

BACILLUS SPHAERICUS A CASE STUDY FOR ANTIBACTERIAL AND PHOTOCATALYTIC EFFECT OF TITANIUM NANOPARTICLES AND MICROCRYSTALLINE TiO₂ ON GRAM-POSITIVE PAINT MICROBES

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Abstract

Currently, there is a massive drift in bacteria as a result of emergence of resistant strains to conventional paint biocides. This study focused on a photodestructive strategy of paint microorganisms using titanium dioxide nanoparticles. The comparative toxicity of microcrystalline TiO₂ and TiO₂ Nanoparticles (NPs) on *Bacillus sphaericus*, a spore forming and environmentally diverse microbe isolated from spoilt paints was investigated. The antibacterial performance of the TiO₂ NPs with 38nm particle size in the inhibition of *Bacillus sphaericus* and their toxic effect on the viability of the organism are illustrated and discussed in comparison with results obtained from the microcrystalline TiO₂. The results show 90% reduction of the *B. sphaericus* cell viability at 10g/L concentrations of TiO₂ NPs under fluorescent light (400-700 nm) after 60 min irradiation. In contrast, a 40% reduction was observed for microcrystalline TiO₂ (as determined by plate count assay) and scanning electron microscopy (SEM). In particular, the toxicity of both microcrystalline TiO₂ and TiO₂ NPs was also observed in the dark although with reduced influence. TiO₂ NPs were found to exhibit bacteriostatic and photocatalytic effect on *B. sphaericus* as exposure time increases. Results of this study suggests that a longer exposure time could impact bactericidal activity. The results of the study showed that photocatalytic inactivation of paint bacteria can be directly linked to time of exposure.

Keywords: Microcrystalline TiO₂; TiO₂ Nanoparticles; Antibacterial Agent; *Bacillus Sphaericus*; Photokilling Phenomenon

Introduction

The ever-increasing trend of bacterial resistance has necessitated the development and use of modern, more sophisticated antimicrobials. This is particularly crucial in manufacturing and industrial settings where preservation from spoilage is required for increased shelf life. Various research works have been involved in the engineering of antimicrobials that target specific microorganisms. Nano-scale particles have emerged as an

unconventional approach to combat multi-resistant microbes. These nanoparticles exhibit higher antimicrobial activity which is enhanced by their high surface area to volume ratio as well as their peculiar chemical and physical properties [1].

Previous reports have demonstrated the photocatalytic UV/TiO₂ system to be efficiently bactericidal against *Escherichia coli* [2], *Agrobacterium tumefaciens* [3] *Streptococcus mutans*, *Candida albicans* [4] *Legionella pneumophila* [5] and some

resistant bacteria such as *Enterobacter cloacae* [6]. The antibacterial activity of TiO_2 against *E. coli* has been reported to be up to 99.99% with visible light illumination [7]. Research has shown that the wide-band-gap TiO_2 in anatase crystalline form is the most useful of all semiconductor photocatalysts. TiO_2 is believed to possess high photocatalytic efficiency as a result of its high quantum yield, high stability toward photocorrosion, insolubility in water, cost effectiveness and low toxicity [8]. The photocatalytic property of TiO_2 can therefore, be harnessed to prevent microbial proliferation and achieve proper disinfection as shown by [9]. However, the photocatalytic ability of TiO_2 depends on certain intrinsic and extrinsic factors which include: particle size, particle preparation technique, surface area, crystal structure, initial reaction concentration, effect of presence of dopants, effect of various operating parameters [10, 11, 12, 13] such as incident light intensity and pH of solution. The field of Nanotechnology is becoming increasingly important with several applications in Science and Technology for efficient and sustainable approaches in the manufacture of new nano-scale materials. TiO_2 NPs have been widely used in treatment of waste water as well as in the production of sunscreens and cosmetics [14, 15]. Titanium dioxide has been the photocatalyst of choice for several decades. Its photocatalytic effects has been extensively investigated and shown to eliminate organic compounds as well as function as disinfectants to kill or inhibit bacterial growth [16]. To better understand

