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ARTICLE TYPE

Room temperature biosynthesis of crystalline TiO₂ nanoparticles using *Bacillus licheniformis* and studies on the effect of calcination on phase structure and optical properties

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Highly crystalline TiO₂ nanoparticles were synthesised in one pot at room temperature using an extremophilic bacteria, *Bacillus licheniformis*. The organism produced extracellular oxidase type of enzyme during the biosynthesis process. The biosynthesised TiO₂ nanoparticles showed anatase phase structure. Further studies were carried out to understand the phase transformation properties of the nanoparticle by subjecting to varying calcination temperature. The morphology, structure and the crystalline phases of the titania nanoparticles were characterized using FTIR, XRD, and HRTEM. The characterization studies clearly indicated the phase transformation of the particles at different temperatures. The optical properties were studied using UV-Vis spectroscopy and Photoluminescence (PL) spectroscopy. The titania nanoparticles exhibited similar structural and optical properties when compared with chemically synthesised nanoparticle. Hence, this ecofriendly, less energy intensive, biosynthetic process may be used as an alternative to chemical approaches for the synthesis of TiO₂ nanoparticle with similar structure, chemical and optical properties.

Key words: biotitania, biosynthesis, phase transformation

Synthesis of TiO₂ (titania) nanoparticles is well reported by many researchers. Physical and chemical methods for synthesizing nanomaterials use toxic chemicals, costly equipments and energy intensive process for achieving crystalline TiO₂ nanoparticles. Considering the hazardous nature and environmental impact of the chemicals being used during the chemical synthesis process,

microbial systems like bacteria and fungi have been viewed as an effective, alternative and ecofriendly approach for synthesizing nanoparticles.¹ Many microorganisms have been reported to synthesis metal nanoparticles under laboratory conditions.^{2,3} However, the selection of microorganism for these studies remains crucial. It is well known that microorganisms in extreme environments like mines, geothermal environments, and other natural metal rich soil biotopes have higher metal resistance when compared to microbes in our normal environment. These metal resistant microbes exert various detoxification mechanisms to survive in these extreme environments.⁴ Microbial synthesis has more advantages like biocompatibility, flexibility, easy processing, ecofriendly and self assemble morphological crystal structures.⁵⁻⁸ As a result, researchers have turned to biological systems with a vision to use microorganisms as possible ecofriendly nanofactory for producing nanomaterials.

TiO₂ nanoparticles have been microbially synthesized using bacteria like *Lactobacillus sp*⁹, *Aeromonas hydrophila*¹⁰ *Bacillus subtilis*¹¹ and fungus like *Fusarium oxysporum*¹², *Sachharomyces cerevisiae*⁹ and *Aspergillus tubingensis*¹³. Among the microorganism bacteria is widely preferred due to its easy fast growth along with its metal resistance properties. The major problem faced during the microbial synthesis process is the inability to achieve high crystalline nanoparticles of defined phase structure and optical property that match with the chemically synthesised TiO₂ nanoparticle. Although there are reported studies of TiO₂ nanoparticles, not many studies have been carried out on the effect of calcination temperature on the

phase structure properties which remains essential for its potential application in the field of environmental, biomedical, self cleaning and other relevant application.

In the present study, we have exploited titanium resistance property of extremophilic, radiation resistant *Bacillus licheniformis* isolated from mine ore sample for synthesis of highly stabled crystalline TiO₂ nanoparticles without any influence of temperature. Generally the titania nanoparticles with high crystalline property are synthesized by subjecting to various high temperatures above 600° C. In this study, we have synthesised anatase phase TiO₂ nanoparticles at room temperature with high crystalline property. After calcination, further phase changes in TiO₂ nanoparticles were determined and studied for its crystalline phase structures, particle size, elemental composition and optical properties.

