Efficient eco-friendly approach towards bimetallic nanoparticles synthesis and characterization using *Exiguobacterium aestuarii* by statistical optimization

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**ABSTRACT**

Biogenic synthesis of bimetallic nanoparticles (gold – AuNp and selenium – SeNp) using inexpensive Tryptophan Enriched Banana Peel Media for the growth of marine isolate (*Exiguobacterium aestuarii* SBG4 MH185868). The response surface methodology is employed for optimizing production conditions. The surface plasmon resonance band showed \( \lambda_{max} \) at 540 nm (AuNp) and 284 nm (SeNp). FTIR and zeta potential analysis confirmed the stability, whereas XRD spectra revealed the nature of nanoparticles obtained at optimum conditions. SEM micrographs showed nanospheres of the following size: AuN, 30 ± 5 nm and SeNp, 50 ± 5 nm. Biocompatibility of Np evaluated by the hemolytic activity showed <20% hemolysis even at highest concentrations (100 µg/ml). AuNp showed the least cytotoxicity, whereas SeNp showed considerable cytotoxicity against the breast cancer cell lines MCF – 7 and MDA-MB-231. Hence, we utilized the environment-friendly growth media for the controlled synthesis of dual Np using single bacterial strain involving feasible steps in downstream processing.

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**Introduction**

Nanotechnology plays a significant role in research and development due to its wide application especially in biomedical research (diagnostics and drug delivery) and optical and electrical properties (1). Extensive human applications of nanoparticles, which are synthesized by chemical and physical methods require expensive energy consuming procedures and cause environmental issues (2). These concerns lead to the idea of preferring green synthesis (microbial synthesis and phytosynthesis) over physicochemical methods as it is also beneficial to the environment and in turn, cost-effective. Microbes are metal resistant due to genes involved in the detoxification of metal ions by various mechanisms like complexation, eflux, or reductive precipitation and flourish in environments with high heavy metal contamination (3). Therefore, it is important to develop sustainable methodologies for nanoparticles synthesis without causing ecological imbalance by maintaining constructive output. Among inorganic metal nanoparticles like gold, selenium, silver, and copper nanoparticles, gold nanoparticles have widespread applications due to their resistance to oxidation, least toxicity, good biocompatibility, and stability in drastic environmental conditions. The NADH- and NADPH-dependent enzymes, denitrification or nitrate reductase or extracellular polymeric substances (EPS), or low-molecular-weight proteins secreted out can be good capping agents in nanoparticle production. These are the vital properties of microorganisms involved in the reduction of Au3+ to Au0 (4,5). Microbial synthesis

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of Np may be extracellular or intracellular, wherein intracellular synthesis is comparatively elaborate as it requires extra downstream processing or purification steps like ultrasonic treatment, treatment with various detergents to release Np from the cells during the process of purification (6). Marine microbes are explored for various applications like bioremediation, biomineralization, bioleaching, and microbial erosion as they are exposed to excessive metal contaminants and pollutants in the marine environment. Hence, metal–microbe interactions serve as potential biofactories for the synthesis of metallic Np (7). Biological reducing agents work as coating molecules around the nanoparticles, making them biocompatible without causing toxicity when used in drug delivery systems (8).

Utilization of Agro-waste such as fruit peels, husk, and bagasse for microbial cultivation reduces the cost of media and can help in bioremediation of environmental waste. For example, worldwide production of banana (Musa paradisiaca) is predicted to be around 72.5 million metric tons, of which 21.77 million metric tons is contributed by India. The enormous amounts of the peel, which are generally discarded as waste, poses the risk of eutrophication due to its high nitrogen and phosphorus content, minerals, and reported concentrations (% of protein (0.90), crude lipid (1.70), carbohydrate (59.00), and crude fiber (31.70). Consequently, a process that accommodates this biowaste would be highly relevant and realistic in solving problems of environmental biowaste accumulation, especially in water bodies (9).

The classical methods of optimization based on changing one factor at a time and keeping the other factors constant are cumbersome and require several optimization procedures by numerous repetitions. However, employing statistical optimizations solve the problems by effective variable selection and their interaction simultaneously between each parameter. This minimizes the time and resources required for the optimization of multiple parameters. Response surface methodology (RSM) is an assembly of statistical and mathematical techniques useful for designing experiments, developing models, and evaluating the effects of variables in which a response of interest is influenced by several variables, and the objective is to optimize this response. It provides information about the correlation and interaction between variables, necessary for designing process optimization by giving multiple responses simultaneously. It usually involves an experimental design such as central composite design (CCD) to fit a second-order polynomial by least square’s technique (10).

Studies referring to innovative nanotechnological applications show that strategies/methods should be implemented for the risk assessment of Np on human health. Np can be toxic when they accumulate in the body; therefore, initial evaluation of biocompatibility has to be carried out (11).