the higher inhibitory and toxic effects of TiO_2 NPs on bacteria as against the bulk TiO_2 , *B. sphaericus* was used as a model organism to investigate this phenomenon. Furthermore, certain drawbacks have been implicated in the use of bulk TiO_2 . A study carried out by [17] reported the degradation of substrates resulting in the delamination of coating when bulk TiO_2 is directly applied on some organic materials and plastics. In addition, since the bactericidal effects of bulk titanium dioxide (TiO_2) photocatalyst is possible only with UV irradiation at levels that are harmful to humans [18], its use in general applications are considerably reduced. It is in this context that the bactericidal activity of bulk titania is compared with TiO_2 nanoparticles under illumination with fluorescent light with a view to overcome the damaging effects of UV light on humans in TiO_2 general applications.

Experimental Protocols

Preparation of Nanoparticle Suspension

Titanium dioxide nanoparticles (80% anatase: 20% rutile) were purchased from Nanophase Technologies Corporation (Romeoville, IL, USA) with specific surface area of $40\text{m}^2/\text{g}$ and advertised particle size of 38nm. The microcrystalline Titanium dioxide (rutile) was supplied by Alfa Aesar Chemicals (Ward Hill, MA, USA). All materials were of analytical grade. To produce TiO_2 stock suspension, 100g of TiO_2 nanoparticles and bulk TiO_2 were each

dispersed in 1L sterile nanopure water to obtain a final concentration of 100g/L. In order to prevent agglomeration of the nanoparticles, the suspension was thoroughly homogenized by sonication for 30 mins and kept in the dark until needed.

Bacterial Isolation and Antibacterial Test

Bacillus sphaericus was isolated from spoilt paints and identified with the Fatty Acid Methyl Ester (FAME) analysis. In this procedure, the cellular fatty acid profiles of the organism was determined by gas chromatography of the fatty acid methyl esters using the microbial identification system (MIS, MIDI Sherlock USA) [19] and 16S-rRNA techniques. Stock cultures were preserved in 10% (v/v) glycerol at -70°C and were freshly subcultured before each experiment. A loopful of culture was harvested and subsequently inoculated into test tubes (18mm x 150mm) containing sterile nutrient broth (NB) (Becton Dickinson). The NB tubes were then incubated aerobically in a shaker at 37°C [20]. The overnight cell suspension was diluted in NB to give a cell concentration of approximately 10^6 – 10^7 colony-forming units (CFU)/mL. The optical density of the culture suspension was determined at 600 nm using a spectrophotometer (Spectronic 20D, Genesys). Bacterial growth inhibition was investigated by inoculating 1 mL of 10^6 CFU/ml of *Bacillus sphaericus* cells into each of the prepared suspensions of bulk TiO₂ and TiO₂ NPs at different concentrations (0, 5 and 10g/L).

Photocatalytic Test and Bacterial Viability Evaluation

The stock TiO₂ suspensions were prepared immediately prior to the photocatalytic reaction and kept in the dark until required. The cell-TiO₂ suspensions were placed in a shaker (200 rpm) with continuous stirring and subsequently irradiated with Agrobrite High output T5 54W 6400K fluorescent lamps (400-700nm) at a distance of 15cm. *B. sphaericus* suspension without TiO₂ was illuminated as control samples. A second control of the TiO₂-cell suspensions in the dark was carried out. Dark control samples were wrapped with aluminum foil and stirred under the same conditions. The survival rate of the test organisms in the presence of TiO₂ nanocrystals and bulk TiO₂ was determined by withdrawing aliquots (1.0mL) of serially diluted samples at intervals of 0, 15, 30, 45 and 60 min. The viable count was performed on Tryptic soy agar (TSA) plates after 2-fold dilutions of the sample in 0.9% NaCl. The TSA plates were aerobically incubated at 37°C for 24 h. The developed colonies after overnight incubation were counted with a colony counter (Hellige Inc. Garden city, NY, USA). All experiments were performed in duplicates.

