

EXTRACELLULAR POLYSACCHARIDE-MEDIATED GREEN SYNTHESIS AND STABILIZATION OF SILVER NANOPARTICLES USING *Bacillus* sp. BPRCHR

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Abstract

In this study, Extracellular polysaccharide from *BACILLUS* SP. BPRCHR was used for the biological synthesis of silver nanoparticle. The extracellular polysaccharide contains more amounts of reducing and strong rheological properties that reduce silver nitrate to silver nanoparticle in a easy step. The biosynthesized silver nanoparticle was characterized by UV-Vis Spectrophotometer; Fourier transformed infra-red Spectroscopy, X-Ray Diffraction (XRD), and Scanning Electron Micrography (SEM). The silver nanoparticle synthesis was confirmed with X-ray diffraction spectrum the intense peaks correspond to (111), (200) and (220), respectively for silver. The extracellular polysaccharide from *BACILLUS* SP. BPRCHR was analyzed for carbohydrate, protein and protein molecular weight by SDS-PAGE. This biological synthesis of silver nanoparticle has strong antimicrobial activity against harmful pathogens that shows inhibition activity of 22mm for *E.coli* 18 mm for *Shigella* and 17.5mm for *Vibrio cholera* by well diffusion method.

Keywords: *Bacillus* sp. BPRCHR, Extracellular polysaccharide, silver nanoparticles, Nanobiotechnology, Antimicrobial activity.

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INTRODUCTION

Metal nanoparticles research gains more importance in current trend due to their small size 10^{-9} times of SI units and cover large surface area, different from those of bulk materials (Mazur, 2004) and diverse applications. Metal nanoparticles are synthesized using several physical, chemical and biological methods. Biological methods of synthesizing nanoparticles gains much more importance due to low cost, eco friendly, single step method, easy scale up for large synthesis and the use of high energy, pressure and toxic chemicals are eliminated by biological synthesis ie., plant leaf (Song *et al.*, 2009) and fruit extract (Jain D, 2009), Bacteria (Saifuddin N 2009), fungi (Bhainsa KC), yeast (Kowshik M *et al.*) and enzymes, Silver and silver nitrate solution was used as a wound healer and antimicrobial agent during the historical period, due to the disadvantage of forming complexes and the effect of the ions remained only for a short time which was then overcome by the use of the silver nanoparticles which are in inert form and also exhibit antimicrobial function (Shankar, 2004).

Polysaccharides are linear or branched polymeric carbohydrate structures joined together by the glycosidic bonds. Polysaccharide surfaces provide the potential for significant surface reduction of silver nitrate to silver nanoparticles. Extracellular

polysaccharide extracted from bacteria provides stability and reducing activity for the synthesis of silver nanoparticle. Polysaccharides like chitosan, curdlan and fucoidan were used for silver nanoparticle synthesis (Thomas Chun-Yiu Leung 2010). In this study extracellular polysaccharide from *Bacillus* SP. BPRCHR (JQ766311) was found to reduce silver nitrate to silver nanoparticle that is stable for long period.

Materials and Methods

Isolation of bacteria and production of extracellular polysaccharide

Serial dilution and pour plate method were used for the isolation of extracellular polysaccharide producing bacteria from the marine sediments of Kovalm beach, India. Based on their mucilage colony growth on the dextrose agar plates six extracellular polysaccharide producing bacteria were isolated and submitted for 16srRNA sequence. Out of six bacteria, one maximum extracellular polysaccharide producing organism was identified as *Bacillus* sp. BPRCHR (JQ766311). *Bacillus* sp. BPRCHR (JQ766311) was inoculated into the sterilized medium containing (g/L) sucrose 20; Tryptone 5; K₂HPO₄ 0.067; KH₂PO₄ 0.083; MgSO₄.7H₂O 5; NaCl 10; CaCl₂ 1; FeCl₃ 0.005; MnCl₂ 0.001; Na₂MoO₄ 0.001; ZnCl₂ 0.001; Distilled water 1000 mL; pH 7.0. The flasks

were incubated at $37 \pm 2^\circ\text{C}$ for three days under static condition.