Bacillus licheniformis (GenBank accession number: JX469598) isolated from uranium mine ore (Narwapahar mines, Uranium Corporation of India Limited (UCIL), Jharkhand, India) was used for this study. For further information on isolation, characterization and metal resistance properties of *Bacillus licheniformis* please refer Selvakumar et al.¹⁴ Titanium precursor (purity > 99.9%), was purchased from Sigma aldrich, India. The media supplements such as Na₂HPO₄·7H₂O (purity > 99%), KH₂PO₄ (purity ≥ 99%), NaCl (purity > 99%), NH₄Cl (purity > 99%), and Glucose (purity = 99%), were purchased from Himedia, India. Tetra-methyl-*p*-phenylenediamine dihydrochloride (purity = 99%) was purchased from Loba, India. All chemicals were used without further purification. During our preliminary studies, we found that the isolate was an extremophile, resistant to uranium (upto 979 ppm), acidic and alkaline pH (pH 2 and 11), temperature (up to 55 °C) and gamma radiation (up to 7.5 kGy). The organism also showed higher resistant to titanium up to a concentration of 0.2 M.¹⁴

The bacteria was grown in a minimal media (composition (g/100 ml): Na₂HPO₄·7H₂O: 6.4, KH₂PO₄: 1.5, NaCl: 0.25, NH₄Cl: 0.5, Glucose: 2.0) containing suitable carbon and nitrogen source and incubated for 36 h at 27±2°C. The organism was screened for resistance towards pH and titanium hydroxide (precursor). The organism which showed higher resistance was selected for the biosynthesis of TiO₂ nanoparticle. The optimized pH and the titanium hydroxide concentration for the microbial synthesis of TiO₂ nanoparticles was 4.5 and 0.1 M respectively. After incubation, a known quantity of 0.1 M

titanium hydroxide (Ti(OH)₄) was added to the culture broth. After 12–48 h, the culture solution was observed for visible coalescent white cluster precipitate at the bottom of the flask which indicates the formation of titania nanoparticles. The pH of the bacterial culture solution was found to be 4.5. The coalescent white cluster precipitate was separated by filtration, centrifuged at 5000 rpm for 30 min, washed, dried at room temperature and used for further studies. The microbial synthesis of the TiO₂ nanoparticles was due to the oxidase type enzymes involved during the microbial reaction. Hence acidic pH and the oxidase enzymes involved in the bacterial culture make the requisite ambience for an oxide nanoparticle synthesis.⁹ The oxidase enzyme assay was performed to qualitatively determine the extracellular synthesis of oxidase enzyme during the reaction.¹⁵ In this assay, the 24 h fresh bacterial culture was taken in the testube and added with 0.1 M of titanium hydroxide (precursor) followed by addition of 1% tetra-methyl-*p*-phenylenediamine dihydrochloride (Kovács oxidase reagent). The reaction was monitored at differet time intervells using UV-Vis absorption spectroscopy at 610 nm and also looked for visible colour changes (Fig. 1 and its insert). The formation of purple colour started after 6 h of incubation with increase in intensity till 24 h and thereafter remained constant. The formation of purple/violet colour confirmed the presence of oxidase enzymes during the reaction and the saturation of the colour was due to the the saturation of enzyme secretion after 24 h.

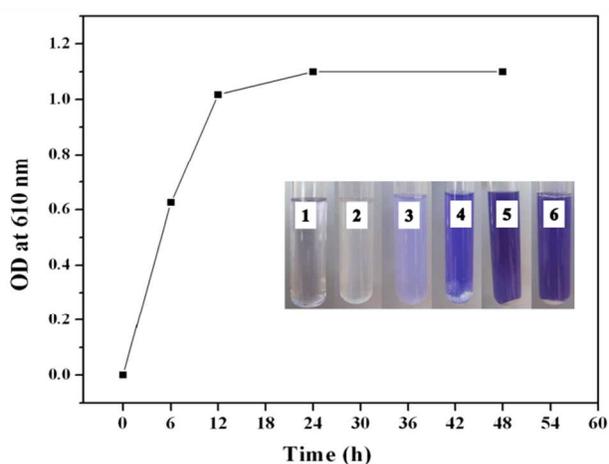


Fig. 1 UV-Vis- Spectrophotometric analysis of the oxidase enzyme assay; inset: Changes in the colour intensity of the bacterial culture at different time intervals 1: Control media, 2: 0 h, 3: 6 h, 4: 12 h, 5: 24 h and 6: 48 h

Phase formation studies with microbially synthesized titania nanoparticles were carried out. In brief, microbially synthesized TiO₂ nanoparticles (Native biotitania) were subjected to calcinations at different temperatures such as 600°C, 700°C and 900°C in a muffle furnace for an hour. The adsorbent materials were labeled accordingly as (i) Native titania, (ii) titania @ 600°C (iii) titania @ 700°C and (iv) titania @ 900°C. The chemically synthesised (sol-gel) titania nanoparticles calcined at 600°C was used as a control to compare the crystalline nature of the titania nanoparticles.