Examination of Cell Morphology by Scanning Electron Microscopy (SEM)

The SEM images of untreated cells (control samples) and cells treated with mixed-phase TiO₂ NPs were obtained. Fixation of all the samples was achieved using 2%

glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1M cacodylate buffer (pH 7.2) for a period of 24 hours at 4°C. Following fixation, the samples were subsequently concentrated onto 0.22 micron filters using a syringe tip filter. Subsequently, the filters were washed thrice in buffer and post-fixed in 1% osmium tetroxide for an hour. Then, two series of washes in buffer was done for 5 minutes each. This was followed by dehydration of the samples in a graded ethanol series up to 100% ultra-pure ethanol followed by substitution into hexamethyldisilazane and left to air dry. After air drying, all samples were arranged on a carbon adhesive coated aluminum stubs and sputter coated (Denton Desk II sputter coater, Denton Vacuum, LLC, Moorestown, NJ) using palladium/gold alloy (60/40). Digital Image capture was achieved with a JEOL 5800LV SEM (Japan Electron Optics Laboratory, Peabody, MA) at 13kV with an O-SIS ADDA II (Olympus Soft Imaging Systems Inc., Lakewood, CO).

Results

The antibacterial activities of the TiO₂ NPs and bulk titania were evaluated by the rate of killing of *B. sphaericus* cells irradiated with fluorescent light based on reduction in the colony forming units (CFU)/mL on agar plate. Figure 1 A shows a bacteriostatic effect of bulk TiO₂ on *B. sphaericus* cells under constant illumination at concentrations of 5 and 10g/L. At a concentration of 5g/L TiO₂, inhibition of *B. sphaericus* cells was apparent from the first

fifteen minutes, this was followed by a slight growth and a further continuous decrease of 30% at the end of 60 min. This result is in line with the report of [21, 22, 23]. At 10g/L, a slight decrease in the number of viable cell was observed in the illuminated samples after 15 min. After 60min, there was a reduction of 40% in cell viability. In the control samples (0g/L) the cells continued to grow within the first 30 min. However, bacterial growth started to decrease after 30 min under constant illumination and after 60 min, there was a bacterial decrease of 33.3%. This probably is because the cells were sensitive to the fluorescent light even though the samples had no TiO₂. It is important to note that the reduction in the bacterial cells of the control samples was lower compared to the samples with 5 and 10g/L. This indicates the antibacterial activity of the bulk titania on the bacterial cells. In the dark, (Fig. 1B) control samples (0g/L) had a decrease in growth in the first 15 min but gradually increased after 45 min. Samples with 5 and 10g/L had a decrease of 28% and 30% respectively after 60 min. This shows a greater cell reduction in the illuminated bulk titania samples than samples in the dark. Fig 1C shows Illuminated samples with nano-scale TiO₂. It was observed that bacterial viability in the control samples (0g/L) started to decrease immediately after 15 min. Thereafter, as the cells tried to adapt, there was a slight growth in the cells which eventually reduced by 50% at the end of 60 min. This reduction could also be attributed to the fluorescent light exposure. Samples with 5g TiO₂ L⁻¹ and 10g/L also had a decrease of

50% and 90% respectively after 60 min. Samples with TiO₂ NPs in the dark showed higher viability of cells (Fig 1D) than samples with bulk titania in the dark (Fig 1B). At 0g/L concentration, there was no decrease in cell viability after 60 min as the cells continued to grow undisturbed. This suggests that illumination plays important role in bacterial cell growth inhibition. However, samples with 5g/L and 10g/L had a decrease of 80% and 77% respectively after 60 min. Overall, the study suggests a higher bacteriostatic effect of the nanoTiO₂ than the bulk TiO₂ under constant illumination. Higher concentrations of TiO₂ and increase in exposure time also resulted in higher antibacterial effect on cells. Previous studies have revealed that high concentrations of nano TiO₂ are required to completely inhibit bacterial growth. This is in agreement with previous research [22, 24, 25] that TiO₂ bactericidal activity increases with increase in dosage and time of exposure. Comparatively, the nano-scale

