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ORIGINAL ARTICLE

Biogenic titanium nanoparticles (TiO_2NPs) from *Tricoderma citrinoviride* extract: synthesis, characterization and antibacterial activity against extremely drug-resistant *Pseudomonas aeruginosa*

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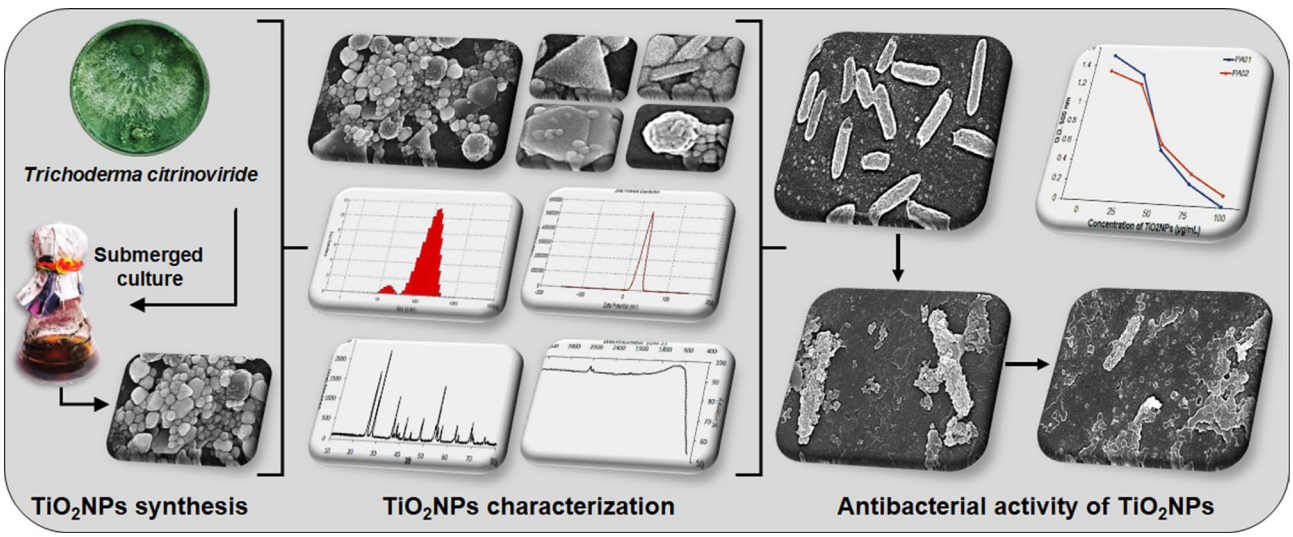
Abstract

Green synthesis of nanoparticles has attracted significant attention as an alternative to chemical synthesis procedure. The bulk availability of plants, microbial biomass and the use of eco-friendly solvents has significantly reduced the cost in addition to the hazards associated with the chemical synthesis of the nanoparticle. In this study, we demonstrated the biosynthesis of titanium nanoparticles (TiO_2NPs) with the extract of *Trichoderma citrinoviridae* as a reducing agent. The physicochemical properties of biogenic TiO_2NPs were studied using FESEM, Zeta sizer, FTIR and XRD. The size (10–400 nm), morphology, crystallinity, zeta potential (29.5 mV), and polydispersity index (0.327) suggested that the biogenic TiO_2NPs were polymorphic, crystalline and stable. FESEM revealed that the synthesized TiO_2NPs were majorly irregular, and some interesting TiO_2NPs structures, i.e., triangular, pentagonal, spherical and rod were also observed. The biogenic TiO_2NPs showed excellent antibacterial activity (100 µg/mL) against planktonic cells of extremely drug-resistant (XDR) *Pseudomonas aeruginosa* clinical isolates. The TiO_2NPs also had better antioxidant potential as compared to standard gallic acid. This study indicates the use of *T. citrinoviridae* for synthesizing biogenic TiO_2NPs and their potential use against XDR bacteria.

