Molecular Bioengineer from a soil bacterium synthesizes gold nanoparticles

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Abstract

Nanoparticles, the extremely small particles ranging in size from few nanometers to about 100 nm have number of applications in various fields. Synthesis of nanoparticles is carried out by chemical and physical methods to yield particles of uniform size and desired properties. However, physical methods consume high level of energy during the synthesis process and chemical methods make use of toxic chemicals that harm the environment. Hence, the development of eco-friendly and biocompatible process for nanoparticle synthesis is required. Biological synthesis of nanoparticles can be carried out using plants, fungi, yeasts, bacteria, algae and animal products. Moreover, biomolecules derived from microorganisms are also known to play an important role in the synthesis of metal nanoparticles, especially silver and gold nanoparticles. In the present work, a soil bacterium capable of producing the enzyme nitrate reductase was used for synthesis of gold nanoparticles. The enzyme was partially purified by salt fractionation and used for gold nanoparticlessynthesis by biochemical reduction of the gold ions.It was found that the partially purified enzyme showed showed higher efficiency of nanoparticles synthesis when compared to crude cell extract or the whole bacterial cells.

***Keywords**: Bacteria, enzyme, nitrate reductase, gold nanoparticles, eco-friendly.*

Introduction

Nanoparticles (NPs) are particles ranging in size from a few nanometers to about one hundred nanometers, and show altered properties on account of enhanced surface area. Innovative approaches are being explored for the production of nanoparticles of desired size, shape and properties with a view to use them for novel applications. NPs have contributed to opening different fronts for designing new materials and assessing their properties by adjusting the size, shape and distribution of their molecules[1]. The metal nanoparticles are of particular interest because of their characteristic physical and chemical properties, especially,NPs of silver, gold, copper, etc. exhibit the well-known localized Surface Plasmon Resonance (SPR) which is not displayed by bulk material of the same metal. A large number of chemical and physical methods are used for the synthesis of metal nanoparticles. However, the chemicals used for synthesis are toxic and cause severe damaging effects on the environment and consequently the living cells [2].Eco-friendly methods of nanoparticle synthesis rely upon biological systems such as whole cells, tissues or cellular components. These methods in addition to being safe, also result in more uniform sized nanoparticles with lower investments on equipment and energy. The biological synthesis of NPs is a safe, dynamic and energy efficient method of producing NPs [3].Use of plant material, animal products, fungi,algae, microbial cells and variety of biomaterials have been investigated as promising alternatives to the chemical and physical methods of synthesis [4]. The synthesis of gold nanoparticles by using bacteria has been reported by Beveridge and Murray [5]. Microorganisms have been used to synthesize a variety of metals, nonmetals, metal oxides and bimetallic nanoparticles for different applications[6].It has been reported that gold nanoparticles generated biologically are more stable than those generated using other methods [7].Metabolites found in living cells, viz. proteins, organic acids, sugars, enzymes and phenolic compounds work as tiny bioengineers to convert metal ions into nanoparticles by different biochemical mechanisms, such as bioreduction, nucleation, complexation, etc. Gold nanoparticles have been employed in a variety of fieldsincludingcell imaging and molecular bioassays,biosensing,photothermal treatment and drug delivery [8].The present studies were focused ona soil bacterial isolate capable of enzymatic reduction of nitrate. The ability of the organism to reduce nitrate to nitrate in the soil was utilized for the reduction of gold ions into nanoparticles of gold. Nitrate reductase enzyme was isolated by salt fractionation process and then used for the bioreduction of gold ions. It was found that the partially purified enzyme had greater potential to synthesize gold nanoparticles when compared to whole cells of the cell-free lysate. Further characterisation of laboratory synthesised gold nanoparticles was carried out to reveal particle size and shape using SEM. A significant amount of gold that is lost in industrial wastewaters can be recovered using biosorption as reported by Pethkar and Paknikar (1998) [9].Selective recovery of precious metals is possible by virtue of the biochemical composition of microbial cell surfaces (Pethkar et al., 2001; Pethkar and Paknikar, 2003a; Pethkar and Paknikar, 2003b; Paknikar et al., 2003)[10, 11, 12, 13].It is speculated that molecular bioengineers such as nitrate reductase will find applications in the production of metal nanoparticles using the metals recovered from industrial wastes.

Material and Methods

Isolation of bacteria from soil samples

A total of 5soil samples(approximately 5g) were collected from various locations in ChhatrapatiSambhajinagar usingcleanand sterile polythene bags. The samples (1g) were added to sterile distilled water (10ml),mixed thoroughly and allowed to stand for about 2 hr. The clear supernatants(0.1ml) were spread on sterile nutrient agar plates and incubated at 37°C for 24hr. Isolated colonies of bacteria were preserved at 2-8°C on nutrient agar slants.

