MICROBIOLOGY

Sustainable and Ecofriendly Nanoparticle Synthesis Using Microbial Strains Isolated From Heavy Metal Rich Industrial Effluent

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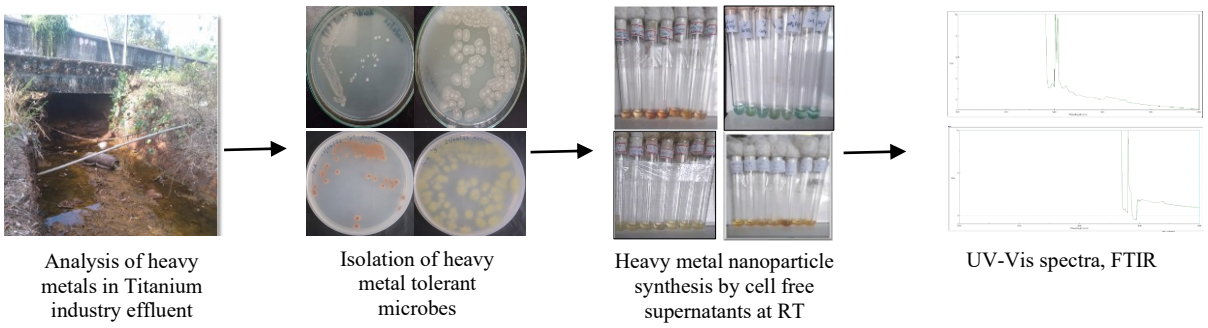
ABSTRACT

Heavy metals present in industrial effluent that are discharged into the environment can leach into water resources and adversely affect human health and ecosystems. Bacteria have evolved defensive mechanisms to counteract heavy metal toxicity. In this study, the concentrations of Cobalt (Co), Copper (Cu), Iron (Fe), Manganese (Mn) and Zinc (Zn) in titanium industry effluent were found to surpass the permissible limits set by three authentic standards for drinking water. Notably, the concentration of manganese exceeded the permissible limit by a staggering factor of 500 times followed by Co, Fe, Zn and Cu. Eight morphologically different bacteria and fungi were isolated from this effluent. The cell free supernatants of six out of these eight cultures produced heavy metal nanoparticles at room temperature over a period of upto 7 days. The presence of nanoparticles was verified by visible colour changes, UV-Vis spectroscopy and FT-IR analysis. The maximum absorption of the Fe, Co, Cu, Zn and Mn nanoparticles was in the UV range between 300 nm- 401 nm. Two of the nanoparticle producers were identified as *Bacillus cereus* and *Curvularia* sp by MALDI-ToF. Our research highlights the ecofriendly and sustainable approach to nanoparticle synthesis and creating value from industrial waste.

Keywords: heavy metal, nanoparticles, sustainable, microbes, Titanium industry effluent

Graphical abstract



1. INTRODUCTION

Heavy metal contamination in the environment is a pressing issue that poses significant risks to both ecological and human health. Unlike other pollutants, heavy metals do not fully degrade; instead, they transform into different states. Once released into the environment, heavy metals can persist for long periods due to their non-biodegradable nature, leading to bioaccumulation in living organisms and potential biomagnification up the food chain [1]. Interestingly, certain microorganisms have developed mechanisms to survive in heavy metal-contaminated environments. These survival strategies include permeability barriers, metal sequestration, where the organism stores the metal in a form that is less toxic; metal reduction, where the organism changes the oxidation state of the metal to a less toxic form; and metal efflux, where the organism actively pumps the metal out of its cells. Some microorganisms can even utilize heavy metals for their metabolic processes, effectively 'cleaning up' the environment through a process known as bioremediation [2].

The application of heavy metal-resistant or tolerant bacteria in the biosynthesis of metal nanoparticles is an emerging field of research with significant potential. The production of nanoparticles by bacteria can be done by three methods: by intact cells, by cell free supernatants or by the use of bacterial cell extracts [3]. Heavy metals such as cobalt, copper, iron, manganese, zinc, chromium and nickel are essential nutrients that can be toxic at high concentrations [2].

