Biosurfactant assisted silver nanoparticle synthesis: a critical analysis of its drug design aspects

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Abstract
Silver nanoparticles exhibit wide dimensional antimicrobial and antifungal properties and therefore they are preferred for their applications in medical and cosmetics sector. They are synthesized by physical, chemical and biochemical methods. Biochemical methods are the best technique because of their non-toxic and energy efficient nature and hence are alternatives for the production of silver nanoparticles. One of the biochemical methods proposed in this work is the use of microbial surfactant for the synthesis of silver nanoparticles. The synthesis of surfactant was performed using Bacillus subtilis. The surfactant thus produced was analyzed by emulsification assay, oil spilling test, and hemolytic test. Silver nanoparticles synthesized from surfactant were observed by UV–Vis spectroscopy in the range of 400–600 nm. The surface plasmon resonance peak was observed at 410 nm corresponding to the peak of silver nanoparticles. The x-ray diffraction pattern showed an intense diffraction peak at 45° with an average particle size of 14 nm. In silico drug designing of surfactin A synthetase C was performed using Schrödinger software for identification of novel drugs. All the two identified ligands, leucine and lysine, exhibited docking with the target protein which shows their potential as drug molecules.

Keywords: surfactant, silver nanoparticles, green technology, B. subtilis, in silico drug design, surfactin A, synthetase C
Classification number: 2.04, 2.09, 4.02, 5.08
method [11]. Biochemical methods such as bacteria, fungi, yeast or plant extracts have also been employed [10].

The use of microorganisms provides an eco-friendly approach for nanoparticle synthesis. Biochemical synthesis also provides a good control over size distribution of nanoparticles [11].

Green synthesis of silver nanoparticles can be approached by five ways [12]. In the polysaccharide method, water and polysaccharide act as stabilizing and/or reducing agents. Silver nanoparticles are formed when starch is used as a stabilizing agent and $\beta-D$-glucose is used as a reducing agent [13]. In the Tollens method, saccharides reduce silver ions in the presence of ammonia. This produces silver nanoparticles of different sizes and shapes [14]. Irradiation of salts of silver and surfactant with laser beams leads to the formation of silver nanoparticles of various size and shape distribution [15]. Doses and dose rates of radiation also control the morphology of formed nanoparticles [16]. In the biological method, the microbial and plant extracts are used as reducing or stabilizing agent. The biomolecules present in the extract, amino acids, polysaccharides, vitamins, enzymes and proteins, act as reducing agents [17]. Nano-plates of silver were synthesized at room temperature by the extracts of Chlorella vulgaris [18].

Biosurfactants are amphiphilic molecules produced on microbial cell surfaces or excreted extracellularly [19]. They are composed of both hydrophobic and hydrophilic moieties. These groups provide biosurfactant the ability to accumulate between two liquid phases and reduce the surface and interfacial tension [20]. Biodegradability, low toxicity, cheap availability of raw materials, resistance to environmental factors such as temperature, pH and ionic strength, biocompatibility and digestibility are the few advantages of biosurfactants over chemical surfactants [21]. They have a variety of applications in agriculture, laundry, biopesticide, petroleum, cosmetics, food processing and medicines [20]. They have antimicrobial, antiviral and anti-cancer activities, and can be used as anti-adhesive agents, immunological adjuvants and in gene delivery [22]. They can be classified as glycolipids, lipopeptides, surfactin, lichenysin, etc [23–26]. Use of biosurfactant for the synthesis of silver nanoparticles is a novel approach with only a few studies currently reported [27, 28].

Herein, a novel, simple and green approach for the synthesis of silver nanoparticles using culture supernatant of Bacillus subtilis and microwave irradiation is described. Bacillus subtilis was chosen as it is omnipresent and is commonly found in various ecological environments like soil, water and air and is non-pathogenic. Microwave radiations were used for heating as they yield nanostructures with smaller size, narrow size distribution and a high degree of crystallization [29]. Microwave irradiation results in shorter reaction times, reduced energy consumption, and better product yields which prevent agglomeration of particles formed. The main objective of this study was the facile and green synthesis of silver nanoparticles using biosurfactant produced by Bacillus subtilis.

