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Exploration of Microbial Diversity of Himalaya Region for Gold Nanoparticles Synthesizing Bacteria

Rajni Kant Thakur*, Poonam Shirkot and Ambika Verma

Department of Biotechnology, Dr. Y.S Parmar University of Horticulture and Forestry, India
**Corresponding author*

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A B S T R A C T

The isolation of gold nanoparticles synthesizing bacteria from natural sources and their identification are very important in terms of discovering new industrial products. Keeping in view, local goldmine at Khaltunala and four hot water springs of Himachal Pradesh were selected as a source for new gold nanoparticles synthesizing bacteria. Therefore, aim of the present study was the isolation and characterization of gold nanoparticles synthesizing bacteria from these sites for synthesis of gold nanoparticles. Eighty five bacterial isolates were isolated from the samples using nutrient agar medium. All the bacterial isolates were studied for various morphological characters. Eighty five bacterial isolates were screened individually for their ability to synthesize gold nanoparticles and only 38 bacterial isolates, from goldmine were shown to possess gold nanoparticles synthesizing activity. Formation of gold nanoparticles was confirmed by colour change of the solution from yellow to purple/red wine colour. An indigenous bacterial isolate capable of gold nanoparticles synthesis was isolate and identified as a *Bacillus licheniformis* GPI-2, based on its morphology and analysis of its16S rDNA gene sequencing. After 6-12 hrs of incubation, monodisperse gold nanoparticles were, obtained. Which were characterized and found to be of 45 nm size. Gold nanoparticles were characterized through FTIR, transmission electron microscope with the size range of 40-45 nm. The process of synthesis of nanoparticles using an indigenous bacterial isolate from gold mine pebbles sample has been reported in this study, leading to the development of an easy bioprocess for synthesis of GNPs with many potential applications. The use of gold compounds and gold nanoparticles, with respect to their potential therapeutic applications such as anti-angiogenesis as anti-malarial agent, anti-arthritic agent, drug delivery, gene transfer, cancer nanotechnology, drug delivery applications, and its optical properties for cancer diagnosis and photo thermal therapy and biosensors, bio imaging, antimicrobial activity, food preservation.

Introduction

Microbial diversity of the Himalaya region is mostly unexplored towards the gold nanoparticles synthesis by bacteria so far. Microbes are primitive organisms which inhabit the earth since origin of life on earth, because of their versatile adaptability they not only survive during millions of the years but they are highly significant in maintaining ecological balance on earth. Explorations of Himalaya region for beneficial bacterial species can potentially provide us novel microbes with newer applications, which may be highly efficient and significant to biotechnology, microbial industry. So in order

to explore the microbial diversity of mid region of Himalaya, we have conducted the survey of different sites like Manikaran, Kasol, Kalath, Vashisht hot water springs, Rhontang, gold mine Khaltunala. The microbial world is vast, with 10^{30} organisms present on the earth, diverse and can only be observed through relatively tiny samples at discrete points in space and time. Biodiversity is related to the number of species, or species richness, along with the richness of activity each species undergoes during its existence through events in the life of its members, plus the non phenotypic expression of its genome. Thus, to study microbial biodiversity, it is necessary to understand interactions between and among species in a given habitat. According to Erwin, 'how these species are grouped as a living unit' in a given ecological unit, is a task that is heroically difficult to complete for microorganisms, without a massive, globally coordinated program of action. The diversity of microbial communities and their ecologic and metabolic functions are being explored across a great range of natural environments: in soils (Whitman *et al.,* 1998; Atlas and Bartha, 1993; Redwood, 1998), air (Fierer *et al.,* 2007) and seas (Schloss and Handelsman, 2006; Roesch *et al.,* 2007; Brodie *et al.,* 2007), on plants (Alonso and Gasol, 2007) and in animals (Frias-Lopez *et al.,* 2008; Sogin *et al.,* 2006) and in extreme environments such as the arctic (Stevens and Ulloa, 2008), deep-sea vents (Fang *et al.,* 2005), uranium-contaminated soil (Michalke *et al.,* 2008), and waste-water treatment discharge (Sharma *et al.,* 2009). In recognition of the role marine microbes play in the biogeochemical processes that are critical to life in all environments on Earth including carbon and nitrogen cycling, the International Census of Marine Microbes. Microbial communities are central to health, sustainable cities, agriculture, and most of the planet's geochemical cycles. Prokaryote communities are also reservoirs for the discovery of new drugs and metabolic processes. As with any reservoir, its size is important. In the world of microbial ecology, we need theory very badly. Almost any consequential microbial community will have 10^{10} to 10^{17} bacteria that could compose more than 10^{7} differing taxonomic groups and countless functional groups. It seems remarkable that we should even contemplate trying to understand such vast systems without recourse to some form of theory. It is now well recognized that, for many decades, microbiologists had grossly underestimated microbial diversity levels by relying on cultivation-based techniques, which capture only a select few microbial taxa capable of growing rapidly under artificial laboratory conditions. Likewise, with few obvious morphological differences delineating most microbial taxa, direct microscopic analyses of environmental samples are of little use for quantifying microbial diversity. By using high-throughput nucleic-acid-based analyses of microbial communities, researchers have gained new appreciation for the breadth and dynamics of microbial diversity in specific habitats, the spatial and temporal variability in the levels of microbial diversity, and the factors driving this variability. Bioprospecting is novel concept for exploiting locally available microbial species in ecosystem. Microbial species can be exploited towards biosynthesis of gold nanoparticles and involve simple experimental conditions such as incubation time, temperature, pH and wavelength (Gade *et al.,* 2008). Microbial diversity is an excellent resource for biotechnological innovations as biomaterials of nanometer dimension form the basis of life. Diversity of life especially microorganisms is being used from several decades as ecofriendly nanofactories for the biosynthesis and production of many useful products such as enzymes, therapeutic proteins, life saving vaccines and now various types, of metal