Nanoparticles were characterized using Fourier transform Infra-Red (FTIR) spectroscopy, X-ray diffraction (XRD), high resolution transmission electron microscopy (HR-TEM) attached with energy dispersive X-ray spectroscopy (EDS), UV-visible absorption spectroscopy and fluorescence spectroscopy. The FTIR spectroscopic analysis was carried out using a Nicolet Avathar- 320 FTIR spectrometer (Nicolet Instruments, Madison) at a scan range of 4000–400 cm⁻¹ with a scanning speed of 2mm/sec. The completely dried samples were treated with spectral grade KBr for pelleting in the ratio of 1: 50 and were used for the FTIR analysis. The phase identification and crystal structures of the nanoparticles were characterized by the X-ray diffraction technique using X-ray diffractometer (XRD-600, Shimadzu, Japan, having CuK α radiation, $\alpha=1.54 \text{ \AA}$) with generator settings of 30 mA; 40 kV; step size 0.05 (2 theta) with scan step time of 10.16 seconds in continuous mode. The HR-TEM images were taken in JEOL- JEM 2100, Japan with an accelerating voltage of 200 kV for TiO₂ nanoparticles. 80KV was used for imaging bacteria. The selected area electron diffraction (SAED) and the energy dispersive X-ray spectroscopy (EDS) of the TiO₂ nanoparticles were also performed. The UV-visible absorption spectroscopic analysis was performed using V-650 UV-Vis Spectrophotometer, JASCO, USA. The fluorescence emission was studied using spectrofluorophotometer (RF-5301 pc model, Shimadzu, Japan). The microorganism before and after exposure to Ti(OH)₄ were imaged using HR-TEM.

The bacteria were rod shaped with peritrichous flagella (Fig. 2a). When these bacteria were exposed to Ti(OH)₄, which on further incubation showed the formation of nanoparticles on the surface of the cell wall (Fig. 2b). The image indicates that the formation of nanoparticle may be due to the synthesis of extracellular oxidase enzymes or biomolecules when the bacteria was exposed to the Ti(OH)₄. The SAED studies confirmed the

formation of TiO₂ nanoparticles on the cell wall surface (Fig.2b insert). The biosynthesis of TiO₂ nanoparticles might be the result of biooxidation of Ti(OH)₄ precursor into TiO₂ nanoparticle.

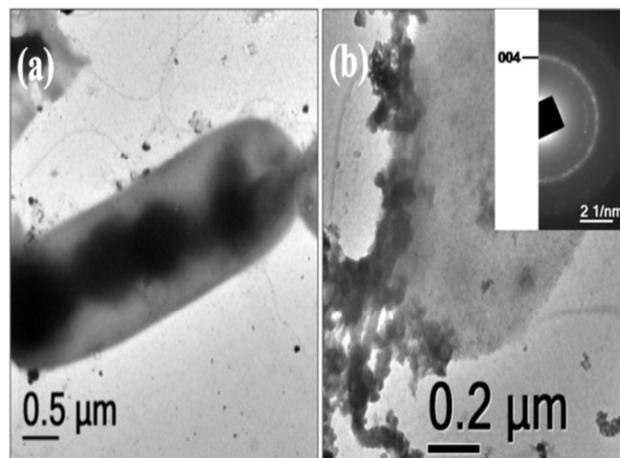


Fig. 2 HRTEM images of bacterium *Bacillus licheniformis* taken before (a) and after the exposure to Ti(OH)₄ (b) (insert: SAED of native biotitania nanoparticles formed)

