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Antibacterial efficacy of synthesized AgNPs from exopolysacharides (EPS) produced by *Bacillus subtilis* of medico-clinical sinks

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Abstract: Nanotechnology is an important field of science which provides the nanoscale materials with anticipated applications in medical, electrical, mechanical, catalysis, molecular computing and structural materials fields. In recent time, bacteria are getting resistant to varied antibiotics based on their wide adaptability nature. In the present study, silver nanoparticles (AgNPs) were synthesized in vitro from the exopolysacharides produced from *Bacillus subtilis*. The appearance of yellowish brown color in the conical flask suggested the formation of Ag-NPs. The supernatant of the EPS changed the solution into yellowish brownish color upon the completion of 12 hrs reaction. The nanoparticles were confirmed by Uv-Vis spectrophotometer, Field emission scanning electron microscopy (FESEM) and Fourier transform infrared (FTIR) analysis. Size of the nanoparticles was recorded within 20nm size by FESM. The Gram negative bacterial pathogens were found more susceptible by the AgNPs compared to Gram positive bacteria.
From the Clinical Editor: In this study, EPS produced from *Bacillus subtilis* was subjected for the synthesis of silver nanoparticles, which was found quite fast, biocompatible, simple and free from any toxic chemicals. The antibacterial efficacy of the EPS nanomaterials showed good antibiotic effect over pathogenic bacteria.
Key words: AgNPs, *Bacillus subtilis*, Exopolysacharide(EPS), FTIR, Uv-Vis Spectrophotometer.

Introduction

Exopolysaccharides are long chain polysaccharides containing branched and repeating units of sugar molecules such as glucose, fructose or rhamnose etc[1]. Due to their high functional properties, these natural biopolymers have been widely used as viscofying, bioflocculating, stabilizing, gelling, and emulsifying agents in the food industry and do not carry a health risk which might make EPS a generally recognized as safe (GRAS) substance[2]. In other way, nanomaterials especially silver nanoparticles have a lot of applications in the field of biotechnology, biomedicine, biosensors, catalyst and therapeutic areas[1,4]. Among the noble metallic nanoparticles, silver nanoparticles (AgNPs) have received the greatest attention due to their wide spectrum of antimicrobial activity towards many Gram positive and Gram negative bacteria including fungi and viruses[4]. Silver has more advantages compared to other antimicrobial agents because of its broad spectrum of inhibitory activity against diverse bacteria, fungi, and viruses[3]. Different methods are available for the synthesis of silver nanoparticles like physical, chemical but recently development of biological method for the synthesis of nanoparticles is simple, quite fast, eco-friendly and free from any solvent or toxic chemicals involvement in the process[5,6]. Synthesis and characterization of nanoparticles is presently an important area of research, as selection of size and shape of nanoparticles provides an efficient control over many of the physical and

chemical properties[1]. Biological method for synthesizing of silver nanoparticles could have application in the field of medicine especially as anti-carcinogenic effect, drug carrier and diagnosis purposes. Different gold and silver nanoparticles (nonmaterial) have been synthesized by the biological method using (fungi, bacteria, algae)[5]. By using biological method, controlled silver nanoparticles has been produced which increases the interest in this field of nanomedicine (development of drug) research[7,8]. Silver has been used from the ancient times in the form of silver nitrate and silver sulfadiazine in order to treat various infections like wounds, burns, ophthalmic problems and also used as a disinfectant[9]. Development of resistance by the bacterial pathogens to the antibiotics has become a major problem in the worldwide in the recent times[5,10]. This present work is an attempt to biosynthesize silver nanoparticles from the exopolysaccharides produced from *Bacillus subtilis* isolated from medico-clinical sinks of Govt. Hospital, Hosur, TN, India. Silver nanoparticles were further characterized by UV-Vis spectroscopy followed by various microscopic characterizations and to evaluate its (silver nanoparticles) efficacy as bactericide to combat the growth of selected bacterial pathogens viz., *Staphylococcus aureus, Bacillus cereus, E. coli,* and *Proteus vulgaris.*