Titanium processing causes severe pollution and has been shown to be rich in persistent organic pollutants and heavy metals [4]. Many investigations have shown the adverse impact of Titanium industry effluents on biota (5, 6, 7, 8, 9, 10] damaging the nearby flora and fauna, as well as causing major health problems among local residents, including skin illnesses, respiratory problems, and cancer [4]. The measures for eliminating these heavy metals are

expensive and impractical for industries. However, many of these metals have various industrial applications which if recycled and reused from the waste water would not only help to address pollution challenges, but also help to create wealth from waste.

With an objective to find an ecofriendly and sustainable approach to mitigate the problem of heavy metal pollution of water resources, we have analysed the heavy metals present in Titanium industry effluent, isolated bacterial and fungal strains from the effluent-contaminated sediments and explored their potential to synthesise nanoparticles of those particular heavy metals in vitro as a means of bioremediation.

2. MATERIALS AND METHODS

2.1. Sample collection and processing

Untreated effluent, treated discharged effluent and sediment samples from different locations in and around a Titanium mineral processing factory in Kollam district, Kerala were collected in sterile bottles and processed for heavy metal analysis and microbial isolation within 3 hours. The physical characteristics of the different effluent samples, such as colour, pH and temperature were analysed on site. The concentrations of cobalt, copper, iron, manganese and zinc in the treated discharged effluent were determined by standard methods as shown in Table 1. These metals were specifically chosen for analysis as their nanoparticles are known to have various applications.

2.2. Isolation of microbes

To isolate microbes, 10 ml or 10 g of the well mixed effluent or sediment sample was vortexed with 90 ml of autoclaved distilled water to make a 10-1 dilution. Serial dilutions of the samples up to 10^{-4} were prepared in autoclaved distilled water and 0.1 ml of each dilution was plated on half strength nutrient agar as this was found to be more suitable for the growth of the organisms. The plates were then incubated at room temperature for 24 - 48 hrs. Eight

morphologically different microbial colonies were randomly selected and then sub-cultured and re-streaked several times to obtain pure cultures. The pure cultures were then stored on half strength nutrient agar plates at 4°C or as 20% glycerol stocks at -20°C.

2.3. Nanoparticle synthesis and analyses

The eight isolates were then tested for their ability to form nanoparticles of the heavy metals that were found in the effluent. For this, 10mM salt solutions of Ferric Chloride ($FeCl_3$), Zinc sulphate ($ZnSO_4.7H_2O$), Manganese Sulphate ($MnSO_4.H_2O$), Copper Sulphate ($CuSO_4.5H_20$), and Cobalt Chloride ($CoCl_2.6H_2O$) each were prepared and autoclaved. These were used as precursor solutions for nanoparticle synthesis. The microbial cultures were inoculated in half strength nutrient broth and incubated at room temperature for 48 hours. The cultures were centrifuged at 4000 rpm for 15 minutes. Then, 800µl of the cell-free supernatants were mixed separately with 200 µl each of 10mM $FeCl_3$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $CuSO_4.5H_20$ and $CoCl_2.6H_2O$ salt solutions. 800 µl of uninoculated half-strength nutrient broth was used as a control. The mixtures of culture supernatants/nutrient broth and salt solutions were kept in the dark at room temperature under static conditions for up to seven days**.** During this period, any colour change observed in the cell-free filtrate after exposure to metal ions indicated that the microbes were nanoparticle producers. Conversely, if the colour of the cell-free filtrate remained unchanged following inoculation with metal ions, the microbes were considered non-producers of nanoparticles. The samples that exhibited colour changes were subjected to UV-visible spectroscopy, during which spectra were recorded between 200 nm and 700 nm using a V-730 UV-Visible spectrophotometer. Subsequently, the mixtures were centrifuged, and the resulting particles were washed with distilled water and ethanol. Finally, the particles were dried in a hot air oven for Fourier Transform-Infra Red (FT-IR) analysis.

