protein was found to be increased.

RESULTS AND DISCUSSION

Nanoparticles degrading properties of inorganic and organic nanoparticle (Cu doped TiO2, 2%, 3%, 4%, 5%,) were tested using *Bacillus amyloliquefaciens* culture. When low concentration of nanoparticles was added, 5% Cu doped TiO2, TiO2 had no apparent effect on microbial growth. When microorganism is grow with nanoparticles synthesized by hydrothermal method growth was seen but the nanoparticles prepared by sol gel method was slightly effected. Final cell density was increase by 20-30%. Nanoparticles synthesized from organic method did not show any effect on the growth of model organism. While nanoparticles made by sol gel method have retarded the growth. The SEM image (fig No 3.3(a)and (b)) shows the surface morphology of nanoparticles. The antibacterial property of Cu doped TiO2 nanoparticles was apparently associated with copper which was seen to be coated 1.36% (Table No. 3.4). The observation indicated that copper and nanoparticles have a synergetic effect on *Bacillus amyloliquefaciens* growth. The combine effect from nanoparticles and toxic ions also been reported by[44], where they observed the toxicity of ionic silver to *Chlamydomonas reinhardtii* was enhance in the presence of nanoparticles. The antibacterial level of Cu doped TiO2 can be alleviated by higher cell density in future studies production of enzyme was checked in presence of organic and inorganic nanoparticles. In case of inorganic nanoparticles *Bacillus amyloliquefaciens* has the potential to we mediate toxic metal nanoparticles. When *Bacillus amyloliquefaciens* were grown in media containing Cu doped TiO2 (2%, 3%, 4%, 5%) it shows maximum enzyme production at 5% concentration of nanoparticles prepared by hydrothermal method.

Table1: Analysis of functional groups in Cu doped TiO2 nanoparticles by FTIR

Wave No.	Band Assignment
3015	O-H Stretching mode(carboxylic acid)
2951	O-H Stretching mode(carboxylic acid)
1192	C-F Stretching mode
408	C-Br Stretching mode
402	C-Br Stretching mode

Table 2 Elemental composition of Cu doped TiO_2nanoparticles by Energy Dispersive X –ray Spectroscopy method

Element	Weight%	Atomic%
O K	79.47	92.17
Ti K	19.18	7.43
Cu K	1.36	0.40
Totals	100.00	100.00

Table 3Effect of Cu-doped TiO2 nanoparticles on the growth of *Bacillus amyloliquefaciens*

Concentration of Nanoparticles	Growth of *Bacillus amyloliquefaciens*	Conclusion
By Sol gel Method		
2%	-	Negative
3%	-	Negative
4%	+	Positive
5%	+	Positive
By Hydrothermal Method		
2%	+	Positive
3%	+	Positive
4%	+	Positive
5%	+	Positive

Table 4Effect of Cu doped TiO2 nanoparticles on the bacterial cell mass

Sr. No.	Concentration of nanoparticles	Total protein content (mg/mg)+SEM
1	2%	190+0.01
2	3%	230+0.02
3	4%	330+0.014
4	5%	400+0.015
5	Control	170+0.003

