Synthesis, Characterization and Optimization of Chicken-Bile Mediated Silver Nanoparticles: A Mechanistic Insight Into Antibacterial and Antibiofilm Activity

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Abstract

The fast-growing urbanization and slow progress in the field of waste management have led to the accumulation of large quantities of animal wastes. Present work focused on the synthesis of low-cost and eco-friendly chicken bile juice mediated silver nanoparticles (BJ-AgNP). Results reveals that bile juices have enough potentiality towards the synthesis of almost uniform sizes (average size < 50 nm) of BJ-AgNPs which remains stable for more than 6 months. Response surface methodology (RSM) successfully demonstrated the optimised condition of AgNP-BJ synthesis. Factor like concentration of salt and bile extract and temperature are significantly responsible for the synthesis of nanoparticle which was further characterized using UV-Vis, TEM, FESEM, XRD, FTIR, TGA, and EDS. The synthesised nanoparticle showed excellent bactericidal activity against both Gram positive and Gram negative bacteria with MIC and MBC of 40 and 50 µg/mL for *Bacillus subtilis* (MTCC-441) and 60 and 60 µg/mL for *Eschecheria coli* (MTCC-1687) respectively. The synthesized nanoparticle also acted as an antibiofilm agent against *Bacillus subtilis*, with ~89% biofilm inhibition efficacy at 4 X MIC, having optimal bacterial concentration of 10⁶ CFU/mL. Therefore, present findings clearly demonstrated that an absolute animal waste could be a valuable ingredient in the field of therapeutic nanoscience.

Introduction

In the recent years, synthesis of nanostructured materials has drawn the attention of scientists due to its unique physical and chemical properties. In order to produce specific sizes of nanoparticles, corresponding metal salts are reduced using various physical, chemical and biological processes (Golubeva et al. 2011; Mythili et al. 2018). Among various mode of nano synthesis biological synthesis has immense importance due to its flexible behaviour as electron donor and protection of nanoparticles along with non-polluting nature. Bio-conjugative material, such as protein, amino acids, biopolymers, carbohydrate etc. were extensively used in the field of nanoscience (Golubeva et al. 2011; Ramesh et al. 2016; Prasher et al. 2020; Espeche Turbay et al. 2021). Very recently, various researchers used plant extract, bacteria, fungi etc, as a green precursor for the synthesis of heavy metal nanoparticle (Saxena et al. 2012; Medda et al. 2015; Guilger-Casagrande and Lima 2019; Ren et al. 2019; Hamouda et al. 2019; Nilavukkarasi et al. 2020). Similarly, a large number of wastes were used including animal derived wastes like hair keratin, eggshell, feather, etc. were also used in nano synthesis (Liang et al. 2014; Lee et al. 2014; Sinha and Ahmaruzzaman 2015; Patnam et al. 2016; Wang et al. 2016; Gopiraman et al. 2017; Gao et al. 2019). But very limited literature are there on the animal derived fluid wastes like bile juices, blood etc. which contains steroidal amphipathic molecules such as –NH and –COOH group from fatty acids, cholesterol, proteins, phospholipids, bilirubin etc. (Nonappa and Maitra 2008; Rahmah 2021). Moreover, these materials may play vital role towards reducing the metals and subsequently stabilised the nanoparticles (Das et al. 2017; Jang et al. 2019). Animals derived waste products including bile is considered as complete waste in the slaughterhouses. However, use of bile is not new in the field of medicinal application. It was reported that black bear's dried bile was used for jaundice treatment (Beuers et al. 2015).

Nanomaterials, an emerging tool can be effectively used as an antimicrobial agent to solve the current problem (Li et al. 2013; Banerjee et al. 2020; Neves et al. 2021). A large number of nanomaterials are being widely used, among them, silver nanoparticles has attracted attention, due to its wide range of applications, starting from antibacterial application, wound healing, food packaging, and textile industries etc.(Dankovich and Gray 2011; Valli and Vaseeharan 2012; Durán et al. 2016; Jin et al. 2018; Riaz Rajoka et al. 2020; Minh Dat et al. 2021). Various reports regarding antibacterial activity of AgNP against both Gram-positive and Gram-negative have been made (Lekeshmanaswamy and Anusiyadevi 2020; Neves et al. 2021). In spite of its high potential antibacterial activity, they show poor stability leading to aggregate in aqueous solution, thus, the antibacterial activity is lost (Ghosh et al. 2012; Esmale et al. 2020; Thi Lan Huong and Nguyen 2021). Therefore, it becomes much important to hunt for novel biological route of silver nanoparticle synthesis which can overcome the above mentioned drawbacks. It is also evident that the size, shape and stability of the nanoparticle largely depends upon the interaction of reducing agent with metal ion, capping agent and metal nanoparticles (Mondal et al. 2019, 2021). Therefore there is urgent need to find a novel precursor for biological synthesis of silver nanoparticle which can overcome the above said drawbacks.