The biooxidation process would have also been augmented by the negative electro kinetic potential of cell wall leading to the binding of cations onto the surface of bacteria. Jha et al.,⁹ reported that acidic pH and oxidoreductases enzymes bound to the *lactobacillus* membrane were responsible for the biosynthesis of TiO₂ nanoparticles. The synthesized TiO₂ nanoparticles were calcined at suitable temperatures to obtain different crystalline phases and compared with chemically synthesized TiO₂ nanoparticle prepared using the sol - gel method.¹⁶

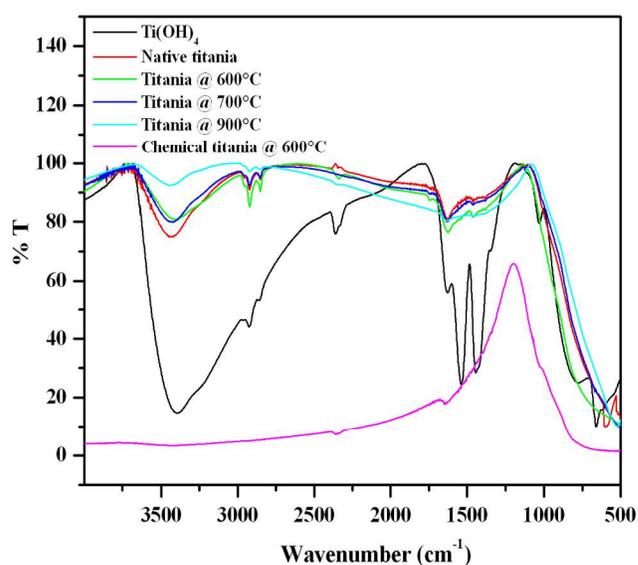


Fig. 3 FTIR spectra of various phases of titania nanoparticles

The FTIR spectra (Fig. 3) of the titanium hydroxide and different phases of synthesized titania samples showed characteristic bands at 3412, 3406, 3402, 3411 cm^{-1} corresponding to the surface water and interacting hydroxyl group involved in hydrogen bonds. The consistent peak at 2363 cm^{-1} in the native TiO_2 sample is apparently due to atmospheric CO_2 asymmetrical stretching vibration. The peaks 1442, 1461 cm^{-1} was observed after calcination, which may be due to the less intense hydrogen bonding due to the loss of broad absorption band from $-\text{OH}$ group.¹⁷ In native TiO_2 , the peak at 605 cm^{-1} is due to the formation of the amide linkages between the bacterial proteins and the TiO_2 formed during the reaction.