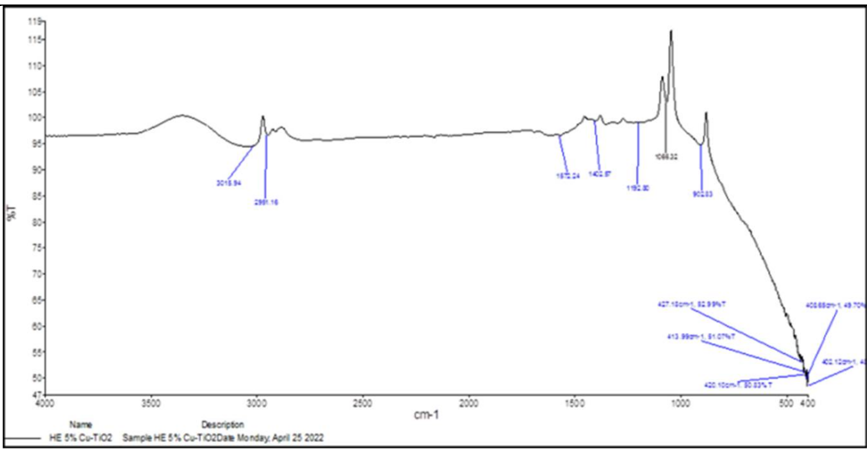

Fig 1. FTIR of Cu doped TiO2

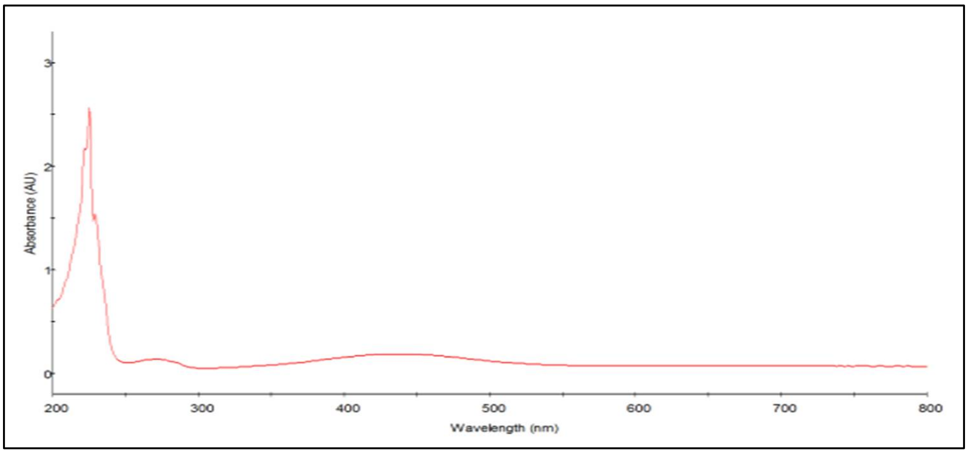

Fig 2. UV Spectroscopy of Cu doped TiO_2

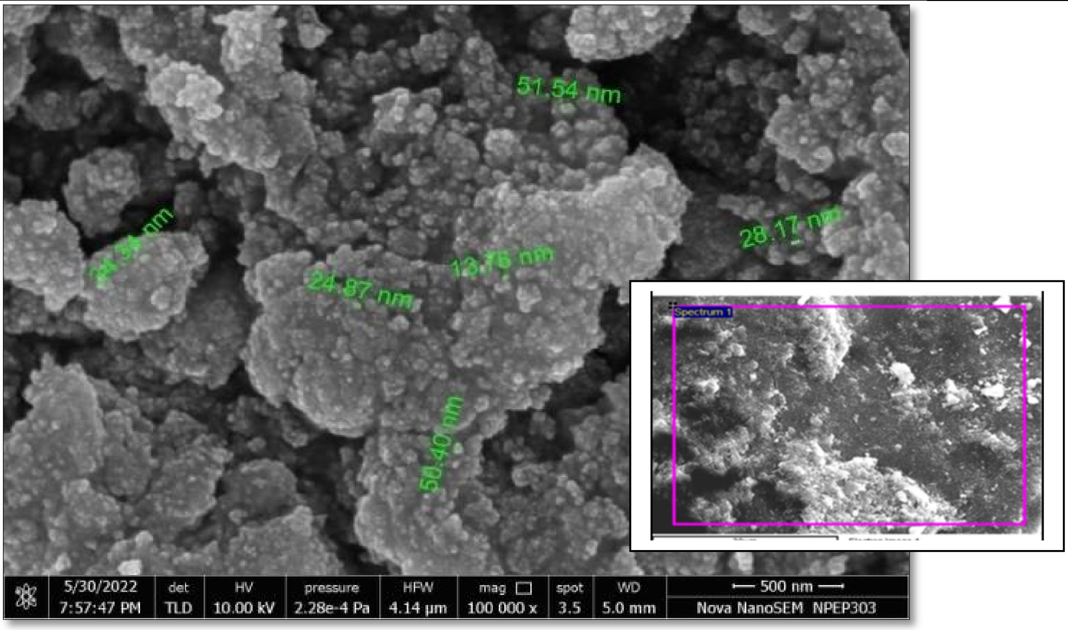

Fig No.3: FE SEM imageof Cu doped TiO2

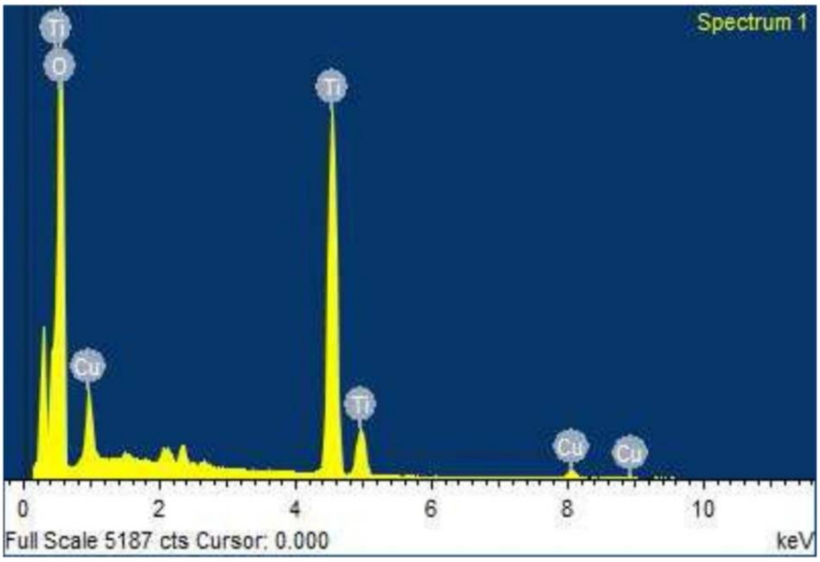

Fig. 4 EDX spectra of Cu-doped TiO2 nanoparticles

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