

Research Article

Capsular Polysaccharide from *Klebsiella Pneumonia* ATCC 70063: Isolation, Green Synthesis of Silver Nanoparticles and study of Antimicrobial Activity

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Abstract: In modern research, the field of nanotechnology/nano-science is the most interesting areas for chemists, biologists/microbiologists for their commercial demand and it emerges rapidly due to its wide application in chemistry, electronics ecology, and medicine as well as in biological fields. Economically preparations of silver nanoparticles using green synthesis path having biological entities are gradually increase. In the investigation, synthesis of Ag-nano-particles were prepared by using capsular polysaccharide, isolated from *Klebsiella pneumoniae* ATCC 70063, and characterized by using UV-VIS spectrum band at 437 nm. The synthesized Ag-nanoparticles were generally found to be very effective as an antimicrobial agent against some important human pathogens such as *E. coli* ATCC 25922, *K. pneumonia* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028, which are affecting mostly and causes serious problems to human beings. These Ag-nanoparticles particle have also sporocidal activity and also responsible for degradation of bacterial DNA, which may be another reason for bactericidal activity of the silver nanoparticles.

Keywords: Green synthesis, Capsular Polysaccharide (CPS), Silver nanoparticles (SNPs), endospore, antimicrobial activity, Human pathogenic bacteria.

Introduction

In recent years nanotechnology has been emerging as a rapidly growing field with numerous applications in science and technology for the purpose of manufacturing new materials. This technology is defined as the design, characterization and application of structures, devices and systems by controlling shape and size at nanometre scale level (1 nm to 100 nm) and has already found practical applications in health and

daily life, such as better drug delivery methods, chemical deposition for environmental pollution cleanup, medical imaging, as well as military purposes. The synthesis of silver nanomaterials/nanoparticles extensively studied by using chemical and physical methods, but the development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. A number of approaches are available for the synthesis of silver nanoparticles for example, reduction in solutions, chemical and photochemical reactions in reverse micelles, thermal decomposition of silver compounds, radiation assisted, electrochemical, sonochemical, microwave assisted process and recently via green chemistry route. Currently, there is a constant need to develop eco-friendly processes for the synthesis of nanoparticles. The focus for this synthesis has shifted from physical and chemical processes towards 'green' chemistry and bio-processes. Biological synthesis process provides a wide range of environmentally acceptable methodology, low cost production and minimum time required.

It has been known that silver and its compounds have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus since ancient times. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells. The bactericidal effect of metal nanoparticles has been attributed to their

small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution.

Here we introduce a novel method for the synthesis of silver nanoparticles by capsular polysaccharide extracted from *Klebsiella pneumoniae* ATCC 70063. We offer evidences to indicate that CPS mediated SNPs can inhibit human pathogenic bacterial growth. First time, we demonstrate that dormant form bacteria (endospore) can effective kill by CPS base SNPs and again we analyze the effect of these SNPs in bacterial DNA.

Materials and Methods

Isolation of Capsular polysaccharide (CPS) from *K. pneumoniae* ATCC 70063:

For the purpose of obtaining capsule at first the bacteria was checked in pure form by streak plate on Mac Conkey agar (HiMedia). Here the pink color of the colony confirmed the presence of *Klebsiella* sp. From the plate single colony was used as inoculum for large scale culture. The bacteria were grown on Tryptone soy broth (HiMedia) for about 17 hour at 37°C. Then the presence of capsule was confirmed by Phase contrast microscopy and by negative staining using nigrosin.

