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Synthesis of nano silver by a marine epibiotic bacterium *Bacillus vallismortis* and its potent ecofriendly antifouling properties

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ABSTRACT

In the present study, biosynthesis of silver nanoparticles (AgNPs) by a newly isolated marine epibiotic bacterium *Bacillus vallismortis* and its potent antifouling (AF) activity was investigated. Biosynthesized AgNPs were primarily confirmed by change in colour from yellow to brown. UV–vis spectrum of the AgNPs showed surface plasmon resonance (SPR) peak at 426 nm. The AgNPs were further characterized using FT-IR spectroscopy, XRD, SEM and HRTEM analysis. The synthesized AgNPs were narrowly polydispersed and spherical in shape with an average particle size of 23 nm. Antibacterial activity inferred that the AgNPs effectively inhibited marine biofilm forming bacterial strains with minimal MIC and MBC values. Moreover, Confocal Laser Scanning Microscopic images evidenced good antibiofilm efficacy of the AgNPs. It had also strongly inhibited fouling microalgal growth at minimal nanomolar (nM) concentration in the range of 0.5–1 nM. Anticrustacean assay using *Artemia franciscana* larvae registered LC_{50} value of 11.59 nM. Further, $EC_{50} < LC_{50}$ and 100% recovery of mussel *Perna indica* in toxicity assay propagated non-toxic nature of the AgNPs. Current study clearly explored the possibility of utilizing AgNPs biosynthesized by *B. vallismortis* in AF coatings to combat biofouling.

1. Introduction

Materials submerged into seawater are quickly colonized by micro (biofilm formation and bacterial adhesion) and macroorganisms (settlement of invertebrates) through a process known as “biofouling” (Clare, 1996). The process of biofouling occurs in a successive event; wherein, the initial conditioning of the submerged surfaces by the adsorption of organic macromolecules is followed by the attachment of marine bacteria, which forms a complex multi-species biofilm (Dobretsov et al., 2009; Jain and Bhosle, 2009). These bacterial biofilms pave way for the subsequent attachment of algal spores, invertebrates (mussels, barnacles and tubeworms) and altogether constitute a mature fouling community (Qian et al., 2007) and in turn causes heavy economic losses towards maintaining marine infrastructure. Earlier, to curb biofouling, tributlytin (TBT) was most widely used in AF paints. But considering its toxic effect on targeted and non-targeted marine organisms their use is strictly prohibited. This scenario has fostered researchers to develop environmentally safe, non-toxic AF agents using nanotechnology (Iyapparaj et al., 2012; Sri Ramkumar et al., 2016).

Nanotechnology is a burgeoning field combining physical, chemical and biological principles to explore the benefit of nanomaterials for the advancement of human life (Ahila et al., 2016). Nanoparticles (NPs) have wide range of applications in the field of biomedicine, drug delivery, diagnostics, biosensing, textile finishing, waste water system and electronics (Sri Ramkumar et al., 2016; Ahila et al., 2016). Of various metal NPs (Ag, Au, Pt, Pd, Cu etc.) studied so far, AgNPs attracted much attention in the field of nanotechnology due to their distinct physico-chemical and biological properties. Also, AgNPs are the subject of intense research in recent years due to their potent antimicrobial properties against 650 different bacterial strains (Alexander, 2009). Silver coatings, especially AgNP deposited surface, are well known to confer bacteriostatic and bactericidal properties on the surfaces (Ren et al., 2014). They are currently used in variety of medical, consumer products including catheters, surgical blades and even FDA-approved food packing (Eby et al., 2009). Indeed, attempts have also been made to

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utilize AgNPs in AF coatings to prevent settlement of biofouling organisms. For instance, Ren et al. (2014) developed polydopamine (PDA)-mediated AgNP coating on a range of generally used industrial materials including glass, polystyrene, stainless steel and paint surface. The resulting AgNPs coatings exhibited significant AF activity against bioadhesion of fouling microalgae both in marine and freshwater environments.

Further, AgNPs can be synthesized by using a variety of methods including physical, chemical and biological methods. Generally, production of AgNPs through conventional physical and chemical methods resulted in production of toxic byproducts with hazard nature. To cull out the problems pertained to conventional methods, synthesizing AgNPs by using biological resources such as those involving microorganisms, plants and, viruses or their biomolecules have emerged as suitable and viable options (Patra and Baek, 2014). Also in recent years, scientists have made intense efforts to utilize microorganisms as possible eco-friendly bio-nanofactory to produce AgNPs mainly due to its growing success, ease handling and genetic modification. Microbial synthesis of metal NPs can be achieved through intracellular or extracellular method. Often intracellular synthesis of NPs requires additional steps *viz*. ultrasound treatment or reactions with suitable detergents to release the synthesized NPs; nevertheless extracellular biosynthesis is economical and it requires simpler downstream processing and also favours large-scale production (Kalimuthu et al., 2008). In view of this many studies have been focused on exploring the extracellular methods for the synthesis of metal NPs. Indeed microbes are known to produce highly attractive and varying size metal NPs with properties similar to that of chemically synthesized nanomaterials (Sri Ramkumar et al., 2016). Among the microorganisms studied, *Bacillus* spp. has received considerable attention towards AgNPs biosynthesis. In particular, studies using culture supernatants of *Bacillus* sp. GP23, *B. licheniformis, B. amyloliquefaciens, B. methylotrophicus, B. pumilus, B. persicus* and *B. subtilis* proved their ability to form extracellular AgNPs very effectively (El-Raheem et al., 2011; Wei et al., 2012; Gopinath and Velusamy, 2013; Wang et al., 2015; Elbeshehy et al., 2015). Among the member of *Bacillus* spp; *Bacillus vallismortis* has been largely explored for numerous biological activities. It was reported that *B. vallismortis* BIT-33 isolated from seawater had promising anticancer activity against colon cancer carcinoma cells (Jeong et al., 2008). Yu et al. (2009) identified a novel thiazole alkaloid with potent antimicrobial property from sponge (*Dysidea avara*) associated bacterium *B. vallismortis* C89. Likewise, Park et al. (2013) isolated a potent *B. vallismortis* BS07 from soil effectively defends plants from pathogenesis and promotes plant growth when it was applied in chilli pepper. A recent study also evidenced the production of organic solvent tolerant thermostable alkalophilic cellulose and thermotolerant xylanase from soil isolate *B. vallismortis* RG-07 (Gaur and Tiwari, 2015). Despite the availability of numerous reports on biological activities of *B. vallismortis*; studies on biosynthesis of AgNPs by this bacterium still stand as lacuna. In view of this, in the present study, the AF activity of extracellular AgNPs biosynthesized by a newly isolated marine epibiotic bacterium *Bacillus vallismortis* is reported.