2.3. Microbial Identification

Gram staining was performed on all the isolates. Two microbes that displayed colour changes with the heavy metal solutions were subjected to identification by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

3. RESULTS

3.1. Physicochemical properties of Titanium industry effluent

The untreated effluent had a fluorescent green appearance, a temperature of 28°C and was extremely acidic with a pH of 0. The lime-treated discharged effluent was pale yellow with a pH and temperature of 3.1 and 26°C. Samples collected less than 200 m away from the factory also had a pale yellow colour and showed an increase in pH up to 3.5 and temperature up to 32°C.

Table 1 shows the concentrations of heavy metals in the collected effluent samples. As these metals can leach into ground water and contaminate water resources, we compared the levels of heavy metals in these collected samples with the drinking water guidelines set by three authentic standards the Bureau of Indian Standards (IS10500: 2012), US EPA (2018) and the World Health Organization (WHO, 2022) [11, 12, 13]. All the metals, except Cobalt, exceeded the permissible levels recommended by these standards. Cobalt has not been considered in any of the drinking water standards, however, based on the WHO, 2003 guidelines [14], cobalt was found to be 20 times higher than the permissible limit.

3.2. Microbiological analysis

Bacteria and fungi were isolated from the discharged effluent by spread plate method and purified by repeated streaking on half strength nutrient agar. To avoid redundancy, eight morphologically different cultures were selected for further studies (Fig. 1). Two of the cultures were microscopically determined to be fungi, while the remaining were Gram positive bacteria.

Two of the microbial isolates which showed good potential for extracellular synthesis of heavy metal nanoparticles were identified by MALDI-TOF analysis as *Bacillus cereus* and *Curvularia sp*.

3.3. Nanoparticle synthesis and analyses

To develop a sustainable and eco-friendly approach to nanoparticle synthesis, the cell free culture supernatants of each of the eight isolates were then tested independently for extracellular production of nanoparticles of Co, Cu, Fe, Mn and Zn at room temperature. For this, the cell free culture supernatants were mixed with 10mM salt solutions of the heavy metals in a 4:1 ratio and incubated in the dark at room temperature for upto seven days. *Bacillus cereus* culture supernatant was found to produce nanoparticles of copper, manganese and zinc in less than 48 hrs, while the *Curvularia* species culture supernatant produced nanoparticles of cobalt, copper and iron within 7 days.

Culture supernatants that showed visible colour changes with at least one heavy metal solution were subjected to UV-Vis spectroscopy in wavelengths of between 200- 700 nm to determine the optical properties of the synthesized nanoparticles. The maximum absorption peaks for the various nanoparticles fell within the UV range between 300-400nm. For Co, Cu, Fe, Mn and Zn nanoparticles, maximum absorption peaks were obtained at 340 nm, 335 nm, 298.5nm, 401nm and 344 nm respectively (Fig. 2). One of the cultures, which was not was able to produce over one gram of cobalt nanoparticles from 25 ml of culture supernatant at room temperature. The presence of Cobalt nanoparticles was confirmed by the presence of two peaks at 669 cm^{-1} and 528 cm^{-1} in FTIR analysis which denoted Co-O stretching vibrations. The peak at 1718 cm^{-1} was due to carbonyl linkages which could arise from the bacteria. These bonding features indicated the presence of Cobalt oxide nanoparticles.

4. DISCUSSION

Many studies on the physicochemical characteristics and the physiological effects of Titanium industry effluents on biota have been carried out in India and abroad. Very few studies have delved into the microbial aspects of such effluents. Koshy in 2013 has isolated heavy metal resistant/tolerant bacterial strains from these effluents [15]. In this study, we were able to isolate 8 morphologically different Gram positive bacteria and fungi. Each of the strains except two were able to synthesise nanoparticles of at least one heavy metal under the conditions provided. Heavy metals are extremely toxic and although metals like cobalt, copper, iron, manganese and zinc are required for normal growth, they can be harmful when their concentrations exceed the permissible limits [16]. Producing pure Cobalt nanoparticles using microorganisms can be tough because of the severe toxicity caused by high levels of Co ions [3]. In this study, the formation of cobalt nanoparticles was verified by visible colour change (light pinkish brown to dark pinkish brown), UV-Vis spectroscopy and FTIR. Further, we were able to biosynthesise nanoparticles extracellularly at room temperature and neutral pH using no extra reagents and minimal processing. Although, the time for production varied from 1 to 7 days depending on the culture and metal used, the use of cell free supernatants and room temperature for nanoparticle synthesis allowed for simpler processing, visualization, downstream purification and characterization of the nanoparticles. Cobalt nanoparticles have many applications in catalysis, energy storage, as contrast agents in biomedicine, in waste water treatment, and as antimicrobial and anticancer agents [17,3]. Several studies have used plant extracts and bacteria to synthesise cobalt nanoparticles, but in all these studies, the use of high temperature from 80 °C to 850 °C and/or other reagents [18, 19, 20, 21, 22] was required to synthesise the nanoparticles. Thus, our study demonstrates an ecofriendly and sustainable approach to nanoparticle synthesis.