nanoparticles including gold nanoparticles (Riddin *et al.,* 2006; Sharma *et al.,* 2012). Gold in different forms, has been used in medicine throughout the history of civilization. The use of gold compounds and gold nanoparticles, with respect to their potential therapeutic applications such as anti-angiogenesis (Mukherjee *et al.,* 2005), as anti-malarial agent (Navarro *et al.,* 1997) and anti-arthritic agent (Tsai *et al.,* 2007)drug delivery (Khan *et al.,* 2014), gene transfer (Shomura *et al.,* 2011), cancer nanotechnology (Cail *et al.,* 2008), drug delivery applications (Ghosh *et al.,* 2008), and its optical properties for cancer diagnosis and photo thermal therapy and biosensors (Amanda *et al.,* 2005), bio imaging (Mohammed *et al.,* 2009), antimicrobial activity (Mohammed *et al.,* 2010) food preservation (Mohammed *et al.,* 2009).

Generally nanoparticles are synthesized through various physical and chemical methods but with poor morphology and these procedures are very expensive and prove to be toxic to environment because of use of toxic chemicals and high temperature used for synthesis process (Rai *et al.,* 2008; Birla *et al.,* 2009). Biological method provides a wide range of resources for the synthesis of nanoparticles. The rate of reduction of metal nanoparticles using biological agents is found to be much faster and also at ambient temperature (Gade *et al.,* 2008*;* Mukherji *et al.,* 2008). Shape and size of nanoparticles can be altered through changing in pH, temperature of reaction mixture (Gericke, 2006). Microorganisms secrete enormous amount of enzymes, which are capable of hydrolyzing metals and thus bring about enzymatic reduction of metal into metal ions (Rai *et al.,* 2009). Enzyme nitrate reductase has been found to be responsible for the synthesis of nanoparticles in case of fungi (Kumar *et al.,* 2007; Kumar *et al.,* 2007). Thus biosynthesis of gold nanoparticles using microbes is less labour intensive, low cost, non toxic and is more ecofriendly. In case of bacteria *Lactobacillus* species during the initial stages of synthesis of nanoparticles, nucleation of clusters of metal ions take place and hence there is electrostatic interaction between the bacterial cell and metal cluster which leads to formation of nanoclusters at the end, smaller sized nanoclusters get diffused through bacterial cell wall (Nair and Pradeep, 2002). The mechanism of extracellular synthesis of nanoparticles using microbes is basically found to be nitrate reductase mediated synthesis (Duran *et al.,* 2005).

The cell wall of the microorganisms plays a major role in the intracellular synthesis of nanoparticles. The cell wall being negatively charged interacts electrostatically with the positively charged metal ions. The enzymes present within the cell wall bioreduce the metal ions to nanoparticles, and finally the smaller sized nanoparticles get diffused of through the cell wall.

Materials and Methods

Survey and selection of the sites

A survey was conducted for selection of various sites of goldmines and hot water springs from different districts of Himachal Pradesh. Goldmine Khaltunala which is three km far from Nauni village in district Solan and four hot water springs *viz*., Manikaran, Vashisht, Kasol and Kalath located in Kullu district of Himachal Pradesh were surveyed and selected. Manikaran is one of hottest water springs of Himachal Pradesh, which is 45 km from Kullu, Kasol is 15 km from Manikaran, lies in the Parvati valley of Kullu district. Kalath is 10 km from Manali and lies in lap of Beas River. Vashisht is 6 km from Manali, located well above the river Beas was surveyed and selected. The geographical locations of these sites along with their altitudes (m) have been described in table 1.

Isolation and identification of gold nanoparticles and bacteria

A survey was conducted for selection of various sites of goldmines Khaltunala, in Solan, Kullu districts of Himachal Pradesh. Eighty seven samples in form of water, soil, biofilm, pebbles, roof topping/stalagamite and rock matting were collected from gold mine sites and four hot water springs *viz*., Manikaran, Vashisht, Kasol and Kalath of Himachal Pradesh. (Map 1, Figure 1) All these samples were kept at 4°C in refrigerator in laboratory till further experimentation. Three different culture media were investigated for isolation of gold nanoparticles synthesizing bacterial isolates *viz.*, Nutrient agar medium, Eosin methylene blue agar medium and Luria bertani medium. One gram soil, Pebbles, stalagmite /1.0 ml water, biofilm samples collected from sampling sites were dissolved in 9.0 ml of sterile water and serial dilution technique was used for isolation of bacterial isolates. The plates were incubated at 37^{o}C for 24-48 hrs for bacterial growth. Turbid cultures were streaked on plates of solidified growth medium. Individual colonies were restreaked repeatedly, and the axenic cultures thus obtained were stored at 4^{o}C.