In the recent years biofilm formation has been considered as a major concern in the field of the drug resistance strategies (Abebe 2020). Biofilms are the association of microorganism usually bacteria or fungi, which can resist the antimicrobial agents as well as the immunity defence mechanism of human leading to infection and other fatalities (Khatoon et al. 2018). The associated microorganism for the biofilm formation usually in the healthcare equipment like catheters and other implants are namely, *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Bacillus sonorensis* (Sanchez et al. 2016; Yilmaz Öztürk et al. 2020; Mohan and Panneerselvam 2021). Biofilm formation takes place with a single microbial cell attachment on the surface of the substratum. After that, it starts forming the conditioning film formed due to self-produced (EPS) insoluble extracellular substance adhered due to electrostatic interaction (Abebe 2020). Multilayer colonization starts after cell to cell interaction and leads to the maturation of the biofilm. This formed bilayer acts as a barrier to the antimicrobial agents leading to failure of the drug therapy (Roy et al. 2017; Singh et al. 2017).

Additionally, morphological modification and controlled processes for AgNPs synthesis have gained attention in recent years. Various reports have been made on the process parameter optimization of silver nanoparticle synthesis to increase the yield of production based on the Surface Plasmon Resonance (SPR) data (Ren et al. 2019). Statistical optimization is a wonderful time reduction technique which is widely used to optimize the operational parameters in nano synthesis (Baghkheirati and Bagherieh-Najjar 2016; Sen et al. 2020; Mondal et al. 2021). Several methods are available for optimization such as Artificial Neural Network (ANN) based (Sen and Mondal 2020), Response Surface Methodology (RSM) (Sen et al. 2020; Azmi et al. 2021) etc. However, the RSM is a potent tool due to its easy operational procedure, good performance for low input data etc. (Sen et al. 2019).

In present study, silver nanoparticles have synthesised from bile juice as a novel agent and the synthesised nanomaterial was characterized by advanced analytical techniques and optimization of

nano synthesis was performed by RSM study. Finally, the efficacy of BJ-AgNP was evaluated through antibacterial activity against both the Gram positive (*Bacillus subtilis*) and Gram negative (*Escherichia coli*) bacterial strains. Metabolic activity, respiratory dehydrogenase enzyme activity, intracellular glutathione activity, protein leakage were observed to identify the therapeutic potential of the synthesised nanomaterial. Live dead assay was further carried out to monitor the viable and dead cell in the BJ-AgNP exposure. Lastly, the synthesised nanomaterial was also used as a biofilm inhibiting agent.

Materials And Methods

Experimental design

The present work is distinctly focused upon two major applications. Bile juice is first collected and extracted, then mixed with silver salt solution in various ratios to determine whether BJ-AgNP is synthesized, which is then optimised and characterized. Finally, the efficacy of the synthesised BJ-AgNP in inhibiting microbial growth and biofilm formation were checked by following various experiments.

Reagents

Chemicals used in the experiments like Silver nitrate (AgNO_3) salt from Sigma-Aldrich (ACS reagent, $\geq 99.0\%$). Methylene blue and other culture media were purchased from HiMedia Laboratories (Mumbai, India). Throughout the experiment, Milli-Q ultrapure water was used. Before conducting the experiments, glassware were cleaned in aqua regia and autoclaved. Reagents were used in the same condition as provided.

Preparation of silver nanoparticle (BJ-AgNP)

Bile induced silver nanoparticles was prepared by using bile juice, which was collected from slaughterhouse, Burdwan town (Latitude $99^\circ 23'15'' 22''$ N Longitude $87^\circ 50'58''$ E) West Bengal, India. Gallbladders were pierced with sterile micro tip and juice extracted in sterile test tube (Borosil Pvt. Ltd, Gujrat, India). Different concentrations of bile juice were taken and volume makeup was done using Milli-Q water, under strong stirring condition. Passed through filter paper (Whatman No 1, USA) then added drop wise in freshly made silver salt solution (1 ppm) in different ratios. Each sets of experiment were conducted under different temperature. Aliquots of the resulting solution are taken in cuvette for UV-Vis analysis. Further, the synthesized BJ-AgNP solution is lyophilized using (Biobase Ltd.) lyophilizer. The dried powder was used for further study.

Optimization of BJ-AgNP synthesis

For optimizing of synthesis condition of BJ-AgNP, in order to gain high yield in respect to the peak intensity of the absorbance (a.u), data analysis was done using Design- Expert (Version 7.0.0) statistical software. The design of the experiment (DOE) was constructed with three parameters (temperature, Bile concentration and salt concentration). Number of experimental run was suggested by face centered central composite design (FCCCD) at three levels. Response indicates the absorbance intensity as SPR

for yield attributes to response having wavelength ranging between 410-430 nm for evident of the BJ-AgNP synthesis.

Characterization of the BJ-AgNP

Surface plasma resonance was estimated using UV-visible spectroscopy (Optizen-POP). Fourier transform infrared (FTIR) spectral analysis was performed using (Cary 630, Agilent Technology). BJ-AgNP solution was lyophilized using BIOBASE, China. Surface morphology and characterization were performed by TEM (JEOL JEM 1200, assisted with Gatan Image Filter) at an accelerating voltage of 120 KV, and FESEM (GeminiSEM 360 ZEISS). Elemental quantification of BJ-AgNP was estimated using EDS (GeminiSEM 360 ZEISS). Dynamic light scattering (DLS) (Malven Instruments) was employed for hydrodynamic size, aggregation and polydispersity of the BJ-AgNP. X-ray diffraction (XRD) analysis for BJ-AgNP was captured with K-beta filter having radius 1.54056 Å. The thermal stability for biogenic AgNP-BJ was examined through thermo gravimetric analyser (PerkinElmer, TGA 8000), whereas temperature range into subambient to 1200°C, 10°C/minute in Nitrogen atmosphere with sampling rate of 20 mL/minute.