Preliminary screening of bacterial isolates

The isolates were grown in nutrient broth and culture supernatants were separated by centrifugation at 10,000 rpm. Cell supernatants (0.5ml) were added to solutions of gold($HAuCl_4$,0.5mM). The mixtures were warmed to 40°C until the color of the solutions changed to deep red. The samples were then analysed for Surface Plasmon Resonance (SPR) by measuring absorbance in the visible range. Bacterial isolates were screened for the production of extracellular/intracellularnitratereductaseenzyme. For this, NADH(2.4 mg/ml) was added to buffered potassium phosphate solutioncontaining the substratepotassiumnitrate(0.2gms/100ml) in test tubes and the tubes were incubated in a water bath at 30°C for 2 min. Sulfinamide(0.8gm/100ml) was added to stop the reaction followed by phenazinemethosulfate(200mg/ml) to remove excess NADH. Red colour formed in the reaction was measured at 540nm against standards.

Identification of selected bacterial isolate/s

Isolates selected in the screening were characterized using routine microbiological practices based on cultural characteristics, morphology, staining reactions and biochemical tests. The organisms were identified up to the species level.

Optimisation of parameters for growth of selected isolate

Bacteria were grown in nutrient broth and parameters like pH (3-9), temperature (8-50°C) and substrate concentration(0.1-10% w/v) were optimized by varying one factor at a time while keeping other factors constant. Growth was measured by recording absorbance at 600nm.

Partial purification of nitrate reductase from selected bacterial isolate

Cells of selected isolate/s were lysedin lysis buffer (containing NaOH and SDS)and centrifuged at 10,000 rpm for 15 min.Crude enzyme extract was obtained by salt fractionation using ammonium sulphate (60% w/v, final concentration). The precipitate was removed by centrifugation at 10,000 rpm for 30 min and dissolved in phosphate buffer (0.05mM, pH 8).Protein concentration was measured byLowry's method [14] with bovine serum albumin (BSA) as standard. The absorbance was measured at 660 nm and protein concentration was computed from standard curve of BSA.

Synthesis of gold nanoparticles using purified nitrate reductase

Preparation of the purified enzyme (0.2u/ml,10ml) was contacted with solution of tetrachloroaurate (0.5mM/100ml) for10 min. The formation of gold nanoparticles was ascertained from the colour change of the reaction mixture from yellow to deep red. The synthesis of gold nanoparticles was confirmed by measuring the SPR in the range of 400-600nm. Samples without enzyme served as negative controls, while those with whole cells of selected bacterial isolate served as positive controls in the experiment.

Characterization of nitrate reductase by SDS-PAGE

Purified nitrate reductase was characterized by SDS-polyacrylamide gel electrophoresis for determination of molecular weight and the subunit composition. Electrophoresis was carried out at constant voltage of 100V for about 90 min with appropriate molecular weight markers and standard protein, viz. bovine serum albumin. Molecular weight of purified enzyme was computed by plotting log of molecular weight of the molecular weight markers and standard protein against the distance travelled by each component.

Results and Discussion

Isolation and characterization of bacteria

From the various soil samples, 38 bacterial isolates were obtained and used in the primary screening for synthesis of gold nanoparticles and presence of nitrate reductase. Amongst the isolates, one isolate, viz. IHM06exhibited high level of synthesis of gold nanoparticles and nitrate reduction as evidenced from the qualitative tests. The isolate was selected for further studies. Cultural, morphological and biochemical tests revealed that the isolate was a strain of *Bacillussubtilis*. Table 1 shows the morphological and biochemical characteristics of selected isolate. The isolate was preserved on nutrient agar slopes until further use.

Table 1. Morphological and biochemical characterization of selected isolate.

Morphological/biochemical Test	Result
Grams staining	Gram positive
Motility test	Motile
Spore staining	Spore positive
Capsule	Positive
Catalase	Positive
Citrate	Positive
Sugar fermentation- glucose	Positive
Sugar fermentation- lactose	Positive
Anaerobic growth	Negative
Nitrate reduction	Positive
Starch hydrolysis	Positive

Preliminary screening of bacterial isolates

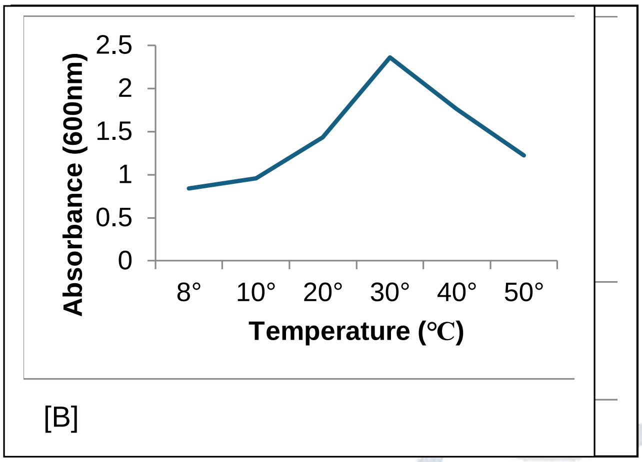
As shown in Figure 2, addition of pure cultures of bacterial isolates to gold solutions resulted in a colour change from yellow to deep red in the case of some isolates. Isolate no. IHM06 i.e. *Bacillus subtilis* displayed highest intensity of red colour when compared to the other isolates.