Graphic abstract



Keywords *Trichoderma citrinoviridae* · Titanium nanoparticles · TiO_2NPs · *Pseudomonas aeruginosa* · Antibacterial activity · Antioxidant activity

Extended author information available on the last page of the article

Introduction

Improvements in green nanotechnology have found prospects in areas of medicine, health-care, agriculture and other commercial sectors [1]. Biogenic nanoparticles (NPs) are now looked upon as a promising and attractive alternative to costly and chemically synthesized NPs that involve the use of hazardous chemicals. Green synthesis procedure with plant and microbial extracts are more safe, reliable, sustainable and eco-friendly. The molecules from plant and microbial extracts act as capping/reducing agents for NPs and, thus, increase their bioactivity, biocompatibility, and environmental sustainability [2].

Among microbes, extensive reports are available on the fungal extract mediated green synthesis of NPs. Fungi-mediated synthesis of metal/metal oxide NPs (Ag, Au, ZnO, CeO, TiO, CuO) is well studied [3–8]. *Trichoderma* is one such genus of fungi in the family *Hypoceraceae*, that are prevalent in all soils and are counted amongst the most laboratory culturable fungi. Various species belonging to the genus Trichoderma are studied for biogenic NPs synthesis. For instance, *T. koningiopsis, T. harzianum, T. viride, T. asperellum, T. longibrachiatum, T. pseudokoningii,* and *T. virens* are used for synthesizing NPs of Ag, Au, Cu and CdS [9–13]. Therefore, due to the laboratory tractable nature, *Trichoderma* sp. may attract significant attention for preparing fungus mediated biogenic metal and metal oxide NPs.

To the best of the authors' knowledge, *Trichoderma* genus is yet to be explored for biogenic synthesis of titanium oxide NPs (TiO_2NPs). TiO_2NPs are important semiconducting transition metal oxides used in paints owing to their excellent optical, magnetic, and electrical properties. They are extensively used in photocatalytic applications to remove toxins or contaminants from wastewater [14]. For many years, TiO_2NPs are looked as an attractive alternative to antimicrobial compounds due to their photocatalytic properties, low cost, biocompatibility, eco-friendly nature, generally recognized as safe (GRAS) status, chemical and thermodynamic stability [15, 16]. Therefore, the TiO_2NPs hold great potential to be used in the areas of food, health, and medicine.

Thus, in the present study, TiO_2NPs were biosynthesized using the extract of an endophytic fungus *Trichoderma citrinoviride* and characterized for size, morphology, surface charge, crystallinity with FESEM, FTIR, UV, zeta sizer, and XRD. Therefore, this investigation could be a source of producing biogenic nano-TiO_2 from *T. citrinoviride* and use them as antioxidants source. Additionally, they displayed antimicrobial property against XDR *Pseudomonas aeruginosa* clinical isolates.

Materials and methods

Isolation of *Trichoderma*

The isolation of endophytic fungus was carried out by the procedure described earlier [17]. The roots of *Sorghum bicolor* (Dehugaon, Pune, India) were washed under running tap water for 10 min followed by surface sterilization with 4% (v/v) sodium hypochlorite. The roots were cut transversely resulting in 2-mm-thick root discs, which were then transferred on Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol 150 mg/L. The PDA plates were incubated at 28 °C for 1 week for the growth of endophytic fungal colonies.

Collection and characterization of *Pseudomonas aeruginosa* clinical isolates

The two clinical isolates of *Pseudomonas aeruginosa* (PA01 and PA02) were collected from the Department of Microbiology B. J. Govt. Medical College, Pune, India. The isolates were characterized by 16S rRNA gene sequencing and the sequences were submitted to GenBank with the accession numbers MK072805 and MK072806. The cultures were further characterized as extremely drug resistant based on their resistance profile evaluated as per CLSI 2017 guidelines (Fig. 1) [18].

