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Research article

An *Aspergillus aculateus* strain was capable of producing agriculturally useful nanoparticles via bioremediation of iron ore tailings

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ABSTRACT

Mining waste such as iron ore tailing is environmentally hazardous, encouraging researchers to develop effective bioremediation technologies. Among the microbial isolates collected from iron ore tailings, *Aspergillus aculeatus* (strain T6) showed good leaching efficiency and produced iron-containing nanoparticles under ambient conditions. This strain can convert iron ore tailing waste into agriculturally useful nanoparticles. Fourier-transform Infrared Spectroscopy (FT-IR analysis) established the at the particles are protein coated, with energy dispersive X-ray Spectroscopy (EDX analysis) showing strong signals for iron. Transmission Electron Microscopy (TEM analysis) showed semi-quasi spherical particles having average size of 15 ± 5 nm. These biosynthesized nanoparticles when tested for their efficacy on seed emergence activity of mungbean (*Vigna radiata*) seeds, and enhanced plant growth at 10 and 20 ppm.

1. Introduction

Iron ore tailings are abundant environmental contaminant from mining sites. World iron ore resources are estimated to be greater than 800 billion tons with more than 230 billion tons of iron (https://minerals.usgs.gov). Brazil is the largest producer of iron ore followed by China, Australia, India and Russia, with these countries in particular having mining contamination problems. About 3220 MT of iron ore was produced globally during 2014, with generation of huge amounts of mining waste. This waste is primarily stored in large impoundments known as tailing dams and is not effectively remediated.

The concentration and availability of metals in the polluted environment in and around mines and other polluting industries, along with the nature of metals and temperature, affect the microbial population living in those conditions. Some fungi are known to thrive in these stressed conditions, as they can tolerate extreme temperature, pH and metal concentrations (Baldrian, 2003; Gavrilescu, 2004; Milova-Ziakova et al., 2016). Some of these fungi have the ability to convert the metal waste material into nanoparticles, thereby neutralising their toxic effects. There are several reports on fungi being applied as bioleaching agents to dissolve metals from waste materials (Bosshard et al., 1996; Dacera and Babel, 2008; Khan et al., 2014; Madrigal-Arias et al., 2015), ores (Biswas et al., 2013; Mishra and Rhee, 2014; Mulligan et al., 2004) and minerals (Amiri et al., 2011; Anjum et al., 2010; Brisson et al., 2016; Hosseini et al., 2007). According to these studies, organic acids such as citric acid, oxalic acid and gluconic acid, exogenously produced by the fungus, can help in the leaching of metals due to their chelating or reducing abilities. This ability of microbes to tolerate high metal ion concentrations and convert this metal waste into nanoparticles, has encouraged scientists to use these microbes as eco-friendly nanofactories for the biological synthesis of nanoparticles (Duran et al., 2005; Khan et al., 2014). Some *Aspergillus* strains have been shown to be resistant to high metal concentrations by modifying their metabolic activities to tolerate these metals (Chakraborty et al., 2013; Kumari et al., 2015; Santhiya and Ting, 2005; Seh-Bardan et al., 2012).

Myconanomining (Fungi mediated bioleaching and conversion of bulk metallic elements/compounds into nanostructures) is considered safe and ecologically benign for the conversion of bulk inorganic (metal based) materials into nanostructured forms. The use of this myconanomining approach for bioleaching and

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biosynthesis of nanoparticles from tailings waste offers several advantages over other environmental biological process, such as: (i) Higher biomass production; (ii) fungal secretome contains large amounts of extracellular proteins with diverse functions; (iii) more biosorption of metallic elements/compounds at low pH; and (iv) high metal reducing activity of secretome. Here, the fungal biomass aqueous extract containing secretome is used for bioleaching from collected iron ore tailings, with the subsequent biosynthesis of nanoparticles for use as plant nutrient Fe.

In this study we isolated several new strains from contaminated iron ore tailing of mining sites and discovered a new strain of *Aspergillus aculeatus* (strain T6) that efficiently converts iron ore tailings into nanoparticles. These particles were then applied to a model plant growth system (mungbean seeds) and show to seed emergence activity.

2. Materials and methods

2.1. Materials used

All chemicals used in this study were purchased from Fischer Scientific (Mumbai, India) and were of analytical grade. Potato dextrose agar, Potato dextrose broth, Mycological peptone, Agar Extra pure used were procured from HiMedia (Mumbai, India) and were sterilized by autoclaving at 120 °C for 15 min at 15 psi before use. Mungbean (*Vigna radiata*) seeds (Type 44 variety) was purchased locally.

2.2. Iron ore tailings collection and analysis

Iron ore tailings (brick red in color) were collected from Codli mines, Goa, India (24°35′58″N 73°49′8″E) in sterile polythene tubes. The Codli mine is one of the largest mining sites in India with an annual production allowance of about 5.5 MT. The collected waste samples were air-dried at room temperature, homogenized, sieved through fine sieve, and stored in dry in the dark.