Morphological characterization

All the bacterial isolates obtained in previous step were further studied for various morphological characters. Various morphological descriptors of colour, size, optical property and elevation of the colonies and various microscopic characteristics studied were gram reaction, shape, arrangement and spore formation.

Quantitative screening of bacterial isolates for gold nanoparticles synthesis ability

Assessment of all eighty five bacterial isolates for their ability to synthesize gold nanoparticles was carried out. One percent concentration of the inoculum (overnight culture) of each bacterial isolate was inoculated into the 50 ml nutrient broth followed by incubation at 37^{o}C for 24-48 hrs at 150 rpm. Supernatant of each bacterial culture was collected by centrifugation at 8500 rpm for 15 minutes at 4^{o}C to study extracellular synthesis of gold nanoparticles. Ten ml of each supernatant was mixed with 10 ml of 1mM solution of $HAuCl_4$ and incubated at 37^{o}C for 240 hrs. Formation of gold nanoparticles was studied 0-240 hrs, with an interval of 12 hrs and confirmed by colour change of the solution from light yellow to red wine/purple colour. This formation of gold nanoparticles was also confirmed by the Spectrophotometer (Spectronic 20, Milton Roy Company) at two different wavelengths of 540 and 560 nm.

Biochemical characterization and molecular characterization

Various biochemical characters were investigated using standard assays. Genomic DNA extraction Mini kit (Real Genomics). Presence of DNA and its quality was checked using 1.0% agarose gel and then was viewed by UV trans-illuminator. After visual confirmation of DNA bands in the gel, photograph of the same gel were taken by gel documentation apparatus, Alphalmager^{TM} (Alpha Infotech Corporation, USA). The DNA of GBI-3 was selectively amplified using PCR technology. Universal primers B27F (5`-AGAGTTTGATCCTGGCTCAG-3'U1492R) and (5`-GGTTACCTTGTTACGACTT-3') for 16S *rrna* gene were used for the experiment. The eluted and purified DNA of GBI-3 was sequenced. The sequences have been submitted to NCBI with accession number KP 219453. To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0 bioinformatics tool. Neighbour-Joining (NJ) technique of

mathematical averages (UPGMA) was used. Nanoparticles obtained were analyzed using various techniques such as, Fourier transform infrared spectroscopy (FTIR and Transmission electron microscope.

Optimization of culture conditions for maximum gold nanoparticles synthesis by selected bacterial isolate

The culture conditions for useful and prized microorganisms are generally optimized to obtain higher yields of their useful products. In the present study the best bacterial isolate was selected by quantitative assay of gold nanoparticles synthesizing activity. The bacterial isolate depicting maximum gold nanoparticles synthesis activity was selected and further investigated to study the effect of different factors such as incubation time, temperature, pH and wavelength on gold nanoparticles synthesis.

Effect of incubation time

Effects of different incubation times for maximum gold nanoparticles synthesis were investigated ranging from 0-72 hrs, and the optimum incubation time leading to maximum gold nanoparticles production of gold nanoparticles was selected.

Effect of incubation temperature

Effect of incubation temperature for maximum gold nanoparticles synthesis was studied at a temperature range of 10-50^{o}C using nutrient broth and the optimum temperature leading to maximum gold nanoparticles production of gold nanoparticles was selected.

Effect of pH

To find optimum pH for maximum gold nanoparticles synthesis by selected bacterial isolate, a pH range of 5.0-7.5 was examined using nutrient broth medium at an optimum temperature and optimum time. The best condition leading to maximum gold nanoparticles production of gold nanoparticles was selected.

Effect of wavelength

Effects of different wavelength for the maximum values of gold nanoparticle synthesis were investigated ranging from 400-650 nm. The optimum wavelength leading to maximum readings of gold nanoparticles synthesis was selected**.**

In vitro synthesis of gold nanoparticles by *Bacillus licheinformis* GPI-2

Fourier transform infrared spectroscopy

Microcup was washed with 100% absolute ethanol. 10 ul sample was filled in a 2 mm internal diameter microcup and loaded onto the FTIR set at 26°C±1°C. The samples were scanned in the range of 4,000 to 400 cm^{-1} using a Fourier transform infrared spectrometer (Thermo Nicolet Model 6700, Waltham, MA, USA). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

Transmission electron microscope

TEM studies were carried out using Jeol 2100 microscope operating at 120 kV accelerating voltage. Samples were prepared by placing a drop of *in vitro* gold nanoparticles solutions on carbon-coated TEM grids. The films on the TEM grids were allowed to dry for 5 min at room temperature before analysis.

Results and Discussion

Isolation of gold nanoparticle synthesizing bacteria was carried out from samples using nutrient agar medium at 37ºC. All the

samples were inoculated in 150 ml flasks containing 50 ml nutrient broth and incubated at 37^{o}C at 50 rpm for 24 hrs. Turbid cultures were streaked on plates of solidified nutrient agar medium (1.5% agar). Individual colonies were restreaked repeatedly and the purified colonies were stored at 4^{o}C in refrigerator till further processing/use. A total of eighty five bacterial isolates were isolated from eighty seven samples. From goldmine a total of 43 bacterial isolates were obtained and rest 42 bacterial isolates were obtained from sample of various hot water springs. Different colony morphotypes have been observed. On the basis of colour, 10.5% morphotypes were found creamish white in colour, rests of them were found white in colour. On the basis of shape 49% isolates showed circular morphotypes, 51% were irregular in shape. On the basis of opacity only 15.3% morphotypes possessed non- opaque colony and rest were opaque.

