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Nano-TiO_2 enhances biofilm formation in a bacterial isolate from activated sludge of a waste water treatment plant

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ABSTRACT

TiO_2 NPs is one of the major components of paints and sunscreens and also acts as a catalyst for waste water treatment. The study demonstrates the toxic effects of TiO_2 NPs on an anaerobic bacterium, *Macrococcus caseolyticus*, isolated from the activated sludge of a waste water treatment facility. The cytotoxicity assessment under UVA, visible light, and dark conditions revealed a dose- and exposure-dependent reduction in viability. The examination of cytotoxicity was performed with membrane permeability assessment through LDH (Lactate dehydrogenase) detection. The formation of exopolymeric substances (EPS) was observed to be dose-dependent, and maximum EPS release was noted under UVA condition. The scanning electron microscopy described the aggregation tendency of biofilm to be more pronounced under UVA condition when treated with TiO_2 NPs as compared to the control biofilm. Uptake of TiO_2 NPs by the biofilm indicated possible remediation of the NPs in waste water by these organisms.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are a major component of cosmetics, paints, and a number of household items. Majorly, these nanoparticles enter waste water as a discharge from residential zones across the globe. Since titania can be photocatalytically activated, it can pose potential threats to the microenvironment if it is not treated where necessary. Ecosystems, where these nanoparticles can enter directly, are more susceptible to these deleterious effects, e.g. freshwater system and other publically accessible water bodies (Yang et al., 2013). Waste water has been considered as major entry point of nanoparticles to the aquatic system. The concentration of TiO_2 NPs in surface water has been reported to be 1 μg mL^{-1}. In waste water treatment plant, the concentration of Ti^{+4} has been estimated to be 181 μg mL^{-1}, and the levels can reach a maximum of 1233 μg mL^{-1} (Kiser et al., 2009; Westerhoff et al., 2011).

Bacteria are considered as a keystone in the ecological niche, where both aerobic and anaerobic bacteria have their functional importance. Bacteria are ubiquitous and are continuously exposed to engineered nanoparticles, and thus, affect the food chain (Adams et al., 2006; Block et al., 1997). *Macrococcus caseolyticus* is one such representative facultative aerobic bacterium isolated from activated sludge of waste water treatment plant. Toxicity of TiO_2 NPs towards anaerobic bacteria has been studied in peridental implants in various species and a concentration-dependent decline in viability has been a major outcome (Vargas-Reus et al., 2012). Reduced effects of TiO_2 NPs due to the formation of biofilm in the anaerobic locales of these peridental explants have been reported. Under stressed conditions, exopolymers are released by bacteria and becomes an integral part of the biofilm. Exo-polymeric substances (EPS) govern properties like surface charge, binding capacity, and protect the microbial cell with van der Waals' forces and electrostatic attraction. Biofilm formed by the microbes produces a shield, and thereby, help in compromising the toxicity (Wuertz et al., 2003). There is an increase in mass transfer in the biofilm due to the limited nutrition supply, which is also responsible for the enhanced biocidal resistance (Wen et al., 2009).

The advantages of anaerobic treatment of waste water as compared to aerobic treatment are gaining remarkable importance because of the properties like: low energy consumption and lesser amount of sludge production (Dutta et al., 2014). The biological and chemical processes to treat waste water in combination with azo dyes under anaerobic condition have been considered important because of their stability, whereas the aerobic process involves the

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biodegradation of the sample (Spagni et al., 2010). Activated sludge involves microbes for the degradation of the organic content in waste water. The waste water treatment tank contains obligate and facultative aerobes. Being in an open system, these bacteria continuously get exposed to different light conditions and environmental changes through the cycle of day (Metcalf et al., 2010).

The present study aims to deal with the outcomes of the interaction of *M. caseolyticus*, a facultative bacterium, with TiO_2 NPs in a waste water matrix under anaerobic conditions. Subsequently, the examination of the cascade of events involved when nanoparticles attaches to the surface of biofilm through the EPS, a major component, and the assessment of biofilm (its thickness and density) as a defense mechanism are done. The possible bio uptake and remediation of the nanoparticles through the biofilm was also investigated. The study was conducted under three conditions as UVA possesses the longest wavelength and reaches to the earth's surface, visible light is an integral part of the solar spectra on the earth, and dark condition is an important phase in the day-night cycle.

2. Materials and methods

2.1. Chemicals procured

All the chemicals and reagents were of analytical grade and were procured from Sigma-Aldrich, India.

2.2. Characterization of collected waste water

The activated sludge collected from waste water treatment plant was situated at Vellore Institute of Technology (VIT University), Vellore, Tamil Nadu, India. The collected waste water was filtered with Whatman no. 1 and subsequently with 0.22-μm membrane filter and was finally steam sterilized. The steriled waste water matrix was stored at 4 °C in a polypropylene bottle until used. The collected waste water under anaerobic condition had a pH of 8.4, conductivity of 2.7 mS, and total dissolved salts of 1.96 ± 0.2 μg mL^{-1}. The waste water medium contains around 0.8 μg mL^{-1} Ti^{+4} ions. The metal content in waste water was analyzed with the help of ICP-OES (Perkin Elmer Optima 5300 DV) (Table 1 supporting information).