The elemental, mineralogical and morphological characteristics of iron ore tailings collected from Codli mines, Goa, India (24°35′58″N 73°49′8″E) were determined by Atomic absorption spectroscopy (AAS) and Transmission electron microscopy (TEM). For the elemental composition of tailings, ~0.5 g finely powdered sample was refluxed with HNO_3 for acid-digestion using Method 3050 B (Arsenic et al., 1996). The sample was later cooled, filtered and analyzed using AAS (Thermo, iCE 3500). The morphology (microstructure) and total elemental composition of the waste sample was analyzed using TEM (TECNAI G^2 T20 TWIN, The Netherlands) equipped with Energy Dispersive X-Ray spectrometer (EDS) (EDAX Inc. The Netherlands). 10 mg of sample was dissolved in 1 mL MilliQ (MQ) water and sonicated for 5 min for disintegration of the microparticles. The sonicated sample (~10 μL) was drop casted on a carbon-coated copper grid and air dried under dark conditions. The prepared grid was analyzed at an accelerated voltage of 200 kV and the TEM micrographs and EDX spectrum images were obtained.

2.3. Isolation of bioleaching fungal strains from tailings and assessment of growth kinetics under batch culture condition

The fungal strains with the highest bioleaching efficacy was isolated from the collected waste sample using a culture enrichment technique. Briefly, 10 g of tailings sample was added to 100 mL of potato dextrose broth (PDB), HIMEDIA, and incubated at 30 °C on a shaker at 140 rpm, for 15 days. After 48 h 100 μL aliquots were taken at regular intervals and plated on potato PDA/HIMEDIA plates and the fungal colonies incubated at 30 °C (Ilyas et al., 2013). Six pure fungal isolates were obtained (T1-T6), with the fungal strain T6 exhibiting the greatest iron leaching ability. The bioleaching efficiency was calculated as follows:

$$\text{Efficiency\%} = (IC - FC)/IC*100$$

where,

IC- Initial concentration of iron in tailings
FC- Final concentration of iron in nanoparticles

The strains, including strain T6, were preserved using 50% glycerol in sterile MilliQ and stored at −80 °C until further used.

Ergosterol is the major sterol present in the cell membranes of filamentous fungi and monitoring its level is a useful method for estimating growth kinetics (Axelsson et al., 1995; Klamer and Baath, 2004; Steudler and Bley, 2015). 50 mg of strain T6 fungal mycelium was taken at regular time interval of 24 h (starting after 48 h). The mycelium was ground using a motor and pestle with liquid nitrogen, followed by the addition of 1 mL of absolute ethanol. This mixture was agitated for 30 s, kept in ice for 1 h and then centrifuged for 5 min at 14,000 rpm. The supernatant was collected and a pellet was suspended in 1 mL of absolute ethanol and treated once again as described above. The two supernatants were pooled together, filtered using 0.22 mm nitrocellulose filters (Millipore, Darmstadt, Germany) and the filtrate was analyzed for Ergosterol using the protocol of Lindblom (Mille-Lindblom et al., 2004) with some modification. Analysis was carried out using HPLC (CBM- 20A, Shimadzu, Kyoto, Japan) equipped with a quaternary pump (LC - 20AT), solvent degasser system (DGU - 20 A5), autosampler (SIL – 20A) and diode array detector (SPDM-20A). Inbuilt software (Shimadzu, LC solution) was used to control the HPLC pump and acquire data from the diode array. A C18 Phenomenex column (Gemini- NX 250 mm × 4.6 mm × 5 μm particle diameter) was used for the analysis. A series of ergosterol standards of varying range 10–50 ppm were prepared in ethanol. The standard peak was obtained with a UV detector set at 282 nm and a runtime of 20 min. The mobile phase was methanol (97%) and water (3%) at a flow rate of 1 mL/min and the injected sample volume was 50 μL.

2.4. Taxonomic characterization of fungal strain T6

2.4.1. Morphological characterization

Fungal strain T6 was initially identified on the basis of monographs (Nyongesa et al., 2015; Samson et al., 2011) and macro as well as micro morphological features. The morphological features of strain T6 were observed using scanning electron microscopy (SEM) (Carl Zeiss, Oberkochen, Germany). The strain T6 was subcultured on PDA and incubated at 25 °C for 4 or 5 days. After incubation, fungal discs were taken and then immersed in fixative solution (modified Karnovsky's fixative containing 2.5% glutaraldehyde, 2.5% paraformaldehyde, 0.05 M Cacodilate buffer and 0.001 M $CaCl_2$) at pH 7.2. Cacodilate buffer was then used to wash the discs (thrice for 10 min each), followed by post-fixation in 1% osmium tetraoxide solution and water for 1 h (Silva et al., 2011). The samples were then washed with sterile MQ water three times and subjected to dehydration in acetone (25%, 50%, 75%, 90% and 100%) for 10 min, followed by critical point drying (CPD) (Emitech K850, Berkshire, U.K.). The sample was later assembled on double-sided carbon tape placed on aluminium stubs and coated with gold-palladium in a sputter coater (Quorum Technologies SC7620, Berkshire, U.K.) and viewed in SEM at an accelerating voltage of 10 kV.

2.4.2. Molecular characterization

For molecular characterization of the fungal strain T6, total