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Phycofabrication of silver nanoparticles and their antibacterial activity against human pathogens

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ABSTRACT

In the present study, the extracellular phycofabrication (synthesis by algae) of silver nanoparticles was demonstrated using algae i.e. *Spirogyra sp.* recovered from the fresh water. The reduction of silver ions present in the aqueous solution of silver sulphate (Ag_2SO_4) was done by the cell filtrate of *Spirogyra sp.* leading to the synthesis of silver nanoparticles. Phycofabrication of silver nanoparticles was confirmed by using characterization tools like UV-Vis spectrophotometer, FTIR, TEM and NTA. The resulting silver nanoparticles were spherical in shape, in the range of 40-80 nm and capped with proteins. Through the experimental studies, it was found that temperature, pH and salt concentration affects the rate of phycofabrication of silver nanoparticles. Antibacterial study of phycofabricated silver nanoparticles was assessed against human pathogenic bacteria. These silver nanoparticles showed better antibacterial activity against Gram positive bacteria i.e. *Staphylococcus aureus* (ATCC-25923) (13 mm) as compared to Gram negative bacteria i.e. *Escherichia coli* JM-103 (ATCC-39403) (11 mm). This is the first report of synthesis of silver nanoparticles by *Spirogyra sp*. using Ag_2SO_4 as a salt. Extracellular phycofabrication of silver nanoparticles by *Spirogyra* sp. was found be easy, simple and eco-friendly method. Copyright © 2016 VBRI Press.

Keywords: Extracellular; human pathogenic bacteria; phycofabrication; silver nanoparticles; spirogyra.

Introduction

Nanotechnology is a highly multidisciplinary field, drawing from applied physics, materials science, colloid science and interface. The metal nanoparticles have novel magnetic, electronic and optical properties, which vary in their size, shape and composition. Metal nanoparticles are more effective because of the high surface area to volume ratio so that a large proportion of metal atoms are in direct contact with their environment **[1]**. Due to novel properties, metal nanoparticles have many applications in different fields like medicine, electronics, agriculture, etc. **[2-4]**.

The biosynthetic method employing different biological systems has received attention as being simple eco-friendly and economically viable compared to the chemical and physical methods used for synthesis of metal nanoparticles **[5]**. Many biological systems like plants [**6**], fungi **[5, 7-9]**, bacteria **[10, 11]**, Callus extract of *Carica papaya* etc. **[12]** have been used successfully for the synthesis of silver nanoparticles. Gade *et al.* **[13]** reported that, in *Opuntia ficus indica* quercetin is responsible for formation of silver nanoparticles.

Similarly, some algae also have been exploited for the synthesis of metal nanoparticles. The brown alga *Fucus vesiculosus* was reported for the bioreduction of Au (III) to Au (0) in to gold nanoparticles of different size and shapes when exposed to dilute hydrometallurgical solutions and leachates of electronic scraps at pH-7 **[14]**. Similarly, Govindaraju and group **[15]** investigated the formation of Ag, Au and Au core and Ag shell nanoparticles from *Spirulina platensis*. Biological reduction and extracellular synthesis of nanoparticles were achieved in 2 hrs at 37^0C at pH 5.6. Similarly, other algae which have been successfully exploited includes; *Sargassum muticum* **[16]**, *Spirogyra varians* **[17]**, *Sargassum longifolium* [**18**] and *Gracilaria crassa* **[19]** etc.

Silver nanoparticles are used in textile fabrics **[20]**, cosmetics and water treatment **[21]**, coating stainless steel in medical devices **[22]** and dental materials **[23]**. Efficacy of silver nanoparticles against bacteria was studied by various researchers **[24, 25]**. Also the combined effect of silver nanoparticles with antibiotics was evaluated against multidrug resistance bacteria **[26]** and pathogenic fungi **[8]**. Due to emerging applications of silver nanoparticles in distinct field there is a need to develop, new cheaper and ecofriendly techniques for synthesis of nanoparticles.

The algae are chlorophyll bearing organism and their colorless relatives which are thalloid i.e. having no true roots, stems and leaves or leaf like organ. By definition algae are simple plants which display a spectrum of photosynthetic pigments and evolve oxygen during the process of photosynthesis **[27]**. Algae may occur anywhere such as fresh water, sea water, soil, on or within plants, on animals, rocks stones, in deserts as well as on permanent snowfields.