Other than their use for the production of silver nanoparticles, biosurfactants can also be used as a source of new drugs. One of the most commonly produced biosurfactant, surfactin is a lipopeptide biosurfactant and has potent antimicrobial activity. The precursor molecule of surfactin, surfactin synthetases, can be utilized as a potential candidate for novel drug design. Surfactin is synthesized by srfA operon. This operon comprises of three genes, srfA, srfB, and srfC which are responsible for template generation, activation of amino acids and termination of peptide chain synthesis [30, 31]. As surfactin is produced by Bacillus subtilis, which is one of the widely found bacterial species, surfactin synthetases can be procured easily for drug molecules. The enzymes surfactin synthetases have not been exploited as suitable drug targets. This study depicts the homology modeling and molecular docking of surfactin A synthetase C as a means of novel drug design.

2. Materials and methods

2.1. Bacterial strain

A strain of Bacillus subtilis was isolated and characterized in the institute’s laboratory. It was streaked onto Luria Bertani (LB) plates, incubated at 37 °C and stored at 4 °C. 100 ml of Nutrient Broth was aseptically inoculated with Bacillus subtilis from the LB agar plates. The culture flasks were incubated at 37 °C for 16 h with shaking at 100 rpm. After 16 h, the cells were transferred to 250 ml culture flasks containing 100 ml of Minimal Media. The composition of Minimal Media (in g 1–1) was as follows: glucose, 2.5; monosodium glutamate, 1; yeast extract, 0.3; MgSO4·7H2O, 0.1; K2HPO4, 0.1; KCl, 0.05. The pH of the medium was 7.0 ± 0.1 [32]. The flasks were incubated at 30 °C with shaking of 100rpm for 120 h.

After the incubation period, the cultures were centrifuged at 10,000rpm for 10 min at 4 °C and the supernatant was collected. The supernatant was subjected to various analytical tests, namely, emulsification index, oil spilling test and hemolytic test, to confirm the presence of biosurfactant. The supernatant was also used as a starting material for the synthesis of silver nanoparticles.

2.2. Hemolytic test

The hemolytic activity of bacteria was used to rapidly isolate biosurfactant producers. Blood agar (Himedia; MP1301) plates were streaked with the 120 h bacterial culture from Minimal Media and incubated at 37 °C for 48 h. The halo around the colony indicated the presence of hemolytic bacteria.

2.3. Emulsification index

This test was performed to determine the emulsifying ability of the biosurfactant producing microorganism. The method was adopted from Cooper and Goldenberg with minor modifications [33]. To examine the Emulsification (E24), castor oil and cell supernatant were mixed in the ratio of 1:1. The solution was vortexed at high speed for 2 min and kept at room temperature for 24 h. After incubation, emulsification percentage was calculated by measuring the emulsion layer formed. The $E_{24}$
was calculated as the percentage of the height of emulsion layer (mm) by the total height of the liquid column (mm)

\[ E_{24} = \frac{\text{Height of emulsion}}{\text{Total height of mixture}} \times 100. \]

2.4. Oil spilling test

A protocol modified from Walter et al was used for oil spilling test. In a petri dish containing 40 ml of distilled water, 15 µl of castor oil was added, 10 µl of cell supernatant was added to the surface of oil film. The formation of halo was an indication of the presence of biosurfactant in the supernatant [34].

2.5. Synthesis of silver nanoparticles

To 50 ml of cell culture supernatant (section 2.1), 50 ml of 1 mM of AgNO₃ was added. The pH was adjusted to 9 using NaOH. The solution was irradiated with microwaves until the change in color was observed. The change in color from yellow to brown was the indication of the presence of silver nanoparticles. The samples were analyzed using UV-Vis spectrophotometer for a wavelength range of 200–1000 nm with a resolution of 1 nm and with nanodrop for wavelength ranging from 300–700 nm. The samples were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was dried at 37 °C for a week to remove moisture content. The powder thus obtained was used for x-ray diffraction analysis.

2.6. X-ray diffraction

X-ray diffraction analysis was performed to detect the crystalline structure of silver nanoparticles. The powder obtained after drying of silver nanoparticle solution was subjected to x-ray diffraction analysis. The XRD patterns were recorded using PANalytical 3 kW X’pert Powder XRD. The data was collected in the scan range 2θ of 4°–120°, with a step size of 0.026°. XRD pattern, thus obtained, was used to calculate the average particle size with the help of Debye-Scherrer formula

\[ D = \frac{0.9 \lambda}{W \cos \theta} \]

where \( \lambda \) is the wavelength of x-ray (0.5141 nm), \( W \) is the FWHM (full width at half maximum), \( \theta \) is the diffraction angle, \( D \) is the particle diameter (size).
2.7 In silico drug design

The three-dimensional structure of surfactin A synthetase C was obtained from Protein Data Bank (www.rcsb.org) with 2VSQ as the PDB ID. The file was uploaded to licensed Schrödinger software. The ligands of surfactin A synthetase C were taken from RCSB database. The structure of the ligands was drawn by 2D Sketcher of Maestro suite. Once the ligands and the target protein were generated, they were prepared using Ligprep and Protein Preparation tool for Maestro software, respectively. After the generation of sites for docking of ligands with the receptor, molecular docking was performed to generate docking scores and glide energies of this interaction.

