Bull. Mater. Sci., Vol. 35, No. 7, December 2012, pp. 1201–1205. © Indian Academy of Sciences.

Biosynthesis of silver nanoparticles by marine bacterium, *Idiomarina sp. PR58-8*

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Abstract. **Metal-tolerant microorganisms have been exploited in recent years to synthesize nanoparticles due to their potential to offer better size control through peptide binding and compartmentalization. In this paper, we report the intracellular synthesis of silver nanoparticles (SNPs) by the highly silver-tolerant marine bacterium, *Idiomarina sp. PR58-8* on exposure to 5 mM silver nitrate. SNPs were characterized by UV-visible spectrophotometry, X-ray diffraction (XRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). UV-visible absorption scan of a 48 h culture exposed to 5 mM silver nitrate revealed a broad peak at 450 nm indicative of the surface plasmon resonance of SNPs. XRD analysis confirmed the presence of elemental silver and the crystallite size was calculated to be 25 nm using Scherrer formula. The average particle size as per TEM analysis was found to be 26 nm. Metal stress is known to induce the production of non-protein thiols (NP–SHs) which sequester metal ions. In this study, the production of NP–SHs was followed from 6–48 h, wherein it was observed that the NP–SH levels in the silver-exposed culture were consistently higher (261% on an average) than in the unexposed culture.**

Keywords. **Nanoparticles; silver; marine; biosynthesis; thiols.**

1. Introduction

Synthesis of nanoparticles has attracted a lot of attention due to their unusual optical (Krolikowska *et al* 2003), photoelectrochemical (Chandrasekharan and Kamat 2000) and electronic (Peto *et al* 2002) properties. Both unicellular and multicellular organisms have been known to produce inorganic materials either intra- or extra-cellularly (Mann 1995). Microorganisms ranging from bacteria to fungi have been used in recent years to develop non-toxic and environment friendly methods to synthesize nanoparticles (Kowshik *et al* 2002; Bhattacharya and Rajinder 2005). Methods of nanoparticle synthesis using microbes offer better size control through compartmentalization in the periplasmic space and vesicles. The rate of intracellular particle formation and therefore, size of the nanoparticles could, to an extent, be manipulated by controlling parameters such as pH, temperature, substrate concentration and time of exposure to substrate (Gericke and Pinches 2006). Additionally, nanoparticles synthesized by microorganisms tend to be stabilized by peptides such as phytochelatins, thus preventing aggregation (Kang *et al* 2008). These short peptides are synthesized in response to heavy metal stress and have been implicated as a universal mechanism to sequester metal ions in bacteria (Pages *et al* 2008), fungi (Guimaraes-Suares *et al* 2007) and plants (Cobbett 2000).

SNPs find applications in nonlinear optics, spectrally selective coating for solar energy absorption, biolabelling (Joerger *et al* 2000), intercalation materials for electrical batteries, as optical receptors, catalyst in chemical reactions, antibacterial capacities (Duran *et al* 2005) and *in vitro* inhibition of HIV-1 (Elechiguerra *et al* 2005). Silver nanoparticles (SNPs) have been biosynthesized using several terrestrial microorganisms such as *Fusarium oxysporum* (Senapati *et al* 2004), a yeast strain MKY3 (Kowshik *et al* 2003), *Psuedomonas stutzeri AG259* (Joerger *et al* 2000), *Lactobacillus sp.* (Nair and Pradeep 2002), *Escherichia coli* (Gurunathan *et al* 2009) etc. Marine environments could be a good source of metal tolerant microorganism as metals are continuously released into the marine environments by volcanoes, natural weathering of rocks and also by numerous anthropogenic acitivities, such as mining, combustion of fuels, industrial and urban sewage and agricultural practices. Recently, they are being explored as potential sources of metal tolerant microorganisms with the ability to synthesize metallic nanoparticles (Agnihotri *et al* 2009). SNPs have been synthesized using marine fungi (Kathiresan *et al* 2009), marine cyanobacteria (Ali *et al* 2011) and marine algal extracts (Venkatpurwar and Pokharkar 2011). However, there are no reports on SNP synthesis by marine bacteria. In this paper, we report the synthesis of SNPs by a marine bacterium, *Idiomarina sp. PR58-8.* This is also the first report on metal tolerance of *Idiomarina* genus. This bacterium synthesized SNPs when silver is added at the time of inoculation unlike the terrestrial bacteria, wherein silver was added in the mid-log phase of growth.