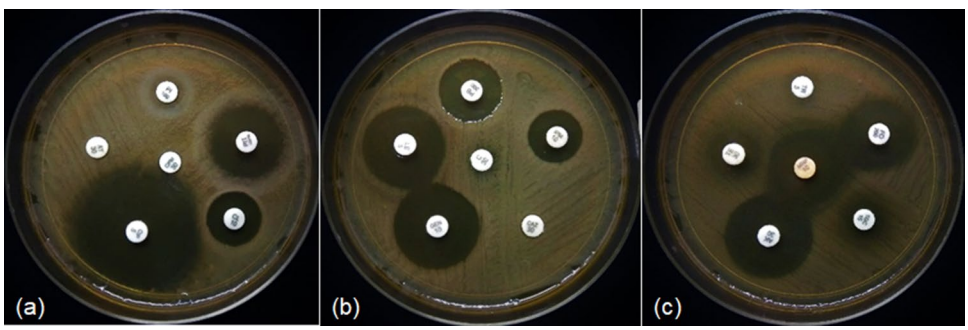


Fig. 1 Resistance profiling of *P. aeruginosa* as per CLSI 2017 guidelines (A representative image of PA01)

Biological synthesis of TiO_2 nanoparticles

For the biosynthesis of TiO_2NPs, *T. citrinoviride* was inoculated in PDB (Potato Dextrose Broth) (HiMedia, Mumbai, India). The flask with *T. citrinoviride* was then kept on a rotatory shaker (150 rpm) at 25 °C for 7 days. The mycelial suspension was then passed through Whatman's filter paper no. 42 and collected separately. The mycelia were extensively washed with sterile distilled water. Five grams of mycelial biomass was dried and powdered followed by suspending it in 100-mL sterile distilled water before overnight incubation. On the next day, the mycelial solution was centrifuged at 10,000 rpm for 15 min at 4 °C, and the cell lysate was used for biosynthesis of TiO_2NPs. Here, 0.2-ml HNO_3 (Himedia, Mumbai, India) was added to 10-mL titanium isopropoxide (Sigma Aldrich, India), 90-mL ethanol (Himedia, Mumbai, India) and 10-mL mycelial cell lysate. The resultant reaction mixture was stirred properly and incubated for 30 min. This was followed by drying and calcination of the incubated reaction mixture in the furnace at 450 °C for 2 h to synthesize TiO_2NPs powder.

Characterization of *Trichoderma* TiO_2NPs

The ultraviolet–visible (UV–Vis) spectrum (Shimadzu Corp., Tokyo, Japan) of TiO_2NPs were recorded at regular intervals and the absorption maxima were recorded between 400 and 700 nm with a resolution of 1 nm (Data not shown).

Morphological details of TiO_2NPs were analyzed using field emission scanning electron microscope (FESEM) (Nova Nano SEM 450, Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the TiO_2NPs were diluted to near transparency in Milli-Q water, plated on a smear-free coverslip and allowed to dry overnight in an oven at 37 °C. The coverslip was then coated with osmium tetraoxide for FESEM analysis.

Zeta potential, hydrodynamic size and PDI of TiO_2NPs were analyzed by zeta sizer (Malvern Zetasizer Nano ZS, UK).

The binding properties of TiO_2NPs to the *T. citrinoviride* extract were studied using Fourier transform infrared spectroscopy (FTIR) (Nicolet Impact 400 FTIR spectrophotometer, Nicolet Instrument Corp., Madison, WI). Briefly, the bio-transformed product present in the cell-free filtrate was freeze dried and diluted with potassium bromide in 1:100 ratio. All measurement was carried out in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. Similarly, the crystallinity of TiO_2NPs was studied using powder X-ray diffraction (XRD, Bruker AXS Inc.).

Antibacterial activity of TiO_2NPs

The *P. aeruginosa* clinical isolates (PA01 and PA02) were treated with different concentrations of TiO_2NPs (0, 25, 50, 75 and 100 µg/mL). Briefly, overnight-grown bacterial suspension of PA01 and PA02 were added to fresh Muller Hinton broth (HiMedia, Mumbai, India) and the O. D. at 600 nm was adjusted to 0.1 before treating with TiO_2NPs. The flasks with TiO_2NPs-treated clinical isolates were kept overnight on shaker incubator (80 rpm) at 37 °C.

The SEM analysis of *P. aeruginosa* (PA01) treated with 100 µg/mL was performed as per the previously reported procedure [19]. The treated sample was subjected to SEM analysis after three different time points, i.e., 0, 30, and 60 min post-treatment. The sample for analysis was prepared as per the procedure described by Dosunmu et al. and the SEM images were taken (Nova Nano SEM 450, Thermo Fisher Scientific, Waltham, MA, USA) [20].