Quantitative screening of bacterial isolates for gold nanoparticles synthesis ability

Eighty five bacterial isolates were screened individually for their ability to synthesize gold nanoparticles. One percent concentration of inoculum (overnight culture) was inoculated into the nutrient broth for gold nanoparticles synthesis followed by incubation at 37^{o}C for 24 hrs at 50 rpm. The supernatant was collected by centrifugation at 8500 rpm, 4^{o}C for 15 mins to investigate extracellular synthesis of gold nanoparticles using the supernatant. 10 ml of supernatant was mixed in 10 ml of 1 mM solution of gold chloride ($HAuCL_4$), prepared with double distilled water and incubated at 37^{o}C for 36 hrs.

Formation of gold nanoparticles was confirmed by colour change of the solution from yellow to red wine/purple colour (Figure-2). This formation of gold nanoparticles was also confirmed by the Spectrophotometer (Spectronic 20, Milton Roy Company) at two different wavelengths of 540 and 560 nm. Out of the total of 85 bacterial isolates, it has been observed that only 38 bacterial isolates, of a total 43 isolated from goldmine have been possess to produce gold nanoparticles synthesizing activity and none of 42 bacterial isolates obtained from hot water springs have been shown to possess gold nanoparticles synthesizing activity. Out of thirty eight gold nanoparticles synthesizing isolates only eleven bacterial isolates has been selected for further studies on the basis of maximum gold nanoparticles synthesis *viz*., GBI-1, GBI-2, GBI-3, GYI-4, GYI-5, GYI-10, GYI-11, GPI-2, GPI-3, GPI-5 and GPI-6.

Statistical analysis of data

On the basis of data analysis using CRD and OPSTAT software package GBI-3 bacterial isolate, the maximum producer of gold nanoparticles possessed the maximum mean of 0.940 at 540 nm, which was found to be higher than rest of the bacterial isolates followed by bacterial isolates GPI-2 and GBI-1, with mean values of 0.880 and 0.870 respectively at 540 nm (Table-2).

Irrespective of bacterial isolates, GBI-3 was giving maximum O.D value at 36 hrs which was found to be the higher then all other isolates. In the interaction effect of maximum producer of gold nanoparticles and time interval the maximum concentration of gold nanoparticles was recorded for GBI-3 after 36 hrs which was statistically at par with treatment combination GPI-2 and GBI-1 at 36 hrs. Again, GBI-3 isolate was the maximum producer of gold nanoparticles with mean value of 0.960 at 560 nm, which was found to be higher than all other isolates followed by GBI-1 and GPI-2, with mean values of 0.900 and 0.880 respectively at 560 nm (Table-3).

Biochemical and molecular characterization

Pure colonies isolated from the different samples were characterized for their morphological and physiological characteristics by various biochemical tests using the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). The screened strain is an aerobic, gram positive, rod shaped with round colonies in shape, 2 mm in diameter with undulated margin, opaque with rough surface. GPI-2 bacterial isolate tested positive for arginine dihydrolase, hydrolysis of esculin, beta galactosidase, phenyl alanine deaminated, degradation of tyrosine acid production from glycerol, salicine, starch, glycogen, lactose, D mannose, maltose, ribose, Sorbitol, sucrose, and found negative for lysine decarboxylase, ornithine decarboxylase, tryptophan, deaminase, hydrolysis of urea, acid production from D and L fucose, methyl beta xyloside, D arabinose, D and L arabitol, adonitol, sorbose, lyxose.

Molecular characterization was carried out using 16S r DNA-PCR technology. Total genomic DNA of the GPI-2 bacterial isolate was extracted successfully using genomic DNA extraction Mini kit (Real Genomics) and was selectively amplified with universal primers for 16S *rrna* gene followed by agarose gel electrophoresis leading to a single clear band. This was eluted, purified and sequenced. The sequence was submitted to NCBI with accession number Genbank KP219455. BLASTn analysis depicted homology of GPI-2 bacterial isolate with other *Bacillus* species. To gain insight of evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0 bioinformatic tool (Tamura *et al.,* 2011). The bootstrap analysis values identified the bacterial isolate GPI-2 as *Bacillus licheniformis* GPI-2 (Figure 3), Multiple sequence alignment of query nucleotide sequence of maximum gold nanoparticles synthesizing indigenous *Bacillus licheniformis* strain GPI-2 was performed with that of the selected nucleotide sequences using Clustal W program and pairwise percent similarity score of these selected fifteen nucleotide sequences obtained from NCBI database with test isolate GPI-2 from goldmine, elucidates that sequence-1. *Bacillus licheniformis* strainGPI-2 showed maximum similarity score of 99% with *Bacillus licheniformis* strain NCDO 1772 16S ribosomal RNA gene partial sequence.