Spirogyra is the best known and most studied alga of Chlorophyceae, is more or less universal in distribution, and its species are recorded from almost all countries from where algal collections are made. There are more than 400 species recorded from the world. It is a freshwater alga found either free floating or rarely attached to some substratum in water. It is also found in pools, ponds, lakes as well as in running water of streams, canals, rivers, etc. In water, filaments of *Spirogyra* form green silky mass. It is commonly called "water silk", "pond scum" or "mermaid's tresses".

The main aim of the present study is to develop rapid, ecofriendly and non-toxic approach for the large scale synthesis of silver nanoparticles. In this context, we used *Spirogyra* for the synthesis of the silver nanoparticles because *Spirogyra* is the simplest filamentous alga found abundantly in the fresh water resources in the pure form. Most important is that it can be grown on simple medium and very easy to handle due its non-toxic nature. Metal ions exposed to algae could be reduced outside the algal biomass leading to extracellular synthesis of silver nanoparticle in the solution.

Experimental

Preparation and maintenance of spirogyra culture

Spirogyra collected from the lake was first observed under the microscope to ensure that the thallus is clean and worth for isolation. Single filament was then transferred into Chu 10 culture medium and placed it in plant tissue culture laboratory under the influence of light for about 10 d.

Synthesis of silver nanoparticles

Spirogyra species maintained, was grown in Brandwien's solution in an Erlenmeyer flask. After sufficient growth of 10 d the biomass was filtered through a Whattman filter paper No. 1 and later resuspended in sterile distilled water and kept for 24 h in an orbital shaker at 120 rpm. After incubation cell filtrate was separated and then challenged with Ag_2SO_4 (1mM) and incubated at different temperatures 0˚ C, 4˚C, 25˚C (room temperature), 37˚C and 70˚C. On the other hand, Ag_2SO_4 solution (1mM) was also treated with cell filtrate with different pH like 3, 5, 7, 9 and 11 to assess the effect of these parameters on synthesis of silver nanoparticles. To maintain the pH acetate buffer for pH 3 and 5, sodium phosphate buffer for pH 7 and carbonate buffer for pH 9 and 11 were used. Also to understand the effect of different salt concentration on synthesis of silver nanoparticles, cell filtrate was treated with different Ag_2SO_4 concentration (0.5 mM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM and 100 mM).

Detection of silver nanoparticles

UV-Vis spectrophotometer analysis

After two hours of incubation, the samples of all the reaction mixture (incubated at different temperatures and pH) were subjected to optical analysis using UV-Vis spectrophotometer (Shimadzu UV-1700, Japan). The spectrum was scanned at the resolution of 1 nm from 200-800 nm for each sample.

Fourier transform infrared spectroscopy (FTIR) analysis

After complete reduction of aqueous silver ions the algal filtrate was subjected to repeated centrifugation at 10000 g for 15 min. The supernatant was replaced by distilled water each time (three times) to concentrate and purify the silver nanoparticles. Absence of untreated free silver ions was determined by addition of sodium chloride to get the pure nanoparticles free from silver ions. The absence of white precipitate confirms the complete removal of unreacted free silver ions from the solution. The resulting suspension was resuspended in 20 ml sterile distilled water. Thereafter, the purified suspension was completely dried at 60 °C. Finally, the dried nanoparticles were analyzed by FTIR (Perkin-Elmer FTIR-1600, USA) in the range 450–4000 cm^{-1} at resolution of 4 cm^{-1}.

Transmission electron microscopy (TEM)

Transmission Electron Microscopy (JEOL-6380A- version 1.1) was performed to determine the shape and size of the phycofabricated silver nanoparticles. The conventional carbon coated copper grids were cleaned using plasma treatment under oxygen for 45 sec. A 5µl of sample was then placed on the grid and then dried at room temperature for 1 h. The samples were inspected by operating at 120 KV. Three images of each sample were taken to have a clear representation of its composition.

NTA analysis

Liquid samples of silver nanoparticle were introduced into a scattering cell through which a laser beam (approx. 40 mW at k = 635 nm) was passed. Particles present within the path of the laser beam were observed via a dedicated non-microscope optical instrument (LM-20, Nano Sight Pvt. Ltd., UK) having CCD camera. The motion of the particles in the field of view (approx. 100 X 100 µm) was recorded (at 30 fps) and the subsequent video and images were analyzed.