Antibacterial activity evaluation

Culture media and strains used in the study

Two bacterial strains were used as test organism for antibacterial study namely, *Eschecheria coli* (MTCC-1687) and *Bacillus subtilis* (MTCC-441) collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Nutrient Agar (N.A) and Nutrient Broth (N.B) of bacteriological grade were used as culture medium. Both the Gram positive and Gram negative bacterium were grown in liquid broth at 37° C and 120 rpm for incubating overnight and then sub-cultured in NB using a culture having OD (optical density)=1, approximately 10⁵ CFU/mL.

Agar diffusion method

Agar well diffusion experiment was carried out using the said bacterial strain (*E.coli* and *Bacillus subtilis*). 100 µL inoculum for both the overnight grown culture were well spread in NA plates and left to dry for 5 minute. Wells (diameter 11 mm) punctured using sterile cork borer (Himedia, Mumbai). Each well was marked and different concentrations of BJ-AgNP were added along with a negative control and raw bile juices were loaded in respective wells. Suspensions of nanoparticles were sonicated in a bath sonicator before loading into the well. After overnight incubation, the zone of inhibition around the wells was measured using a scale. The experiments were conducted in triplicates for each bacterial strain, and the mean and standard deviation were calculated in the results section (Thi Lan Huong and Nguyen 2021).

Broth dilution method

Broth dilution method was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of BJ-AgNP against *Escherichia coli* (MTCC 1687) and

Bacillus subtilis (MTCC 441). 1% v/v inoculum was given in 200 µL NB broth, which taken in 96- micro well plate reader (Tecan Infinite M200 Pro) at 600 nm.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination

MIC and MBC for both *E. coli* and *B. subtilis* against BJ-AgNP, were estimated by following the standard micro broth dilution assay. In short, 1% v/v of bacterial culture was inoculated in 200 µL of LB broth taken in each microtitre well. BJ-AgNP was added in increasing concentration and the plate is incubated overnight at 37° C and 120 rpm. To check the growth of the treated bacterial cell, OD was taken at 600 nm. The lowest concentration of BJ-AgNP showing $OD_{600} < 0.1$ were reinoculated without treatment into fresh LB broth and allowed to incubate further for 12 hours at 37° C and 120 rpm. The lowest concentration of AgNP-BJ that completely killed the bacteria (i.e., wells with $OD_{600} < 0.1$ value) was considered as the MBC value.

Time dependent in vitro killing assay (growth kinetics)

Bacterial growth pattern in presence of the treatment (BJ-AgNP) can be observed following the experiment. 200 µL cell suspensions (10^6 CFU/mL) were taken in 96 microwell plate and treated with various concentration of BJ-AgNP at 37°C for 12 hours. Absorbance (600 nm) was monitored at an interval of 30 minute using microwell plate reader (Tecan Infinite M200 Pro). Data analysis and graph plotting were done using Origin 9.1 software.

Measurement of intracellular Glutathione (GSH) activity

Glutathione (GSH) acts as an antioxidant and protects cell from oxidative stress related damage. GSH upon oxidation, converts to oxidized glutathione(GSSG) which fails to protect cells from oxidative damage, eventually oxidizes vital cellular components like protein, nucleic acid and lipid, finally leads to cell death (Ulrich and Jakob 2019). Intracellular GSH activity was measured following standard Ellman's assay, as reported in previous literature (Parandhaman et al. 2015). The *E. coli* and *B. subtilis* (10^6 CFU/mL) were treated with increasing concentrations of AgNP-BJ for 6 h at 37° C and 200 rpm. Bacterial cells suspensions were centrifuged at 4000 rpm for 10 min, washed with PBS and finally lysed, supernatant was collected after re-centrifugation and mixed with 50 mM Tris-HCl and 100 mM 5, 5-dithiobis (2-nitrobenzoic acid). The mixture was kept in dark place for 30 min incubation. Absorbance analysis was done at 412 nm using UV-visible spectrometer. The loss of glutathione were compared with control using the following formulae

$$GSH(\%) = \left(1 - \frac{OD \text{ at } 412nm \text{ of treated}}{OD \text{ at } 412nm \text{ of control}}\right) \times 100$$

Measurement of Metabolic Activity

The metabolic activity was estimated by Alamar blue assay methods, herein the cells get converted to a pink fluorescent dye resofurin from purple resazurin (nonfluorescent). Cell treatment combination of 1 X MIC to 4 X MIC, were kept for 6 h at 37° C and 200 rpm for dose exposure. *E. coli* and *B. subtilis* are incubated at 37° C with 25 µL of 50 µg/mL resazurin solution for 1 h. The metabolic conversion of pink coloured resofurin from resazurin was measured spectrophotometrically at 571 nm (Rampersad 2012a).

Measurement of respiratory dehydrogenase enzyme (LDH) activity

The LDH enzyme activity was measured using Pierce LDH Cytotoxicity Assay Kit. Both control and treated bacterial cells of *E. coli* and *B. subtilis* incubated for 6 h at 37°C and 200 rpm. Cells were centrifuged at 5000 rpm for 5 min and supernatant was discarded, pellet was washed with PBS and LDH reaction solution was added. Then 30 min incubation in dark was followed for red formazan production. Stop solution added to stop the reaction and absorption taken at 490 nm. Results obtained after comparing the % LDH activity of the treated cell with that of control (Roy et al. 2019).