Extraction of Extracellular polysaccharides

The *Bacillus* sp. grown culture flask was centrifuged at 10,000 g for 20 min the supernatant were mixed with 3 volumes of ice-cold ethanol and held at 4°C for 12h to precipitate the extracellular polysaccharide. The precipitate was collected and dried at 80°C , diluted with deionized water and precipitated again with ice cold ethanol in the ratio (1:3) and dried to a constant weight at 80°C .

Extracellular polysaccharide analysis

The extracted EPS were analyzed for carbohydrate, protein and protein molecular weight. Carbohydrate was estimated by phenol-sulphuric acid method Dubois et al using glucose as standard. The amount of protein was estimated by (Lowry, O.H) with bovine serum albumin as standard. SDS-PAGE was performed on 10% gels according to the Laemmli method (Laemmli, 1970). After electrophoresis, the gels were silver-stained for visualization of protein and glycoprotein bands, respectively. The extracted EPS, and bacterial cells of *Bacillus* sp BPRCHR were visualized by scanning electron microscopy (SEM; Hitachi S3400n).

Synthesis of silver nanoparticles

The extracted EPS 100 mg was measured and diluted in 10ml distilled water and transferred to the conical flask containing 100 ml of 1mM silver nitrate solution at pH 7.5 and incubated on the orbital shaker at a speed of 120 rpm for 48 hours at 30°C in dark condition. The control sample of 1mM silver nitrate solution without extracellular polysaccharide was incubated along with the experimental flask.

Characteristics of synthesized silver nanoparticles

The synthesized silver nanoparticles were confirmed and studied by the following instrumentations, the UV-Visible absorption spectra of the silver nanoparticles were recorded using Varian, Cary 100 Concentration with a resolution of $\pm 1\text{nm}$ between 200-800nm of the 12, 24 and 48 hours. The lyophilized samples was subjected to FTIR spectroscopy analysis using Perkin-Elmer Co., USA model spectrum with samples (0.3-0.5 mg) were ground in about 80mg of spectral-grade KBr (sigma) and pressed into pellets under about 5-6 tons cm^{-2} pressure with the help of a hydraulic press. The spectral measurements were carried out in the absorbance mode. The measurements were carried out in the mid-infrared range from 4000 to 400 cm^{-1} with plain KBr pellet was used as the background reference. The lyophilized silver nanoparticle structure was analyzed by scanning electron microscopy (SEM; Hitachi S3400n). The crystalline nature of the sample was analyzed at University of Madras using X-ray diffraction spectroscopy (XRD; SEIFERT JSO Debyelex 2002 model). The X

radiation used was copper (K-Alpha 1) radiation ($\lambda = 1.54056\text{ \AA}$) operating at a voltage of 40 KV and a current of 20 mA. The scanning rate was maintained at $1.6^\circ/\text{min}$ and the diffracted intensities were recorded from 10° to 70° at 2θ angles.

Application of silver nanoparticles as antimicrobial agent

Antibacterial activity of biologically synthesized silver nanoparticles was analyzed by agar well diffusion method on MHA plates against pathogenic strains such as *Salmonella* dysentery, *E.coli* and *Shigella*. The pathogens were maintained on nutrient agar medium and stored at 4°C . The pathogens were inoculated in MHB and incubated overnight at 37°C to make a uniform suspension. The Pathogens were lawn cultured on MHA plates by using sterilized cotton swabs. In each of these plates, wells were cut out using standard cork borer (7 mm diameter) and kept in refrigerator for two hours and the MHA plates were brought down to room temperature. Using micropipette, 100 μl of silver nitrate solution (100 $\mu\text{g/ml}$) as control, 100 μl of silver nanoparticle (100 $\mu\text{g/ml}$) and 100 μl of distilled water were added to separate wells and kept in refrigerator at 4°C for 2 hours and later the plates were incubated for 24 hours at 37°C . Silver nanoparticle 100mg/L concentration were added to sterile MHA agar, by pour plate method, pathogenic strain *E.coli* suspension 0.1ml was added to sterile MHA with silver nanoparticle was poured on to it at bearable temperature, a control plate without silver nanoparticle is also analyzed along experiments and the plates were incubated at 37°C for 24 hours.