Antibacterial study by disc diffusion method

Antibacterial activity of silver nanoparticles against the Gram positive (*Staphylococcus aureus*- ATCC-25923) and Gram negative bacteria (*Escherichia coli*- ATCC-25923) was evaluated by disc diffusion method according to NCCLS guidelines. The bacterial cultures having the load of 1 X 10^8 CFU/ ml were used to evaluate the antimicrobial activity.

Results and discussion

The colorless algal filtrate on addition of Ag_2SO_4 (1mM) changes to dark brown color. (**Fig. 1** inset). The appearance of the brown color was apparent indication of the formation of the silver nanoparticles in the reaction mixture. The color change of the cell filtrate was noted by the visual observation. The change in color was due to the excitation of the surface plasmon resonance **[9, 19]**. Time dependent increase in the intensity of surface plasmon resonance was observed in *Spirogyra* sp. The formation of silver nanoparticles was further confirmed by optical spectroscopy. UV–Vis spectra recorded after 2 h of

incubation are shown in **Fig. 1**. It showed typical plasmon resonance at 422 nm expected for spherical silver nanoparticles. The effect of different temperatures on the rate of synthesis of silver nanoparticles was studied on the basis of the UV-visible spectrophotometer analysis. Algal filtrate after treatment with Ag_2SO4 were kept at 0° C, 4°C, 25°C (room temperature), 37°C and 70°C with respective control (in triplicate).

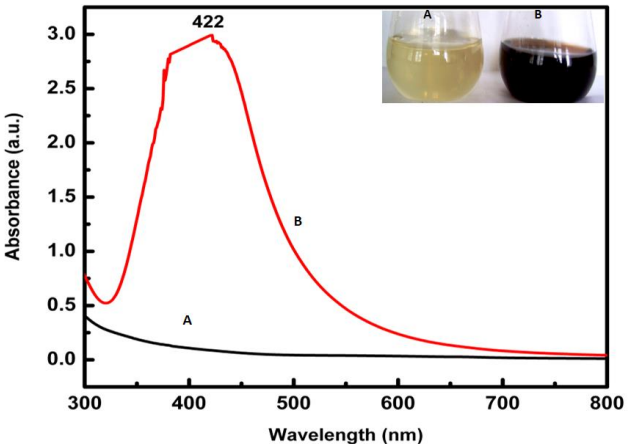

Fig. 1. UV-Vis spectra of (A) Algal (*Spirogyra* sp.) filtrate (control) **(**B**)** Silver nanoparticles synthesized after treating algal filtrate with aqueous silver sulfate showing absorbance at about 422 nm. [Inset Fig. **(**A**)** Algal filtrate before and **(**B**)** Algal filtrate after treatment with aqueous silver sulfate].

The UV-Visible spectrum recorded revealed that the absorbance at 70˚C was found to be optimum for the synthesis of silver nanoparticles. The synthesis of silver nanoparticles was not reported at the 0˚ C, while 4˚C, 25˚C and 37˚ C favors the synthesis of the silver nanoparticles (**Fig. 2**). These results showed resemblance with the finding reported by Bawaskar *et al.* **[7]**. The authors reported that 37˚C was optimum temperature for the synthesis of silver nanoparticles using a fungus *F. culmorum*, but synthesis of polydisperse nanoparticles was reported at 70˚C, while no synthesis observed at 0˚C.