5. CONCLUSIONS

Eight morphologically different microbial strains were isolated and purified from heavy metal rich titanium industry effluent. All the isolates except two, were able to biosynthesize heavy metal nanoparticles at room temperature, which was verified by visible colour changes, UV-Vis spectroscopy and FTIR. Importantly, we were able to synthesise the nanoparticles extracellularly using cell-free supernatants at ambient temperature and neutral pH using only the heavy metal salt solution and no additional reagents or high temperatures. This method represents a significant advancement in the field, offering a more sustainable and environmentally friendly approach to nanoparticle synthesis. This research also highlights the untapped potential of industrial effluents as sources of microbial strains capable of nanoparticle biosynthesis. Further research is needed to fully understand the mechanisms underlying this process and to optimize the yield and properties of the biosynthesized nanoparticles.

6. ACKNOWLEDGMENTS

The authors acknowledge the seed money sanctioned to SN for the project entitled "Isolation of Bacteria from Titanium Mineral Processing Effluent and Evaluation for Nanoparticle Synthesis" (Sanction order FC/E3/SM20/2022) provided by Fatima Mata National College (Autonomous), Kollam for carrying out this work.

7. AUTHORS' CONTRIBUTIONS

SN conceived and designed the study, analysed and interpreted the data and wrote the manuscript. SM and AJ acquired and analysed the data.

8. FUNDING

The authors acknowledge the financial assistance provided by Fatima Mata National College (Autonomous), Kollam.

9. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

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Table 1: Concentration of heavy metals in the discharged Titanium industry effluent and comparison with permissible limits of these heavy metals in drinking water.

S. No.	Heavy metal	Test method	Concentration (mg/L)	BIS (2012)-(IS 10500: 2012-second revision) (mg/L)	WHO (2022) guideline value (mg/L)	USEPA (2018) (secondary drinking water regulations) (mg/L)
1	Cobalt	GCKL/QS/C/7.2/115	1.017	Not mentioned	0.05*	Not mentioned
2	Copper	IS 3025 (Part 42)	3.5	0.05	2	1.3
3	Iron	IS 3025 (part 53): 2003 (UV Method)	4.0	0.3	Not of health concern at levels causing acceptability problems in drinking-water	0.3
4	Manganese	IS 3025-1999	40.0	0.1	0.08	0.05
5	Zinc	IS 3025 (Part 49)	12.2	5	Not of health concern at levels found in drinking-water.	5