2.3. Stability of TiO_2 NPs in waste water under anaerobic condition

A stock suspension of 100 μg mL^{-1} of TiO_2 NPs was made in ultrapure de-ionized water and was sonicated at 130 W for 10 min (Sonics Vibra Cell, USA). A working concentration of 0.25, 0.5, and 1 μg mL^{-1} TiO_2 NPs was prepared in filtered and sterilized waste water by adding suitable amount from stock suspension. The samples were purged with nitrogen to maintain de-oxygenated state. The size of nanoparticles was expressed in terms of effective diameter, and their stability was checked at 6, 12, 24, and 48 h of time interval under UVA, visible light, and dark condition with a particle size analyzer (90 plus particle size analyzer, Brookhaven Instruments Corporation, U.S.A).

2.4. Isolation and characterization of bacteria

Isolation of bacteria was done from the activated sludge of VIT sewage treatment plant situated at VIT University. The collected waste water samples were serially diluted under anaerobic conditions by mixing 1 mL of waste water to 9 mL of sterile de-ionized water in the test tubes till 10^{-9} (9 times) dilution was achieved. The anaerobic chamber maintains a rate of 85% (v/v) N_2, 10% (v/v) H_2, and 5% (v/v) CO_2 as the chamber utilizes a cycle of nitrogen and mixed gas containing carbon dioxide and hydrogen for asserting the anaerobic conditions. Further, for each dilution, nutrient agar plating was done and maintained in an anaerobic chamber. After a period of 48 h, the observed bacterial colonies were sub-cultured to obtain pure strain. The most prominent bacterial colony was selected for the experiments. Morphology of this bacterial colony was established by Gram staining. Phenol-chloroform and dye terminator method was used for extracting the genomic DNA (Green et al., 2012). Sequences were analyzed using BLAST and aligned utilizing CLUSTAL W with the help of the neighbor joining method, and thereafter, the phylogenetic tree was constructed. The anaerobic isolate was identified as *Macrococcus caseolyticus* (VITAN-0502), and the Genbank accession ID obtained was KT923490.1.

The bacterial samples were isolated from waste water collected from home University's sewage treatment plant (activated sludge), and therefore, no specific authorization was required. After collection of the sample, all experiments were conducted in laboratory, and extreme care was taken not to contaminate the ecosystem.

2.5. Assessment of cell viability

The selected anaerobic bacterial isolate from waste water was examined for the effect of TiO_2 NPs under UVA, visible light, and dark condition. The bacterial culture was grown in a nutrient broth for a period of 48 h; the samples were purged with nitrogen till the dissolved oxygen concentration became zero to maintain the anaerobic condition. The harvested cells were then centrifuged at 7000*g* for 10 min, and the pellet was washed with sterilized waste water matrix. An inoculum bacterial cell concentration of 5×10^8 cells mL^{-1} was maintained throughout the experiments. A working concentration of 0.25, 0.5, and 1 μg mL^{-1} of TiO_2 NPs was used, and the assays were conducted in closed serum bottles. The conditions used for the experiments were UVA (350 nm, 18 W, 1 mW/cm^2), visible light, and dark for an exposure period of 12, 24, and 48 h. The serum bottles were kept in an incubating shaker at 120 rpm, 37 °C (Scigenics Biotech, Obiteck). A standard plate count assay was followed. The decrease in viability of the treated samples was calculated with respect to the number of colonies observed in control samples. The control samples were considered as 100% with respect to the treated samples.

2.6. Estimation of membrane integrity with lactate dehydrogenase (LDH)

The control cells (absence of nanoparticles) and cells treated with 0.25, 0.5, and 1 μg mL^{-1} of TiO_2 NPs were used for the quantification of extracellular LDH. The suspension was purged with nitrogen and incubated for 12, 24, and 48 h of time intervals under UVA, visible light, and dark conditions. The suspension was centrifuged for 7000*g*, 10 min, and the supernatant was collected. The harvested aliquot of supernatant was treated with 30 mM sodium pyruvate and 2.8 mL of 200 mM Tris HCl reagents according to the standard assay (Brown et al., 2001). A decline in absorbance at 340 nm was measured using a UV-Vis spectrophotometer (HITACHI-2910) to assess the consumption of NADPH, which corresponds to the LDH activity.

2.7. Quantification of EPS (exopolymeric substances)

Exopolymeric substances are the structural and functional fraction of biofilm, these are complex organic molecules liberated by microbes. The bacterial loop full culture was inoculated in 100 mL nutrient broth medium and purged with nitrogen to