Protein Leakage Assay

E. coli and *B. cereus* cells (10⁶ CFU/mL) were treated with increasing concentrations of BJ-AgNP for 6 h at 37°C and 200 rpm. The bacterial cells were pelleted at 5000 rpm for 5 min and the supernatant was collected to measure the amount of protein leaked by following the standard Bradford assay (Kim et al. 2017; Roy et al. 2019).

Antibiofilm study

To know the efficacy of the synthesised BJ-AgNP against the biofilm formation, the experiment was conducted using *E. coli* (MTCC-1687). Biofilm were grown with 48 hrs culture in a 96 microwell plate with increasing dose of BJ-AgNP treatments and control. The media was decanted after complete incubation and washed with sterile PBS (Phosphate buffer saline) solution and air dried. Excess stains were solubilised using 95% ethanol and OD was taken at 590 nm using a microwell plate reader (Tecan Infinite M200 Pro). During the inhibition calculation the following formula was followed

$$\%Inhibition = \frac{Control\ absorbance_{575nm} - Treatment\ absorbance_{575nm}}{Control\ absorbance_{575nm}} \times 100 \quad (2)$$

Results And Discussion

Synthesis and optimization of BJ-AgNP

A wine red colour solution was formed after mixing bile juice with 100 ppm of silver nitrate solution in the ratio of 1:9. This is perhaps due to the Surface Plasmon resonance (SPR) of silver nanoparticle (Mondal et al. 2021). The synthesis of nanoparticle was monitored after varying factors like temperature, amount

of bile and concentration of silver nitrate solution, which is shown in Fig. 1, and data was recorded following optimization study. From Fig. 1a, it is seen that varying bile concentration and temperature interfered the BJ-AgNP synthesis. Moreover, bile and salt concentration are responsible factor for nanoparticle formation which is clearly showed in Fig. 1b. The silver salt concentration and temperature interaction corresponding BJ-AgNP synthesis shown in Fig. 1c. Therefore, results revealed that bile concentration is the regulatory factor which largely affects the yield of silver nanoparticles. This may be due to its composition, including bilirubin and fats (cholesterol, fatty acids, and lecithin) which collectively helps towards the reduction of Ag^+ ions (Mandal et al. 2002). On the other hand, the synthesis of AgNPs was recorded highest at higher temperature (near boiling point). At higher temperature bile molecules undergoes thermal degradation which helps to make the availability of free electron in the medium and subsequently reduction of Ag^+ to Ag^0 . Therefore, temperature plays an important role in nanoparticle synthesis (Jiang et al. 2010). Moreover, the synthesised nanoparticle were protected from agglomeration by bile containing biomolecules. The outcomes of optimization study were depicted in Table S1. From Table S1 it is clear that the model is significant ($P < 0.001$) with very high F value (97.57). Modelling study also revealed that the silver nano synthesis is strongly influenced by temperature ($P < 0.0001$) and bile concentration ($P < 0.0001$). On the other hand higher desirability (1.00) was achieved under the following conditions: temperature, (99.73° C) bile concentration (5.70 $\mu\text{L/mL}$ (v/v)) and salt concentration (0.30 mg/L) (Fig. 1.d).

Characterization of BJ-AgNP

UV Vis study

In order to investigate the optical response of bile juice mediated AgNPs which is the results of intense surface plasmon resonance at visible region, UV-Vis absorption spectroscopy was initially performed (Sharifi-Rad et al. 2021). The distinct colour change of the mixture was observed when the silver nitrate solution was mixed with chicken bile juice. This colour change is a clear indication for the formation of the silver nanoparticles which is again confirmed from the shift of λ_{max} peak in the UV-Vis spectrum (Fig. 2a) (Saad et al. 2021). In the present study two sharp spectral bands were recorded at 430 and 400 nm for BJ-AgNPs and raw bile juice, respectively (Fig. 2a). Moreover, symmetrical spectral also revealed that the synthesised AgNPs are uniform in shape (Yılmaz Öztürk et al. 2020).

FESEM, EDX, TEM and DLS study

Field Emission Scanning Electron Microscopy (FESEM) clearly revealed the crystalline spherical structure of BJ-AgNP (Fig. 3a). Figure 3a also showed that the synthesised BJ -AgNP, are well dispersed and mostly spherical in shape. Moreover, from figure 3a, it is also clear that about $25.76\% \pm 3.77$ particles are within 40 nm. The size distribution patterns of nanoparticles are depicted in Fig. 3b. The EDX study demonstrates the clear signature of silver metal (Fig. S2). Similarly, dynamic light scattering (DLS) study also supports the above results. The dimension of particles is depicted in Fig. 2c. The nanoparticles size ranges from 30-45 nm with average particle size 40 ± 1.55 nm. The polydispersity index was recorded 0.181 and Zeta potential (ζ) ranges from -14.74 to -41.79 mV. This high value of Zeta potential clearly

suggest the higher stability of synthesised nanoparticles. Present findings are in accordance with previous literature (Hebeish et al. 2013; Haque et al. 2017). Transmission electron microscopy (TEM) study also supports the above outcome (Fig. 3b). Selected area electron diffraction (SAED) pattern again demonstrated the polycrystalline structure of the BJ-AgNP, with miller indices (111), (200), (220), and (311)., corresponds to face-centered cubic (FCC) structure (Fig. 2d) (Yılmaz Öztürk et al. 2020).

