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Technical note

Bacterial exopolysaccharide (EPS)-coated ZnO nanoparticles showed high antibiofilm activity and larvicidal toxicity against malaria and Zika virus vectors

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ABSTRACT

In this study, a novel and effective approach was performed to synthesize ZnO nanoparticles (ZnO NPs) using the exopolysaccharides (EPS) from the probiotic strain *Bacillus licheniformis* Dahb1. EPS acted as reducing and stabilizing agent for the formation of EPS-ZnO NPs by co-precipitation method. Structural characterization was investigated by a surface plasma resonance centered at 375 nm in UV–vis spectrum. FTIR spectrum exhibited functional groups with strong absorption peak at 3814.7–420 cm^{-1}. XRD showed the crystalline nature of EPS-ZnO NPs. TEM showed that the EPS-ZnO NPs were hexagonal in shape, with size within the range of 10–100 nm. The presence of Zn was confirmed by EDX analysis. Antibacterial activity of EPS-ZnO NPs was demonstrated as 100 µg/ml significantly inhibited the effective growth control of Gram-negative (*Pseudomonas aeruginosa* and *Proteus vulgaris*) and Gram-positive (*Bacillus subtilis* and *Bacillus pumilus*) bacteria. Light microscopy and confocal laser scanning microscopy evidenced that the antibiofilm activity of EPS-ZnO NPs was higher against Gram-negative bacteria over Gram positive bacteria. EPS-ZnO NPs also inhibited the biofilm growth of *Candida albicans* at the concentration of 75 µg/ml. The hemolytic test showed low cytotoxicity of EPS-ZnO NPs at 5 mg/ml. In addition, EPS-ZnO NPs achieved 100% mortality against third instars mosquito larvae of *Anopheles stephensi* and *Aedes aegypti* at very low doses. Moreover, histology studies revealed the presence of damaged cells and tissues in the mid-gut of treated mosquito larvae. The multipurpose properties of EPS-ZnO NPs revealed in the present study can be further considered for pharmaceutical, parasitological and entomological applications.

1. Introduction

Exopolysaccharides (EPS) are biological macromolecules that protect bacterial cells from harsh environments by their metabolic by-products [1]. They play a multifunctional role in bioactive natural product science, leading to various biochemical and medical applications [2]. The high molecular weight of EPS allowed their binding activity with nanoparticles to stabilize the suspension and to inhibit the aggregation of NPs, due to their adsorption onto NPs surface [3,4]. Nano-sized particles of semiconductor materials have gained increased attention in recent years due to their desirable properties and applications [5]. Among them, metal oxide NPs received considerable attention and they are being incorporated into variety of products based on catalytic capacity, as well as optoelectronic and antimicrobial properties [6].

Nowadays, researchers are focusing on the synthesis of nanoparticles using various metals such as zinc, gold, silver, platinum and palladium [7–10]. Among all these metal nanoparticles, ZnO nanoparticles (ZnONPs) are recognized as one of the most promising ones due to their wide utilization in catalysis, sunscreen production, as well

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as cosmetics, coatings, pigments and food additives [11,12]. Most ZnO nanoparticles used commercially have some advantages if compared to other nanoparticles, due to their non-toxicity, low cost, stability and well known antibacterial and antifungal properties [13–16]. Therefore, ZnONPs are largely used in consumer products and industrial applications. The increased use of such materials may lead to their release into the environment [17]. ZnONPs have been synthesized by several different methods, such as sol gel techniques [18], wet chemical method [19], green chemistry [20] and microwave-assisted method [21]. Chemical and physical methods are quite expensive and require extensive labour and time. Alternatively, biological materials represent a safer and greener option to synthesize metal nanoparticles at extra/intercellular level because, they are often cheap, reliable and eco-friendly [22]. Furthermore, large quantities of secondary waste are generated resulting from the addition of chemical agents for precipitation and reduction in the chemical nanosynthesis processes, while this does not happen in green based nanosynthesis routes [23,24]. Recently, some natural polysaccharides or their derivatives such as cellulose, chitosan, starch, and sulfated chitosan have been applied to produce ZnONPs [25–27]. Microbial polysaccharides have higher reducibility because of their diverse chemical constituents and complex structures [28]. Therefore, a focus on active microbial exopolysaccharides (EPS) is needed, which special reference to strains able to produce highly EPS residues into the external environment. In addition, polysaccharides are non-toxic, biocompatible, easily biodegradable, and abundantly present in natural sources. Many researchers have used various natural biopolymers to reducing and stabilizing the metal nanoparticles [29–31].

In recent years, increasing evidences suggested that some polysaccharides isolated from cultivable sources have antioxidant capabilities and low cytotoxicity due to their remarkable functional properties, these natural biopolymers have been widely used as viscofying, bio-flocculating, stabilizing, gelling, and emulsifying agents by the food industry [32]. To the best of our knowledge, this is the first study reporting on the synthesis and characterization of Zn NPs using a bacterial exopolysaccharide (EPS) from a probiotic strain of *Bacillus licheniformis* Dahb1. Besides this, the antibacterial, antibiofilm and antifungal activities of the EPS-ZnONPs were examined *in vitro*. Lastly, the larvicidal activity of EPS-ZnONPs was studied on mosquito larvae of *Anopheles stephensi* and *Aedes aegypti*, and histological damages triggered by NP-based treatment in mosquito larvae from the two species mentioned above were analyzed.