Antioxidant activity

The antioxidant activity of TiO_2NPs was performed according to the procedure given by Santhoshkumar et al. (2014) [21]. Briefly, different concentrations of TiO_2NPs (10, 20, 30, 40, 50, 75 and 100 µg/mL) and standard Gallic acid were taken in different test tubes. To this, 1 mL of freshly prepared DPPH (1 mM) (HiMedia, Mumbai, India), solubilized in methanol was added and vortexed thoroughly. Finally, the solution was incubated in dark for 30 min. The absorbance of stable (standard) DPPH was recorded at 517 nm. The DPPH (containing no sample) was used as a control and was prepared using the same procedure. The activity was expressed as the percentage (%) inhibition calculated using the equation of:

$$\text{DPPH radical scavenging activity (\%)} = (\text{Ac—As})/\text{Ac} \times 100,$$

where Ac is the absorbance of the control (DPPH radical + methanol), and As is the absorbance of the sample (DPPH radical + TiO_2NP/gallic acid).

Results and discussion

Identification of fungi and submerged culturing

Fungal colonies of *T. citrinoviride* were separated and identified based on the morphology (Fig. 2a) and reproductive characters with standard identification manual [22].

Physicochemical characterization of green synthesized TiO_2NPs

The physicochemical characterization using UV–vis, FESEM, zeta sizer, XRD and FTIR revealed the details like

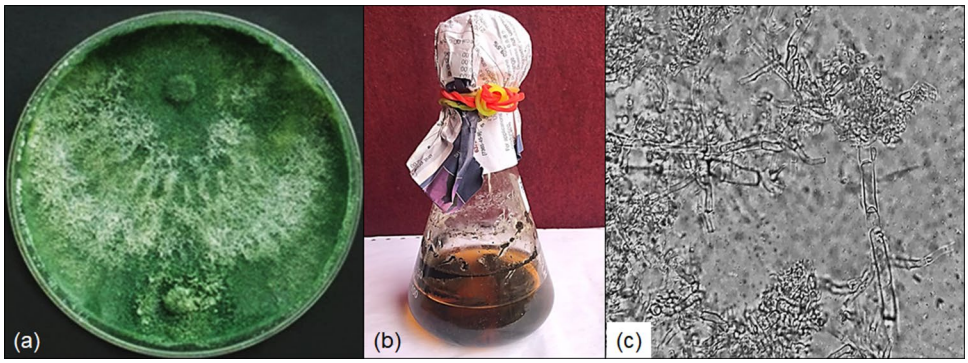


Fig. 2 *Trichoderma citrinoviride*: **a** separated and identified based on the morphology, **b** submerged culture, **c** mycelial microscopy

size, shape, morphology, surface charge, crystallinity, stability and dispersivity of biogenic TiO_2NPs. The strong absorbance recorded at 400 nm was considered as the initial sign for the successful synthesis of TiO_2NPs.

FESEM of TiO_2NPs synthesized from *T. citrinoviride* extract revealed the morphological details, predominantly comprising of irregular, triangular, pentagonal, spherical and rod-shaped particles with the size ranging between 10 and 400 nm (Fig. 3). We suppose that the size of TiO_2NPs should be further modified and controlled by varying the concentration of *T. citrinoviride* extract. It will be interesting to see if monomorphic particles like spheres can be synthesized or isolated from the mixture for a particular application. For instance, spherical TiO_2NPs of 25–200 nm are used in sunscreens to prevent erythema [23]. For NPs, the most common shape is spherical; however, pentagonal, triangular and rod shapes are more effective against bactericidal activity due to the exposed planes [24]. Furthermore, even if the NPs have identical surface areas, the shape of NPs is important as the planes having high atom density facets increase reactivity.