TiO₂ show more toxicity than the bulk titania. Scanning electron microscopy (SEM) of the *B. sphaericus* cells (Fig. 2A and B) showed that the cell morphology was intact before and after treatment with TiO₂ and therefore, TiO₂ NPs were only bacteriostatic at 10g/L after 60 min. Higher concentrations at increased time of exposure will be required for complete bactericidal effect. Viable cell count however, showed a reduction in cell viability, which suggests a bacteriostatic effect of the TiO₂ NPs. Fig. 2 (A) shows *Bacillus sphaericus* cells without TiO₂ NPs. However, it is apparent from Fig. 2 (B) that the cell morphology was still intact even after 10g/L concentration of TiO₂ NPs at 60 min. This also indicates that a longer exposure time and probably a higher concentration of TiO₂ NPs are required for cell disruption and bactericidal effect.

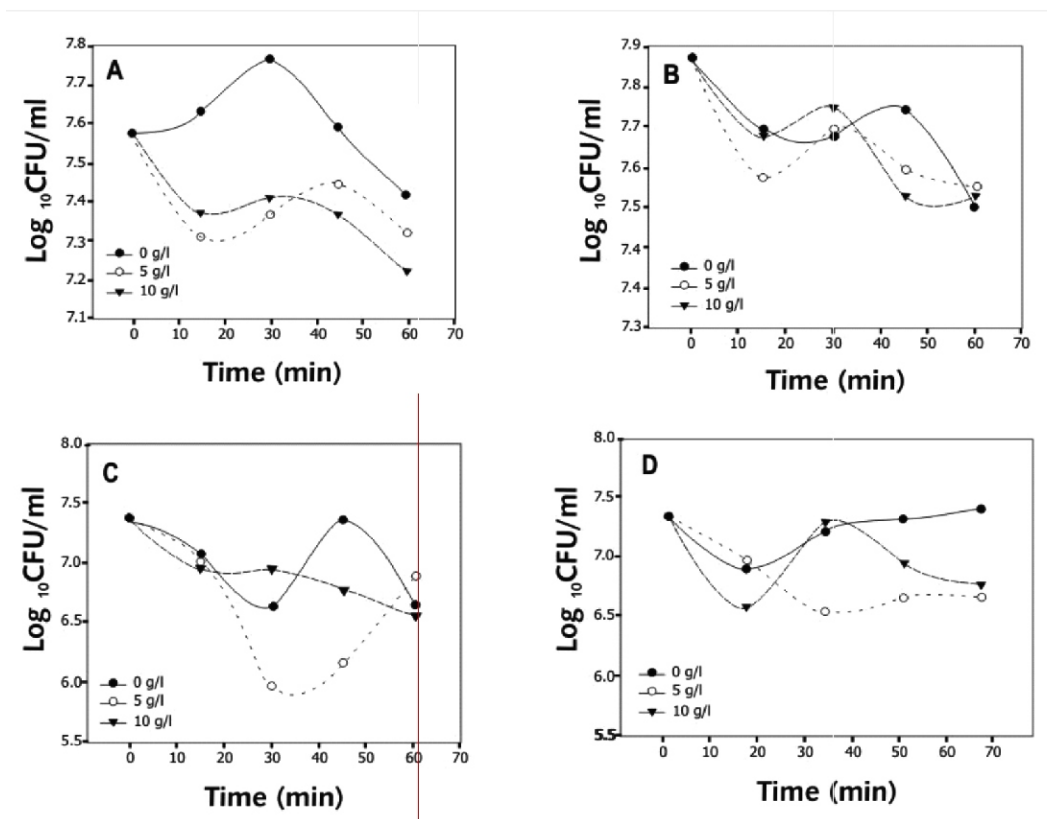


Fig. 1. Growth curves of *Bacillus sphaericus* inoculated with 10^6 CFU of bacteria in the presence of different concentrations of nanoscale and bulk TiO_2 (A) Using Microcrystalline TiO_2 under Fluorescent light, (B) dark control (C) Using Nanoscale TiO_2 under Fluorescent Light, (D) dark control