The capsuled forming bacteria are grown large scale in Tryptone soy broth from extracting the capsular polysaccharide. The capsular polysaccharide is extracted by a modification of the procedure described by Anderson and Smith, the outline of which is, the organism from broth was harvested by centrifugation at 10,000 Xg for 30 minutes. The capsular polysaccharide (CPS) was precipitated from culture using hexadecyltrimethylammonium bromide (Cetavlon) in such a way that its final concentration reaches to 5mM. Then the precipitate was collected and dissolved in equal volume of 1 M CaCl₂ and 25% ethanol. Then the mixture was stirred at room temperature for 60 min. The precipitated nucleic acid was removed by centrifugation at 10,000 Xg. The CPS was precipitated from the resultant supernatant by addition of equal volume of 80% ethanol. Thus crude CPS obtained by centrifugation at 10000 Xg for 10min. The precipitate materials is dissolved in distilled water and dialyzed through DEAE cellulose bag against distilled water for 12 hours to remove low molecular weight materials. The dialyzed CPS was lyophilized and 25 mg lyophilized materials is

dissolved in minimum volume of water and was purified through a sephadexG-100 gel permeation column (50x1.5 cm) using water as eluent with a flow rate of 0.5 ml/min. A total 100 test tubes (2ml each) were collected and monitor spectrophotometrically at 490 nm using phenol-sulphuric acid method. Test tubes 35-56, yields desired CPS and freeze dried.

Synthesis of Ag-nanoparticles

The silver nanoparticles are prepared by as usual way by using purified capsular polysaccharide of *Klebsiella pneumoniae* ATCC 70063. The process performed by adding 20 ml of 0.5% of CPS into 20 ml of aqueous solution of 1 mM AgNO₃ for reduction of Ag⁺ ions into Ag. The total mixture was stirred with magnetic bar for 3 hours in heating condition at 70°C. The brownish color of the solution indicates the presence nanoparticles and is confirmed by UV-Visible spectrophotometer.

Analysis of effect of CPS based Ag-nanoparticles on bacteria

The antimicrobial activities of Ag-nanoparticles

The antimicrobial activities of CPS based Ag-nanoparticles was done on human pathogenic *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028 by standard cup plate method. The Luria Bertain (LB) broth media are used for overnight culture of pathogenic strain of bacteria at 37°C under shaking condition (150 rpm). The young cultures of inoculum (100µl) of each bacterium were spread in LB agar plates. Now in agar media four wells were bored using a borer of diameter of 0.7cm. The four well are filled with 200 µl each of Concentrated CPS based Ag-nanoparticles (C, 500µg/ml), Diluted CPs based Ag-nanoparticles (D, 250µg/ml), Reduced silver (R, 500µg/ml) and Crude capsular polysaccharide (P, 500µg/ml) respectively. The plates were incubated at 37°C for overnight.

Bacterial Growth Curve

The effect of CPS based silver nanoparticles on growth of different pathogenic bacterial strain (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028) were done in Luria Bertain (LB) broth at 37°C under shaking condition (150 rpm) using CPS-SNPs (final concentration 200ng/ml) at different time intervals (2 to 24

hours). The growth of *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028 in broth media was indexed by measuring the optical density (at $\lambda=600\text{nm}$) at regular intervals using UV-Vis spectrometer. Whereas control does not contain any exposure of silver nanoparticles synthesized from Capsular polysaccharide extract of *K. pneumoniae* ATCC 70063.