The current study focuses on the formulation of low-cost media by utilizing banana peel extract to cultivate marine bacterial isolates to synthesize dual nanoparticles without compromising on their quality. This is a novel and exclusive model of nanoparticles generation, wherein the same bacterial total cell proteins (TCPs) are used for AuNp and cell-free supernatant (CFS) for SeNp synthesis. The nanoparticles obtained by this approach were characterized for various structural properties, stability, and storage conditions using common preservatives and evaluated for their biocompatibility for further applications without causing ecological issues.

Materials and methods

Chemicals

All the chemicals and media components used in this present study were procured from Hi-Media (Mumbai, India) and Sigma-Aldrich (USA).

Isolation and screening of isolates for a synthesis of dual nanoparticles

Marine water samples were collected from Rushikonda Beach, Vishakhapatnam, Andhra Pradesh, India, and transported at ambient temperature to the laboratory. The samples were diluted in sterile saline (0.9% w/v), and isolates were obtained by spread plate technique on Zobell’s Marine Agar (ZMA) at 37°C for 48 h. Bacterial isolates were used for the synthesis of AuNp and SeNp. All the bacterial isolates were cultivated in Luria Broth (LB) containing 1 mM tryptophan at 37°C for 24 h. The culture supernatant was separated by centrifugation at 6000× g for 10 min at 4°C. The cell pellets (log 8 CFU/ml) were washed with sterile distilled water and added to 10 ml of deionized water with 1.5 mM chloroauric acid (HAuCl₄), and supernatant obtained was used for the synthesis of SeNp using 1.8 mM sodium selenite (Na₂SeO₃) (12,13).

Identification of marine isolate

The morphological and physiological characterization of the finest quality producing G4 isolate was performed according to the methods described by Bergey’s manual of determinative bacteriology; 16S rRNA sequence was amplified and sequenced by Macrogen Inc (Korea). The species similarity was analyzed by
NCBI-BLAST and phylogenetic tree constructed by MEGA-Version X (14).

Utilization of banana peel extract as growth media

Tryptophan enriched banana peel (TEBP) media optimization

The fresh banana peels were collected and washed thoroughly with brine solution followed by sterile distilled water. Excess water was drained by placing on paper towel, and peels were then subjected to hot air drying at 50°C for 12–18 h until no further change in the weight was observed. The dried peel was made into fine powder and stored at room temperature until further use. The banana peel extraction (BPE) was performed by adding the powder to 100 ml distilled water in a 500 ml conical flask with constant stirring on a hot plate set (80°C). The optimization studies were carried out using different concentrations of BPE (2–10%w/v), pH (5–8), tryptophan (0.05–1 mM), sodium chloride (2–16% w/v), and incubation time.

Downstream processing

Optimization of parameters for dual nanoparticles synthesis

The optimization for quality nanoparticles synthesis was based on a metal salt concentration (HAuCl4/Na2SeO3), pH, and incubation time, which was optimized by the CCD model.

Experimental design

Limitations confronted during biogenic nanoparticles synthesis are mainly stability and aggregation due to physicochemical properties like pH, temperature, source, and time (15). We designed two sets of experiments using three factors, three-level CCD using RSM (16), each with 20 runs (Table 1). Set I was designed for AuNp synthesis keeping TCP as constant (log 8 CFU/ml) with varying pH (A1), HAuCl4 concentration (B1), and incubation time (C1). The same pattern of design was followed for SeNp synthesis for set II. CFS remains constant with varying conditions of pH (A2), Na2SeO3 concentration (B2), and incubation time (C2).

The approach for designing experiments was based on the change of one variable at a time (COVT). Based on the study by Noginov et al. (12), the SPR band (200–700 nm) was measured for the characterization of biogenically synthesized nanoparticles, and this was used as a response (Y = RS(Np) for the experimental runs performed. The responses (λmax) obtained from RSM-CCD were subjected to the second-order multiple regression analysis and analysis of variance (ANOVA) given in Table 2, which explains the overall behavior of the system as well as the justification of the significance and adequacy of the developed regression model using the least square regression methodology. Each experimental run of the CCD matrix was analyzed, and the response was correlated with three input variables by following the quadratic polynomial Equation (1).