Materials and Methods

Isolation of biofilm bacteria

The clinical sample was collected from the wash basin sinks of the Govt. Hospital, Hosur, TN, India by swab method and brought to the Microbiology laboratory, M.G.R. College, Hosur with proper care in order to avoid the contamination. The biofilm bacterium, *Bacillus subtilis* was isolated and identified based on routine biochemical tests[11] and expertise of the authors. The bacterial strain was further subcultured and stored at 4°C in nutrient agar slants as stock cultures. The strain was grown in nutrient broth for maintenance in order to experiment for the production of EPS.

Production of Exopolysacharides

The isolated bacterium was inoculated in a standardized medium "Yeast mannitol glucose broth" as this medium supports the growth rate of bacteria & also good production rate EPS. The flask containing 100 ml of the above said medium (YMGB) was inoculated with the bacterium and the flask was incubated at 37°c overnight. After overnight incubation 100 µl was transferred to 500 ml of a fresh media in a conical flask. It was carried out in an aseptic condition; the flask was incubated for 5 days at 37°c.

Extraction of EPS

Sample from the flask was separated and concentrated to small volumes. The EPS was then precipitated from the supernatant by addition of equal volume of alcohol. The mixture was agitated during addition of alcohol to prevent local high concentration of the precipitate and left over night at 4°c before centrifuged at 7000 rpm for 20 min. After centrifugation, the precipitate was collected in petriplates and dried at 60°c and it was followed by the extraction of EPS.

Synthesis of AgNPs employing EPS

The solution of EPS (20 ml) was mixed with a 10mM aqueous solution of $AgNo_3$ prepared freshly in deionized water under stirring conditions. The mixture was stored at room temperature for 3 months in a dark place. After 12 hr of incubation, the colorless solution turned yellow followed by brown, which confirmed the formation of AgNPs.

Characterization of silver nanoparticles

These biologically synthesized silver nanoparticles from EPS were characterized by UV-Vis spectrophotometric analysis in the range of 300- 600 nm to check the absorption peak using a quartz cuvette with the control as the reference and checked the optical density values. The surface Plasmon resonance absorption peaks were observed and recorded. These biologically synthesized silver nanoparticles were kept at the room temperature for three to four months to check their stability as well as its activity again by recording the absorption peak using UV-Vis spectrophotometer analysis.

Field emission scanning electron microscopy (FESEM) analysis was used to determine the surface morphology and particle size of the silver nanoparticles. The AgNPs samples were sonicated and later on

centrifuged at 15000 rpm for 20 minutes. Before the process for FESEM analysis, the samples were further sonicated to get the uniformity and better observation. The supernatant were discarded and pellet was washed with the Milli-Q water for three to four times. The sample were transferred into the Petriplate and dried for about two hours at 50^0c, after that the sample were subjected to FESEM analysis.

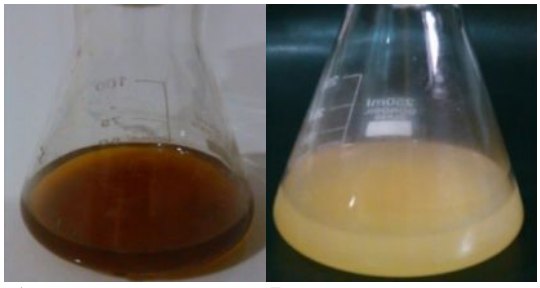
The synthesized silver nanoparticles were characterized by Fourier Infrared spectroscopic analysis by scanning the spectrum in the range of 500 – 4000 cm^{-1}. FTIR analysis was used to determine the molecules, proteins, functional groups involved in the reduction of Ag^+ into Ag^0 and stabilized them as a capping agent present inside the fungal filtrate solution. For FTIR analysis, sample was prepared as silver nanoparticles solution with the acetone in the ratio of 1:5 and subjected to centrifugation at 10000 rpm for 20minutes. Later the supernatant was discarded and 1-2 ml of acetone was added to the pellet and poured onto a petriplate. Acetone was then allowed to evaporate and then dried. The petriplate was scrapped and powder form of nanoparticles was obtained. Three milligram of the sample was taken and mixed well with potassium bromide (KBr) and pressed into a disc shape. The sample was placed into the sample holder and FTIR spectra were recorded.