Effect on Endospore

Bacterial endospore is dormant structures that is formed in unfavorable condition by certain group of bacteria (e.g. *Bacillus* sp.) and are resistance to most of the antibacterial agent. So destruction of endospore is now a challenging filed in biological world by natural way. The spores of bacteria were purified by a method as demonstrated by TjakkoAbeetal with few modifications. For example spores of the *Bacillus subtilis* were prepared on a nutrient-rich, chemically defined sporulation medium designated Y1 medium, which contained the following components (final concentrations): D-glucose (10 mM), L-glutamic acid (20 mM), L-leucine (6 mM), L-valine (2.6 mM), L-threonine (1.4 mM), L-methionine (0.47 mM), L-histidine (0.32 mM), sodium-dl-lactate (5 mM), acetic acid (1 mM), FeCl_3 (50 μM), CuCl_2 (2.5 μM), ZnCl_2 (12.5 μM), MnSO_4 (66 μM), MgCl_2 (1 mM), $(\text{NH}_4)_2\text{SO}_4$ (5 mM), Na_2MoO_4 (2.5 μM), CoCl_2 (2.5 μM), and $\text{Ca}(\text{NO}_3)_2$ (1 mM). The medium was buffered at pH 7.2 with 100 mM potassium phosphate buffer. Furthermore, spores were prepared on modified G medium; the medium contained 0.2% yeast extract, CaCl_2 (0.17 mM), K_2HPO_4 (2.87 mM), MgSO_4 (0.81 mM), MnSO_4 (0.24 mM), ZnCl_2 (17 μM), CuSO_4 (20 μM), FeCl_3 (1.8 μM), and $(\text{NH}_4)_2\text{SO}_4$ (15.5 mM) and was adjusted to a pH of 7.2. This medium was expected to contain approximately 14 mM amino acids, based on a 70% protein content of the yeast extract. Cultures were incubated at 30°C with shaking at 225 rpm, which resulted in >99% free spores in both media, after incubation for 48 h followed by incubation at 55°C for 1 hour. The presence of endospore was confirmed by endospore staining using malachite green and counted by hemocytometer. The spores were then harvested, washed repeatedly and treated with CPS based silver nanoparticles to analyze the sporocidal activity. For example the

2ml of spore culture (1.37×10^7 CFU/ml) are treated with 400ng of CPS based silver nanoparticles and incubate for specific time intervals (0 hour, 1 hour, 24 hour, and 48 hour). The treated spore solutions are plated in nutrient agar medium and incubate at 37°C for 24 hours under inverted condition to count the colonies number.

Effect on Bacterial DNA

Total DNA was extracted from bacterial (*E. coli*) by thephenol/chloroform method. DNA concentration was measured with the Bio-Photometer (Eppendorf). 2ml of diluted DNA sample (200ng) was prepared and 20 μl of CPS based Ag-nanoparticles (500 $\mu\text{g}/\text{ml}$) was added to it. Then the sample was left undisturbed at 37°C for 10 min and analyzed the effect using UV-Vis spectroscopy taking same quality and quantity of DNA as control in sterilized distilled water. Again the degradation effect on DNA of CPS based silver nanoparticles confirmed by taking 5 μl of DNA solution (400 $\mu\text{g}/\text{mL}$) were mixed with 200 μl of a suspension containing the nanoparticles (500 $\mu\text{g}/\text{mL}$) and incubated at 37°C for 10 minutes. After incubation, an aliquot was sampled and analyzed by electrophoresis run at 100 V for 2 h in 1% agarose gel. The gels were stained with ethidium bromide and images were acquired.

Result and Discussion

Isolation of Capsular Polysaccharide (CPS)

The capsule forming bacteria are grown in MacConkey agar plates (Fig 1A) and capsule forming ability was analyzed by Phase contrast microscope as well as by negative staining using nigrosin (Fig1B,C). The capsular polysaccharide extracted from *Klebsiella pneumoniae* ATCC 70063 by cultivation in Tryptone soy broth and subsequently precipitated by Cetavlon. The CPS are further purified by dialyzed through DEAE cellulose bag against distilled water for 12 hours and finally we get Capsular polysaccharide (CPS) in a concentrated form (500 $\mu\text{g}/\text{mL}$) through a sephadexG-100 gel permeation column (50x1.5 cm) using water as eluent with a flow rate of 0.5 ml/min.