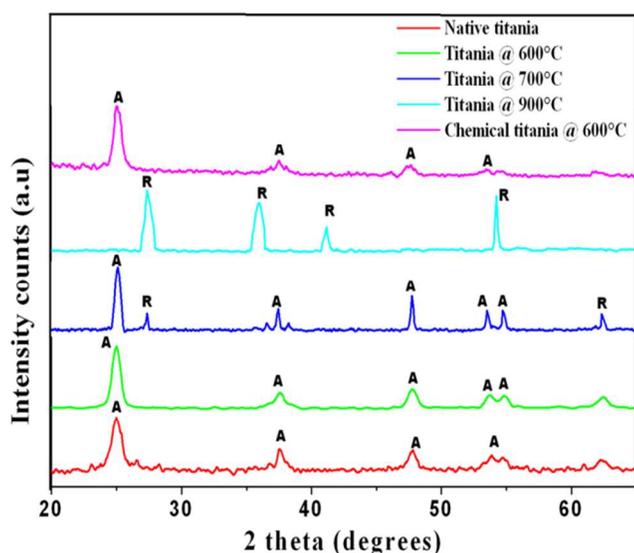


Fig. 4 XRD pattern of various phases of titania nanoparticles

XRD results (Fig. 4) show that the titania samples possess either anatase or rutile or a mixture of both these phases. Native titania shows the peaks corresponding to anatase phase, indicating proper bio conversion of titanium hydroxide by the microorganism into TiO_2 nanoparticles. The nanoparticle had anatase phase crystals with a body centered tetragonal crystal structure with space group $I4_1/amd$ (no. 141) and cell dimensions $a = 3.758\text{\AA}$ and $c = 9.513\text{\AA}$ (JCPDS card no 21-1272). The anatase crystal structure obtained after the biological synthesis as comparable to anatase phase TiO_2 synthesized after heating at 600°C. This is the first study to show that crystalline anatase phase TiO_2 nanoparticles can be synthesized using the microbial synthesis process. When calcined at 600°C, the native TiO_2 nanoparticle retains its anatase phase. At 700°C, the native showed a mixed phase formation having both anatase and rutile; Whereas at 900 °C, a complete rutile phase was observed with a primitive tetragonal crystal structure having space group

$P4_2/mnm$ (no. 136) and cell dimensions $a = 4.593\text{\AA}$ and $c = 2.959\text{\AA}$ (JCPDS card no 21-1276). The chemically synthesized TiO_2 nanoparticles calcined at 600°C showed anatase phase similar to that of 600°C calcined titania.

The HR-TEM image of synthesized titania nanoparticle with different phases is shown in Fig. 5 (a-d). The Fig. 5a shows the native titania synthesized using *Bacillus licheniformis*. These spherically shaped nanoparticles were evenly distributed with an average size of 16.3 ± 5.5 nm. The high resolution image (Fig. 5 a1) of the nanoparticle using SAED (shown as insert in Fig 5 a1) pattern indicates the crystalline phase formation of native titania with anatase crystal structure without any calcinations temperature. When the native titania was calcined at 600°C, the nanoparticles retained its anatase crystalline phase with a particle size of 51.2 ± 13 nm (Fig 5 b, b1, b1 insert). On further increase of temperature, mixture of anatase and rutile crystals appeared with particle size 52.2 ± 12 nm at 700 °C and at 900°C, pure rutile crystalline nature of particles appeared with particle size 60 ± 8 nm (Fig. 5 c, c1, c1 insert, d, d1 and d1 insert). The histogram of the particle size distribution is shown in supplementary figure S1. The size of the particle increased with increase in the calcination temperature. The size of the chemical titania nanoparticles (control) was found to be 41 ± 9 nm and had anatase crystal structure (Fig. 5 e and 5e insert). The purity of the synthesized titania samples was studied by EDS and confirmed the presence of Ti and O elements corresponding to TEM images. (see supplementary figure S2).

Fig. 6 shows the UV-visible absorption spectra of synthesized different phases of titania nanoparticles. The optical absorption spectra show red shift with increase in calcination temperature, which might due to increase in particle size. The optical band gap energy of the corresponding samples was calculated to be 3.76, 3.53, 3.44, 3.28 and 3.56 eV for native titania, titania @ 600°C, titania @ 700°C, titania @ 900°C and chemical titania @ 600°C respectively. The band gap of the nanoparticles decreased by increasing the calcination temperature. This might be due to the increased particle size and crystal defects formed in the particles as at higher temperature moves the absorption edge to higher energies. The shift of the excitation maximum for anatase crystal structure is higher than rutile crystal structure, signifying that there are fewer defect sites in the titania having both anatase and rutile structure which exhibit the characteristic band-edge absorbance properties.¹⁸