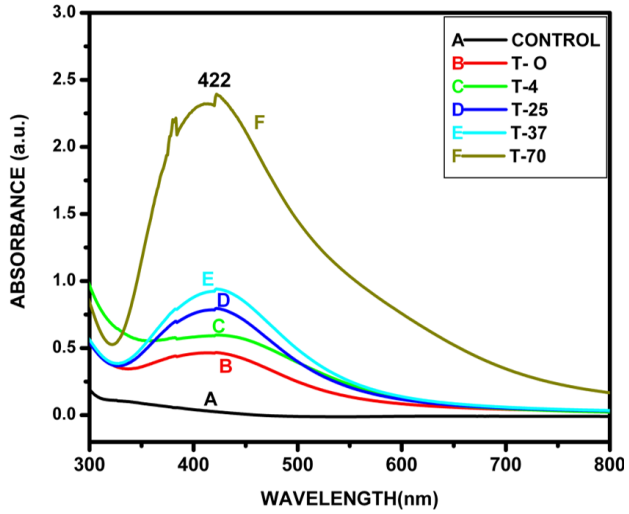

Fig. 2. UV-Vis spectra of silver nanoparticles synthesized from algal (*Spirogyra* sp.) filtrate at different temperature [Spectrum **(A)** Control, (B) 0^0C, (C) 4^0C, (D)25^0 C, (E)37^0C, **(**F**)** 70^0C].

From this it can be proved that temperature required for synthesis of nanoparticles varies depending on biological agents used for the synthesis of silver nanoparticles.

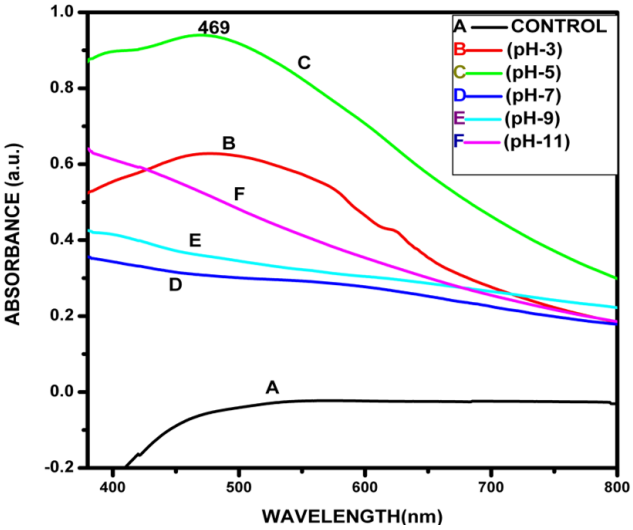

Fig. 3. UV-vis spectra of silver nanoparticles synthesized from algal (*Spirogyra* sp.) filtrate at different pH [Spectrum (A) Control, (B) pH 3, (C) pH 5, (D) pH 7, (E) pH 9, (F) pH 11].

Gradual increase in colour intensity and surface plasmon absorbance of filtrate was observed with rise in temperature, which indicates the increase in synthesis of silver nanoparticles. While studying the effect of different pH, pH-11 and pH-9 were found be better for the phycofabrication of the silver nanoparticles. From the work, it was noticed that the acidic pH supports the synthesis of silver nanoparticles only to a certain extent and form aggregation of nanoparticles, but the alkaline pH favors the better and stable synthesis of nanoparticles. The neutral pH was also found to support the phycofabrication of the nanoparticles (**Fig. 3**). The present study confirmed that increase in pH accelerates the reduction time of silver ions as well as stabilizes the silver nanoparticles by adsorbing on it. But on the other hand it showed controversy with results reported by Banu and Rathod [**28**].

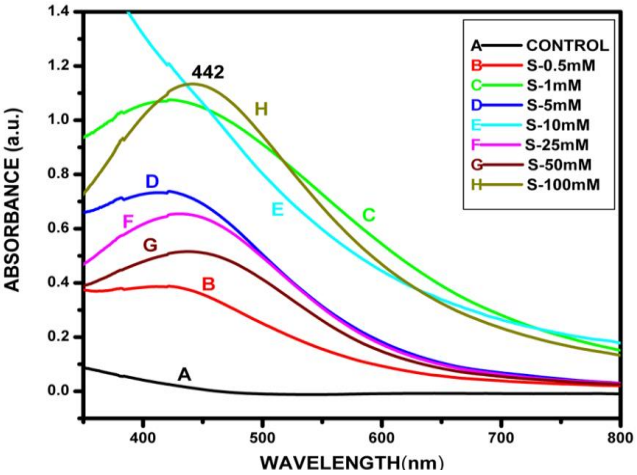

Fig. 4. UV-Vis spectra of silver nanoparticles synthesized from algal (*Spirogyra* sp.) filtrate by treating with different $AgSO_4$ concentration [Spectrum **(A)** Control, **(B)** 0.5 mM, **(C)** 1mM, **(D)** 5 mM, **(E)** 10 mM, **(F)** 25mM, **(G)** 50 mM, **(H)** 100 mM].