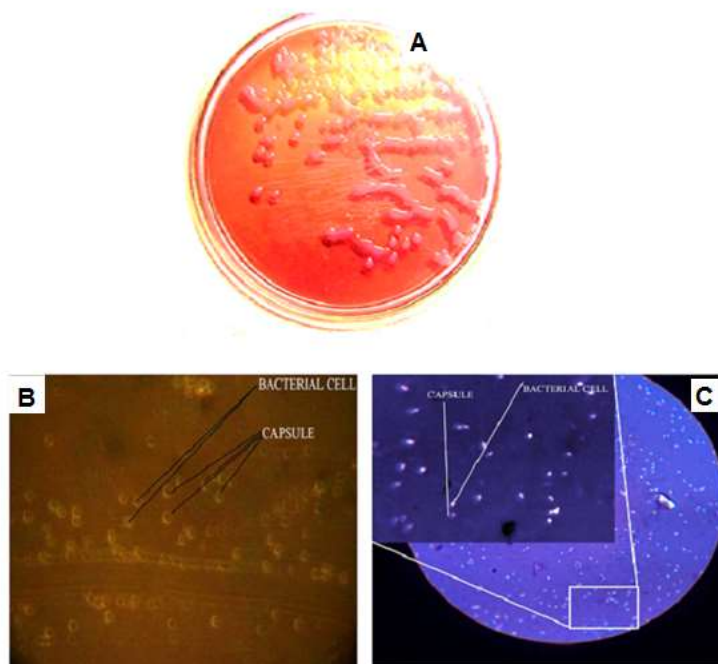


Fig1: Capsule forming bacteria are grown in Mac Conkey agar plates (A), Capsule forming bacteria under Phase contrast microscopic (100x) (B), Negative staining using nigrosin (100x) (C).

Synthesis of Ag-nanoparticles

It is well known that silver nanoparticles exhibit reddish/brownish color in water; this color arises due combined vibration of free electrons of silver nanoparticles in resonance with light wave, which give rise to a surface plasmon resonance (SPR) absorption band in the visible region of electromagnetic radiation. The synthesis of Silver nanoparticles by reduction of the aqueous metal ions during exposure of 20 ml of 0.5% of capsular polysaccharide (CPS) extract from *K. pneumoniae* ATCC 70063 into 20 ml of aqueous solution of 1 mM AgNO₃ detected by color change from yellow-

green to brownish (Figure-2A). In case of negative control (silver nitrate solution alone), no change in color change was observed. The silver nanoparticles synthesis further confirmed by UV-Vis spectra. UV-Vis absorption spectrum of silver nanoparticles in the presence of Capsular polysaccharide extract is shown in figure 2B. The Surface Plasmon band in the silver nanoparticles solution remains close to 437nm throughout the reaction period, suggesting that the nanoparticles were dispersed in the aqueous solution with no evidence for aggregation in UV-Vis absorption spectrum.

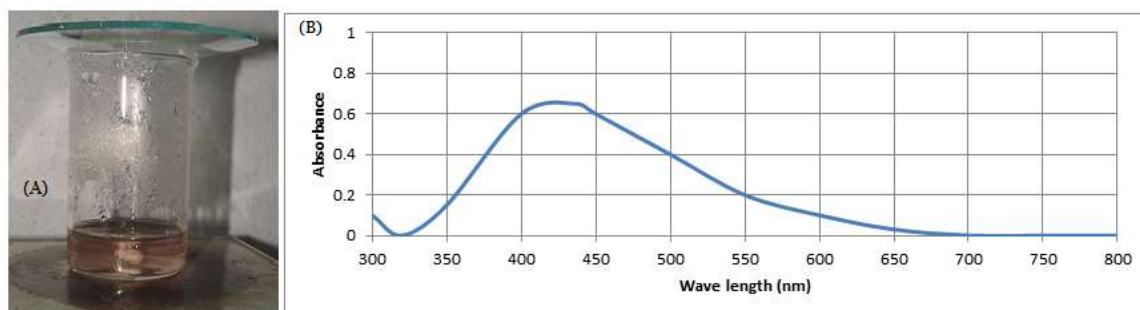


Fig2: Ag-nanoparticles synthesized from CPS from *K.pneumoniae* (A), UV-VIS spectrum of the Ag-nanoparticles (B).