The hydrodynamic size distribution (Z-average) and zeta potential of the TiO_2NPs were found to be 10–396 nm and 29.5 mV, respectively (Fig. 4a, b). The zeta potential close to + 30 mV suggests that the biogenic TiO_2NPs are strong cationic and would remain stable in the suspension for a

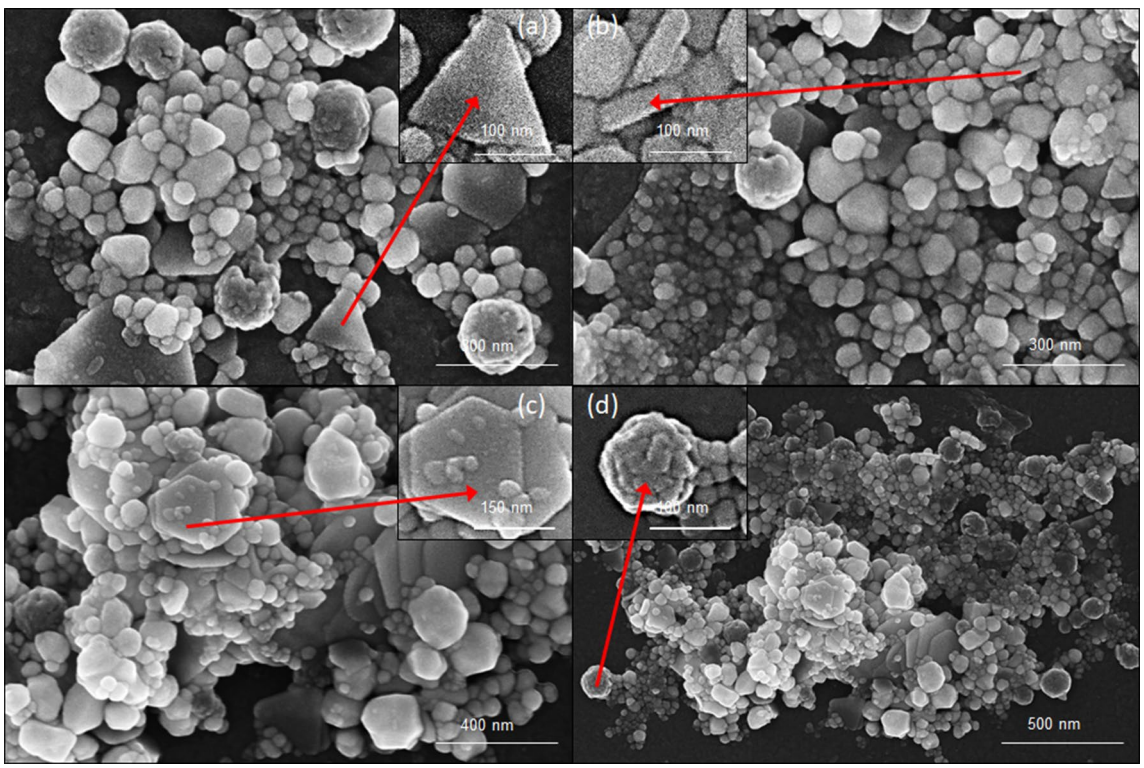


Fig. 3 SEM images of TiO_2NPs (10–400 nm) showing a mixture of shapes; for example, **a** Triangular, **b** Rods, **c** Pentagon, **d** Spherical

longer period. Further, the low polydispersity index (PDI) value of 0.327 indicated high particle homogeneity. The high zeta potential and low PDI assured stability of the TiO_2NPs.

XRD of synthesized TiO_2 showed numerous peaks (Fig. 4c) indicating the presence of two or more phases of TiO_2 in the sample. The observed 2θ values were comparable with JCPDS card no. 21–1272 and 21–1276 of anatase and rutile TiO_2. The peaks at 2θ—25.17, 37.70, 36.81, 39.17, 47.91, 55.01, 62.59, 68.92, 70.49 and 75.09 belong to the anatase phase (JCPDS card No. 21–1276); while, peaks at $2\theta = 27.40$, 35.91, 38.25, 41.19, 56.45, 63.93, and 69.91 belong to rutile phase of TiO_2 (JCPDS, no. 21–1276). Apart from peaks of these two phases, extra peaks were not present in the XRD data indicating that a sample of TiO_2 is pure. From XRD data, crystal parameters of both phases were calculated and they match the JCPDS of rutile ($a = 4.6026$ Å $c = 2.9661$ Å crystal volume = 62.83 Å3) and anatase phase ($a = 3.7752$ Å $c = 9.5359$ Å crystal volume = 135.91 Å3) of TiO_2 [25, 26]. The crystallite size of both phases was calculated by Debye–Scherrer's formula and it is found to be 53 and 66 nm, respectively, for anatase and rutile phase.