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2. Materials and methods

2.1 *Culture isolation and identification*

The bacterial culture used in this study was isolated from soil samples at the banks of the Mandovi River in Goa, India. The soil was collected in sterile containers and stored at 4°C. The soil (1 g) was suspended in 100 ml of saline and agitated at 170 rpm for 30 min. The sample was then appropriately diluted and plated on Zobell Marine 2216 Agar. The plates were incubated at 30°C for 24 h and the colonies obtained were purified by re-streaking on the isolation medium. Purified bacterial culture was identified based on sequencing of the 16S rDNA region. Briefly, bacterial universal primers, 27F and 1429R were used against the genomic DNA template and PCR was performed. The amplified 16S rDNA fragments were sequenced. The sequence data was aligned using ClustalX (version 2.0.12) and analysed to find the closest homologs for the microbe. The phylogenetic tree was prepared using NJ-plot.

2.2 *Silver resistance study*

The silver resistance study was performed by growing the *Idiomarina sp. PR58-8* in Zobell Marine Broth 2216 containing 0·1 mM of silver nitrate. The flasks were incubated for 24 h and the culture was transferred to fresh medium containing a higher concentration of the metal salt. A concentration range of 0·1 to 9 mM was used to determine the tolerance levels. Elemental analysis was done using atomic absorption spectrophotometer, Shimadzu AA-6300.

2.3 *SNP synthesis and characterization*

Idiomarina sp. PR58-8 was grown in Zobell Marine Broth 2216 containing 5 mM silver nitrate for 48 h under agitation at 120 rpm. The optical absorbance measurements of the culture growing in 5 mM silver nitrate were taken on a UV visible spectrophotometer (Shimadzu UV 2450) in the range 200–800 nm. Cell pellets obtained from 48 h cultures were washed with deionized water and lyophilized. XRD measurements of the SNPs were taken on a Rigaku Miniflex from 20–80° and the scan speed was 0·2/min using a Cu-Kα radiation of wavelength, 1·5408 Å. The crystallite size was calculated using Scherrer's equation,

$$D = k\lambda / B \cos\theta,$$

where D is the crystallite diameter in Å, k the shape constant (0·9), λ the X-ray (Cu Kα) wavelength in Å, θ the diffraction angle and B (in radian) is the half width measured for the XRD peak. Transmission electron microscopy (TEM) was performed using Phillips CM 200 electron microscope. Lyophilized cell pellet was cast on carbon coated copper grid and observed under TEM. Scanning electron microscopy and energy dispersive analysis of X-rays (SEM-EDAX) of the dried cell pellets was carried out on a JEOL JSM 6360 LV.

2.4 *Quantitation of thiols*

The concentration of T–SH and NP–SH was determined with 5, 5′-dithio-*bis* (2-nitrobenzoic acid) (DTNB; Sigma) according to Sedlak and Lindsay (1968). For T–SH assay, 50 μL aliquots of the cell-free extracts were mixed with 150 μL of 0·2 M *Tris*, pH 8·2, 10 μL of 0·01M DTNB and 790 μL of absolute methanol (Merck). After 15 min, the mixtures were centrifuged (3000 g, 15 min) and the absorbance was measured at 412 nm (Shimadzu UV-2450 UV/VIS Spectrometer) against a reagent blank, using reduced glutathione as standard. To determine NP–SH, aliquots of 500 μL of the cell-free extracts were mixed with 400 μL of milli-Q water and 100 μL of 50% trichloroacetic acid (Merck). The mixtures were gently shaken for 12 min and centrifuged (3000 g, 15 min). Subsequently, 200 μL of the supernatant was mixed with 400 μL of 0·4 M *Tris*, pH 8·9 and 10 μL of 0·01M DTNB and absorbance was measured within 3 min of DTNB addition. The concentration of protein bound thiols (PB–SH) was calculated by subtracting the NP–SH from T–SH concentrations.