The antimicrobial activities of Ag-nanoparticles

The antibacterial activity of CPS based SNPs were done on human pathogenic *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028 by standard cup plate method, depicted in figure-3. The nanoparticles synthesized by green route are found highly toxic against multi drug resistant human pathogenic (Fig 3). The antibacterial activity of silver nanoparticles was compared for various microorganisms using the diameter of inhibition zone (Table1). The diameter of

inhibition zone (DIZ) reflects magnitude of susceptibility of the microorganisms. The strains susceptible disinfectants exhibit larger DIZ, whereas resistant strains exhibit smaller DIZ. The data support the sensitivity of bacterial strain towards CPS based nanoparticles in comparison to Crude capsular polysaccharide and Reduced silver, which shows no zone of inhibition. Again the data show that *E. coli* ATCC and *Salmonella typhimurium* ATCC 14028 is 25.5% and 17% more sensitive than *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734.

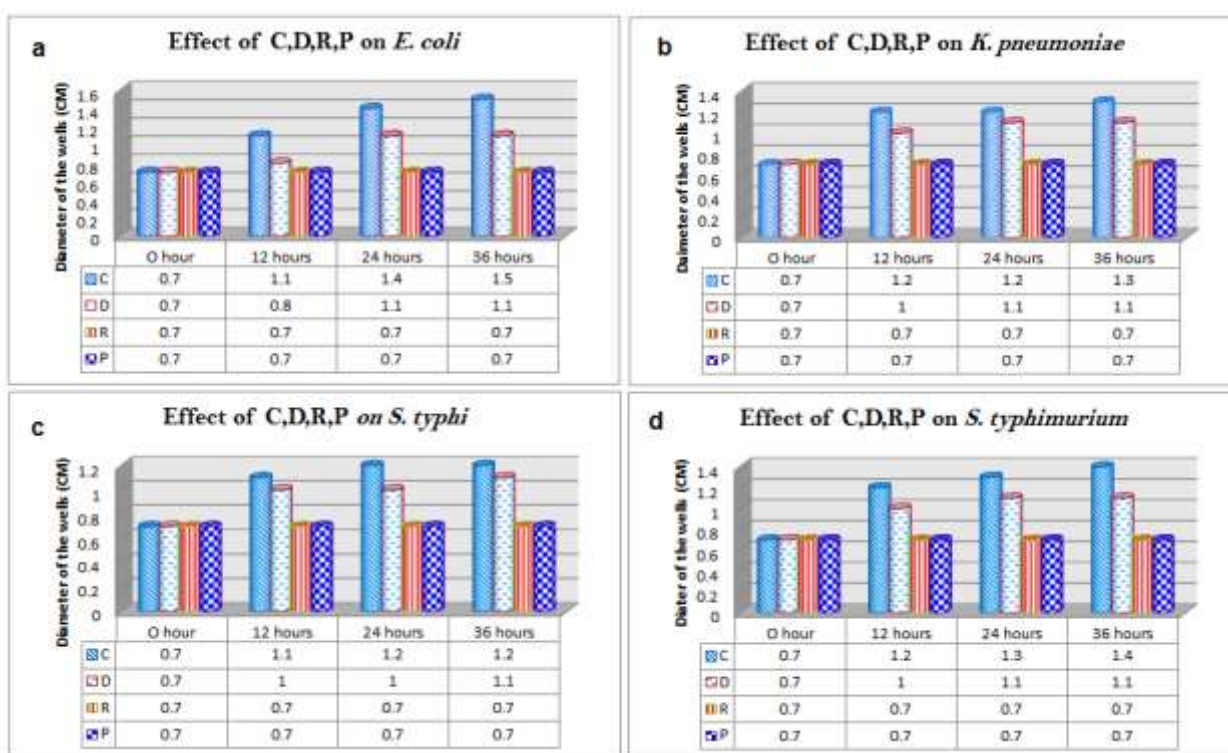


Fig3: Effect of Concentrated CPS based Ag-nanoparticles (C, 500 μ g/ml), Diluted CPs based Ag-nanoparticles (D, 250 μ g/ml), Reduced silver (R, 500 μ g/ml) and Crude capsular polysaccharide (P, 500 μ g/ml) on *E. coli* ATCC 25922 (A), *K. pneumoniae* ATCC 70063 (B), *Salmonella typhi* MTCC 734 (C) and *Salmonella typhimurium* ATCC 14028 (D)

Table 1: The diameter of inhibition zone (DIZ) of antibacterial activity of CPS based SNPs against human pathogenic *E. coli* ATCC 25922 (a), *K. pneumoniae* ATCC 70063(b), *Salmonella typhi* MTCC 734 (c) and *Salmonella typhimurium* ATCC 14028 (d).