Figure 1. Conversion of gold ions into nanoparticles of gold as evidenced from change in colour from yellow to red in the presence of selected bacterial isolate IHM06 (identified as *Bacillus subtilis*).

Preliminary screening revealed that *B. subtilis* was capable of producing the enzyme nitrate reductases evidenced from a change in colour of potassium nitrate solution to red and absorption at 540 nm. Hence this organism was selected with a view to utilizing the reducing power of the enzyme for converting gold ions into nanoparticles of gold.

Optimisation of parameters for growth of selected isolate

The optimum pH for growth of *B. subtilis*was found to be in the range of 6-8 with maximum growth at pH7 (Figure 2, A). The optimum temperature was about 35°C (Figure 2, B), while the optimum substrate concentration was 0.8%(w/v) as shown in Figure 2, C.



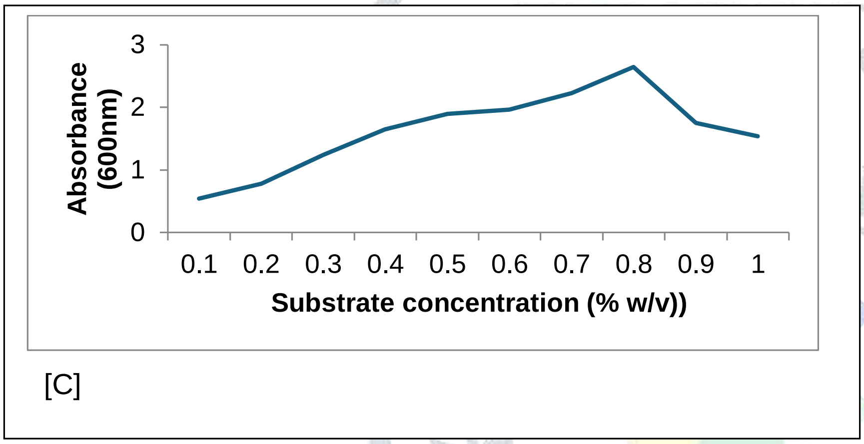


Figure 2. Optimization of parameters for the growth of selected isolate *B. subtilis*, [A] effect of pH on growth of the isolate, [B] effect of temperature on growth of the isolate, [C] effect of substrate (nitrate) concentration on growth of the isolate.

Partial purification of nitrate reductase from selected bacterial isolate

Ammonium sulphate fractionation yielded crude enzyme fraction with total protein content of 5 mg. The total activity of the crude fraction was to the tune of 6.93 units, specific activity of 1.137 u/mgandfold purification was to the tune of 3x as compared to cell free supernatant (Table 2). The separation using ion-exchange chromatography yielded total protein content of 1.5 mg and total activity of 3.96 units with specific activity of 2.64 u/mg. The purity level was thus enhanced to about 7 fold.

Samples	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Fold Purification	Yield (%)
Crude extract	100	0.1122	0.3	0.374	11. 22	-	100
Ammonium sulphate	25	0.2275	0.2	1.137	6.9375	3.040	61.8
Ion-ex (DEAE cellulose)	15	0.2643	0.1	2.643	3.9645	7.066	35.30

Table 2. Purification of nitrate reductase enzyme from B. subtilis by salt fractionation and ion-exchange chromatography using DEAE-cellulose.

Synthesis of gold nanoparticles using purified nitrate reductase

It was observed that exposure of gold ions to the purified enzyme from *B. subtilis*showed SPR peak at 540 nm indicating the synthesis of gold nanoparticles (Figure 3). Several authors have reported characteristic SPR values in the range of 520-550nm [14]. Interestingly, the absorbance value for the purified enzyme was higher than that for the whole bacterial cells.The results justify the use of purified enzyme preparations for bioengineering the synthesis of gold nanoparticles. The extracellular biosynthesis of stable gold nanoparticles (AuNPs) using bacterial culture supernatants containing the cofactor NADH and electron carrying NADH dependent enzymes by the bioreduction of Au^{3+}ions to Au^{0}has been reported earlier [15]. Our results point to the usefulness of purified enzyme preparations for synthesis of monodisperse metal nanoparticles.