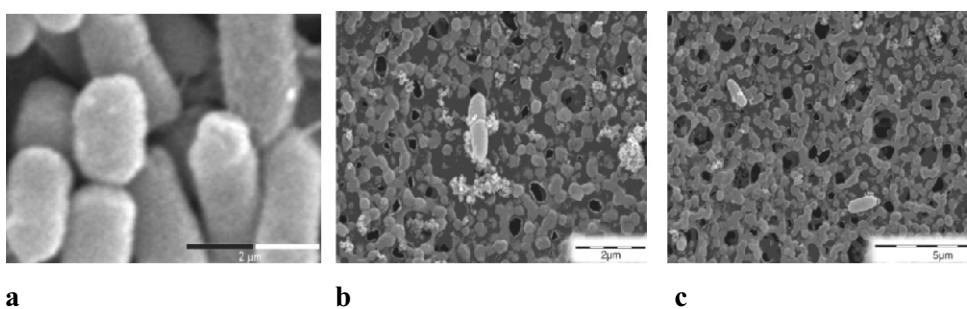


Fig. 2. Scanning electron micrographs (SEM) showing (a) cell morphology of *Bacillus sphaericus* without TiO_2 NPs, (b and c), with 10 g/L TiO_2 NPs After 60 min of fluorescent light irradiation.

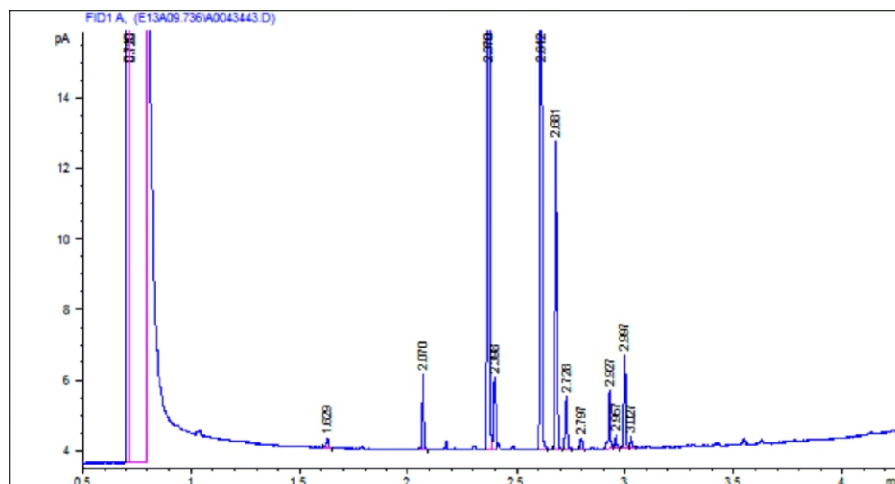


Fig. 3. Chromatogram of the fatty acids of *Bacillus sphaericus*

Discussion

The present study showed that increased concentration of TiO_2 dose and irradiation time achieves higher photokilling and antibacterial activity. This agrees with previous studies [6] who reported 99.9% bactericidal effect of TiO_2 NPs on *Staphylococcus typhimurium* after 40 mins exposure at 0.1 g/ L. However, the size of the nanoparticles, the type of illumination and the organism in question are major factors in the bactericidal activity and may have resulted in the slight differences in the results obtained in this study compared with that of [6]. The TiO_2 cell suspension in the dark also showed a reduction in cell viability compared to the samples exposed to fluorescent lights which emphasizes the impact of light in the inhibition process. This finding is in line with work done by [26]. It is apparent from the survivability curve that

there was a decline in the viable cell at the end of 60min compared to the start of the experiment. Generally, paints contain a great diversity of bacteria, with many of the organisms belonging to different genera. The application of the FAME-based identification approach to characterize microbial community and diversity in spoilt paints provided a meaningful ecological survey of the heterotrophic microbial community structure. The variety of fatty acids present in bacteria exerts a strong influence on the classification of bacteria [27] and may be a useful tool to determine microbial diversity in community of heterotrophic organisms. The attraction and uniqueness of the FAME analysis hinges on the fact that microorganisms possesses different kinds of fatty acids in diverse combinations. In addition, these fatty acids can be readily analyzed by gas chromatography [28]. The Sherlock