3. Results and discussion

During the screening of microorganisms for synthesis of nanoparticles, we obtained a marine bacterium, *Idiomarina sp. PR58-8* that synthesized SNPs intracellularly. Based on

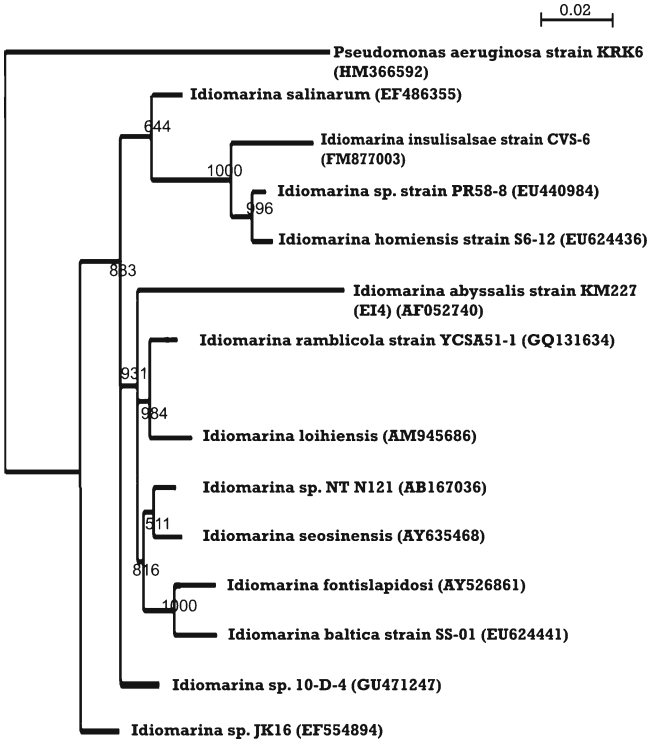

Figure 1. Phylogenetic position of marine bacterium in *Idiomarina* genus using NJ plot. The tree was rooted using *Pseudomonas aeruginosa strain KRK6* as the outgroup. Bootstrap values from 1000 samplings are shown near the branches.

nucleotide homology and phylogenetic analysis (figure 1), the bacterium was identified to be *Idiomarina sp. PR58-8* (GenBank Accession Number EU440984). Nearest homolog was found to be *Idiomarina homiensis.* Salt was found to be an obligate requirement and a minimum of 1% NaCl was required for growth. The culture could tolerate up to 15% NaCl which is similar to the levels of salt tolerance reported for *Idiomarina abyssalis KM227* and *Idiomarina zobelli KMM231* (Ivanova *et al* 2000).

This bacterium was found to tolerate up to 7 mM of silver nitrate in Zobell Marine Broth 2216 and accumulated more than 90% of silver intracellularly. *Idiomarina sp. PR58-8* grown on liquid media containing silver salt turned brownish-black after 48 h indicative of silver accumulation by the biomass while the unexposed culture was golden yellow in colour (figure 2). Characterization of the intracellularly accumulated silver revealed the presence of SNPs. UV-visible absorption scan of the 48 h culture grown in 5 mM silver nitrate revealed a broad peak at 450 nm due to the surface plasmon resonance of SNPs (figure 3). Observation of a broad surface plasmon peak in the 400–450 nm range is a characteristic of SNPs (Kowshik *et al* 2003). XRD analysis of lyophilized cell pellets is shown in figure 4. The peaks at Bragg angles of 38·11°, 44·27° and 64·42° correspond to elemental silver (3C-syn) in the ICDD. The crystallite size as determined by Scherrer formula was determined to be 25 nm. Due to the high salt content of the system, NaCl peaks were also seen. XRD of lyophilized Zobell Marine Broth 2216 containing 5 mM silver nitrate did not show any of the signature silver peaks. TEM micrographs showed that most of the particles were in the 26 nm size range (figure 5). Energy dispersive X-ray analysis (EDAX) showed presence of silver

Figure 2. Colour change from golden yellow to brownish-black observed when *Idiomarina sp. PR58-8* is grown in 5 mM silver nitrate after 48 h.

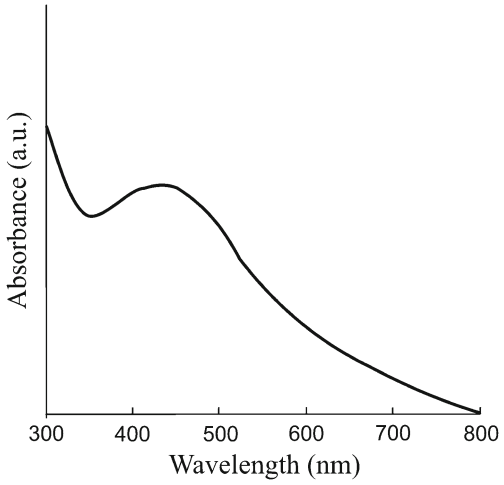

Figure 3. UV-visible absorbances scan of *Idiomarina sp. PR58-8* growing in 5 mM silver nitrate after 48 h.