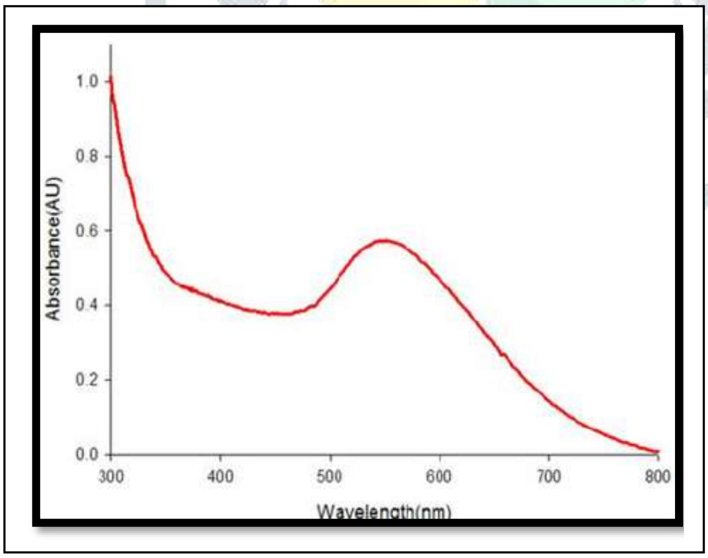


Figure 3. Characteristic surface plasmon resonance (540nm) of gold nanoparticles synthesized using purified nitrate reductase from *B. subtilis*.

Characterization of nitrate reductase by SDS-PAGE

Structural characterization of cytoplasmic nitrate reductase from *B. subtilis* by SDS-PAGE revealed single subunit of the enzyme with molecular weight of 6.6 KD (Figure 4).The results corroborate well with earlier investigation on the enzyme [16]. Chu et al. [17] described the biochemical and molecular features of nitrate and nitrite reducing enzymes from *Bacillus megaterium* and reported that the cytoplasmic enzyme has similar single-subunit structure. According to existing knowledge, nitrate reductase exists in three forms in bacteria, viz. membrane bound, periplasmicenzymeand the cytoplasmic enzyme [18]. However, since the purpose of the experiments was to use the reducing capability of the enzyme to convert gold ions into nanoparticles, no special efforts were made in order to separate the three types of nitrate reductases from *B. subtilis* cells. Further experiments on the studies of enzyme kinetics and molecular docking are underway for the elucidation of mechanism of gold nanoparticles synthesis. Attempts will also be made in order to evaluate to toxicity of gold nanoparticles and applications in biomedical field.

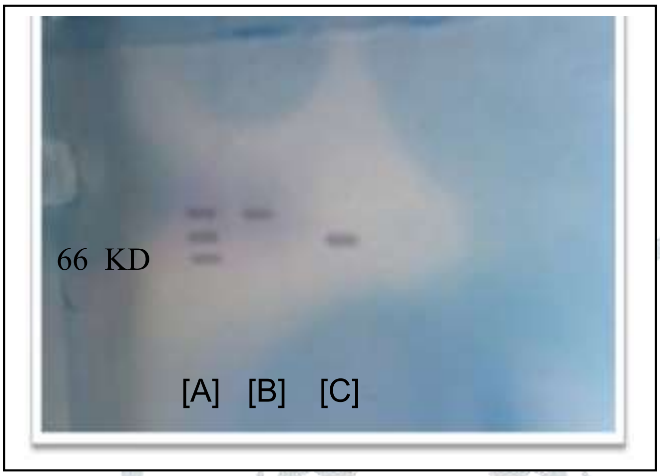


Figure 4. Characterization of purified nitrate reductase from *B. subtilis* by SDS-PAGE, lane1-Molecular weight markers, lane2- purified nitrate reductase from *B. subtilis*, lane3- standard protein (bovine serum albumin). The purified enzyme fraction coincides with the molecular weight marker of molecular weight 66KD.

Conclusions

Biological agents such as plants and plant products as well as microorganisms are valuable tools for the reduction of metal ions to yield stable nanoparticles since they are eco-friendly and economical. In the present studies, a soil isolate, identified as *Bacillussubtilis* was found capable of gold nanoparticle using the intracellular enzyme nitrate reductase.By virtue of the fixed molecular size and shape of the biological bioengineer, it was deemed fit for production of monodisperse gold nanoparticles. Optimization of enzyme production was carried out by controlling the environmental and growth conditions.Since, enzyme-based processes are more controllable and predictable as compared to whole cells the intracellular enzyme nitrate reductasewas purified by salt fractionation followed by ion-exchange chromatography and then used for the enzymatic synthesis of gold nanoparticles.The enzyme was purified to about 7 fold with specific activity of 2.643 u/mg and yield of 35%. Purified enzyme showed high efficiency of gold nanoparticles synthesis compared to culture supernatants and crude enzyme preparations.

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