FTIR spectrum of synthesized TiO_2NPs was analyzed in a range of 400–4000 cm^{-1} (Fig. 4d). It displays strong IR absorbance below 1200 cm^{-1}, which belong to Ti-O-Ti stretching frequency. The stretching vibrations observed near 1400–1600 cm^{-1} and 3000–3100 cm^{-1} suggest the presence of aromatic group carbonyl groups like C=C and C–H, respectively. Peaks observed at 1670–1820 cm^{-1} can be attributed to the carbonyl group (C=O). Thus, the FTIR spectra confirmed the synthesis of TiO_2NPs and the capping of functional groups from *T. citrinoviride* extract.

Antibacterial activity of TiO_2NPs against *P. aeruginosa*

The antibacterial activity was aimed to analyze the concentration of TiO_2NPs required to inhibit the growth of extremely drug-resistant *P. aeruginosa* clinical isolates (PA01 and PA02). The bacterial cultures grown overnight showed a decrease in cell density with increase in the concentration of TiO_2NPs (Fig. 5). Both PA01 and PA02 strains showed a similar trend and no bacterial growth was observed at 100 µg/mL of TiO_2NPs. The SEM analysis of bacterial culture (PA01) at three time points (0, 30 and 60 min) showed bacterial cell lysis after TiO_2NPs treatment (Fig. 6).

When we talk about medicine, the indiscriminate use of antibiotics has generated the problem of antimicrobial resistance resulting in millions of fatalities every year. As a result, WHO has listed a few critical priority pathogens which require immediate attention [27]. One such pathogen is drug-resistant *Pseudomonas aeruginosa* that causes nosocomial infections, cystic fibrosis, pneumonia, sepsis and, therefore, is one of the reasons for mortality in terminally ill patients in ICUs. The biogenic TiO_2NPs can become an attractive alternative to antibiotics or can be used synergistically to potentiate the ailing antibiotics [28].