3. Results and discussion

The hemolytic activity is a qualitative screening for detection of biosurfactant-producing microorganisms. A clear zone around the colonies (figure 1(a)) indicates the presence of bacterium producing biosurfactant. The surface active molecules rupture cell membrane resulting in red blood cell lysis and halo or clear zone around the cells. As blood agar is a complex medium, it is difficult to test the biosurfactant productivity of the culture. However, this has not been considered as a reliable method for the detection of biosurfactant activity [35].

Oil spilling test was used as a preliminary indication for biosurfactant production. This method is simple, cost-effective, fast to implement and requires easily accessible reagents. This test determines the potential of a microorganism for biosurfactant production based on halo diameter. Greater the halo diameter, higher is the potential of a microorganism to produce biosurfactant. As the current study only dealt with the detection of biosurfactant the halo diameter was not measured. Figure 1(b) shows the halo formation by biosurfactant on oil layer, thereby, spilling it.

The emulsification of castor oil produced by biosurfactant from \textit{B. subtilis} was assessed through visual observation. High-speed vortexing of castor oil and cell supernatant for 2 min resulted in the formation of a foamy layer at the medium-castor oil interface which was stable for 24 h (figure 1(c)). The layer confirmed the presence of biosurfactant produced by \textit{B. subtilis}. The emulsification index by biosurfactant was depicted in table 1.

The heating of the cell culture supernatant with AgNO$_3$ resulted in a color change from yellow to dark brown (figure 2), indicating the formation of silver nanoparticles. The excitation of surface plasmon vibrations in nanoparticles gave the solution a characteristic brown color and provided a spectroscopic signature of their formation. Several hydroquinones with excellent redox properties were reported that act as electron shuttle in metal reductions. Thus, it was evident that electron shuttle or others reducing agents released by \textit{Bacillus subtilis} are capable of reducing silver ions to silver nanoparticles [36].

The presence of silver nanoparticles was further confirmed by spectrophotometric analysis. The solution containing nanoparticles was scanned for wavelengths ranging from 200–1000 nm with UV-Vis spectrophotometer (figure 2(a)). The same solution was also scanned for wavelengths 300–700 nm using Nanodrop (figure 2(b)). The absorbance peak of the synthesized nanoparticles was observed around 400 to 530 nm (figure 3). This result is in agreement with the previous studies. The reason for the change in color and the strong absorption of the solution in the range of 400–530 nm can be explained as follows. The conduction band and valence band of silver nanoparticles lie close to each other and the electrons can move freely between them. These free electrons produce a surface plasmon resonance (SPR) which occurs due to oscillations of electrons over the surface of silver nanoparticles in resonance with light waves. The electrons are polarized by the electric field of incoming waves. This creates a net charge difference between electrons and the heavier ionic core of silver nanoparticles resulting in a dipolar oscillation of all electrons. The resonance of the frequency of electromagnetic

![Graph](image1.png)

**Figure 4.** The x-ray diffraction pattern (Intensity versus 2θ) of the synthesized silver nanoparticles.

| Ligand ID | Ligand name | Docking score | Glide energy (kcal/mol) |
|-----------|-------------|---------------|-------------------------|
| LEU       | Leucine     | −3.807        | −24.950                 |
| GOL       | Glycerol    | −4.556        | −11.934                 |

Table 2. Docking outcome of surfactin A synthetase C with different ligands.
field with the electron motion results in a strong absorption. This is the reason for the observed color [37]. The absorption depends on the particle size, dielectric constant of the medium and the chemical surroundings. As the particle size increases from 10 nm to 100 nm, the absorbance peak increases from 400 nm to 500 nm and broadens in width.