In vitro synthesis of gold nanoparticles by indigenous *Bacillus licheniformis* strainGPI-2

Extracellular biosynthesis of gold nanoparticles was carried out using supernatant of *Bacillus licheniformis* strainGPI-2, treated with 1mM gold chloride solution and incubated at 37^{o}C for a time period of 0-240 hrs. Biosynthesis absorption spectra of gold nanoparticles which was indicated by colour change of solution from yellow to red wine (Figure 2) and was further confirmed spectrophotometrically. UV-VIS absorption spectra and the time of incubation course and increase in formation of gold nanoparticles took place up to 36 hrs and remained stable up to 48 hrs and then the values declined up to 240 hrs. Gold nanoparticles formation clearly revealed the gold nanoparticles formation initiated after 6 hrs and studies at two different wavelengths of 540 nm and 560 nm (Figure 4a and 4b). On the basis of statistical analysis of data obtained at two wavelengths 540 nm and 560 nm, it was observed that optical density values were higher at 560 nm (Tables 2 and 3) and gold nanoparticles also showed more stability at 36-48 hrs, thus 560 nm wavelength was found superior over 540 nm and was selected for further experiments.

Optimization of culture conditions for maximum gold nanoparticles synthesis by *Bacillus licheniformis* strain GPI-2

The bacterial isolate *Bacillus licheniformis* GPI-2depicting maximum gold nanoparticles synthesis activity was further optimized to study the effect of different factors such as incubation time, temperature, pH and wavelength on gold nanoparticles synthesis. Effect of pH on biosynthesis of gold nanoparticles by *Bacillus licheniformis* GPI-2bacterial supernatant of was studied for 0-72 hrs using 1mM gold chloride solution at a pH range of 5.0, 6.0, 6.8, 7.5, 8.0 (Fig. 5a) and it was observed that maximum gold nanoparticles took place at pH: 6.8. Thus pH has been found to be an important parameter affecting gold nanoparticles synthesis. Variation in pH during exposure to gold ions had an impact on the size, shape and number of particles produced per cell (Kaithresan *et al.,* 2009). Gold nanoparticles formed at pH 6.8 were predominantly triangles, spherical, hexagons, circular in shape. Whenever pH increases, more competition occurs between protons and metal ions for negatively charged binding sites (Sintubin *et al.,* 2009). Effects of different incubation times for maximum gold nanoparticles synthesis were investigated from 0-72 hrs. The optimum incubation time of 36 hrs, leading to maximum gold nanoparticles production was observed (Figure 5b). Effect of incubation temperature for maximum gold nanoparticles synthesis was studied at a temperature range of 10-50^{o}C +using nutrient broth and optimum temperature of 37^{o}C leading to maximum gold nanoparticles synthesis was observed (Figure 5c). It has also been reported that incubation time for maximum gold nanoparticles formation ranges from 30 to 37^{o} C (Lengke *et al.,* 2006). Effect of different wavelengths for the maximum optical density values of gold nanoparticles synthesis was investigated in the range of 400-650 nm and an optimum wavelength of 560 nm was found to be leading to maximum values of optical density of gold nanoparticles synthesis (Figure 5d).

Characterization of *in vitro* synthesis of nanoparticles by *Bacillus licheniformis* strain GPI-2

FTIR measurements were carried out to identify the possible biomolecules protein responsible for the capping and efficient stabilization of the gold nanoparticles synthesized by *Bacillus licheniformis* GPI-2. FTIR spectrogram has shown presence of four peaks 3280.18, 2380.99, 2109.12 and 1636.32 (Figure 6). The FTIR spectra reveal the presence of different functional groups. Wavenumber between 3235 and 3280 cm^{-1} Indicates for hydrogen bond lengths between 2.69 to 2.85A^{o}. Alkynes C-C triple bond stretch is found at 2109 cm^{-1}. Peak at 1636 cm^{-1} corresponds to the N-H bend of primary amines due to carbonyl stretch. Amide 1 is most intensive absorption band in protein. It is primarily governed by the stretching vibration of the C=O (70-80%) and CN stretching groups (10-20%) frequency 1600-1700 cm^{-1}. In the amide I region (1700−1600 cm^{-1}), each type of secondary structure gives rise to a somewhat different C=O stretching frequency due to unique molecular geometry and hydrogen bonding pattern. N-H Stretch of primary and secondary amines, amides. Amide A is with more than 95% due to N-H stretching vibration. This mode of vibration does not depend on the backbone conformation but is very sensitive to the strength of a hydrogen bond. Peak maximum around 1650 cm^{-1} corresponds to proteins alpha helical structure. Half width of alpha helix band depends upon on the stability of the helix. When half width of about 15 cm^{-1} then we have more stability of helix and transition free energy of more than 300 cal/ml.