FTIR, XRD and TGA analysis

The FTIR spectral signature of both raw bile juice and bile mediated AgNPs were recorded for the identification of functional group (Garibo et al. 2020). The possible chemical profile of bile revealed that the presence of alkyl halide, aldehyde, amines and aromatic compounds with transmission peaks at 1250, 2863, 1053 and 1405 cm^{-1} , respectively (Fig. 3a). However, bile mediated silver nanoparticles (BJ-AgNP) showed three distinct peaks at 3735 cm^{-1} , 1650 cm^{-1} , and 1405 cm^{-1} which corresponds the stretching frequencies of free hydroxyl $-\text{OH}$, $-\text{C}=\text{O}$ and $\text{C}=\text{C}$ (aromatic), respectively. These functional group could be responsible for the synthesis of nano particle as well as capping of Ag^0 (Kleiner et al. 2002). This explores the dual nature of bile juice. Figure.3a also revealed that peaks at 1393.75 cm^{-1} shifted to 1405.55 cm^{-1} assigning the $\text{C}=\text{C}$ aromatic (weak) stretching, 2373.61 cm^{-1} shifted to 2379.86 cm^{-1} for vibrational of $\text{H}-\text{C}=\text{O}$: $\text{C}-\text{H}$ stretch (aldehydes), 2863.19 cm^{-1} shifted to 2869.44 cm^{-1} for $\text{H}-\text{C}=\text{O}$ (alkenes) vibrational stretching, 1065.27 cm^{-1} shifted to 1053.47 cm^{-1} for $\text{C}-\text{N}$ stretch (aliphatic amines) (Slavin et al. 2017). Moreover, the FTIR spectrum of the formed biofilm and biofilm treated with AgNP-BJ, are represented in Table S3. From the Table S3 it is clear that peaks at 2959.02 cm^{-1} shifted to 2929.16 cm^{-1} corresponds to $-\text{C}-\text{H}$ stretching. Similarly, peaks at 856.25 cm^{-1} (corresponds $\text{C}-\text{S}-\text{H}$ bending of thiol groups), 2337.5 cm^{-1} (alkynyl $\text{C}=\text{C}$ stretch, 2373.61 cm^{-1} (aldehydes vibrational stretching) and 1543.05 cm^{-1} (for $\text{N}-\text{O}$ asymmetric stretch, nitro compounds). Previous reports also supports that those peaks are responsible for the deformation of the β -(1,4)-glycosidic bond of the peptidoglycan layer of the bacterial cell wall, leading to the inhibition of the bacterial growth (Nikonenko et al. 2000; Feo and Aller 2001; Slavin et al. 2017). The spectral signature of BJ-AgNP formation is remarkably changed when BJ-AgNP interacted with formed biofilms (Fig. 2b).

Another analytical technique, XRD analysis revealed that the synthesised BJ-AgNP has distinct six measured d-spacing at 21.38° ($d_{\text{hkl}} = 2.11 \text{ \AA}$, FWHM = 0.152°), 21.95° ($d_{\text{hkl}} = 2.05 \text{ \AA}$, FWHM = 0.134°), 24.29° ($d_{\text{hkl}} = 1.86 \text{ \AA}$, FWHM = 0.155°), 30.12° ($d_{\text{hkl}} = 1.53 \text{ \AA}$, FWHM = 0.045°), 31.87° ($d_{\text{hkl}} = 0.036 \text{ \AA}$, FWHM = 0.015°) and 37.82° ($d_{\text{hkl}} = 0.056 \text{ \AA}$, FWHM = 0.071°) which corresponds to miller indices of (111), (200), (220), (311), (222) and (300), respectively (JCPDS # 01-087-0719) (Fig. 3c) (Yılmaz Öztürk et al. 2020). The average size of the synthesized BJ-AgNP was also calculated by following the Scherrer equation (Sen et al. 2020), and the average size of 41.81±1.66 nm.

Similarly, TGA is one of the important characterization tool which confirmed the presence of organic capping layer over silver nanoparticles. Weight loss depends on moisture loss and organic compounds decomposed that exist in the BJ-AgNP compound (Paterlini et al. 2021). The current results of BJ-AgNP

and bile juice were shown in Fig. 3d. Moreover, from Fig. 3d, it has been found that the synthesised BJ-AgNP, losses 15.39% weight when temperature increased from 400°C to 600°C. Similarly raw bile loosed 37.46% weight at 500°C. Therefore, TGA study clearly revealed that the synthesised BJ-AgNP are moderately stable. This is perhaps due to the presence of strong organic capping agents (Thi Lan Huong and Nguyen 2021).