\[
Y = \beta_0 + \sum_{i=1}^{n} \beta_iX_i + \sum_{i=1}^{n} \beta_iX_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij}X_iX_j \tag{1}
\]

where the parameter is the model constant, \(\beta_i\) is the linear coefficient, \(\beta_{ij}\) is the quadratic coefficient, \(\beta_{ij}\) is the cross-product (interaction) coefficient, \(X_i (i = A, B, C)\) are the independent variables, \(Y\) is the predicted response (λmax = 200–700 nm), and \(n\) is the number of variables. The three-

| Run | A1 – pH | B1 – HAuCl4 | C1 – Incubation time | Raunp (nm) |
|-----|---------|-------------|----------------------|------------|
| 1   | 7.98651 | 2.43244     | 9.64857              | 533        |
| 2   | 7.98651 | 0.767555    | 9.64857              | 553        |
| 3   | 5.01349 | 2.43244     | 20.3514              | 549        |
| 4   | 6.5     | 1.6         | 15                   | 550        |
| 5   | 6.5     | 1.6         | 15                   | 541        |
| 6   | 7.98651 | 0.767555    | 20.3514              | 548        |
| 7   | 7.98651 | 2.43244     | 20.3514              | 534        |
| 8   | 5.01349 | 0.767555    | 20.3514              | 548        |
| 9   | 5.01349 | 2.43244     | 9.64857              | 550        |
| 10  | 6.5     | 1.6         | 15                   | 534        |
| 11  | 5.01349 | 2.43244     | 9.64857              | 549        |
| 12  | 6.5     | 1.6         | 15                   | 532        |
| 13  | 6.5     | 1.6         | 6                    | 534        |
| 14  | 6.5     | 1.6         | 24                   | 533        |
| 15  | 4       | 1.6         | 15                   | 546        |
| 16  | 6.5     | 1.6         | 15                   | 537        |
| 17  | 6.5     | 0.2         | 15                   | 543        |
| 18  | 6.5     | 1.6         | 15                   | 537        |
| 19  | 9       | 1           | 15                   | 543        |
| 20  | 6.5     | 3           | 15                   | 550        |

| Set: II – SeNp Synthesis | Run | A2 – pH | B2 – Na2SeO3 | C2 – Incubation time | Rsenp (nm) |
|--------------------------|-----|---------|--------------|----------------------|------------|
| 1 | 6.5 | 1.75 | 48 | 284 |
| 2 | 7.98651 | 2.49325 | 62.7205 | 296 |
| 3 | 6.5 | 1.75 | 24 | 276 |
| 4 | 6.5 | 1.75 | 72 | 284 |
| 5 | 6.5 | 1.75 | 48 | 280 |
| 6 | 6.5 | 1.75 | 48 | 284 |
| 7 | 5.01349 | 1.00675 | 62.7205 | 268 |
| 8 | 6.5 | 1.75 | 48 | 276 |
| 9 | 4 | 1.75 | 48 | 242 |
| 10 | 6.5 | 1.75 | 48 | 279 |
| 11 | 7.98651 | 1.00675 | 33.7295 | 280 |
| 12 | 5.01349 | 2.49325 | 62.7205 | 272 |
| 13 | 7.98651 | 1.00675 | 62.7205 | 301 |
| 14 | 9 | 1.75 | 48 | 298 |
| 15 | 5.01349 | 2.49325 | 33.7295 | 266 |
| 16 | 7.98651 | 2.49325 | 33.7295 | 294 |
| 17 | 6.5 | 1.75 | 48 | 298 |
| 18 | 5.01349 | 1.00675 | 33.7295 | 258 |
| 19 | 6.5 | 3 | 48 | 296 |
| 20 | 6.5 | 0.5 | 48 | 288 |
dimensional graphs and the prediction values were used to obtain the optimum conditions required for better quality synthesis of AuNp and SeNp.

Data analysis

The design of statistical experiments, analysis of the results, and prediction of the optimum responses ($\lambda_{\text{max}}$) were done by applying the statistical software Design-Expert Version 11.0.0 (State-Ease Inc., Minneapolis, MN, USA).

Verification of the model

The process parameters for biosynthesis of AuNp and SeNp were numerically optimized by the desirability function of RSM. The responses were determined under the optimized conditions recommended by the model. Validation of the designed model was done by comparing the model predicted response with the experimental response.

Characterization of nanoparticles

UV–VIS spectrophotometry

The synthesized AuNp and SeNp using the TCP and CFS, respectively, were characterized by UV-Visible spectroscopy (Epoch, India) for their surface plasmon resonance (SPR) ranging from 200 to 700 nm (16) at a resolution of 1 nm.

Fourier transform infra-red (FT-IR) spectroscopy

Run samples with variable interactions were checked for the presence of functional groups using FT-IR analysis (SHIMADZU, India) at a resolution of 4 cm$^{-1}$ in KBr pellets and analysis ranging from 400 to 4000 cm$^{-1}$ (17).
**X-ray diffraction (XRD) spectrum**

The biosynthesized Nps were dried, and the powdered sample was used for XRD analysis (18) to confirm their crystalline nature (Philips X-pert pro, India) at 40 kV and 30 mA current with 2.2 KW Cu anode radiation ($k = 1.540 \text{Å}$).

**Zeta potential measurement**

The effective surface charges on the AuNp and SeNp at different variables were measured using zeta analyzer (Horiba scientific, nanoparticle analyzer SZ-100) (19).

**Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis**

The morphological analysis of the particle was carried out using SEM (Carl Zeiss, AG, India) at different magnifications. EDX analyses of elemental Au and Se were carried out by the same instrument from 0 to 12keV (20).

**Cryopreservation of nanoparticles for long-term stability**

The Nps were stored at either room temperature (25°C) in the refrigerator (4 –10°C) or in the freezer (−20°C) for a span of 6 months. Nps were formulated as described previously and then diluted into sterile distilled water and 1% (w/v) tween, glycerol, dextrose, poly ethylene glycol (PEG-4000/6000) along with control without any preservative. Before freezing, the addition of 1% (w/v or v/v) glycerol, tween, dextrose, and PEG 4000/6000 as cryoprotectants were used either suspended or centrifuged or by drying of Np followed by incubation (21).

**Biocompatibility of nanoparticles**

**Cell viability assay**

The effect of AuNp and SeNp on breast cancer cell lines was evaluated by performing the cell viability assay as described by Mosmann (22). Approximately 15,000 cells per well (MDA-MB 231 and MCF-7) were seeded in a flat-bottomed 96-well plate and incubated in a 5% CO$_2$ incubator at 37°C for 24 h, followed by serum starvation for 8 h. The cells were then treated with the increasing concentration of AuNp and SeNp (0.5–200 µg/ml) and after 24 h of incubation, and MTT assay was performed.

**Hemolysis assay**

Fresh human blood samples were obtained from healthy volunteers. The Np suspensions in PBS (250–0.03 mg/ml) were added in 250 µl of blood samples. Positive (100% lysis) and negative (0% lysis) control samples were prepared. The samples were incubated at 37°C for 24 h. After 24 h, the samples were centrifuged at 1500 x g for 5 min. Oxyhemoglobin absorbance has been measured spectrophotometrically at 540 nm (23). The percentage of hemolysis was calculated using Equation (2):

$$\text{Hemolysis (\%)} = \frac{\text{SampleOD} - \text{NegativeControlOD}}{\text{PositiveControlOD} - \text{NegativeControlOD}} \times 100$$

(2)

**Results and discussion**

**Biosynthesis of nanoparticles**

The biogenic synthesis of nanoparticles is an eco-friendly method to develop environmentally sustainable nanoparticles as an alternative to existing chemical methods. As specified by Senapati et al. (24), biotransformation of metal salts by microbes plays an important role in the biosynthesis of metal nanoparticles. The specific mechanism involved in the reduction of metal salts to the nanoparticles need to be studied further; however, it is known that membrane proteins or the reductases inside a bacterial cell can reduce the chloroauric acid to AuNp without causing agglomeration of nanoparticles. Also, extracellular protein fraction secreted out into the supernatant was able to reduce sodium selenite to selenium nanoparticles (5). We isolated seven (G1–G7) bacterial strains from marine water samples based on their ability to synthesize both extracellular AuNp and SeNp. A visible color change was observed from pale yellow to red or purplish red is a characteristic property of AuNp synthesis as reported earlier by Nangia et al. (25), Bhambure et al. (26) and colorless to reddish orange in case of SeNp. These color changes correlated with the observations made by Shah et al. and Wadhwan et al. (27,28). Among the seven isolates, G4 was shown to produce good quality nanoparticles in shorter duration compared with other isolates, showing $\lambda_{\text{max}}$ at 540 nm for AuNp (Supplementary Fig. 1A, C) and 280 nm for SeNp (Supplementary Fig. 1B, D). Isolates G1 and G7 were able to produce AuNp, but not SeNp.

**Identification of microorganism**

The isolate G4 was identified by biochemical and morphological examinations followed by 16s rRNA sequencing (Macrogen Inc, Korea). The 16s rRNA sequence was aligned against recognized sequences in GeneBank.
and results showing homology of 99% with *Exiguobacterium* sp. The phylogenetic tree given in Figure 1 was constructed using MEGA-X software tool using the neighbor-joining method (14). The sequence was submitted to NCBI and has been recorded under the name and accession number *Exiguobacterium aestuarii SBG4 MH185868* (E. aestuarii SBG4 MH185868).

**Optimization of TEBP media for dual nanoparticles synthesis**

To formulate a growth medium for *E. aestuarii SBG4 MH185868* having a cost-effective benefit for efficient Np production, an optimized TEBP medium containing (pH 6.5) dried BPE (8%; serving as both carbon and nitrogen source) enriched with tryptophan (0.2 mM) and NaCl (10%) was screened. Optimum synthesis conditions were taken based on the UV–VIS spectrum (Supplementary Fig. 2 A–D – AuNp and 2 E–H – SeNp) and nanoparticles synthesized from 24 h incubated culture (Supplementary Fig. 2 I and J). The synthesis time for AuNp using TCP and CFS for SeNp was at 24 and 72 h, respectively. Therefore, reduction of synthesis time is one of the parameters in statistical optimization. The synthesized AuNp and SeNp were observed to have desirable physical and chemical properties, required to be considered as good quality nanoparticles (29,30).