They synthesized silver nanoparticles using Rhizopus stolonier and observed that pH-7 was optimum for the synthesis of silver nanoparticles but at pH-8 no synthesis was observed. In case of effect of different salt concentration on synthesis of silver nanoparticles, it was found that the synthesis of the silver nanoparticle increases with the increase in the concentration of salt. The optimum synthesis was observed when the filtrate was treated with 100 mM of salt Ag_2SO4 (**Fig. 4**). While the salt concentration 0.5 mM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM also showed the synthesis in increasing order. This provides evidence that the synthesis of silver nanoparticles is directly proportional to salt concentration.

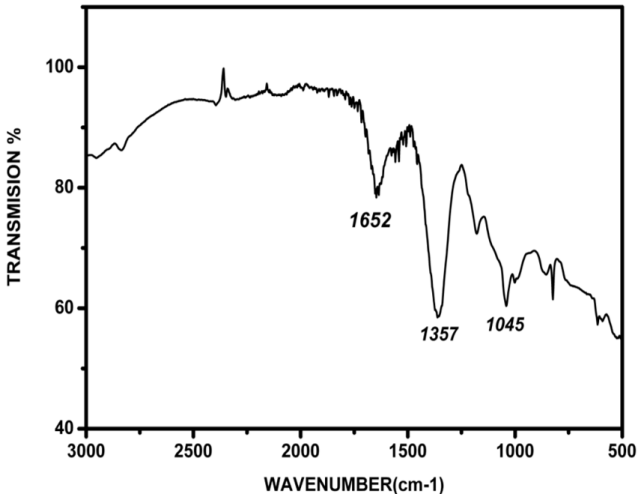

Fig. 5. FTIR spectrum for algal cell filtrate after treatment with 1mM silver sulfate solution. Showing the peaks at 1045, 1357 and 1652 cm^{-1} which was corresponds to functional groups -C-O-C-, residual nitrate solution, amide I and amide II groups respectively.

The FTIR spectrum of algal cell filtrate after treatment with $AgSO_4$ showed strong peaks at 1045, 1357 and 1652 cm^{-1}. This peak corresponds to different functional groups (**Fig. 5**). The peak at 1045 cm^{-1} can be assigned to -C-O-C- or -C-O- **[6, 29]**. The peak at 1652 cm^{-1} corresponds to the presence of amide I and amide II, which arises due to the carbonyl stretch and -N-H- stretch vibration **[16, 30]**.

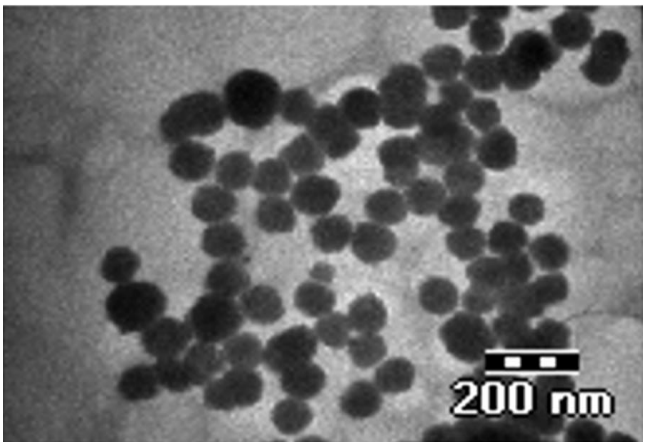

Fig. 6. TEM micrograph of silver nanoparticles synthesized by *Spirogyra* sp. Clearly showing the spherical particles size in the range of 70-90 nm.

Fourier transform infrared (FTIR) spectroscopic measurements revealed the fact that the protein is the possible biomolecules responsible for the reduction and capping of the biosynthesized nanoparticles. Transmission Electron Microscopy (**Fig. 6**) analysis finally confirmed the synthesis of spherical and polydispersed silver nanoparticles in the reaction mixture. The particles were in the range of 70-90 nm.