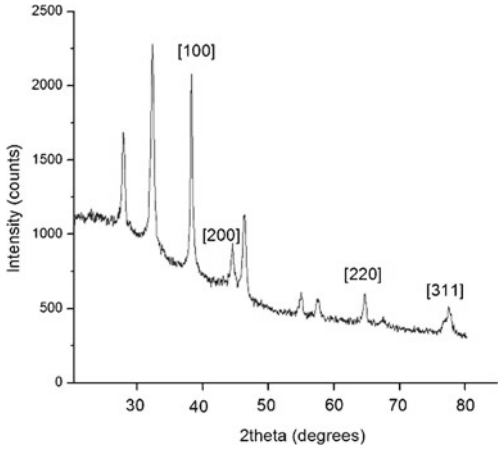

Figure 4. XRD of *Idiomarina sp. PR58-8* grown in presence of 5 mM silver nitrate for 48 h.

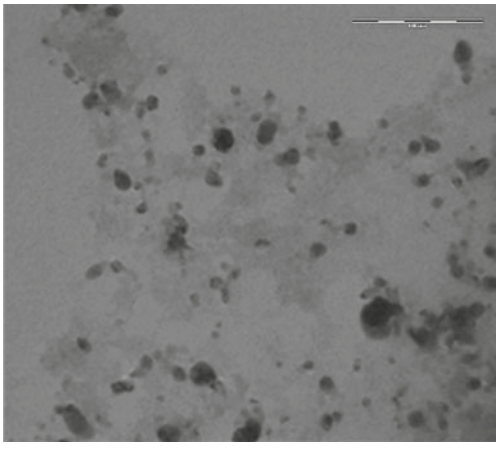

Figure 5. TEM image of lyophilized cells of *Idiomarina sp. PR58-8* grown in presence of 5 mM silver nitrate for 48 h.