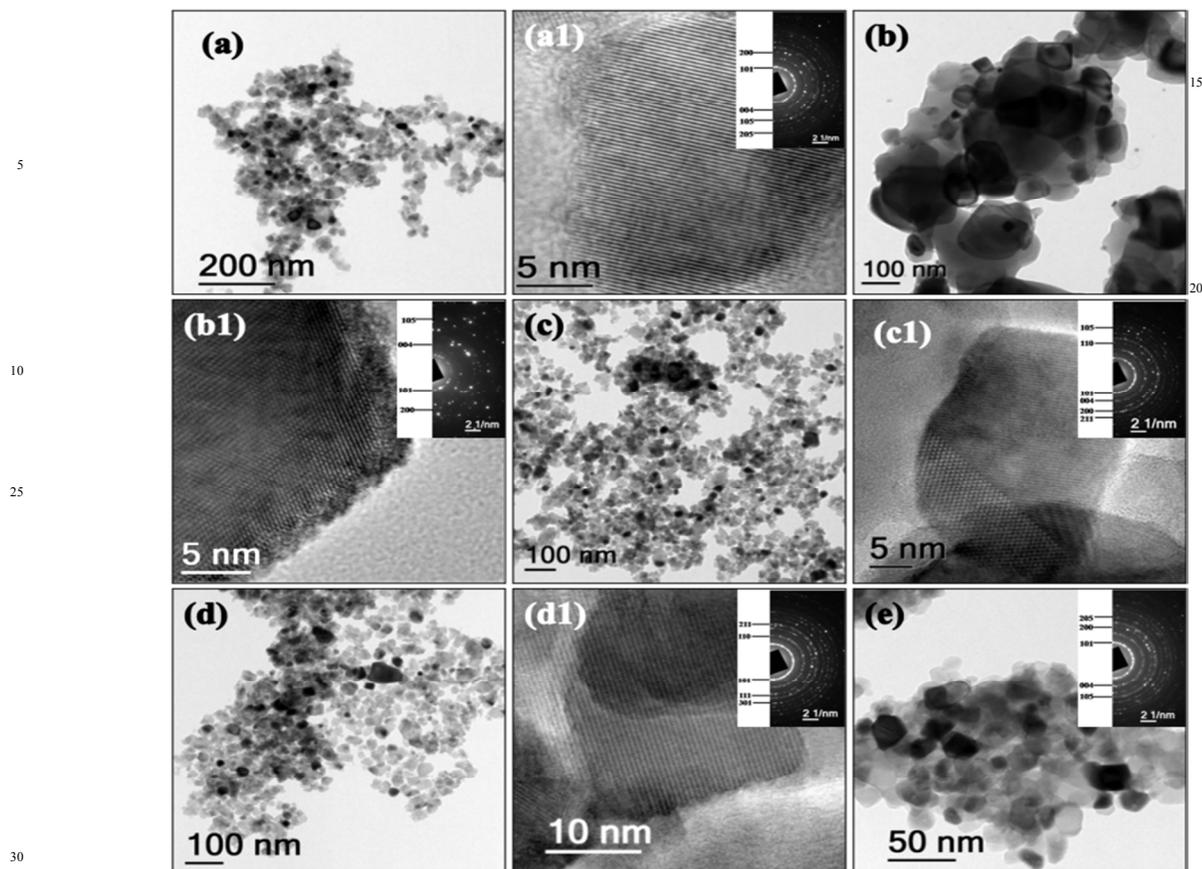


Fig. 5 HRTEM images of (a, a1) native titania, (b, b1) titania at 600°C, (c, c1) titania at 700°C, (d, d1) titania at 600°C and (e) chemical titania at 600°C.

The native titania possess high crystalline structure without any heat treatment has a great merit for the microbial synthesis. By tuning the native titania nanoparticles with different temperatures, the optical property differs accordingly and this may provide excellent optoelectrical properties.