**Downstream processing optimization**

**Statistical analysis and validation of the model**

For statistical analysis and validation of Np production by optimized TEBP media, the experiments were conducted in two sets (I and II). Optimum conditions for AuNp and SeNp synthesis and responses were recorded for analysis.

Set-I – Optimization of AuNp synthesis: (A1) pH (4–9) of the reaction solution, (B1) HAuCl4 concentration (0.2–3 mM), and (C1) incubation time (6–24 h); Set-II – Optimization of SeNp synthesis (A2) pH (4–9) of the reaction solution, (B2) Na2SeO3 concentration (0.5–3 mM), and (C2) incubation time (24–72 h) and their response-absorption maxima of the trials are represented in Table 1.

The λmax of R<sub>AuNp</sub> (≥ 540 nm) was confirmed in the treatment runs 10, 12, 16, and 18 under the optimum conditions for quality AuNp, which were at pH 6.5, HAuCl4 at 1.6 mM, and incubation time of >15 h.

The response equation used for AuNp synthesis.

\[
R_{AuNp} = 538.032 + -0.342816*A_1 + 0.642355*B_1 \\
+ -0.782156*C_1 + 3.875*A_1B_1 \\
+ 2.875*A_1C_1 + 4.875*B_1C_1 + 3.17153*A_1^2 \\
+ 3.52509*B_1^2 + -1.07111*C_1^2 
\] (3)

The λmax of R<sub>SeNp</sub> (~284 nm) was demonstrated in the treatment runs 1, 4, 5, and 6. The optimum parameters were pH 6.5, Na2SeO3 at 1.75 mM concentration, and 36 h of incubation for quality SeNp.

\[
R_{SeNP} = 283.536 + 14.7311*A_2 + 2.52286*B_2 \\
+ 6.45956*C_2 + -0.375*A_2B_2 \\
+ 1.47157*A_2C_2 + -4.83515*B_2C_2 \\
+ -5.00927*A_2^2 + 2.7689*B_2^2 \\
+ -4.16837*C_2^2 
\] (4)
Equations (3) and (4) indicate how individual variables (quadratic) or double interaction affects the AuNp synthesis. The negative coefficient values indicate no significant effect, whereas positive coefficient values show a significant influence on dual Np synthesis. The adequacy of the models was justified by analysis of variance (ANOVA), and estimated optimum conditions for nanoparticles synthesis were computed and given in Table 2. The $R^2$ value of $R_{\text{AuNP}}$ at 3.20 and $R_{\text{SeNP}}$ at 11.07 implies that the model is significant for AuNp and SeNp, respectively. The model $F$-value is the ratio of mean square of the individual term to the mean square of the residual. The $F$ value is the probability of $F$-statistics value and is used to test the null hypothesis. The parameters having $F$-statistical probability value $<0.05$ are said to be significant, and values $>0.1000$ indicate that the model terms are not significant (31). The values obtained in our study are in statistical agreement with model $p$ values of 0.049 for AuNp and 0.0004 for SeNp.

In Set-I (AuNp), $B_1$, $C_1$, $A_2^2$, and $B_1^2$ are significant model terms. The lack-of-fit $F$-value of 0.1163 and a $p$-value of 98.17% imply that the lack of fit is not significant. Optimized parameters for AuNp synthesis involve ($A_1$) pH, ($B_1$) HAuCl$_4$ concentration, and ($C_1$) incubation time. $A_2$, $C_2$, and $A_2^2$ are significant model terms for SeNp (Set-II) synthesis, where ($A_2$) pH, ($B_2$) Na$_2$SeO$_3$ concentration, and ($C_2$) incubation time are the optimized conditions for SeNp synthesis. The lack-of-fit $F$-value of 0.03 and a $p$-value of 89.65% imply that lack of fit is not significant.

A well-fitted relationship between the experimental and predicted response values was established on the basis of the $R^2$ value, indicating the relevance to the model for biosynthesis of AuNp and SeNp. For AuNp, predicted $R^2$ of 0.3589 is in reasonable agreement with the adjusted $R^2$ of 0.5235. The ratio of 5.045 indicates an adequate signal. The SeNp predicted $R^2$ of 0.7303 is in reasonable agreement with the adjusted $R^2$ of 0.8267; ratio of 12.579 indicates an adequate signal (Table 2). By this model, we can say that a clear correlation between input and output variables could be drawn. The diagnostic case statistics of the study predicted coefficient of determination value (Pred $R^2$) and the adjusted coefficient of determination values (Adj $R^2$) is in reasonable agreement, thus confirming the significance of the used quadratic model. Finally, these criteria indicated the adequacy of the polynomial model’s accuracy and general availability (Supplementary Table 1).