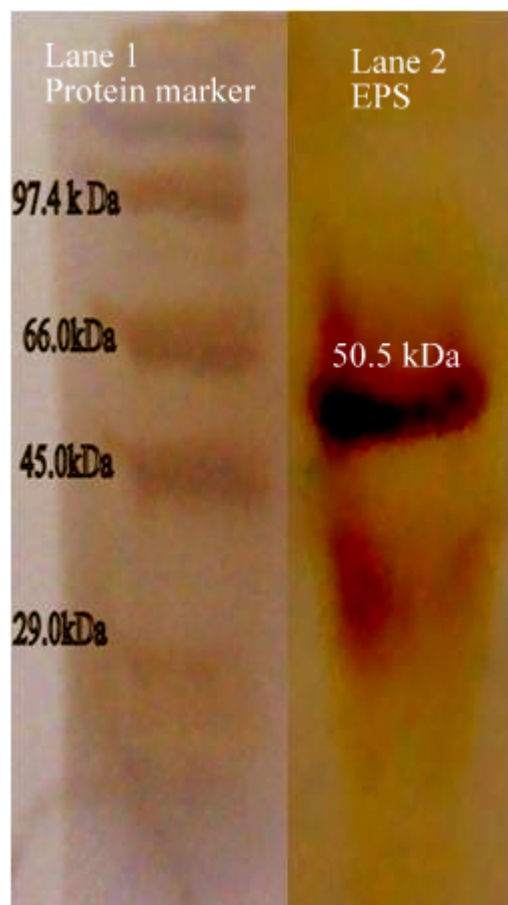
Results and Discussion

Extracellular polysaccharide analysis

The extracted EPS from *Bacillus* sp BPRCHR (JQ766311) was found to be white porous and water-soluble polymer. The carbohydrate and protein analysis showed 250 $\mu\text{g/mg}$ and 240 $\mu\text{g/mg}$ respectively. The SDS-PAGE results in fig showed 50.5 kDa of protein in lane 2 against the standard protein marker in lane 1 of 97.4 kDa which ran separately. Previous results show 66 kDa of protein from red microalgae polysaccharide (Roshan Prakash Shrestha (2004)).

SDS-PAGE

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Lane 1 97.4 Protein molecular marker, Lane 2: Extracellular polysaccharide from *Bacillus* sp. BPRCHR

Biosynthesis of silver nanoparticle

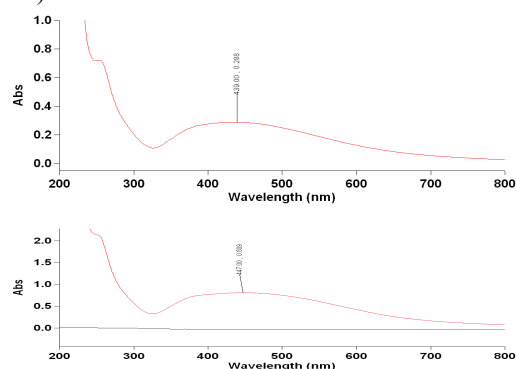
In this study, we attempted to use the extracellular polysaccharide from *Bacillus* sp. BPRCHR (JQ766311) to produce silver nanoparticle. Reddish brown color solution was formed after 24 hours and the color change was not observed in the control flask (Fig.1). The intensity of dark brown color was increased after 48 hours; this is due to the excitation of surface Plasmon vibrations (Duran N, Marcato PD) (Jae, Y.S. and Beom, S.K 2009.) (Henglein, A., 1993.) (Sastry, M., Mayya, K.S) (Sastry, M., Patil, V.)