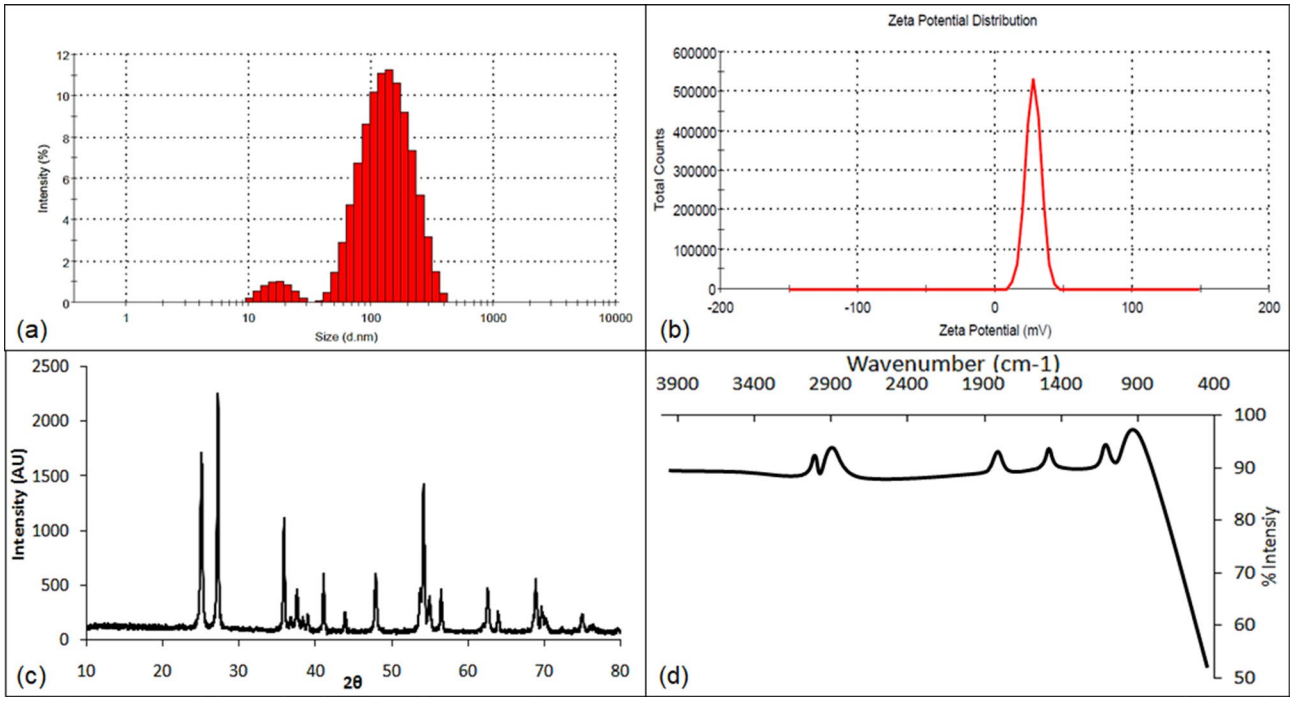


Fig. 4 Characterization of TiO_2NPs: **a** Size distribution (10–500 nm), **b** Zeta potential (29.5), **c** X-ray diffraction pattern, **d** FTIR spectra

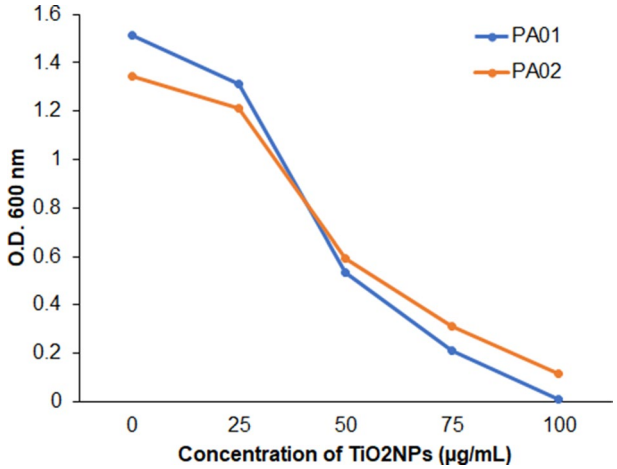


Fig. 5 Antibacterial activity of TiO_2NPs against extremely drug-resistant *P. aeruginosa*, the optical density of overnight treated **a** PA01, and **b** PA02 with different concentrations of TiO_2NPs

Reports on chemically synthesized TiO_2NPs suggest that the concentration of biogenic TiO_2NPs (100 µg/mL) required to inhibit the growth of XDR *P. aeruginosa* is 3.5-fold less than the chemically synthesized TiO_2NPs (350 µg/mL) [28, 29]. This suggests the potency of biogenic TiO_2NPs over chemically synthesized ones. However, the TiO_2NPs have shown varying toxicity to human cell types [30], and therefore, the complete toxicity assessment of *T. citrinoviride* TiO_2NPs on different cell types is recommended before it is considered for human use.

TiO_2NPs synthesized by both biogenic and chemical means have shown antibacterial properties [7, 31]. The bactericidal effect of TiO_2NPs is generally attributed to the ROS-mediated decomposition of bacterial cell wall and membrane. Due to the presence of hydroxyl group, TiO_2NPs can dissolve the bacterial outer-membrane causing electrolytic leakage [32]. Apart from ROS, the shape of TiO_2NPs (e.g., pentagonal, triangular and rod) may have also played a major role towards their antibacterial potential. The NPs with corners and planes are more surface reactivity as compared to spherical and irregular NPs [24]. However, the other plausible mechanisms that may have resulted in the inhibition of bacterial growth are also reported elsewhere [33]. But looking at the overall outcome, these TiO_2NPs can be tested against other bacterial pathogens and even in combination with antibiotics for their synergistic antimicrobial potential.

Antioxidant potential

The DPPH (α, α-diphenyl-β-picrylhydrazyl) is the most stable free radical that can be used to quantify the ROS scavenging activity of antioxidant compounds towards it. The anomalous electron of the nitrogen atom in the DPPH is reduced by accepting the hydrogen atom from antioxidants to the resultant hydrazine [34]. The *T. citrinoviride* extract and the TiO_2NPs showed better antioxidant activity as compared to gallic acid standard (Fig. 7). The DPPH scavenging activity of TiO_2NPs was found to increase in a dose-dependent manner.