In both the experimental sets, one parameter was kept constant for validating the variable interactions. The effect of pH (4.0–9.0) vs. HAuCl$_4$ concentration (0.7–2.43 mM), with constant incubation time, was studied using RSM (Figure 2(A, D)). It was observed that $\lambda_{\text{max}}$ for AuNp has increased beyond 550 nm with an increase in pH and HAuCl$_4$ concentration, which denotes non-uniform morphology resulting in red shift toward near-infrared region as reported by Sastry et al. (32). Figure 2(B, E) indicates the influence of pH vs. incubation time on Np synthesis, when compared with changes shown by variations of pH on $\lambda_{\text{max}}$ and incubation time did not seem to have any effect on $\lambda_{\text{max}}$ but the quantity of nanoparticles production increased during 9–12 h. The 3D plot clearly exhibited that the values below and above the optimum conditions for HAuCl$_4$ concentration and incubation time resulted in redshift (Figure 2(C,F)). According to the studies conducted by Daniel and Astruc (20), the characteristic of SPR band strongly depends on the uniform shape, size stability, and diameter of AuNp. In the present study, the optimization of the AuNp synthesis condition tends to show a reduction in redshift, i.e. $\lambda_{\text{max}} < 550$ nm, which inclines more toward blue shift. This pattern of reduction in redshift was also reported by Huang et al. (33) in their studies of nanoparticle synthesis employing plant extracts. Microbial synthesized Np have reported to vary in size and stability; however, we observed that optimization of synthesis conditions leads to the production of particles by *Exiguobacterium sp.*, which were uniform in shape, size, and stability.

Figure 3(A, D) for SeNp ($\lambda_{\text{max}}$) represents the effect of pH (4–9) and Na$_2$SeO$_3$ concentration (0.5–2.49 mM), while the incubation time was kept constant at the central value. The observed $\lambda_{\text{max}}$ reached 284 nm at optimum conditions, while the pH and Na$_2$SeO$_3$ concentration increased the $\lambda_{\text{max}}$ as the parameters deviated from optimum values. Figure 3(B,E) shows the influence of the interaction of pH and the incubation time, and incubation time did not affect the SPR band as time of incubation increased, but the concentration of nanoparticles was less until 24 h and reached maximum at optimum time of 36 h. Figure 3(C,F) signifies the Na$_2$SeO$_3$ concentration and incubation time. All the 3D plots clearly show that interactions above and below optimized values resulted in an increase of $\lambda_{\text{max}}$ above 284 nm, which is known as red shift as stated by Lin and Wang (34) due to the increased particle size. The Np quality obtained was similar to chemically synthesized Np obtained in studies by Shankar et al. (29).

Generally, wide variations in size and shape of the biologically synthesized Np show multiple SPR bands as reported in the study by Fesharaki et al. (30) in Np synthesized by *Klebsiella pneumoniae*. However, in our study, we observed single SPR band at 284 nm, which is rare in biological synthesis.
The predicted optimum conditions specified in Table 3 determined from the CCD model for AuNp were pH 6.5, HAuCl₄ concentration (1.6 mM) and incubation time (>15 h) obtaining Np at λmax 530 ± 5 nm. The optimized conditions for SeNp synthesis were pH of 6.5, Na₂SeO₃ concentration of 1.75 mM, and incubation time of 36 h. Hence, by optimizing the conditions for synthesis, we could control the shape and size of nanoparticles by reducing the time with appropriate concentration of metal salts and maintaining suitable pH conditions.

Characterization of nanoparticles

**FTIR spectrum analysis**

The spectra from FTIR analysis of biogenically synthesized AuNp and SeNp reveal the probable functional groups (carboxyl, hydroxyl, amines, or amide), which stabilize the Np. The IR spectrum shows intense peaks at 3448.84, 2359.02, 2343.59, and 1629.90 cm⁻¹ for AuNp. The broadband at 3448.84 corresponds to strong stretching vibration of O–H group of water molecules, whereas 2359.02 and 2343.59 can be attributed to alkyl C–H stretch. The peak at 1629.90 cm⁻¹ was assigned to C=O stretching of amide I of a peptide linkage, whereas the stretch for AuNp was found around 669.32 cm⁻¹. The bands at 3421.83, 1637.62, 1629.90, and 522.73 cm⁻¹ correspond to stretching and vibrational bending of O–H, N–H, C=O, and M–H (metal-hydrogen stretch) comparable peaks were observed in previous studies (35,36). These bands specify the presence of reducing groups, indicating different bacterial proteins responsible for the reduction of AuNp and SeNp in Figure 4(A,B) where no variations were observed even when interactions of variables are varied in RSM-CCD runs. The thin coating of proteins and secondary metabolites act as capping agents charge nanoparticles suspended by reducing agglomeration.