Characterization of silver nanoparticle

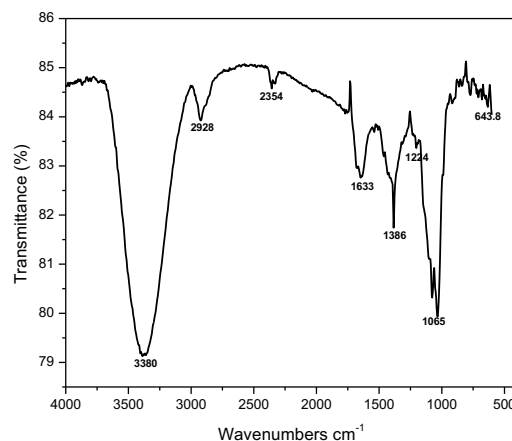
The incubated 12, 24, 48 hours silver nanoparticle samples were scanned in UV-visible spectrophotometer at 200-800 nm. The absorbance peak exhibited at 12 hours samples showed a peak at 424 nm, and 24 hours at 439 nm and 48 hours at 448 nm respectively Fig 3.

The UV-visible spectrophotometer results also correspond with the results of (Dinesh kumar 2011). The silver nanoparticle solution was stable for more than 4 months and the surface plasmon band remains close to 430nm throughout the reaction period and indicates that the particles are dispersed in the

aqueous solution and extracellular polysaccharide act as a strong stabilizing agent for silver nanoparticle. (Saifuddin. N. C.,) (Shankar,S.S., A). (Jain. D. H., D. Kumar)



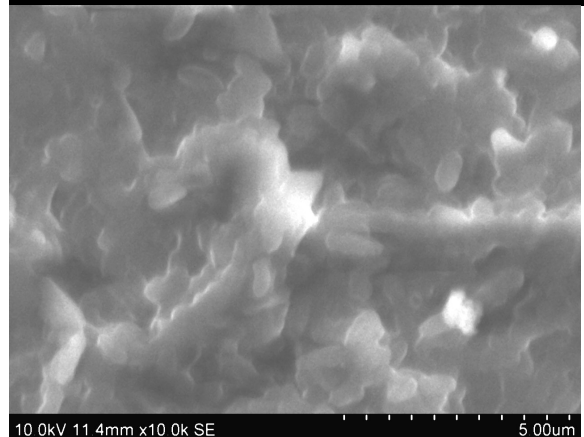
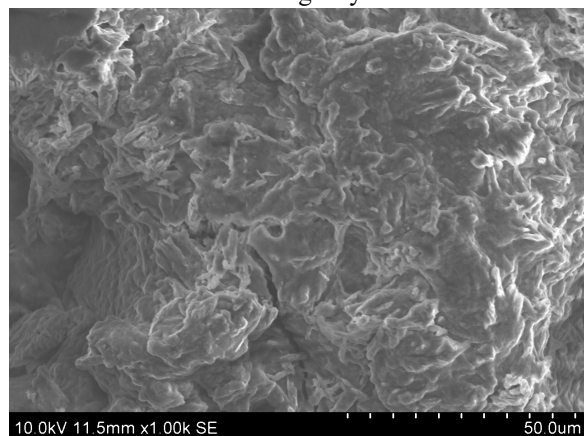
Fourier transform infrared spectroscopy results of synthesized silver nanoparticle shown in fig confirms the presence of peaks near 3380 cm^{-1} , 2928 cm^{-1} and 2854 cm^{-1} assigned to OH stretching and aliphatic – CH_3 and CH_2 stretching, 2354 cm^{-1} for phosphor group, 1386 cm^{-1} confirms the presence of C-N stretching of amines that proves the presence of protein in the sample. Peak at 1633 cm^{-1} (-NHCO of amide), 1224 cm^{-1} for ester carbonyl, phenol group, 1065 cm^{-1} proves the presence of C-O stretching of polysaccharides, Si-O asymmetric stretch and 643.8 cm^{-1} for CH. FTIR results proves the presence of polysaccharide group, protein for the formation of silver nanoparticle which corresponds to the results of (Gole A Dash C) (Dinesh kumar S)