Antibacterial Analysis

The silver nanoparticles were checked for its antibacterial activity by disc diffusion method[12]. The antimicrobial activity of the prepared silver nanoparticles from EPS produced by *Bacillus subtilis* was tested against the pathogenic clinical bacteria (Gram positive & Gram negative) such as *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Proteus vulgaris*. The $AgNO_3$ and Ofloxacin (5mcg) were taken separately as control parallel to the AgNPs to find a comparative assessment of the antibiotic efficacy over the pathogenic bacteria. The zone of inhibition was measured after overnight incubation at 37^0C.

Results and Discussion

The exopolysacharides from the isolated bacterium, *Bacillus subtilis* was employed in the present study for the biosynthesis of silver nanoparticles. The appearance of a yellowish brown color in the conical flask suggested the formation of AgNPs[5,10]. The supernatant of the in EPS culture changed the solution to a brownish color upon the completion of the 12 hr reaction with Ag+ (Fig 1). Control without silver ions showed no change in color when incubated under the same conditions. Many metals can be treated as free-electron systems; these metals, called plasma, contain equal numbers of positive ions (which are fixed in position) and conduction electrons (which are free and highly mobile)[5]. Under the irradiation of an electromagnetic wave, the free electrons are driven by the electric field to oscillate coherently. These collective oscillations of the free electrons are called plasmons. These plasmons can interact, under certain conditions, with visible light in a phenomenon called surface plasmon resonance (SPR)[13]. SPR plays a major role in the determination of optical absorption spectra of metal nanoparticles, which shifts to a longer wavelength as the particle size increases[14]. The AgNPs were characterized by UV-Vis spectroscopy. The technique outlined above has proved to be very useful for the analysis of nanoparticles[5,13,14]. As illustrated in Fig 2, Uv-Vis spectra, a strong surface plasmon resonance were centered at approximately 410nm indicated the presence of silver nanoparticles. The exact mechanism of silver nanoparticle synthesis has not been clearly known but several reports suggested that these silver ions required NADH – reductase enzyme for their reduction into AgNPs, which was secreted by the extracellular environment, might have been involved in the reduction of silver nitrate ions into silver nanoparticles[14].