**X ray diffraction (XRD) analysis**

Next, the XRD spectrum for AuNp and SeNp were analyzed as follows. AuNp shows distinct diffraction peaks around 2θ (degrees) of 38.25°, 44.42°, 64.88°, and 77.57°, which were indexed as the (211, 200, 220, and 311) planes of AuNp, respectively (Figure 4(C)). These sharp Bragg peaks might have resulted due to the crystalline nature of Np (37). The average size of the AuNp was determined using Scherrer’s formula, which reveals the size of Np obtained by E. aestuarii SBG4 MH185868 were in the
range of 10–50 ± 5 nm. Similar XRD pattern was observed in biogenic synthesized AuNp by *Escherichia coli* (38) and cell filtrate of *Penicillium brevicompactum* (39). The broader pattern (Figure 4(D)) without any sharp Bragg’s peaks indicates that the nature of SeNp produced is amorphous. In the previous biosynthetic studies carried out with *Bacillus sp.*, characteristic peaks were observed at 23.680, 29.788, and 43.9. Our findings were consistent with these reports (40) and *Acinetobacter sp. sW30* (28).

**Zeta potential of nanoparticles**
The zeta potential is a vital parameter for understanding the charge, stability, and the dispersion of synthesized Np, which indicates the repulsive forces present between the Np and helpful to give evidence about the long-term stability of these biosynthesized AuNp. The high repulsions between the nanoparticles due to the increased surface charge without allowing aggregation of the particles imply greater zeta potential, which in turn shows high stability of nanoparticles. We observed that AuNp stability under optimum conditions was in accordance with zeta potential charges obtained for AuNp synthesized by soybeans proteins (41). The high stability at pH 6.5 (−38.3 mV), poor stability for pH 4 (−15.5), and moderate stability at pH 5 and 9 (−20.5 mV) are represented in zeta potential graphs (Figure 5(A–D)). SeNp (Figure 5(E–G)) showed high stability of −48.5 mV at pH 6.5 and poor stability at pH 7.0 and 9 of −15.5 and +11.6mV, respectively, and results were in line with SeNp by *E. coli* (42) report. These observations were

![Figure 3. Response surface plots and contour plots for SeNp synthesized by E. aestuarii SBG4 MH185868 CFS SPR – λmax vs. pH and selenium salt (A, D), pH and incubation time (B, E), and Selenium salt and incubation time (C, F).](image)

| Table 3. Optimum conditions for quality synthesis of dual nanoparticles. |
|-------------------------------------------------|
| Factor | Name | Level | Low level | High level | $R_{AuNp}$ | $R_{SeNp}$ |
|--------|------|-------|------------|------------|------------|------------|
| $A_1$  | pH   | 6.50  | 5.01       | 7.99       | Predicted mean = −538.032 |
| $B_1$  | chloroauric acid (mM) | 1.60 | 0.7576 | 2.43 | Std. Dev. = 5.02939 |
| $C_1$  | Incubation time (h) | 15.00 | 9.65 | 20.35 | |
| Factor | Name | Level | Low level | High level | $R_{AuNp}$ | $R_{SeNp}$ |
|--------|------|-------|------------|------------|------------|------------|
| $A_2$  | pH   | 6.19  | 5.01       | 7.99       | Predicted mean = −274.207 |
| $B_2$  | Sodium selenite (mM) | 1.26 | 1.01 | 2.49 | Std. Dev. = 6.22899 |
| $C_2$  | Incubation time (h) | 36.43 | 24.00 | 72.00 | |
further confirmed by the results obtained from SEM imaging, which showed polydisperse particle aggregation at pH values outside the optimum range.

**SEM and EDX**

The SEM analysis of nanospheres produced by *E. aestuarii SBG4 MH185868* at different pH values was performed. At pH 5.0 and 6.5 (Supplementary Figure 3A, Figure 6(A)), the Np did not show any aggregate formation, whereas at pH 4.0 and 9.0, AuNp were observed to aggregate (Supplementary Figure 3B, C). The formation of selenium nanospheres was obtained at pH 6.5 (Figure 3(B)). Further as the pH increased from pH 7.0 to 9.0 (Supplementary Figure 4A,B) in SeNp, the particle size was observed to be increasing due to the formation of an aggregate. SEM micrographs were used to determine diameter of particles (~100) and to characterize the size distribution of the particles in each batch. AuNp shows optimum average size of 30 ± 5 nm at pH 6.5 and increased size of 80 – 130 ± 5 nm for pH 4, 5, and 9, and SeNp shows optimum average size of 40 ± 5 nm at pH 6.5 and increased size of 80 – 180 ± 5 nm at pH 7.0 and 9.0. All the particle sizes were represented along with respective SEM micrographs. The EDX spectra of dual nanospheres confirm the presence of elemental Au and Se (Figure 3(A,B)), and these results are supported by the previous studies ([28,43]).

