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Research article

Native hypersaline sulphate reducing bacteria contributes to iron nanoparticle formation in saltpan sediment: A concern for aquaculture

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ABSTRACT

A hypersaline dissimilatory sulphate reducing bacterium, strain LS4, isolated from the sediments of Ribander saltpan, Goa, India was found to produce (Fe_2O_3) maghemite nanoparticles. The presence of maghemite nanoparticles was also detected in the same sediment. Strain LS4 was isolated anaerobically on modified Hatchikian's media at 300 psu, growing optimally at 30 °C, 150 psu salinity and pH 7.8. Based on biochemical characteristics and 16S rRNA sequence analysis, the strain LS4 belongs to genus *Desulfovibrio*. This isolate synthesized iron oxide nanoparticles in vitro when challenged with $FeCl_3$ & $FeSO_4$ in the growth medium. The biological nanoparticles were characterized to be Fe_2O_3 nanoparticle of 19 nm size by X-ray diffraction, transmission electron microscopy, fourier transform infrared spectroscopy, scanning electron microscopy and energy-dispersive x-ray spectroscopy. Maghemite nanoparticles (5.63 mg g^{-1}) were isolated from the saltpan sediment by magnetic separation which showed similar characteristic features to the Fe_2O_3 nanoparticle produced by strain LS4 with an average size of 18 nm. Traditionally Goan saltpans were used for aquaculture during the non-salt making season, thus effects of these nanoparticles on Zebra fish embryo development were checked, which resulted in developmental abnormalities and DNA damage in a dose dependent manner. With the increasing nanoparticle concentration (0.1 mg.L^{-1} to100 mg.L^{-1}), the mortality rate increased with a decrease in the hatching rate (93.05 ± 2.4 to 25 ± 4.16%) and heart rate (150–120 beats per minute). The nanoparticle exposed embryos developed malformed larvae with a characteristic of pericardial edema, curved body, curved notochord, curved tail and curved tail tip. These results suggest that strain LS4 might be playing a role as a contributor in the formation of iron oxide nanoparticle in the Ribander saltpan sediment, however; its high concentration will have a negative impact on aquaculture in these saltpans.

1. Introduction

Nanoparticle production by biological methods were relatively advantageous (Shah et al., 2015) as it adheres to the Green Chemistry approach (Duan et al., 2015) and eliminates problems, such as the use of toxic chemicals, stringent conditions and higher cost of physicochemical methods (Revati and Pandey, 2011). Sulphate reducing bacteria (SRB) carryout anaerobic metal reduction by producing sulphide and precipitate metals generally as metal sulphide nanoparticles viz. CdS (White and Gadd, 1998; Utgikar et al., 2002), CuS (White and Gadd, 2000), ZnS (Labrenz et al., 2000; Moreau et al., 2007), CoS, NiS, CrS (White and Gadd, 1996) and FeS (Watson et al., 2000). Extracellular proteins of SRB limit the dispersal of nanoparticles produced by them and promote nanoparticle aggregation to prevent nanoparticle transportation in the environment (Moreau et al., 2007). *Desulfovibrio* sp. is the most studied SRB for production of metallic nanoparticles such as Ni, Pd, Pt, CrS, MgS, FeS, TeS and Au nanoparticle (Capeness et al., 2015; Lloyd et al., 1999; Lengke and Southam, 2006). Sakaguchi et al.

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(1993, 2002) reported a magnetosome producing SRB *Desulfovibrio magneticus* strain RS1, which produces intracellular magnetite particles of gregite (Fe_3S_4) or magnetite (Fe_3O_4).

Hypersaline environments normally support and favour the growth of halophiles which produce various stable and unique biomolecules viz. lipase, amylase, gelatinase, protease for various applications (Moreno et al., 2009, 2013). Saltpans are man-made extreme environment with hypersaline conditions and exist as a niche for different halophiles. Through different ways various metals gain entry in to the saltpans (Pereira et al., 2012) and get concentrated with the evaporation process. The study site, Ribander saltpan, is fed by the tidal influx from the Mandovi River estuarine waters containing metal effluents from the ferro-manganese ore mining activities (Kerkar and Das, 2017), barge traffic (Pereira et al., 2013) and sewage disposal (Mani et al., 2012). High iron concentration was detected in the Mandovi estuarine water (Goa state pollution control board, 2016). Ambient iron concentration regulates the sulphate reducing activity in the mangrove ecosystem adjacent to Ribander saltpan by influencing the SRB (Attri et al., 2011).

During the salt making season ie, pre-monsoon and post-monsoon seasons, salinity gradient pond systems are created in saltpan to harvest salt crystals. During non-salt making season (monsoon), the saltpans remain submerged in estuarine waters and traditionally utilized for aquaculture, to breed fishes, shrimps and prawns (Mani et al., 2012). Bacteria from these hypersaline niches have adapted to be metal tolerant and exhibit various metal detoxification processes like exopolysaccharides production, biosorption, bio-precipitation etc. (Llamas et al., 2012; Malik, 2004; Pereira et al., 2012) and deposit metal salts in the underlying sediments.