Antimicrobial activity

Primarily, agar cup diffusion method was applied to evaluate the antimicrobial activity of synthesised BJ-AgNP. Diameters of the zone of inhibition were measured after 24 h of incubation at 37°C and the results are depicted in Fig S4. The zone of inhibition obtained with 2X MIC doses of BJ-AgNP, were 2.5 and 2.1 cm for *E. coli* and *B. subtilis*, respectively and non-significant zone of inhibition was recorded for raw bile juice (Fig. S4). The Zone of inhibition is probably due to damage of bacterial cell walls through penetration of silver nanoparticles (Liao et al. 2019). The diameter of the zone of inhibition is very less and marginal difference was observed between 1X MIC and 2X MIC concentration. This may happened due to the aggregation of the BJ-AgNP in the agar medium.

Similarly, results of MIC and MBC value of BJ-AgNP against *E. coli* and *B. subtilis* were depicted in Table S4. The MIC and MBC values of BJ-AgNP against *E. coli* were recorded as 60 and 60 µg/mL and for *B. subtilis* as 40 and 50 µg/mL respectively (Table 1). These results clearly revealed that BJ-AgNP could have lower efficiency against *E.coli* compared to *B. subtilis*. Almost similar findings was recorded by Bruna et al. 2021 (Bruna et al. 2021) Antibacterial potentiality was also examined by MBC/MIC ratio and it was recorded as ≤ 1.2 for both *E. coli* and *B. subtilis* which clearly suggests the potentiality BJ-AgNP is an active bactericidal agent (Table 1) (Roy et al. 2019). Study also highlighted the time dependent growth inhibition and complete reduction was observed at 9th hour of exposure (Fig. S3).

Table 1

MIC, MBC, and MBC/MIC ratio of AgNP-BJ

Organisms	MIC value (µg/mL)	MBC value (µg/mL)	MBC/MIC ratio
<i>E. coli</i>	60	60	1
<i>B. subtilis</i>	40	50	1.25

The growth kinetics of *E. coli* and *B. subtilis* were shown in Fig. 5a and 5b respectively. From the figure 5a and 5b it is clear that complete inhibition occurred at 90 and 80 µg/mL of BJ-AgNP for *E. coli* and *B. subtilis* respectively. Antibacterial efficacy of BJ-AgNP against *E. coli* was also determined after 12 h of exposure and results shows following trend of reduction with respect to control as: 20 mg/L (61.66%) > 40 mg/L (51.45%) > 60 mg/L (12.33%) >80 mg/L (5.39%) > 100 mg/L (1.29%). Similarly, for *B. subtilis* showed that the growth reduction trend as; 20 mg/L (31.29%) > 40 mg/L (30.39%) > 60 mg/L (1.22%) > 80 mg/L (3.59%) > 100 mg/L (0.79%) after 12 h.This information also suggested that the growth curves

gradually declines with increasing the concentration of BJ-AgNPs. This is possibly due to direct intervention of BJ-AgNP resulting in the degradation of enzyme, DNA and inactivation of cellular proteins (Nikaido 2003). Recent study suggested that nanoparticle induced cell death is due to generation of reactive oxygen species (ROS) which subsequently damages protein and other biomolecules (Rohde et al. 2021).

Mechanism of Antibacterial Action

Metabolic Arrest

In present study, by observing the colour change of non-fluorescent purple dye resazurin to resofurin (pink fluorescent) which is the indicator of metabolically active cells load, depicted in Fig. 6(a). From Fig. 6(a) it was clearly observed that metabolic activity of both the bacterial strains (*E. coli* and *B. subtilis*) are greatly hampered under different concentrations of BJ-AgNP. At 4 X MIC, *E. coli* and *B. subtilis* showed 53.44% and 62.26% reduction in metabolic activity respectively. Therefore, it is clearly established that BJ-AgNP upon interacting with bacterial cells impeded the cellular respiration leading to metabolic arrest and ultimately cell death occurs (Rampersad 2012b)

Inhibition of lactate dehydrogenase activity (LDH)

The LDH activities under different concentration of nanoparticle treatment in two studied bacterial species (*E. coli* and *B. subtilis*) were presented in Fig. 6(b). Fig. 6(b) highlighted that reduction of LDH% increases from 33.26–72.34% with increasing the MIC concentration from 1X to 4X for *E. coli*. On the other hand, *Bacillus subtilis* also exhibited similar reduction of LDH% (55.73%) level at 4X MIC. Results clearly depicts that BJ-AgNP treatment on bacterial cells has greatly affected the LDH activity. Extensive membrane damage under nano treatment could be the possible cause that inhibited the respiratory enzyme (Roy et al. 2019).

Intracellular glutathione oxidation

In the present study, percent loss of GSH activity was evaluated with respect to control and it is presented in Fig. 6 (c). Figure 6 (c) indicate a progressive loss of GSH activity for both *E. Coli* and *Bacillus subtilis* with 25.79% and 22.79% at 4 X MIC treatment of BJ-AgNP respectively. This phenomenon can be attributed by the fact that the entry of BJ-AgNP inside the bacterial cell due to the small size of BJ-AgNP. Disruption of lipid bilayer occurs which may be the additional cause of ROS generation along with the membrane damage (Fahey et al. 1978). Therefore, it can be said that BJ-AgNP possesses an overall improved antibacterial efficacy.