Table1a

Sample	DIZ (Cm) against <i>E. coli</i> with respect to time			
	0 hour	12 hours	24 hours	36 hours
C	0.7	1.1	1.4	1.5
D	0.7	1.8	1.1	1.1
S	0.7	0.7	0.7	0.7
P	0.7	0.7	0.7	0.7

Table1b

Sample	DIZ (Cm) against <i>Klebsiella pneumoniae</i> with respect to time			
	0 hour	12 hours	24 hours	36 hours
C	0.7	1.2	1.2	1.3
D	0.7	1	1.1	1.1
S	0.7	0.7	0.7	0.7
P	0.7	0.7	0.7	0.7

Table1c

Sample	DIZ (Cm) against <i>Salmonella typhi</i> with respect to time			
	0 hour	12 hours	24 hours	36 hours
C	0.7	1.1	1.2	1.2
D	0.7	1	1.1	1.1
S	0.7	0.7	0.7	0.7
P	0.7	0.7	0.7	0.7

Table1d

Sample	DIZ (Cm) against <i>Salmonella typhimurium</i> with respect to time			
	0 hour	12 hours	24 hours	36 hours
C	0.7	1.2	1.3	1.4
D	0.7	1	1.1	1.1
S	0.7	0.7	0.7	0.7
P	0.7	0.7	0.7	0.7