2. Materials and methods

2.1. Isolation and identification of metal resistant bacteria

For the present study, seaweed *Ulva lactuca* was collected from the Kanyakumari coast (Lat.8°08′12.39”N; Long.77.55°18′06”E), Tamilnadu, India and brought to the laboratory in an ice box. The epibiotic bacteria present on the surface of seaweeds were isolated by following the method of Kanagasabhapathy et al. (2006). The isolated epibiotic bacterial strains were then individually cultured on Zobell marine agar (ZMA) plates supplemented with 1–5 mM concentration of silver nitrate ($AgNO_3$) for 48 h at 37 °C and observed for bacterial growth. Based on the results, the epibiotic bacterium SS7 which showed higher resistance to $AgNO_3$ was used for further study (data not shown). The selected epibiotic bacterium SS7 was identified by polyphasic characteristics as described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The identity of SS7 was further authenticated by 16S rRNA sequencing and then deposited in NCBI GenBank.

2.2. Extracellular biosynthesis of AgNPs

The candidate bacterium SS7 was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of Zobell marine broth (ZMB) and incubated for 24 h at 37 °C. The culture broths were then centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was used for the synthesis of AgNPs. In brief, the supernatant was mixed with $AgNO_3$ at a 1 mM final concentration and placed in a shaker incubator (120 rpm) for 24 h at 37 °C in dark condition for extracellular synthesis of AgNPs. The supernatant without addition of $AgNO_3$ was maintained separately as positive control. Simultaneously, ZMB without microorganism but with 1 mM $AgNO_3$ was used as negative control. The bioreduction of Ag^+ ions into AgNPs was detected initially by visual observation of colour change from pale yellow to dark brown.

2.3. Characterization of AgNPs

Characterization of AgNPs was carried out by following Vijayan et al. (2014). In detail, the biosynthesized AgNPs was characterized by UV–vis spectrophotometer (Techcomp, UV-2301 III, Hong Kong) instrument scanning in the range of 200–800 nm at a resolution of 1 nm. The FT-IR spectrum (FT-IR Nicolet Thermo spectrophotometer iS5, USA) of the supernatant and synthesized AgNPs was recorded using KBr pellets and the spectrum was collected at a resolution of 4 cm^{-1} in wave number region of 400 − 4000 cm^{-1}. Crystalline structure of AgNPs was determined by X-ray diffraction (XRD) analysis. XRD analysis was carried out by X'Pert PRO Analytical X–ray diffractometer (PANalytical, Netherlands). A drop of colloidal AgNPs were coated on a copper plate, dried in a hot air oven, and examined using Scanning Electron Microscopy (SEM − Carl Zeiss, Germany) equipped with Energy Dispersive X-ray spectroscopy (EDX-JEOL, JSM- 5610) analysis. The morphology and size of the AgNPs was further authenticated by High Resolution Transmission Electron Microscope (JEOL JEM 2100 HRTEM) operated at the accelerating voltage of 200 kV. A drop of AgNPs was coated on carbon coated copper grid of 200 mesh size and dried for 5 min prior to observation. In addition to that the selected area electron diffraction (SAED) pattern was also performed.

2.4. Antimicrofouling activities

2.4.1. Antibacterial assay

The antibacterial activity of the biosynthesized AgNPs was determined through agar well diffusion method (Ramasubburayan et al., 2014). The test marine biofilm forming bacterial strains used in the present study were isolated from panels submerged in seawater. In brief, overnight cultures of biofilm forming bacterial strains were prepared and aseptically spreaded on plates. A total of five wells, each of 6 mm diameter were punched over Muller Hinton Agar (MHA) plates using a sterile gel puncher. Then wells were loaded with different concentrations of colloidal AgNPs (20 µL −0.25 nM, 40 µL − 0.50 nM, 60 µL − 0.75 nM, 80 µL − 1 nM and 100 µL − 1.25 nM) and incubated at 37 °C for 24 h. The growth inhibitory activity around each wells were recorded (mm). The assay was carried out in triplicate.

2.4.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs

MIC and MBC of AgNPs was determined by following Marechal et al. (2004) with little modification. For this, various concentrations (0.25-1.5 nM) of colloidal AgNPs were prepared and added to six wells of 96-well polypropylene plates. Then 0.1 mL of biofilm bacterial

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