Protein Leakage

The percentage of protein leakage was recorded from the two target species (*E. coli* and *B. subtilis*) under treated and control conditions are depicted in Fig. 6d. The result revealed that maximum protein leakage was recorded at 4 X MIC for *E. coli*. On the other hand, nearly 35% protein leakage is enhanced when *Bacillus subtilis* cells treated with 2 X MIC concentrations with respect to MIC concentration. On the other

hand, TEM images (Fig. 4e and 4f), clearly supported BJ-AgNP is responsible for damaging the cell. From the TEM image it is also clear that fluid dispersion and protein leakage is due to the damage of the peptidoglycan layer (Roy et al. 2019).

Finally, trypan blue exclusion assay has been introduced to observe the cell viability under different concentration of BJ-AgNP and the microscopic results were shown in Fig. 4. The live cells for both bacteria under controlled experiment were depicted in Fig. 4a and 4b. The cells of *E. coli* and *B. subtilis* were taken in glass slides and it is observed that 2X MIC concentration is quite effective to kill the cells (Fig. 4c and 4d).

Biofilm inhibition assay

The result of the biofilm inhibitory assay with respect to *E. coli* was demonstrated in Fig. 5c and 5d. Present result revealed that BJ-AgNP exhibited 49.73% inhibition at 1 X MIC with respect to the control. Meanwhile, 65.24% of inhibition was observed at 2 X MIC with respect the control. However, at 4 X MIC, almost 89% inhibition of the biofilm formation with respect to control was recorded. The result indicates that BJ-AgNP successfully crossed the extracellular polymeric matrix of biofilm to ultimately invade bacterial cells by causing membrane damage. The intensity of the stained preformed biofilm plate gradually decreases with the increase in the concentration of the BJ-AgNP treatment, depicting that the synthesised BJ-AgNP have a strong antibiofilm property shown in dose dependent manner.

The antibacterial activity of silver nanoparticles synthesised from various biogenic sources has been presented in Table 2. From Table 2 it is clear that the size of the nanoparticle is directly relate to the minimum inhibitory concentration (MIC) of the nanoparticles. Data revealed that smaller the size of AgNPs, lower is the MIC value (7.8-40 µg/ml). This is probably due to higher level of silver ions rereleased from smaller size of AgNPs (Sotiriou and Pratsinis 2010) (Table 2). Present study results exhibited moderate MIC against *E. Coli* (60 µg/ml) and *B. subtilis* (40 µg/ml) (Table 2). AgNP (22.46 nm) synthesised from *A. rigidula* extract when applied to on *E. Coli* (ATCC11229) showed MIC value of 62.5 µg/mL (Escárcega-González et al., 2018). Similarly, AgNP synthesis (20–40 nm) from red algae *Gelidium corneum* extract showed MIC value of 0.26 µg/mL for *E. coli* (ATCC25922)(Yılmaz Öztürk et al. 2020). The synthesised BJ-AgNP showed better antibacterial activity than many of the listed nanoparticles (Table 2).

Table 2
Comparative table for current study relevant with previous biogenic silver nanoparticle and effect on bacterial stain

Synthesis	Average particles Size	Bacteria stain	MIC	References
Chemical Purchase	120 nm	<i>N. gonorrhoeae</i> (ATCC49226)	500 µg/mL	(Li et al. 2013)
Extract of <i>Salvadora persica</i> bark	50 nm	<i>E. coli</i> (ATCC 25922)	100 µg/mL	(Miri et al. 2016)
Extract of <i>Salvadora persica</i> bark	50 nm	<i>S. aureus</i> (ATCC 25923)	400 µg/mL	(Miri et al. 2016)
Ear leaves of <i>Z. mays</i> L.	45.26 nm	<i>E. coli</i> (ATCC 43890)	50 µg/mL	(Patra and Baek 2017)
Ear leaves of <i>Z. mays</i> L.	45.26 nm	<i>S. Typhimurium</i> (ATCC 43174)	25 µg/mL	(Patra and Baek 2017)
Ear leaves of <i>Z. mays</i> L.	45.26 nm	<i>B. cereus</i> (ATCC 13061)	25 µg/mL	(Patra and Baek 2017)
Ear leaves of <i>Z. mays</i> L.	45.26 nm	<i>S. aureus</i> (ATCC 49444)	12.5 µg/mL	(Patra and Baek 2017)
Ear leaves of <i>Z. mays</i> L.	45.26 nm	<i>S. Typhimurium</i> (ATCC 43174)	50 µg/mL	(Patra and Baek 2017)
Rice straw biomass	77.3 nm	<i>E. coli</i> (ATCC25922)	16 µg/mL	(Li et al. 2017)
Rice straw biomass	77.3 nm	<i>P. Aeruginosa</i> (ATCC27853)	8 µg/mL	(Li et al. 2017)
Rice straw biomass	77.3 nm	<i>S. aureus</i> (ATCC29213)	32 µg/mL	(Li et al. 2017)
Rice straw biomass	77.3 nm	<i>B. subtilis</i> (ATCC6633)	16 µg/mL	(Li et al. 2017)
Casein hydrolysate	30 nm	<i>V. natriegens</i>	2.4 µg/mL	(Dong et al. 2019)
Pu-erh tea leaves extracts	4.06 nm	<i>E. coli</i> ATCC 25922	7.8 µg/mL	(Loo et al. 2018)
Pu-erh tea leaves extracts	4.06 nm	<i>K. pneumoniae</i> ATCC 13773	3.9 µg/mL	(Loo et al. 2018)
Pu-erh tea leaves extracts	4.06 nm	<i>S. Typhimurium</i> ATCC 14028	3.9 µg/mL	(Loo et al. 2018)