Table.1 Geographical location of selected sites

Sr. No.	Sites	Districts	Sample codes	Altitude
1.	Goldmine (Biofilm)	Solan	GBI-1	
			GBI-2	
			GBI-3	
			GBI-4	
			GBI-5	
			GBI-6	
			GBI-7	
			GBI-8	
			GBI-9	
			GBI-10	
2.	Goldmine (Roof topping)	Solan	GRI-1	
			GRI-2	
			GRI-3	
			GRI-4	
			GRI-5	
			GRI-6	
			GRI-7	
			GRI-8	
			GRI-9	
			GRI-10	
			GRI-11	
3.	Goldmine (Yellow soil)	Solan	GYI-1	1000 m
			GYI-2	
			GYI-3	
			GYI-4	
			GYI-5	
			GYI-6	
			GYI-7	
			GYI-8	
			GYI-9	
			GYI-10	
			GYI-11	
			GYI-12	
4.	Goldmine (Pebbles)	Solan	GPI-1	
			GPI-2	
			GPI-3	
			GPI-4	
			GPI-5	
			GPI-6	
			GPI-7	
			GPI-8	
			GPI-9	
			GPI-10	
5.	Manikaran (MS1)	Kullu	MS1I-1	1735m
			MS1I-2	
			MS1I-3	

			MS1I-4	
			MS1I-5	
			MS1I-6	
			MS1I-7	
6.	Manikaran (MS2)	Kullu	MS2I-1	
			MS2I-2	
			MS2I-3	
			MS2I-4	
7.	Manikaran (MS3)	Kullu	MS3I-1	
			MS3I-2	
			MS3I-3	
8.	Manikaran (MS4)	Kullu	MS4I-1	
			MS4I-2	
			MS4I-3	
9.	Vashisht (VS1)	Kullu	VS1I-1	
			VS1I-2	
			VS1I-3	
			VS1I-4	
10.	Vashisht (VS2)	Kullu	VS2I-1	2050 m
			VS2I-2	
			VS2I-3	
			VS2I-4	
			VS2I-5	
			VS2I-6	
11.	Kalath (KaS1)	Kullu	KaS1I-1	
			KaS1I-2	
			KaS1I-3	
			KaS1I-4	
12.	Kalath (KaS2)	Kullu	KaS2I-1	2050 m
			KaS2I-2	
			KaS2I-3	
			KaS2I-4	
13.	Kasol (KS1)	Kullu	KS1I-1	
			KS1I-2	
			KS1I-3	
			KS1I-4	
			KS1I-5	1640 m
			KS1I-6	
			KS1I-7	
14.	Kasol (KS2)	Kullu	KS2I-1	
			KS2I-2	

Table.2 Statistical analysis of effect of time (hrs.) on gold nanoparticles synthesis at 540 nm O.D

Time in (hrs) / Isolates	0	12	24	36	48	60	72	84	96	108	Mean A
GBI1	0.100	0.570	0.830	1.290	1.170	1.033	1.000	0.95	0.900	0.850	**0.870**
GBI2	0.100	0.40	0.770	1.200	1.100	0.943	0.923	0.907	0.860	0.820	0.800
GYI10	0.130	0.280	0.690	1.333	1.130	1.067	1.067	1.050	0.950	0.900	0.860
GPI3	0.170	0.310	0.517	1.007	0.960	0.927	0.897	0.877	0.830	0.800	0.730
GYI5	0.100	0.470	0.760	1.267	1.100	1.017	1.013	0.960	0.920	0.880	0.850
GYI11	0.100	0.350	0.737	1.267	1.170	1.000	0.970	0.927	0.880	0.820	0.820
GYI4	0.170	0.310	0.720	1.267	1.170	1.017	0.993	0.923	0.880	0.830	0.830
GBI3	0.170	0.640	0.937	1.333	1.230	1.133	1.067	1.020	0.950	0.890	**0.940**
GPI2	0.130	0.250	0.667	1.300	1.230	1.133	1.100	1.067	0.960	0.900	**0.880**
GPI5	0.170	0.230	0.723	1.133	1.050	0.95	0.937	0.900	0.860	0.830	0.780
GPI6	0.130	0.320	0.773	1.233	1.090	1.067	1.033	0.973	0.910	0.860	0.840
Mean B	0.130	0.370	0.738	1.249	1.130	1.026	1.000	0.959	0.900	0.850	

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.032	0.016	0.012
Factor(B)	0.031	0.016	0.011
Factor(A X B)	0.102	0.051	0.036

Table.3 Statistical analysis of effect of time (hrs) on gold nanoparticles synthesis at 560 nm O.D

Time in (hrs) / Isolates	0	12	24	36	48	60	72	84	96	108	Mean A
GBI1	0.200	0.570	0.840	1.330	1.230	1.070	1.000	0.950	0.910	0.860	**0.900**
GBI2	0.230	0.410	0.780	1.200	1.100	0.990	0.930	0.910	0.860	0.830	0.820
GYI10	0.200	0.290	0.700	1.330	1.200	1.100	1.100	1.050	0.950	0.910	0.870
GPI3	0.270	0.320	0.520	1.010	0.990	0.940	0.910	0.880	0.840	0.800	0.750
GYI5	0.200	0.470	0.760	1.300	1.170	1.050	1.020	0.970	0.920	0.890	0.870
GYI11	0.200	0.360	0.740	1.270	1.200	1.000	0.970	0.930	0.890	0.840	0.840
GYI4	0.270	0.320	0.720	1.270	1.230	1.030	0.990	0.890	0.880	0.830	0.840
GBI3	0.270	0.640	0.940	1.330	1.270	1.170	1.070	1.020	0.960	0.910	**0.960**
GPI2	0.230	0.250	0.680	1.300	1.230	1.100	1.030	1.000	0.960	0.910	**0.880**
GPI5	0.230	0.240	0.730	1.170	1.090	0.990	0.920	0.890	0.870	0.830	0.800
GPI6	0.200	0.330	0.780	1.230	1.130	1.100	1.030	0.980	0.920	0.870	0.860
Mean B	0.230	0.380	0.750	1.250	1.170	1.050	1.000	0.950	0.910	0.860	