Scanning electron microscopy (SEM) figure shows small spherical structure of uniform dispersed silver nanoparticle with diameter of 12-52 nm. Figure 2 and 3 show the SEM images of extracellular polysaccharide reduced silver nanoparticles at various magnifications. The SEM image in figure confirms the amorphous, porous structure of the extracted EPS by ice cold ethanol precipitation. Figure 2 shows the

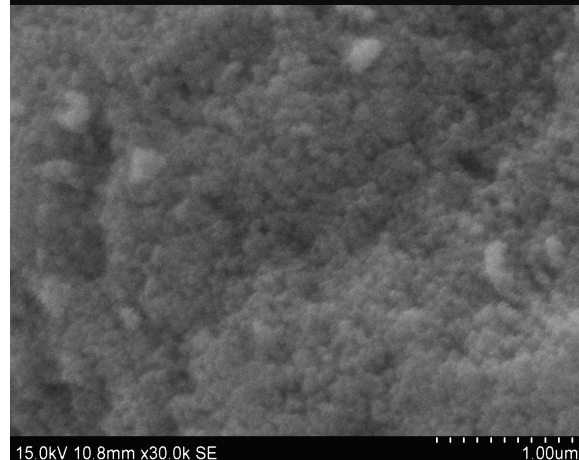
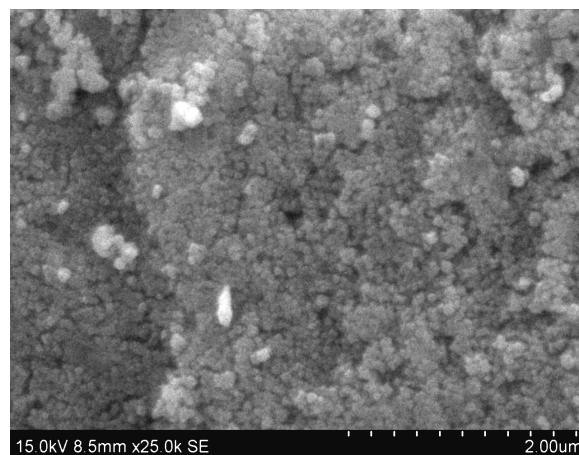
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clear bacterial rod-shaped cell of *Bacillus* sp BPRCHR held with mucilage layer.



Extracellular polysaccharide form
sp. BPRCHR rod shaped cells surrounded
Bacillus sp. BPRCHR
mucilage layer of EPS

Bacillus
by



Biosynthesized silver nanoparticles at
Biosynthesized silver nanoparticles at higher lower
magnification (×25,000)
magnification (×30,000)

X-ray diffraction analysis

The dry powders of the silver nanoparticles were used for XRD analysis. The diffracted intensities were recorded from 10° to 70° at 2 theta angles. The diffraction pattern in Figure 3 at 38.24, 44 and 64, which can be indexed to (111), (200) and (220), respectively for silver (Jeevan *et al.*, 2012)