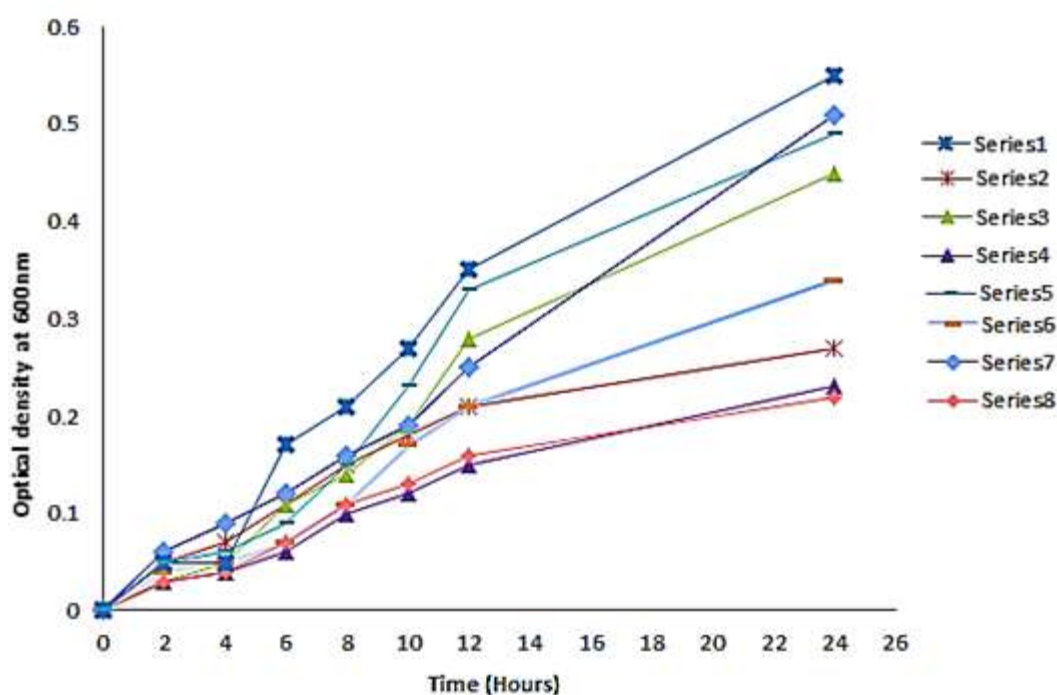
Bacterial Growth Curve

The growth curves of *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734 and *Salmonella typhimurium* ATCC 14028 treated with SNPs were shown in Fig. 4 by measuring optical density at 600 nm (Table 2). In presence of 200ng/ml of CPS based SNPs, the growth curves of each of the bacterium decreased (lag phase, exponential phase, and stationary phase), however decline phases in each growth curve could not be revealed because we only assayed the total numbers of bacteria, including

live and dead ones, based on the value of OD₆₀₀. In absence of nanoparticles the growth curve reached exponential phase quickly but treatment with CPS based SNPs decrease the growth rate of each bacterium and most immersed effect are found in case of *E. coli* ATCC 25922 and *Salmonella typhimurium* ATCC 14028. The experiment proved that silver nanoparticles generated by treatment of capsular polysaccharide from *K. pneumoniae* ATCC 70063 exhibits strong antibacterial activity due to their well-developed surface which provides maximum contact with the environment.

Table 2: Measurement of optical density at 600 nm of Bacterial growth under treated with CPS based SNPs and untreated SNPs.

Time (Hour)	Optical Density					
	Control <i>E.coli</i>	Treated <i>E.coli</i>	control <i>K. pneumoniae</i>	Treated <i>K. pneumoniae</i>	Control <i>S. typhi</i>	Treated <i>S. typhi</i>
0	0	0	0	0	0	0
2	0.05	0.05	0.03	0.03	0.05	0.04
4	0.05	0.07	0.05	0.04	0.06	0.05
6	0.17	0.11	0.11	0.06	0.09	0.07
8	0.21	0.15	0.14	0.1	0.15	0.11
10	0.27	0.18	0.19	0.12	0.23	0.17
12	0.36	0.18	0.28	0.16	0.33	0.21
24	0.55	0.23	0.45	0.23	0.49	0.29

**Fig. 4:** Bacterial growth curve under treated with CPS based SNPs (Even Series) and untreated (odd series). *E. coli* ATCC 25922, (Series 1,2); *K. pneumoniae* ATCC 70063, (Series 3,4); *Salmonella typhi* MTCC 734 (Series 5,6); and *Salmonella typhimurium* ATCC 14028 (Series 7,8).

Effect on Bacterial Spore

Spore are resistant form of bacteria that create a threat in medical world. We first prepared the concentrated culture of *B. subtilis* spore and confirmed by staining with malachite green (Fig 5A). To analyze the sporocidal activity of silver nanoparticles we first time demonstrate that CPS

based SNPs responsible immensely decreased *B. subtilis* spore in time dependent manner. The data show that the CPS based SNPs responsible for 80% and more than 90% decrease the CFU count of bacterial spore in 24 and 48 hours respectively (Fig 5B), where the vegetative cell of *B. subtilis* kills near about 100% (data not shown).

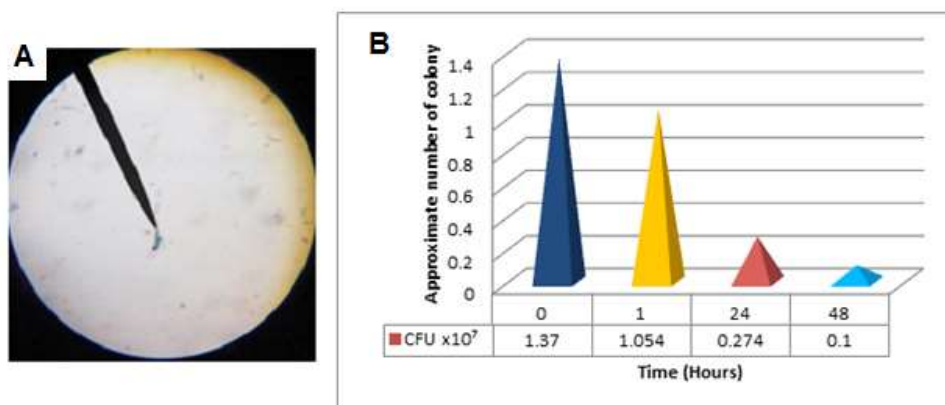


Fig 5: *B. subtilis* spore by staining with malachite green (A), Sporocidal activity of CPS based SNPs (B)