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.035	0.017	0.012
Factor(B)	0.032	0.017	0.011
Factor(A X B)	0.102	0.051	0.036

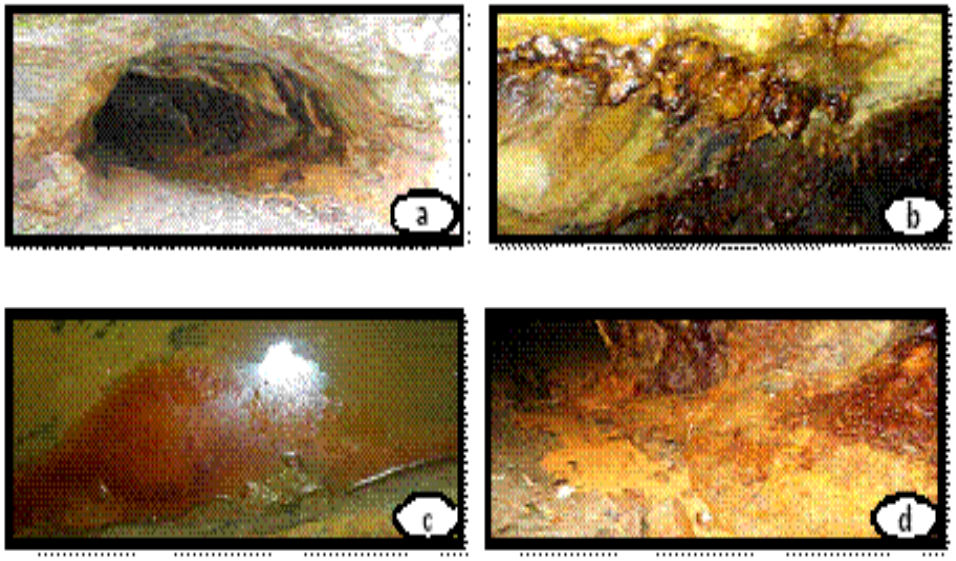


Figure 1: Sample collection sites: Khalturala gold mine from where sample were collected, representing (a) pebble (b) rooftopping (c) biofilm (d) yellow soil

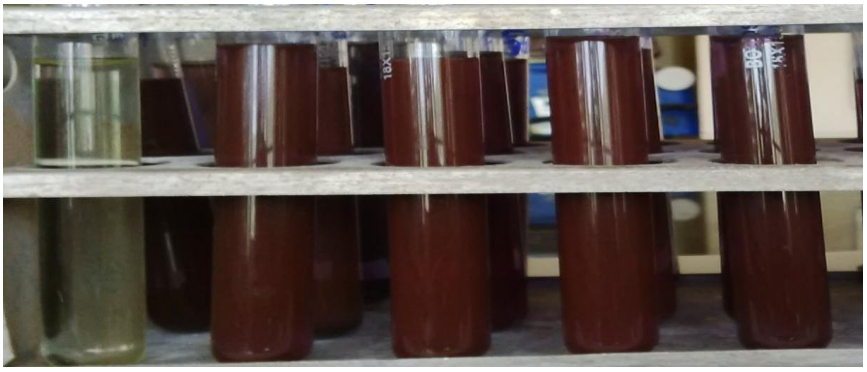


Figure:2 Biosynthesized gold nanoparticles in a colloidal dispersion using the supernatant of *Bacillus licheniformis* GPI-2