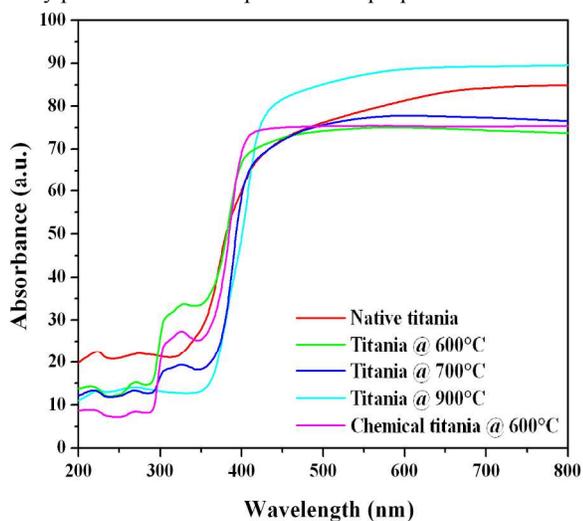


Fig. 6 UV-visible absorption spectrum of various phases of titania nanoparticles

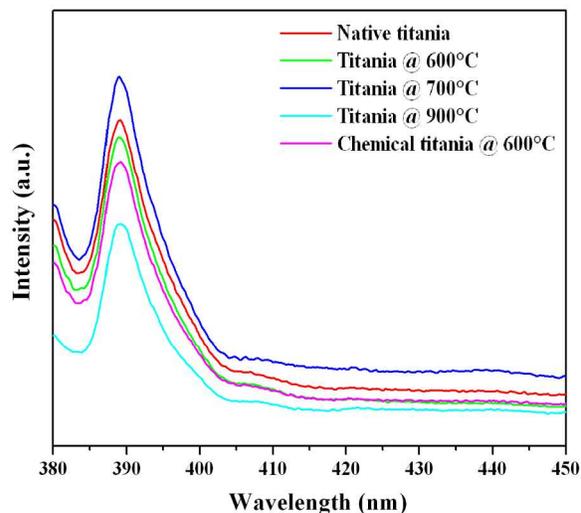


Fig. 7 Photoluminescence spectrum of various phases of titania nanoparticles (λ_{ex} = 360 nm)

Photoluminescence (PL) spectroscopy was used for the study of emission properties of prepared samples. Fig. 7 illustrates the photoluminescence property of prepared titania samples with different phase structures by the excitation wavelength (λ_{ex}) at 360 nm. The synthesized titania and

chemical titania nanoparticles showed PL emission at 388 nm. The native nanoparticles showed higher intensity when compared to the nanoparticles calcined at 900°C which showed lower PL intensity. The PL intensity of the TiO₂ nanoparticles decreased with an increase in the calcination temperature. Tripathi et al¹⁸ reported that on increase in calcination temperature, the number of oxygen vacancies in pure phases reduces and hence the intensity decreases accordingly. In case of titania nanoparticles calcined at 700°C, the sudden increase in the intensity may be attributed to the mixed phase of anatase and rutile. The mixed crystalline phases in the same material is responsible for the rapid increase in the oxygen vacancies created on the surface of the TiO₂ nanoparticle which has significant impact on the luminescence of TiO₂ nanoparticle. Titania calcined at 900°C showed the lowest PL intensity compared to all other nanoparticles which might be due to the decreased oxygen vacancies on the surface of the pure rutile phase TiO₂ nanoparticles. These results are in substantial agreements with those of Tripathi et al¹⁸ and Wang et al¹⁹. The chemical titania nanoparticles showed similar luminescence properties when compared with biologically synthesized titania nanoparticles. PL spectra of titania nanoparticles also clearly evidence the properties such as surface states (defects) and self-trapped excitons behaviors.¹⁹

Conclusions

A one pot titania nanoparticles with pure anatase phase was prepared using extremophilic *Bacillus licheniformis* and the crystal nature of the particles were studied. The native titania showed good crystalline nature without any calcination. The structural and optical properties of the nanoparticles were similar to that of the sol gel synthesized TiO₂ nanoparticle. Although characterizations of mechanical, electrical and biological properties are still in progress, we believe that the biosynthesized titania nanoparticle shows excellent promise in sensors, environmental and many biomedical applications.