A **B**

(A) With $AgNo_3$ treatment (B) Without $AgNo_3$ treatment

Fig 1: Synthesis of silver nanoparticles from EPS of *B. subtilis*

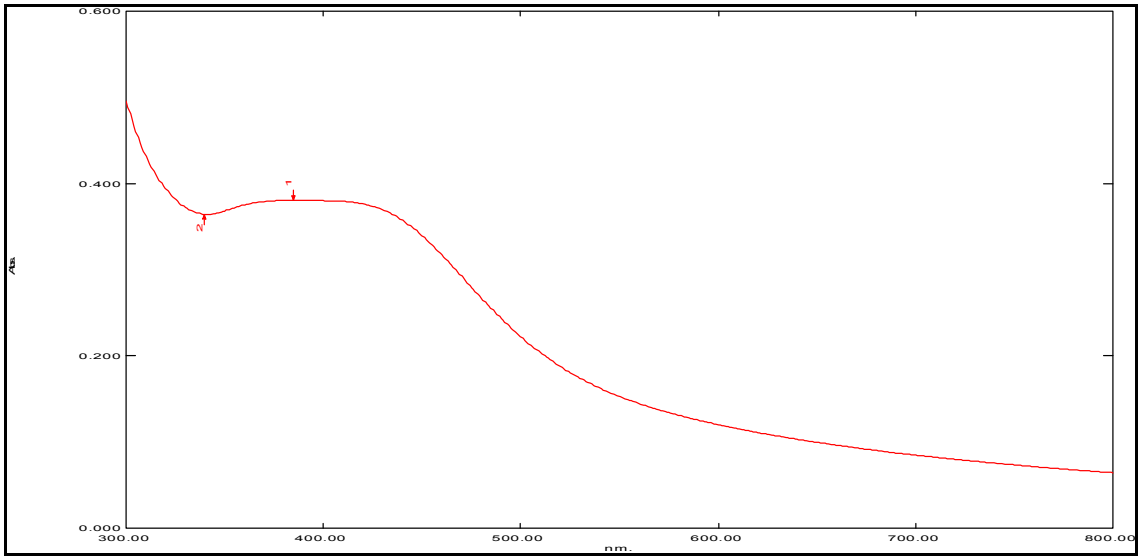


Fig 2: Uv–Vis spectrum of AgNPs synthesized from EPS of *B. subtilis*

FTIR analysis was used to identify the molecules, proteins and functional groups involved in the reduction of silver ions into silver nanoparticles (Table 1 & Fig 3). The FTIR analysis showed that the absorption peaks located at 3441.01and 3417.86 cm^{-1}(N-H stretch of amines), 1614.42 cm^{-1}(C-O stretch of amides), 1196.87 cm^{-1} (C-C(O)-C stretch [all others] of esters), 1138.00cm^{-1} (C-C stretch of ketones),1097.50 cm^{-1} (C-O stretch of alcohols), 867.73cm^{-1} (C-H bend [meta] of aromatic amines) and 601.79 cm^{-1} (Acetylenic C-H bend of Alkynes). The other peaks found to have involvement in the EPS AgNPs adsorption were at 1400.32 and 534.28 cm-1 due to the presence of tertiary –OH stretch in carboxylic acid and S-S disulfide stretches in amino acids respectively.

Table 1: Functional groups recorded from EPS silver nanoparticles

Sl. No.	Wave number (Cm^{-1})	Functional Group
1.	3441.01, 3417.86	N-H stretch of amines
2.	1641.42	C=O stretch of amides
3.	1195.87	C-C(O)-C stretch (all others) of esters
4.	1138.00	C-C stretch of ketones
5.	1097.50	C-O stretch of alcohols
6.	867.73	C-H bend (meta) of aromatic amines
7.	601.79	Acetylenic C-H bend of Alkynes

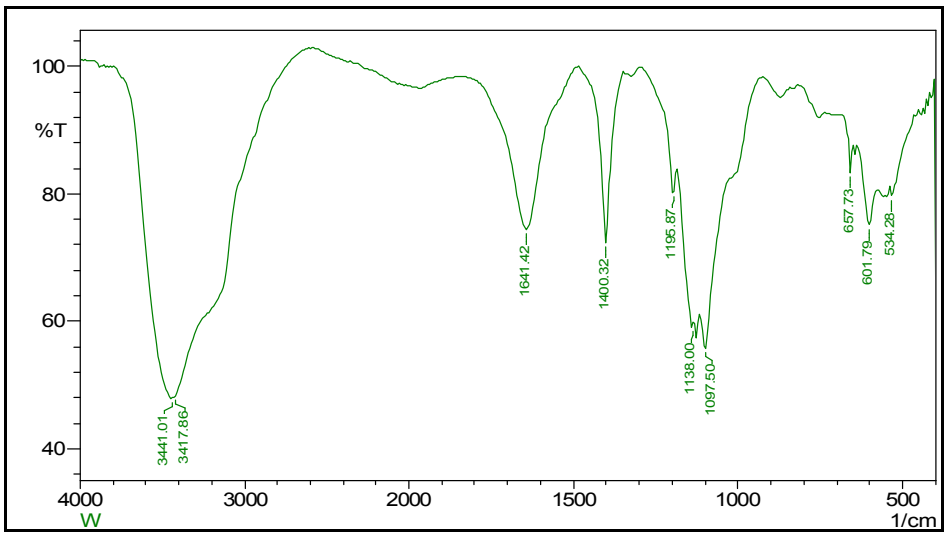


Fig. 3: FTIR analysis of silver nanoparticles synthesized from EPS of *B. subtilis*