Synthesis	Average particles Size	Bacteria stain	MIC	References
Pu-erh tea leaves extracts	4.06 nm	<i>S. Enteritidis</i> ATCC 13076	3.9 µg/mL	(Loo et al. 2018)
<i>A. rigidula</i> extract	22.46 nm	<i>E. coli</i> (ATCC11229)	62.5 µg/mL	(Escárcega-González et al. 2018)
Red algae <i>Gelidium corneum</i> extract	20–40 nm	<i>E. coli</i> (ATCC25922)	0.26 µg/mL	(Yılmaz Öztürk et al. 2020)
Red algae <i>Gelidium corneum</i> extract	20–40 nm	<i>C. albicans</i> (ATCC14053)	0.51 µg/mL	(Yılmaz Öztürk et al. 2020)
<i>Carya illinoensis</i> leaf extract	20.34 nm	<i>E. coli</i> (ATCC 25922)	16 µg/mL	(Javan bakht Dalir et al. 2020)
<i>Carya illinoensis</i> leaf extract	20.34 nm	<i>S. aureus</i> (ATCC 25923)	128 µg/mL	(Javan bakht Dalir et al. 2020)
<i>Carya illinoensis</i> leaf extract	20.34 nm	<i>L. monocytogenes</i> (ATCC 7644)	64 µg/mL	(Javan bakht Dalir et al. 2020)
<i>Carya illinoensis</i> leaf extract	20.34 nm	<i>P. aeruginosa</i> (ATCC 27853)	32 µg/mL	(Javan bakht Dalir et al. 2020)
<i>Chicken Bile</i>	40 nm	<i>E. coli</i> (MTCC 1687)	60 µg/mL	Current Study
<i>Chicken Bile</i>	40 nm	<i>B. subtilis</i> (MTCC 441)	40 µg/mL	Current Study

Conclusions

According to the current study, BJ-AgNP were successfully prepared using chicken bile juice as waste material, exhibiting spherical shape, high crystallinity, average size of 40 nm, and high stability. For nanoparticle synthesis, statistical optimization was successfully applied to minimize process parameters. It is highly effective against Gram positive (*B. subtilis*) and Gram negative bacteria (*E. coli*) with MIC values of 40 and 60 µg/mL, respectively. Results also revealed that synthesized BJ-AgNP kills bacteria through metabolic arrest, protein leakage, intracellular glutathione oxidation, and inhibition of lactate dehydrogenase activity. In these studies, bacterial membrane damage, oxidative damage, and a loss of metabolic activity are clearly demonstrated. BJ-AgNP inhibited biofilm formation almost to 90% at 4 X MIC concentration, as demonstrated by the biofilm inhibition assay. Therefore, from the present findings it can be concluded that, BJ-AgNP can act as a promising antimicrobial agent against both Gram positive and Gram negative pathogenic bacteria. Moreover, it can be used as a potent antibiofilm agent. Finally it can be concluded that the bile juice could be an effective ingredient towards synthesis of noble metal nano synthesis. However, further research should be focussed in the area of other animal waste towards biomedical applications.

Declarations

Ethical approval: Not applicable

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Authors Contributions:

Anupam Mondal: Investigation, Conceptualization, Methodology, Formal analysis, Writing - Original Draft.

Kamalesh Sen: Conceptualization, Writing-Review & Editing, Investigation. **Arghadip**

Mondal: Investigation, Characterization. **Priyanka Debnath:** Conceptualization & Analysis. **Naba Kumar**

Mondal: Conceptualization, Resources, Writing -Review & Editing, Supervision.

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Competing of interest:

There is no competing of interest in favor of the manuscript, & publication

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Figures

Figure 1
Optimization study from RSM output of silver nanoparticle synthesis, (a) parameter interaction of bile conc. vs. temperature, (b) salt concentration vs. bile concentration, (c) salt concentration vs. temperature, and (d) desirability study for predicted output.

Figure 2

(a) FTIR analysis for Bile, AgNP-NJ, Biofilm silver loaded, Biofilm, (b) UV-Vis analysis (c) TGA for AgNP-BJ and (d) XRD pattern analysis.

Figure 3

Characterization of AgNP-BJ, (a) FESEM, (b) TEM (c) DLS for particle distribution and (d) SAED for crystallographic experimental.

Figure 4

Antibacterial activities (a) control for *E. coli*, (b) control *B. cereus*, (c) treatment 2 X MIC for *E. coli*, (d) treatment 2 X MIC for *B. cereus*, (e) treatment 2 X MIC for TEM of *E. coli*, and (f) Control condition of *E. coli*.

Figure 5

(a) Growth kinetic for *E. coli* respect on treatment combination of AgNP-BJ, (b) Growth kinetic for *B. cereus* respect on treatment combination of AgNP-BJ, (c) study of Biofilm viability percentage, and (d) visibility of antibiofilm activity; Error bar indicated that 5 % level of significant.

Figure 6

(a) Metabolic activity in percentage *E. coli* and *B. cereus*, **(b)** % of LDH activity, **(c)** % loss in GSH activity and **(d)** protein leakage of concentration at different treatment, Error bar indicated that 5 % level of significant.

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