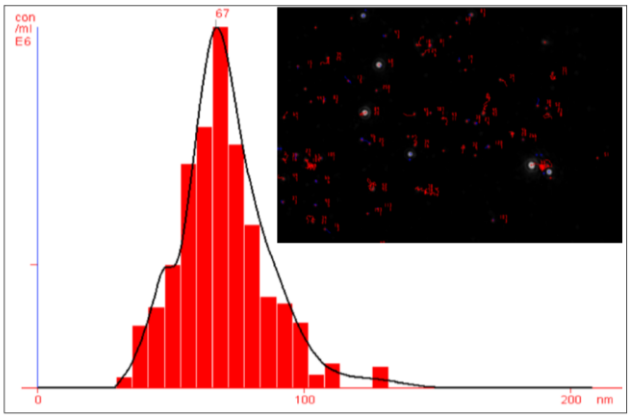

Fig. 7. Particle size distribution histogram of Silver Nanoparticles analyzed by Nanoparticle Tracking Analysis (NanoSight LM 20).
[**Inset Fig** Brownian motion of silver nanoparticles captured by CCD camera of NanoSight LM 20.]

NTA analysis by Nanosight LM-20 was also carried out to strengthen the results obtained from TEM. It actually includes Nanoparticle Tracking and Analysis (NTA) which measure the average size and its distribution of nanoparticles. NTA allows individual nanoparticles in a suspension to be microscopically visualized and their Brownian motion to be separately, but simultaneously analyzed (**Fig. 7**). From which the particle size distribution can be obtained on a particle-by-particle basis. **Fig. 7** shows the particle size distribution in the range 14 - 90 nm but average size having 67 nm and concentration of 1.044×10^{11} particles/ml. These results corroborate the findings by Rai *et al.* **[5]** and Montes-Burgos and group **[31]**. Metallic silver has been used since primitive times, as a potential antimicrobial agent **[3, 4, 32]**.

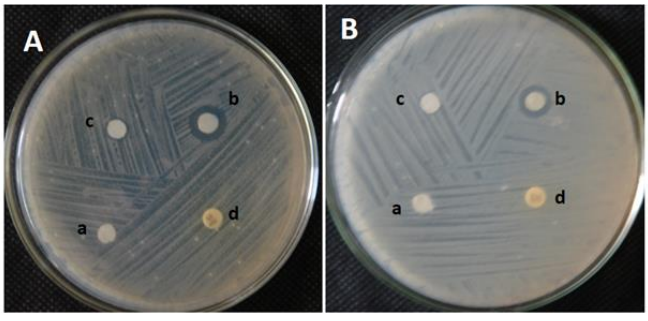

Fig. 8. Antimicrobial activity of silver nanoparticles against (A) *S. aureus* and **(**B**)** *E. coli* a-Algal filtrate, b- silver nanoparticles, c- 1 mM $AgSO_4$, d- Antibiotic (Ampicillin).

Hence, in the present study, we evaluated the efficacy of phycofabricated silver nanoparticles against Gram positive (*Staphylococcus aureus* - ATCC-25923) and Gram

negative bacteria (*Escherichia coli* - ATCC-25923) (**Fig. 8**). The silver nanoparticles thus synthesized from *Spirogyra* sp. showed potential antibacterial activity against *S. aureus* (13 mm) as compared to *E. coli* (11 mm). This result corroborates the earlier findings **[9, 33]**. The authors in their respective studies found that silver nanoparticles synthesized from *F. acuminatum* and *A. niger* respectively showed remarkable activity against *S. aureus* followed by *E. coli*. This is the first report of synthesis of silver nanoparticles by *Spirogyra* sp. using Ag_2SO_4 silver sulphate as a salt.

Conclusion

From the present study, it can be concluded that *Spirogyra* sp. is capable of synthesizing silver nanoparticles extracellularly with minimum efforts and at low cost. Generally, $AgNO_3$ is the common precursor used for the synthesis of silver nanoparticles; but in the present study concludes the use of Ag_2SO_4 as precursor for synthesis of silver nanoparticles using *Spirogyra* sp. by optimization of physical parameter like temperature, pH, and salt concentration, large scale synthesis of stable silver nanoparticles is possible. Phycofabricated silver nanoparticles showed potential antimicrobial activity against Gram positive and Gram negative bacteria and thus can be used in the formulation of nanoparticles independently as well as in combination with antibiotics as new generation antimicrobial.

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