Field emission scanning electron microscopy (FESEM) was used to understand the surface topology and the size of silver nanoparticles and it showed that the silver nanoparticles synthesized from EPS were spherical and well dispersed with the diameter ranges within 20 nm (Fig 4).

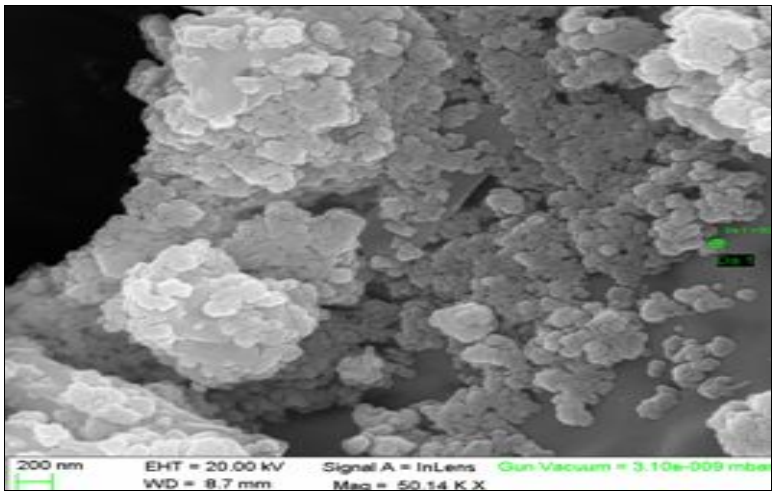


Fig. 4: FESEM analysis of AgNPs synthesized from EPS.

The antimicrobial activity of silver nanoparticles synthesized from EPS produced by *B. subtilis* against different clinically isolated pathogens viz., *Staphylococcus aureus, Bacillus cereus, E. coli,* and *Proteus vulgaris* were found satisfactory in the present study (Table 2). Overall, the Gram negative bacterial pathogens were found susceptible by the AgNPs compared to Gram positive bacterial pathogens. Similar result was found by the previous workers who reported that the Gram negative bacterium *E. coli* showed a greater inhibition zone compared to that of the Gram positive bacteria *Bacillus cereus* and *Streptococcus pyogenes*[4,15,16,18], which was probably due to their thick cell walls.

Table 2: Antibacterial effect of AgNPs synthesized from EPS of *B. subtilis*.

Sl. No.	Pathogenic Bacteria	Dilution of AgNPs/ Zone of inhibition (mm)			Control	
		10µl	15µl	20µl	15µl $AgNO_3$	Ofloxacin (5mcg)
1	*S. aureus*	09	11	14	09	12
2	*B. cereus*	08	10	16	10	09
3	*E. coli*	10	14	23	10	13
4	*P. vulgaris*	11	13	21	09	14

Conclusion

The synthesis and characterization of AgNPs employing EPS produced by *B. subtilis* were evaluated for their antibacterial efficacy. Formation of the AgNPs was first confirmed by visual observation on colour change and followed by UV-vis spectroscopy. Analysis of FTIR further confirmed the identity of the molecules, proteins and functional groups involved in the reduction of silver ions into silver nanoparticles. The EPS stabilized AgNPs showed strong signals in the silver region confirming the presence of elemental silver and the crystalline nature of the AgNPs. The AgNPs were stable for up to 90 days at room temperature. The Gram negative bacterial pathogens were found susceptible by the AgNPs compared to Gram positive bacterial pathogens in our present study.

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