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Notes and references

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1. K. B. Narayanan, N. Sakthivel, Biological synthesis of metal nanoparticles by microbes. *Adv in Colloid and Inter Sci.*, 156(2010) 1–13
2. D. Mandal, M. E. Bolander, D. Mukhopadhyay, G. Sarkar, P. Mukherjee, The use of microorganisms for the formation of metal nanoparticles and their application. *Appl Microbiol Biotechnol.*, 69(2006) 485–492
3. U. Shedbalkar, R. Singh, S. Wadhvani, S. Gaidhani, B. A. Chopade, Microbial synthesis of gold nanoparticles. Current status and future prospects *Adv in Colloid and Inter Sci.*, 209(2014) 40–48
4. M. R. Bruins, S. Kapil, F. W. Oehme, Microbial resistance to metals in the environment. *Ecotoxicol Environ Saf.*, 45(2000) 198–207
5. C. Jayaseelan, A.A. Rahuman, S. M. Roopan, A. V. Kirthi, J. Venkatesan, S. K. Kim, M. Iyappan, C. Siva, Biological approach to synthesize TiO₂ nanoparticles using *Aeromonas hydrophila* and its antibacterial activity. *Spectrochim Acta A Mol Biomol Spectrosc.*, 107(2013) 82–90.
6. D. R. Lovely, J. F. Stolz, G. Nord, Phillips, Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism, *E.J.Nature.*, 330 (1987) 252–254
7. S. K. Das, A. R. Das and A. K. Guha, Gold Nanoparticles: Microbial Synthesis and Application in Water Hygiene Management. *Langmuir.*, 25(2009) 8192–8199.
8. Prashant, M.; Nisha, K. R.; Sudesh K. Y. Biosynthesis of nanoparticles: Technological concepts and future applications. *J.Nanopart Res.*, 10 (2008) 507–517.
9. K. Jha, K. Prasad, Kulkarni, Synthesis of TiO₂ nanoparticles using microorganisms. *Colloids Surf B.*, 71(2009) 226–229
10. C. Jayaseelan, A. A. Rahuman, S. M. Roopan, A. V. Kirthi, J. Venkatesan, S. K. Kim, M. Iyappan, C. Siva, Biological approach to synthesize TiO₂ nanoparticles using *Aeromonas hydrophila* and its antibacterial activity., *Spectrochim Acta A Mol Biomol Spectrosc.* 107 (2013) 82–9
11. A. V. Kirthi, A. A. Rahuman, G. Rajakumar, S. Marimuthu, T. Santhoshkumar, C. Jayaseelan, G. Elango, A. Abdul Zahir, C.

- Kamaraj, A. Bagavan ,Biosynthesis of titanium dioxide nanoparticles using bacterium *Bacillus subtilis*., *Mater. Lett.* 65 (2011) 2745–2747. 40
12. V. Bansal, D. Rautaray, A. Bharde, K. Ahire, A. Sanyal, A. Ahmad, M. Sastry, Fungus-mediated biosynthesis of silica and titania particles., *J. Mater. Chem.* 15 (2005) 2583–2589 5
13. A Tarafdar, R Raliya, WN Wang, P Biswas, JC Tarafdar, Green Synthesis of TiO₂ Nanoparticle Using *Aspergillus tubingensis*., *Advan. Sci., Engi. Med.*, 5(2013) 943-949
14. R. Selvakumar, S. Aravindh, J. Ravichandran, U. K. Mudali, C. Anandbabu, Baldev Raj, Impregnation of S-layer protein isolated from extremophilic *Bacillus licheniformis* NARW 02 onto titanium phosphate ceramic enhances uranium removal from aqueous solution., *Current sci.* 106 (2014) 1719-1725 10
15. N. Sones, M. Sekimachi, E. Kutoh, Identification and Properties of a Quinol Oxidase Super-complex Composed of a *bcl* Complex and Cytochrome Oxidase in the Thermophilic Bacterium PS3., *Biol. Chem.* 262 (1987) 15386-15391 15
16. S. Prakash, Ramasamy and B. S. Murty, Influence of bias voltage on dielectric relaxation of nanocrystalline anatase TiO₂ using modulus formalism. *J. Appl. Phys.*, 109(2011) 084116 20
17. J. Maira, J. M. Coronado, V. Augugliaro, K. L. Yeung, J. C. Conesa, and J. Soria, Fourier Transform Infrared Study of the Performance of Nanostructured TiO₂ Particles for the Photocatalytic Oxidation of Gaseous Toluene. *J. Catalysis.*, 202(2001) 413– 420 25
18. K. Tripathi, M. K. Singh, M. C. Mathpal, S. K. Mishra, A. Agarwal, Study of structural transformation in TiO₂ nanoparticles and its optical properties, *Journal of Alloys and Compounds.*, 549 (2013) 114–120 30
19. X. Wang, Z. Feng, J. Shi, G. Jia, S. Shen, J. Zhou and C. Li, Trap states and carrier dynamics of TiO₂ studied by photoluminescence spectroscopy under weak excitation condition, *Phys. Chem. Chem. Phys.*, 12 (2010) 7083–7090 35

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