The present work was initiated with a hypothesis that, the hypersaline strains of SRB in the Ribander saltpan might have adapted different strategies to deal with the incoming ionic iron of estuarine water. In the current study, biological route of iron nanoparticle biosynthesis by hypersaline SRB was investigated and linked to the presence of naturally occurring iron nanoparticles in these saltpans. Since the study site is traditionally used for aquaculture during monsoon season, the nanoparticle effect on fish embryo development was assessed using Zebra fish, as a model organism for environmental toxicity screening.

2. Materials & methods

2.1. Sampling site and culture isolation

SRB strain designated as LS4 was isolated on modified Hatchikian's medium (composition in supplementary information S4) (Kerkar and Lokabharathi, 2011) at 300 psu salinity from sediment of Ribander saltpan (32feet 15°29′ 54″N 73°50′ 44.6″E) Goa, India. Cells of strain LS4 were anaerobically cultured and maintained in modified Hatchikian's broth in screw cap test tubes and screw capped bottles at 30 °C in static condition to determine the physiological, biochemical and molecular level characterization of the bacterium.

2.2. Bacterial growth condition and effect of pH & salinity

Growth rate of SRB strain LS4 was calculated by counting the number of cells using a haemocytometer under phase contrast microscope (Lawrence and Mayo N-800M) and by measuring the increase in optical density (OD) at 480 nm. Simultaneously increase in sulphide concentration was measured (Harithsa et al., 2002). To assess the optimum pH for growth of strain LS4, media was prepared at different pH values ranging from pH 5 to pH 10. For salinity optimization, media was prepared with salinities ranging from 0 to 400 psu. After an incubation of 28 days the growth and sulphide content was measured.

2.3. Biochemical and molecular characterization of the SRB

Utilization of electron donors and electron acceptors were tested in 25 ml glass vials containing growth medium supplemented with a 5 mM final concentration of sterile stock solution of substrates (lactate, acetate, pyruvate, fumarate, formate, ethanol, propionate, glucose, fumarate, benzoate and malate). Growth was detected with an increase in sulphide concentration on the 14th day of incubation. For physiological characterization motility, catalase, oxidase, presence of desulfoviridin and cytochrome were tested as described in Bergey's manual of systematic bacteriology. Cell structure was acquired with ZEISS EVO 18 Scanning Electron Microscope. For molecular characterization 16S rRNA gene was amplified using universal primer 27F and 1492R (Lane, 1991), purified PCR product was sequenced. Further the sequence was aligned using NCBI GenBank BLAST utility. The evolutionary history was inferred using neighbour joining method on Mega7 software tool.

2.4. Bacterial nanoparticle (BNP) synthesis

For nanoparticles synthesis, SRB strain LS4 was grown anaerobically in 100 ml of 150 psu (optimum salinity for its growth) liquid Hatchikian's media supplemented with 5 ml of filter sterilized iron salt solution (prepared with Ferric chloride and Ferrous sulphate in 3:2 M ratio) and purged with nitrogen to make the medium anoxic. The medium was inoculated with 20 ml of 10^6 cells.ml^{-1} LS4 culture and incubated at 30 °C anaerobically in static condition. After 35 days of incubation the bio-transformed products settled at the bottom were collected by centrifugation at 14000 rpm for 15min and processed for their characterization. The supernatant was checked for total protein content measured with Qubit protein assay kit by following manufacturer's instruction.

2.5. Isolation of sediment nanoparticle (SNP)

The sediment sample collected from the Ribandar saltpan was dried in an oven at 60 °C for 24 h. With the help of a bar magnet, magnetic particles were separated from the dried sediment.

2.6. Characterization of BNP and SNP

Data on morphology of BNP and SNP were obtained by transmission electron microscopy (TEM) using an FEI, TECNAI G2 F30, S-TWIN microscope operating at 300 kV equipped with a GATAN Orius SC1000B CCD camera. X-ray diffraction (XRD) pattern of BNP and SNP were obtained using a Rigaku Miniflex II desktop X-ray diffractometer and compared in International centre for diffraction data (ICDD) database. Scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDS) were performed to know the elemental composition of BNP and SNP on a JEOL-JSM-6360 LV SEM operated at 15–20 keV, equipped with OXFORD INCA 200 Energy Dispersive Spectrometer. Fourier transform infrared spectroscopy (FTIR) spectrum for BNP and SNP were recorded on a Shimadzu FTIR IR AFFINITY-1.

2.7. Zebra fish embryo maintenance and exposure to nanoparticles

Viable eggs of zebra fish were collected and rinsed thrice with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgCl_2$) prepared as per Brand et al. (2002) with pH 7.2–7.3,