* Based on WHO, 2003 guidelines

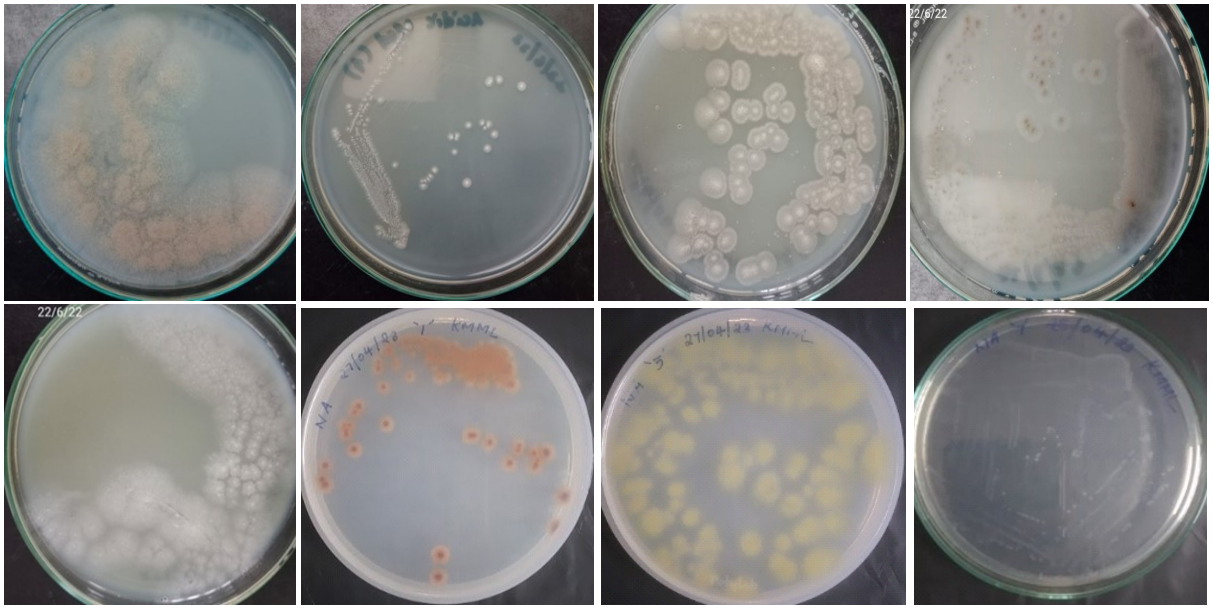

Figure 1: Purified microbial isolates from Titanium industry effluent on half strength nutrient agar medium.

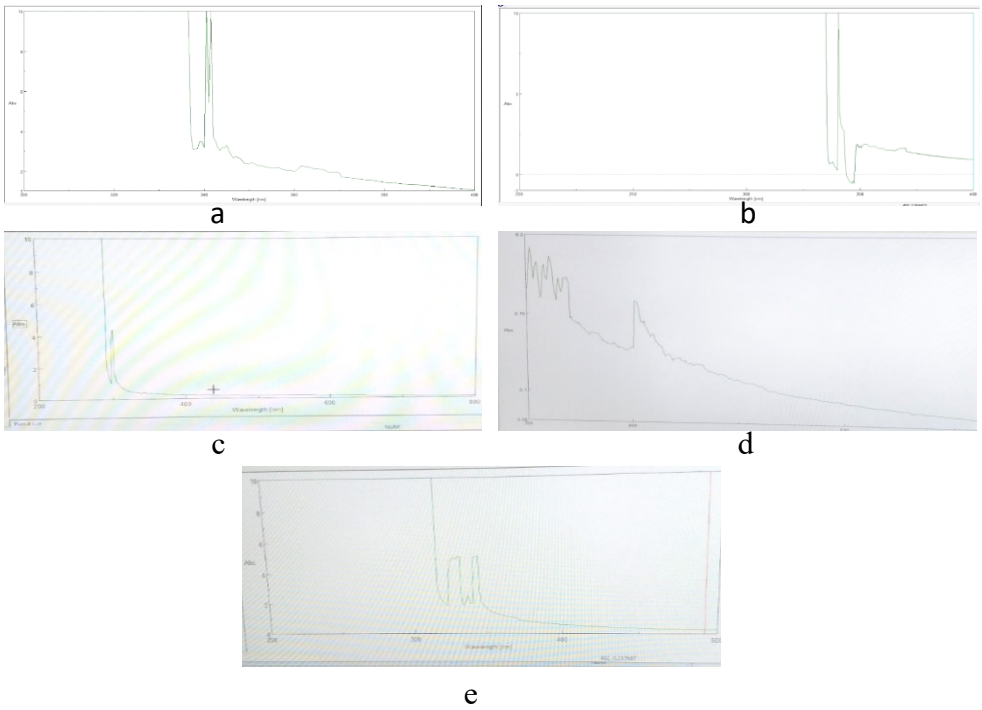

Figure 2: UV-Vis spectra of Co (a), Cu (b), Fe (c), Mn (d) and Zn (e) nanoparticles obtained from culture supernatants of the microbial isolates obtained from Titanium industry effluent.