Effect on Bacterial DNA

The effect of CPS based Silver nanoparticle on bacterial DNA is given in Fig6 and Fig7. The nanoparticles is responsible for degradation of the of bacterial DNA into mononucleotide or oligonucleotide level and for that reason it shows

hyperchromic effect (Fig6) when analyzed by UV-Visible spectrophotometer and also responsible for disappearance of the DNA band in 1% agarose gel electrophoresis (Fig7). This result also predicts that the bactericidal effect of CPS based SNPs also due to degradation of the genomic DNA.

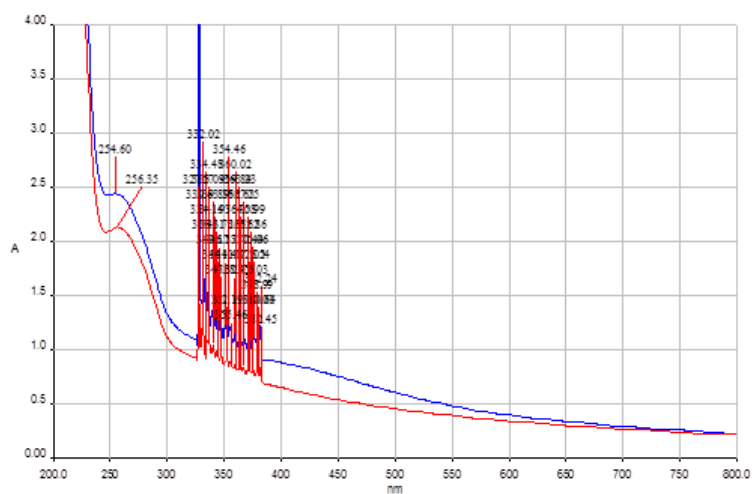


Fig 6: Hyperchromic effect of DNA on treated with CPS based SNPs.

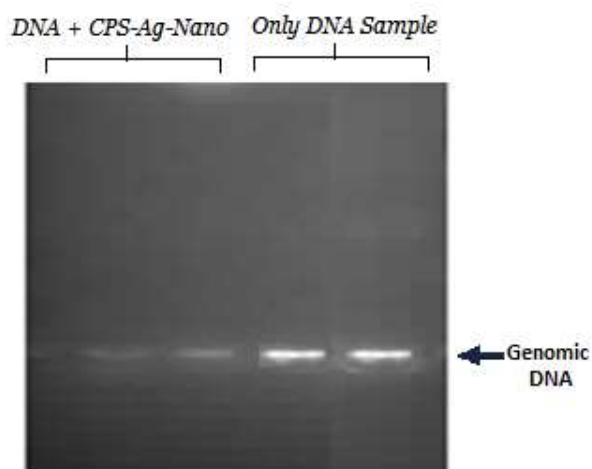


Fig 7: Gel electrophoresis of untreated and treated DNA sample.

Conclusion

The green synthesis of silver nanoparticles using Capsular polysaccharide extracted from *K. pneumoniae* ATCC 70063 is an ecofriendly and simple process and also economic one. The CPS based SNPs responsible for destruction of different multi drug resistant (MDR) human pathogenic bacteria. In conclusion it has been demonstrated that bacteriocidal activity of CPS based SNPs are not only due to surface leakage, membrane damage but also due to degradation of bacterial DNA. Our research also proved that the green synthesis SNPs also responsible for sporocidal activity, which may create a goal in future to treat medically important spore forming pathogenic bacteria.

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