Conclusion

The present work demonstrated the possibility of using *T. citrinoviride* for the biogenic synthesis of TiO_2NPs. The physicochemical characterization revealed that the TiO_2NPs are polymorphic and stable. Further through the basic antibacterial studies, we demonstrated that the biogenic TiO_2NPs can inhibit the growth of extremely drug-resistant *P. aeruginosa* clinical isolates in a concentration and time-dependent manner. The TiO_2NPs also showed better antioxidant potential as compared to the *T. citrinoviride* extract and gallic acid. Our finding provides an idea about the excellent prospects for *Trichoderma* sp. to produce TiO_2NPs using green synthesis approach in the coming years. Owing to the ubiquitous nature, ease of laboratory culturing and rapid growth, *Trichoderma* genus can become a preferred choice for synthesizing biogenic TiO_2NPs. Thus, the outcome

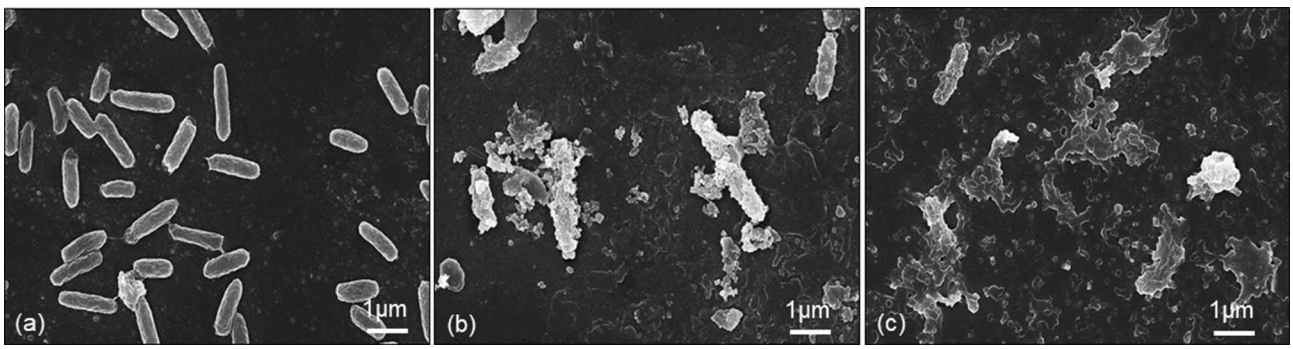


Fig. 6 SEM images of *Pseudomonas aeruginosa* treated with 100-µg/mL TiO_2NPs taken at; **a** 0 min, **b** 30 min, **c** 60 min. Scale—1 µm

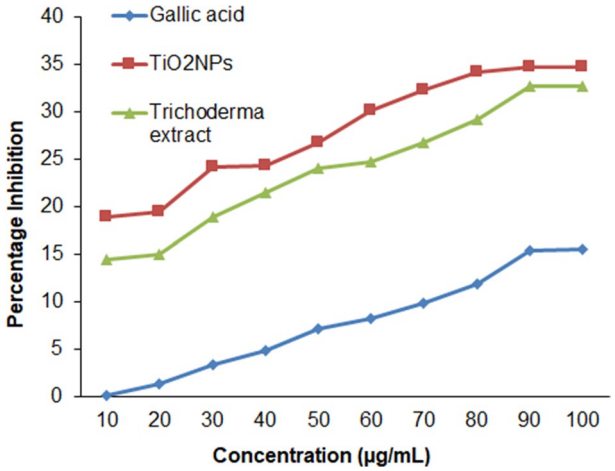


Fig. 7 DPPH free radical scavenging activity of Gallic acid, TiO_2NPs and *Trichoderma citrinoviride*

would benefit mainly the researchers working at the interface of nanotechnology and mycology, pharmaceutical industries with a focus on repurposing NPs as antibiotics, wastewater treatment plants, skincare products and other allied nano-based industries.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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