The x-ray diffraction analysis patterns of the silver nanoparticles synthesized from the current study is depicted in figure 4. The XRD patterns of silver nanoparticles show intense peaks \((2\theta)\) at 40.0°, 44.10° and 67.0°, which are found normally for silver nanoparticles [38, 39]. These peaks correspond to (111), (200), and (220) planes, respectively and its comparison with JCPDS (file no: 89-3722) indicate it to have face-centered cubic structure [40, 41]. The findings also indicate that the synthesized silver nanoparticles are a mixture of silver and silver oxide nanoparticles as the peaks are little shifted for the silver nanoparticles peak suggesting the formation of oxides. The peaks at 28.54 and 31.69 are indicative of the presence of silver oxide nanoparticles [42, 43]. From this study, considering the intense diffraction peak at 45°, the average particle size as calculated by Debye-Scherrer formula was found to be around 14 nm.

Molecular docking was performed for surfactin A synthetase C using licensed version Maestro of Schrödinger software. Two ligands, leucine and glycerol (obtained from RCSB database), were docked with surfactin A synthetase C. Both these ligands were analyzed for docking score and glide energy and they exhibited positive interactions with the synthetase. The docking scores and glide energy of these interactions are mentioned in table 2. Docking score provides a rough estimate of ligand at its active site and glide energy is the free energy resulting from ligand’s activity at the target site. The yellow lines in the three-dimensional structure of ligand-protein interaction diagram denote the hydrogen bonds. The blue and red colors depict nitrogen and oxygen atoms, respectively. In ligand interaction diagram (obtained from Schrödinger software), purple solid lines are the hydrogen bonds between ligands and target protein backbone and the purple dotted lines are the hydrogen bonds between ligands and protein side chain. The amine group of leucine interacts with threonine and proline of surfactin A synthetase C at 1115 and 1113 positions, respectively and the hydroxyl group interacts with glutamine at 1141 position and threonine at 1115 position (figure 5(a)). This interaction resulted in a docking score of \(-3.807\) and slide energy of \(-24.950\) kcal/mol (table 2). Similarly, the hydroxyl group of glycerol exhibited interaction with glycine at 1059 position, another glycine at 1114 position, arginine at 1142 position, and threonine at 1115 position.
position and leucine at 1267 position (figure 6(a)). Docking score of $-4.556$ and glide energy of $-11.924$ kcal/mol was observed as a result of this interaction (table 2). These results show that the surfactin A synthetase C have a strong interaction with the ligands.

Surfactin synthetase, a multi-subunit enzyme complex for surfactin synthesis, is an operon composed of three genes: srfA, srfB, and srfC. These enzyme subunits can themselves act as potential drug target sites. The classical route of drug development requires a number of different experimental set up for screening of bacterial extract, identification of pharmaceutically active compound and determination of compound structure which leads to high cost of chemicals, reagents and instruments used. After in vitro testing of drugs they should be tested on animals to study their in vivo efficacy, thereby adding the cost of experiment. In order to overcome the above problems, in silico modeling of these drugs should be performed prior to any experiments. The in silico modeling of drugs provides the advantage of structure prediction at high speed [44]. Once the molecules of the drug are established, they can be used as novel drug molecules for surfactin synthetase. In this study, surfactin A synthetase C was used as a target molecule and the ligands identified for this compound were leucine and glycerol.

After the discovery of novel drugs, nanotechnology can also be applied to efficient drug design strategies. The coating of drug molecules with nanoparticles provides the advantage of small size which helps it to penetrate microcapillaries easily [45]. This study provides a green method for the production of silver nanoparticles using biosurfactant by Bacillus subtilis. After their production and characterization, they can be utilized for microencapsulation of leucine and glycerol, the ligands for surfactin synthetase. These encapsulated drugs can then be delivered inside cells at the target site, surfactin A synthetase C (figure 7).

4. Conclusion

In conclusion, biosurfactants produced by B. subtilis has shown potential for the production of silver nanoparticles. Using cell filtrate in combination with microwave irradiation provides a method of rapid synthesis of nanoparticles. This helps in developing a green technology/approach for nanoparticle synthesis. The extracellular synthesis offers the advantage of ease of access to the substrate, unlike the intracellular synthesis where an additional processing step for the release of nanoparticles is required. Although the synthesis of nanoparticles using bacterial supernatant provides an advantage of being safe it is unable to control the size of nanoparticles thus produced. Hence, the biochemical mechanism by which these nanoparticles are synthesized inside the bacterial cell needs to be elucidated. The future applications of these nanoparticles can be in encapsulating the ligands for surfactin A synthetase C. These encapsulated ligands can penetrate the cell membrane and act at the target site and therefore can be used as drug delivery agents.

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Disclosure statement

The authors declare no conflict of interest in the submission of this manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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