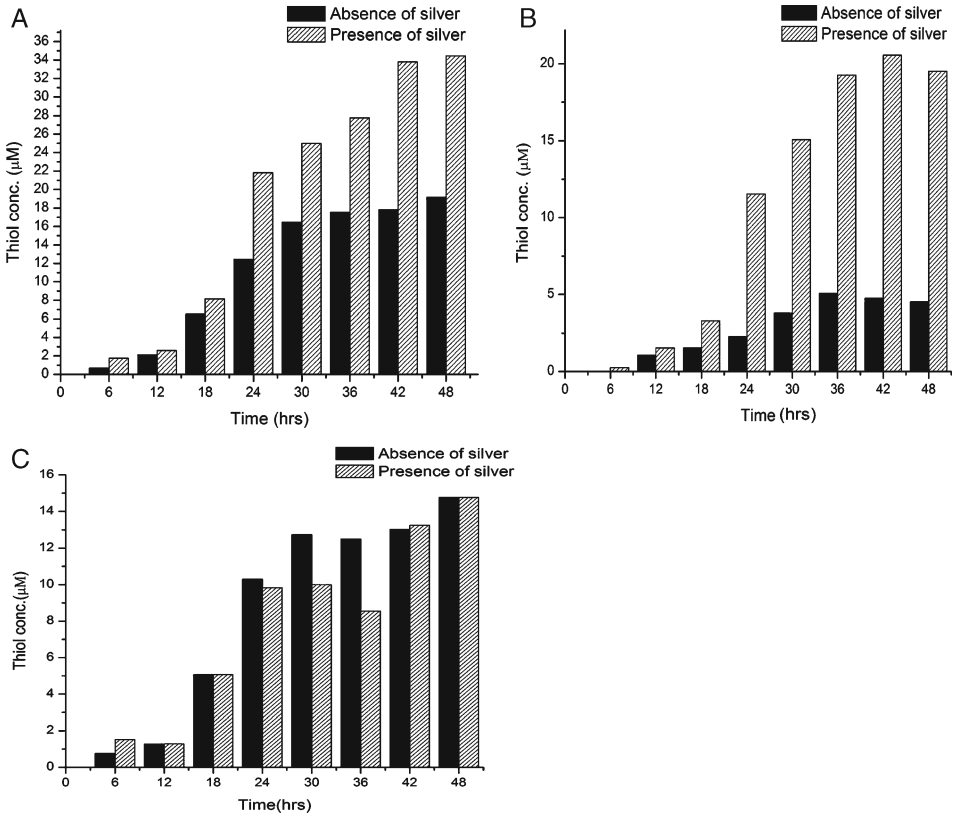

Figure 6. Comparison of intracellular thiol levels for cells grown in presence and absence of silver nitrate with respect to time. Levels of (**a**) total thiols, (**b**) non-protein thiols and (**c**) protein-bound thiols.

which constituted up to 6% of the sample. The other elements present were C, O, N and S which were constituents of the microbial cells.

Bacteria respond to oxidative stresses such as exposure to heavy metals by inducing the intracellular expression of thiol peptides which sequester the metal ions (Pages *et al* 2008). Capping of nanoparticles by thiol peptides is known to occur in the biosynthetic system (Kang *et al* 2008). Hence, the intracellular thiol response of *Idiomarina sp. PR58-8* to 2 mM silver nitrate was studied over a period of 48 h. It was observed that the silver-exposed culture responded to the silver stress by increasing its intracellular levels of T–SH (figure 6a). This increase was prominent after 18 h of growth, averaging an increase of 70·5% over the unexposed culture (control). The increase in T–SHs could largely be attributed to the induction of NP–SHs as shown in figure 6b. The NP–SH levels showed a 261% increase on an average over the control. An increase in NP–SH levels in bacteria and fungi is reported to occur in response to metal stress (Guimaraes-Suares *et al* 2007; Pages *et al* 2008). The levels of PB–SHs did not change significantly in response to silver and were found to be equal or lower than that observed for cells grown in the absence of silver (figure 6c). The lower levels of PB–SHs in exposed cultures could be explained by a sulfur shift in the focus of the bacterial proteome towards synthesis of glutathione. A similar trend was observed for cadmium-exposed yeast wherein the yeast adjusts its proteome by reducing the synthesis of sulfur-rich proteins and diverting most of the assimilated sulfur towards glutathione synthesis, thereby reducing PB–SH levels and increasing levels of NP–SHs (Fauchon *et al* 2002). Thus, NP–SHs appear to play a key role in silver tolerance of *Idiomarina sp. PR58-8.*

4. Conclusions

Here we report the synthesis of intracellular SNPs by a marine bacterium, *Idiomarina sp. PR58-8* which was found to be highly silver tolerant. This is the first report on heavy metal resistance and synthesis of metal nanoparticles in the *Idiomarina* genus. An advantage exhibited by the marine bacterium, *Idiomarina sp. PR58-8* is that the marine bacterium synthesizes SNPs when silver is added at the time of inoculation as against terrestrial bacteria such as *Lactobacillus sp*. and *Escherichia coli* wherein silver was added in the mid-log phase of growth. This could be attributed to the high silver-tolerance of *Idiomarina sp. PR58-8* and eliminates the requirement of growth phase monitoring during synthesis of SNPs. UV-visible absorbance scan of the 48 h culture from 300–800 nm revealed a broad peak at 450 nm, a characteristic of SNPs. XRD of lyophilized cell pellets

obtained from 48 h cultures corresponded to silver (3C-syn) in the ICDD. TEM showed presence of SNPs in the 26 nm size range which upon purification could be applied in biolabelling, antimicrobial coatings etc. The bacterium was found to respond to silver stress by inducing the expression of NP–SHs at extremely high levels (261% on average) over the control, peaking at 42 h. Thus *Idiomarina sp. PR58-8* is a promising microorganism for metal accumulation and metal nanoparticle synthesis.

Acknowledgements

(MK) thanks DST, India, for financial support (SR/FT/L-94/2005). The authors thank Dr Rahul Mohan, NCAOR, Goa, for helping with the SEM-EDAX measurements and SAIF, IIT Bombay, for TEM analysis. (SS) thanks BITS Pilani, K K Birla Goa campus, Goa, for a Research Fellowship.

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