microbial identification system identified bacteria solely by computer comparison of the unknown isolates's fatty acid methyl ester (FAME) profiles with the profiles of many known bacterial species included in a database using pattern recognition software. Fatty acids 15:0, 16:0, iso- and anteiso-branched fatty acids were particularly observed in the isolate. This is in line with the work of [29, 30] who reported that saturated fatty acids 16:0 are generally observed in all bacteria while the iso- and anteiso-branched fatty acids were identified in the Gram-positive bacteria. Previous research also showed that 15:0-iso has been reportedly found in Gram-positive bacteria [31]. The results of this study suggests that the FAME microbial identification system is a reliable technique for bacterial identification.

Conclusion

The use of mixed-phase TiO₂ NPs exhibited enhanced antibacterial and photokilling properties towards *B. sphaericus* cells compared to the bulk TiO₂ under fluorescent light illumination. Antibacterial effects increased with increase in TiO₂ concentration and time of exposure. The potentials of this technique can therefore be harnessed in our living environments to achieve disinfection and elimination of resistant microbes. The results offers promising potentials of photocatalysis via readily available fluorescent lights.

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References

- Zheng X, Chen Y, Wu R. Long-term effects of Titanium dioxide nanoparticles on nitrogen and phosphorus removal from waste water and bacterial community shift in activated sludge. *Environ. Sci. Technol.* 2011; 45, 728-7290.
- Joost U, Juganson K, Visnapau M, Mortimer M, Kahru A, Nommistre E, Joost U, Kisand V, Ivask A. Photocatalytic antibacterial activity of nano-TiO₂ (anatase)-based thin films: Effects on *Escherichia coli* cells and fatty acids. *J. Photochem. Photobiol. B: Biol.* 2015; 178-185.
- Aminedi R, Wadhwa G, Das N, Pal B. S h a p e dependent bactericidal activity of TiO₂ for the killing of Gram-negative bacteria *Agrobacterium tumefaciens* under UV torch irradiation. *Environ. Sci. Pollut. Res.* 2013; 20, 6521-6530.
- Özyildiz F, Güden M, Uzel A, Karaboz I, Akil O, Bulut H. Antimicrobial activity of TiO₂-coated orthodontic ceramic bracket against *Streptococcus mutans* and *Candida albicans*. *Biotechnol. Bioproc. Eng.* 2010; 15, 680-685.
- Cheng YW, Chan RCY, Wong PK. Disinfection of *Legionella pneumophila* by photocatalytic oxidation. *Water Res.* 2007; 41, 842-852.
- Ibanez JA, Litter MI, Pizarro RA. Photocatalytic bactericidal effect of TiO₂ on *Enterobacter cloacae*. Comparative study with other Gram-negative bacteria. *J. Photochem. Photobiol. A Chem.* 2003; 157, 81-85.
- Shieh K J, Li M., Lee YH, Sheu SD, Liu YT, Wang YC. Antibacterial performance of photocatalyst thin film fabricated by deflection effect in visible light. *Nanomed.* 2006; 2, 121-126.
- Dalrymple OK, Stefanakos E, Trotz MA, Goswami DY. A review of the mechanisms and modeling of photocatalytic disinfection. *Appl. Catal. B. Environ.* 2010; 98, 2738.
- Kuhn KP, Chaberny IF, Massholder K, Stickler M, Benz VW, Sunntag H, Erdinger L. Disinfection of surfaces by photocatalytic oxidation with titanium dioxide and UVA light. *Chemosphere.* 2003; 53, 717.
- Ohko Y, Hashimoto K, Fujishima, A. Kinetics of photocatalytic reactions under extremely low-intensity UV illumination on Titanium dioxide thin films. *Phys. Chem. A.* 1997; 101(43), 8057-8062.
- Ogorevc JC, Pirc ET, Matoh L, Bukovec P. Antibacterial and photodegradative properties of metal doped TiO₂ thin films under visible lights *Acta Chim. Slov.* 2012; 59, 264-272.
- Ten YN, Wong CL, Mohamed AR. An overview on the photocatalytic activity of nano-doped TiO₂ in the degradation of organic pollutants. *ISRN Mat. Sci* 2011; 2011, 1-18.
- Wu Y, Zhang J, Xiao L, Chen F. Properties of carbon and iron modified TiO₂ photocatalyst synthesized at low temperature and photodegradation of acid orange 7 under visible light. *Appl. Surf. Sci.* 2010; 256(13), 4260-4268.
- Nel A, Xia T, Madler I, Li N. Toxic potential of materials at the nano level. *Science.* 2006; 311, 622-627.
- Robertson TA, Sanchez WY, Roberts MS. Are commercially available nanoparticles safe when applied to the skin? *Biomed. Nanotechnol.* 2010; 6: 452-468.
- Sunada K, Watanabe T, Hashimoto K. *Environ. Sci. Technol.* 2003; 37, 4785-4789.
- Schmidt H, Naumann M, Muller TS, Akarsu M. Application of spray techniques for new photocatalytic gradient coatings on plastics. *Thin Solid Films.* 2005; 502, 132-