2. Materials and methods

2.1. Materials

Zinc acetate [Zn $(CH_3COO)_2$], sodium hydroxide (NaOH), ethanol (C_2H_5OH), methanol (CH_3OH), acridine orange (235474) and crystal violet (c3886), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were purchased from Sigma Aldrich, India. Nutrient broths (NB) and Luria Bertani agar (LBA) were purchased from HiMedia (India). All the chemicals used for the experiments were of analytical grade. All the solutions were primes using deionized water. The glasswares were properly washed, sanitized and autoclaved for experimental purposes.

2.2. Microbial strains

The probiotic strain *Bacillus licheniformis* Dahb1 (HM235407.1) used in this study was from the stock culture maintained in our laboratory on Luria-Bertani (LB) broth with sterile glycerol at −20 °C (20% v/v) [33]. Microbial cultures Gram positive *Bacillus subtilis* (KT763078.1), *Bacillus pumilus* Dahb3 (HQ693273.1) and Gram negative *Pseudomonas aeruginosa* Dahp1 (HQ4006631), *Proteus vulgaris* Dahp2 (HQ116442.1) bacteria were collected from Department of Animal Health and Management laboratory culture collections.

2.3. Extraction of EPS

The bacterial exopolysaccharide (EPS) was extracted from the probiotic strain *B. licheniformis* Dahb1 according to the method by Fang et al. [34] with minor modifications. Briefly, the fresh culture of *B. licheniformis* Dahb1 was inoculated into 500 ml of nutrient broth medium and grown at 37 °C for 72 h. The culture was then heated for 30 min at 100 °C to inactivate the enzymes and then centrifuged at 8000 × *g* for 10 min to separate the cells. The supernatant fluid containing EPS was precipitated by adding three volume of ice cold 95% ethanol (20 °C) and placed at 4 °C for overnight. After centrifugation at 8000 x g for 10 min, the precipitated EPS was washed twice with distilled water and the pellet was retained and dried in desiccator. The crude EPS powder obtained were used for the synthesis of ZnONPs.

2.4. Synthesis of EPS-ZnONPs

EPS-coated zinc oxide nanoparticle (EPS-ZnONPs) was synthesized by co-precipitation method, following the procedure by Raliya et al. [35] and Vijayakumar et al. [36]. Briefly, 0.5 g of EPS crude powder was added to the aqueous solution (0.02 M) of zinc acetate and NaOH (2 M) under vigorous stirring at 37 °C, which resulted in the formation of a white suspension. The white product obtained was centrifuged at 8000 × *g* for 10 min and washed 3 times with distilled water and absolute ethanol. The precipitate was collected, washed and dried at 70 °C, for further analysis.

2.5. Characterization of EPS-ZnONPs

The surface plasma resonance of EPS-ZnO NPs was measured by double beam UV–vis spectrophotometer (Labtronics, Model LT-2900) in the range of 200–400 nm. The crystalline phases of EPS-ZnONPs were checked using X-ray diffractometer (Bruker, D_2-Phaser). The XRD spectrum was recorded from 5° to 80° 2θ angles using CuKα radiation operated at 10 kV and 30 mA. The FTIR spectral analysis of EPS-ZnONPs was recorded on Perkin Elmer (spectrum GX) with a resolution of 4 cm^{-1} in 4000-400 cm^{-1} using KBr pellets of sample. The surface state, morphology and structure of EPS-ZnONPs were recorded using scanning electron microscopy (SEM) (JEOL Model JSM-6390LV). The EPS-ZnONPs size, shape and selected area electron diffraction (SAED) of EPS-ZnONPs were observed by transmission electron microscopy (TEM) (JEM 2100F) using carbon coated grids. The elemental confirmation of EPS-ZnONPs was determined by energy dispersive X-ray spectroscopy (EDX) (EDX Jeol JSM-6510) analysis. The stabilization of EPS-ZnONPs was estimated through zeta potential (90 Plus Particle Size Analyzer, Brookhaven Instruments Corporation, using Zeta Plus software). The measurement of zeta potential was based on the stability of the particles under electric field.

2.6. Antioxidant activity of EPS-ZnONPs

The antioxidant activity of EPS-ZnONPs was determined by DPPH [1,1-diphenyl-2-picryl hydrazyl] radical method, as previously described by Das et al. [5] and Nagajyothi et al. [37] with some modifications. Briefly, the reaction mixture containing EPS-ZnONPs at different concentrations (i.e., 20, 40, 60, 80, and 100 μg/ml) was added to 100 μl of DPPH solution (0.2 mM, dissolved in 95% methanol, v/v) in 96 well microtiter plates. The mixture was incubated for 30 min at room temperature in dark place. The absorbance was measured at OD_{517nm}.

The DPPH radicals scavenging ability of EPS-ZnONPs was calculated as follows:

$$\text{Scavenging activity (\%)} = (1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}) \times 100$$