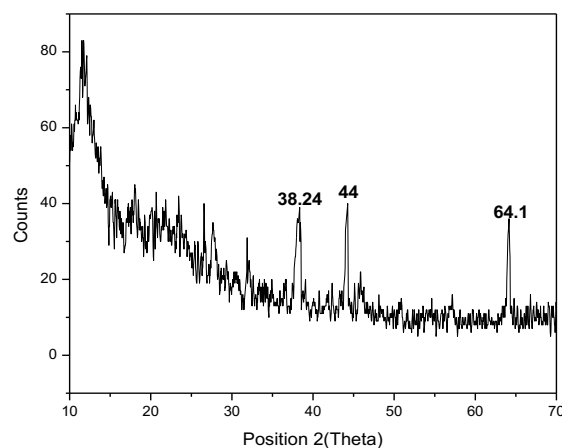


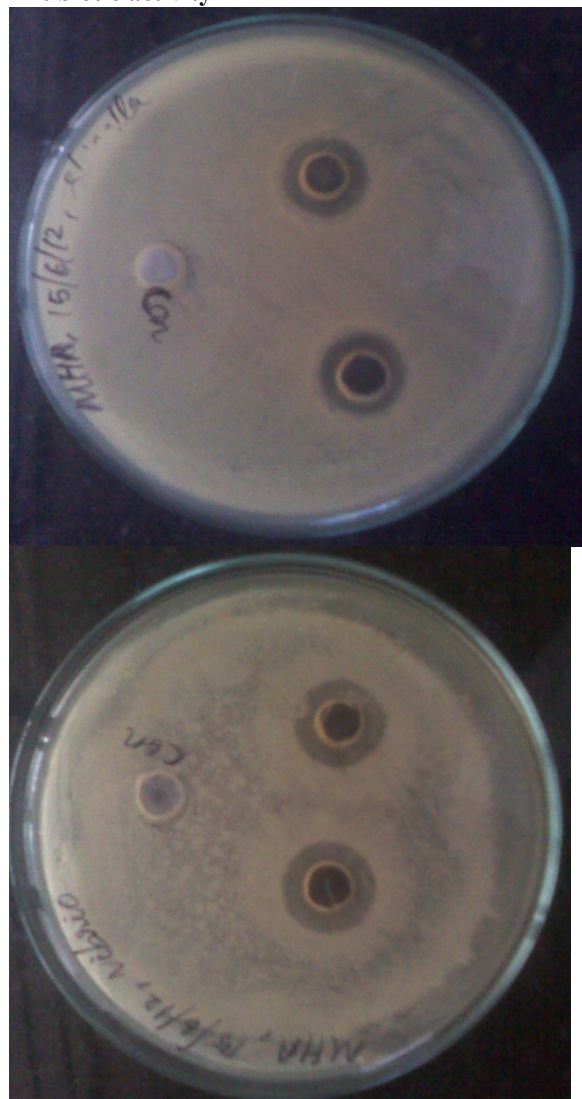
Figure 3 X-ray diffraction analysis

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Antimicrobial activity of silver nanoparticles

The antimicrobial activity of biologically synthesized silver nanoparticle against pathogenic organisms *E.coli*, *Shigella dysenteriae* and *Vibrio cholera* were analyzed using well diffusion method. In Fig 1 the highest inhibition zone was measured 22mm against *E.coli* in 3rd well with silver nanoparticle formed with 150mg EPS concentration. 2nd well showed 18mm with 100 mg concentration. In fig 2, inhibitory zone was measured 17.5 mm in 2nd and 3rd wells against *Shigella dysenteriae*. In fig 3. 18mm inhibitory zone was measured in 2nd and 3rd well against *Vibrio cholerae*. The lower activity was found in poured with silver nitrate as control. Fig 5. The pour plate method for *E.coli* showed no colonies for Silver nanoparticle 100mg/L concentration with MHA agar, fig b. a control plate without silver nanoparticle shows no number of *E. coli* colonies. This result confirms antimicrobial activity that corresponds to reference (Song H Y et al)

Antibiotic activity



Shigella sp.
Vibrio sp.



Salmonella dysentery
E. coli

Conclusion

In conclusion, the biosynthesis of silver nanoparticle by the extracellular polysaccharide bioreduction method has been demonstrated, and for the first time Extracellular polysaccharide extracted from the *Bacillus* sp BPRCHR used for the bioreduction of silver nitrate in to silver nanoparticle. This method was found to be reliable and eco-friendly process, the extracellular polysaccharide provides stability for the nanoparticle and the characterization of synthesized silver nanoparticle were studied using UV-VIS Spectrophotometer, XRD, FTIR, SEM techniques. The synthesized silver nanoparticle was sensitive to *E. coli*, *Shigella*, *Vibrio* these results prove that biosynthesized silver nanoparticle has strong antimicrobial activity. Such eco-friendly nanoparticle

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can be scaled up for large production of noble metal nanoparticles which can be applied as bactericidal, nano biotechnology and biomedical applications.

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