- 137.
- Erdođan EE, Sahin F, Namli A. Phospholipid fatty acids analysis-fatty acid methyl ester (PLFA-FAME) changes during bioremediation of crude oil contamination soil. *Afr. J. Biotechnol.* 2013; 12(44), 6294-6301.
- Skocaj M, Filipic M, Petkovic J, Novak S. Titanium dioxide in our everyday life; Is it safe? *Radiol. Oncol.* 2011; 45(4), 227-247.
- Pal A, Pehkonen SO, Yu LE, Ray MB. Photocatalytic inactivation of Gram-positive and Gram-negative bacteria using fluorescent light. *J. Photochem Photobiol.* 2007; 186, 335.
- Matsunaga T, Tomoda R, Nakajima T, Wake H. *FEMS Microbiol. Lett.* Photoelectrochemical sterilization of microbial cells by semiconductor powders. 1985; 29, 211-214.
- Matsunaga T, Tomoda R, Nakajima T, Nakamura N, Komine T. Continuous sterilization systems that uses photo semiconductor powders. *Appl. Environ. Microbiol.* 1988, 54, 1330-1333.
- Ireland JC, Klostermann P, Rice EW, Clark RM. *Appl. Environ. Microbiol.* 1993; 59, 1668.
- Vohra A, Goswami DY, Deshpande DA, Block SS. Enhanced photocatalytic inactivation of bacterial spores on surfaces in air. *J. Ind. Microbiol. Biotechnol.* 2005; 32, 364-370.
- Galvez JB, Ibanez PF, Sixto MR. Solar photocatalytic detoxification and disinfection of water: Recent overview. *J. Solar. Energ. Eng.* 2007; 129, 12-12.
- Saito T, Iwase T, Morioka T. Mode of photocatalytic bactericidal action of powdered semiconductor TiO₂ on mutants *streptococci*. *J. Photochem. Photobiol. B Biol.* 1992; 14, 369-379.
- Hacck, S. K., Garchow, H., Odelson, D. A., Forney, L. J. and Klug, M. J. Interpretation of fatty acid methyl ester profiles of model bacterial communities. *Appl. Env. Microbiol.* 1994; 60(7): 248
- Cavigelli, M. A., Robertson, G. P. and Klug, M. J. Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. *Plants and Soil.* 1995; 170:99-113.
- Pelz O, Chatzinotas A, Andersen N, Bemasconi S. M, Hesse C, W.R. Abraham, Zeyer J. Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to a specific microbial population, *Archives of Microbiol.* 2001; 175:270-281.
- Keinanen M. M, Martikainen P. J., Korhonen LK, Suutari M. H. Microbial community structure in biofilms and water of a drinking water distribution system determined by lipid biomarkers, *Water Sci. Technol.* 2003; 47:143-147.
- Olsson S, Persson P. The composition of bacterial populations in soil fractions differing in their degree of adherence to barley roots. *Appl. Soil Ecol.* 1999; 3(12):205- nb215