**Cryopreservation of nanoparticles for long-term stability**

The long-term stability of Np upon storage at 4°C, −20°C, and −80°C up to 6 months was studied (Supplementary Table 2). Tween-20, glycerol, dextrose, PEG – 4000, and PEG – 6000 were used as cryoprotectants. Among all the cryoprotectants, glycerol is proved to be good at all temperatures for suspended Np, when compared with pelleted or dried samples followed by Tween-20, which proved to be suitable for storage and handling of Np at 4°C and −20°C. Dextrose, PEG-4000, and PEG-6000 were found to be less useful for persevering both
suspended and dried samples under all three conditions, as they result in aggregation. Therefore, the present preservation process is easier to handle compared with previous methods suggested by Abdelwahed et al. (21).

Comparative cost study of media and quality of nanoparticles
The Np production was initially performed using bacterial culture grown in LB media supplemented with tryptophan and NaCl, which generally costs around Indian Rupees 5000 (INR) for 1 liter of media. To provide an inexpensive C and N source for growth, we used TEBP composing of BPE containing low concentration of tryptophan (0.2 mM) and 10% NaCl, which resulted in superior quality Np production. By replacing LB medium with TEBP, we could bring down the cost of production of Np to 40%. As banana peel is zero cost, which is a biowaste, and approximate cost for

Figure 5. Zeta potential of nanoparticles synthesized by E. aestuarii SGB4 MH185868 pH conditions A–D – 4.0, 5.0, 6.5, 9.0, respectively, for AuNp and E–G – 6.5, 7.0, 9.0 (pH), respectively, for SeNp.

Figure 6. SEM micrographs and EDX spectrum of biosynthesized nanoparticles SEM micrographs of AuNp and SeNp were taken using (instrument). AuNp showed optimum size at 30 ± 5 nm at pH 6.5, whereas SeNp showed optimum size of 50 ± 5 nm at same pH value. EDX spectra of the nanospheres confirm the presence of elemental gold and selenium.
other supplements is around INR 2000 for the same quantity. The BPE media used to culture bacteria for Np production in this report required minimum processing wherein drying and powdering of banana peel was convenient for storage without any preservation process.

**Analysis of cytotoxicity of nanoparticles**

MDA-MB231 and MCF-7 cell lines were exposed to AuNp and SeNp (0.5–100 µg/ml). The concentrations used correspond to the range generally used for in vitro cancer studies. In terms of acute cytotoxicity of SeNp, dose-dependent toxicity was observed, where maximal effects were found for concentration ranging from 25 to 100 µg/ml SeNp. A significant decrease in cell viability of MDA-MB-231 (52.08%) and MCF-7 (28.75%) cell lines was observed (Figure 7(A,B)). As stated by Huang et al. (33), a decrease in cell viability may be due to apoptosis induced by SeNp. This needs further evaluation for the application in cancer treatment (22,44). AuNp showed minimum cytotoxicity at the above concentrations, indicating that as per previous studies AuNp only serves as a vehicle for binding drug targets (45) and has no cytotoxic effects in the concentration range as mentioned earlier.

**Hemolytic activity**

The utilization of nanoparticles for intravenous drug delivery needs to check for the degree of nanoparticles interactions with blood cells and evaluate the hemolysis. Hemolytic activity of AuNp (0.03–250 mg/ml) and SeNp (0.03–200 mg/ml) was evaluated by the addition of Np to whole blood, and the percentage of hemolysis was calculated using Equation (2) in experimental and control samples. Figure 7(C) shows the hemolysis by high concentrations of AuNp (250 mg/ml) and excessive hemolysis by SeNp at 200 mg/ml (Figure 7(D)), which decreased as the concentration range reduced, while 0.03 mg/ml shown ≤20% hemolysis. Less than 0.03 mg/ml did not show any change from negative control (without Np) at OD 540 nm. Hemolysis levels <20% are generally regarded as biocompatible; therefore, this demonstration records low hemotoxicity even at concentration ≥0.1 mg/ml and is considered, as reported in the studies conducted by Kumar et al. (23).

**Conclusion**

The study highlights threefold advantages of Np production using the same bacterial strain. First, we could reduce the cost of Np production by 40% by
utilizing an easily available biowaste. Second, *E. aestuarii SBG4 MH185868* grown in the optimized TEBP medium could be used for the biosynthesis of dual Np of optimum physicochemical properties by RSM optimization. Finally, an interesting feature of this optimization was that the duration of synthesis of the Np was shortened. Due to the aforementioned advantages, these Np can be further utilized in several applications such as designing probes for protein detection or as drug delivery system by tagging anticancer or antimicrobial compounds.

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No potential conflict of interest was reported by the authors.

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