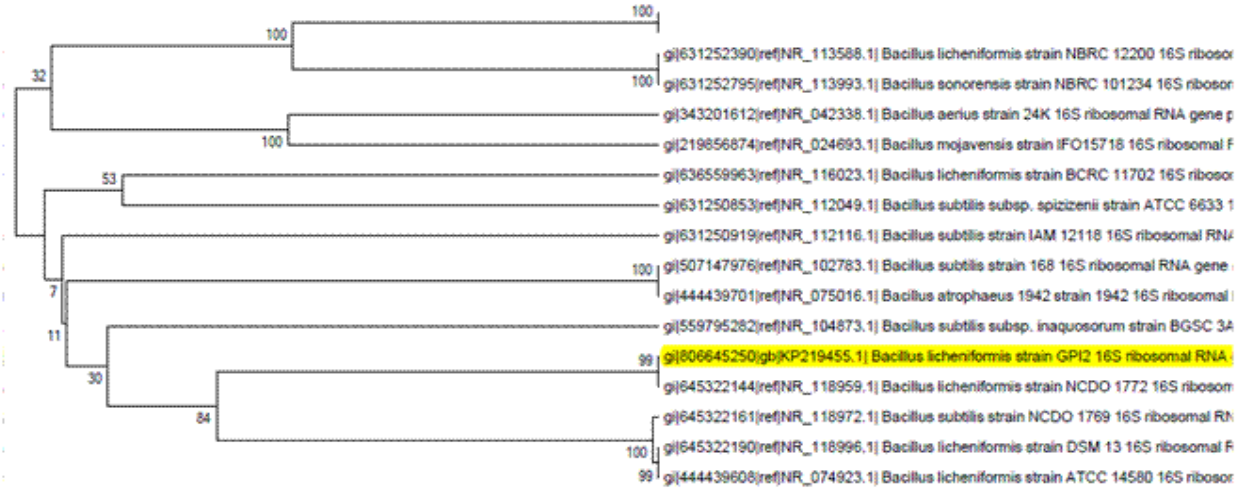
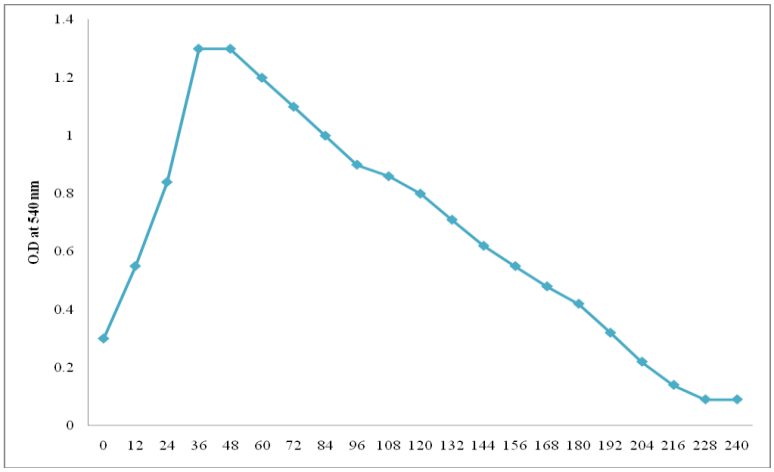
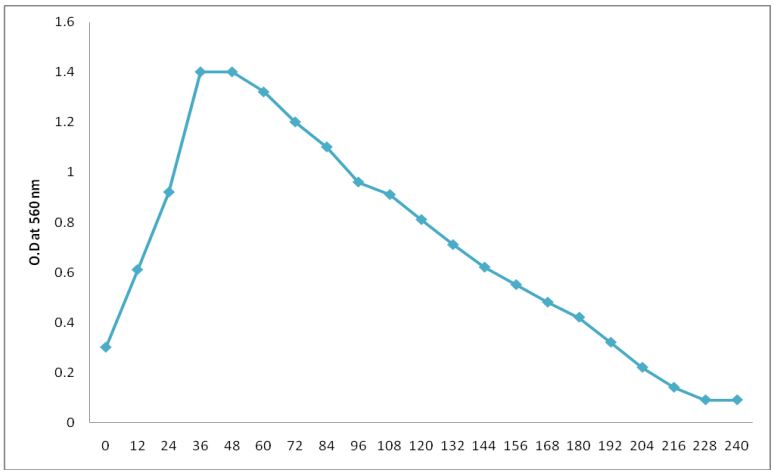


Figure: 3 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Bacillus licheniformis* GPI-2

Fig.4a, b UV-Vis absorption spectra of gold nanoparticles after incubation of supernatant of Bacillus licheniformis GPI-2 with 1mM sodium chloride for time period (0-240 hrs) at 6.8 pH





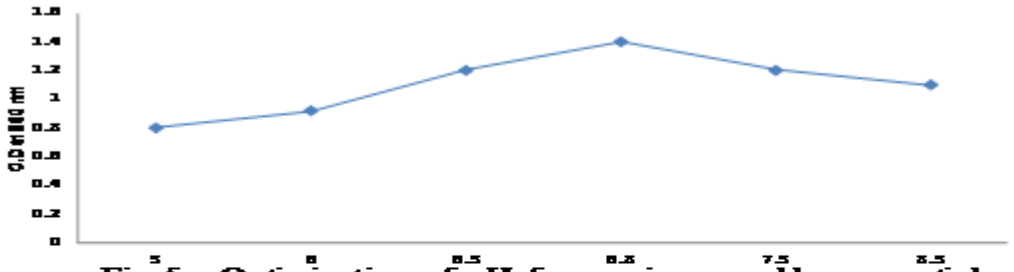


Fig 5a: Optimization of pH for maximum gold nanoparticles synthesis

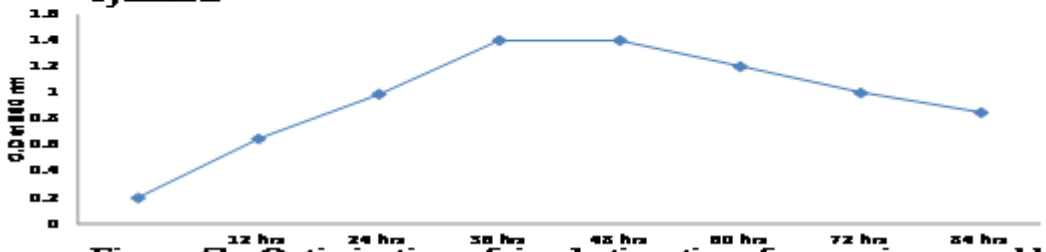


Figure 5b: Optimization of incubation time for maximum gold nanoparticles synthesis

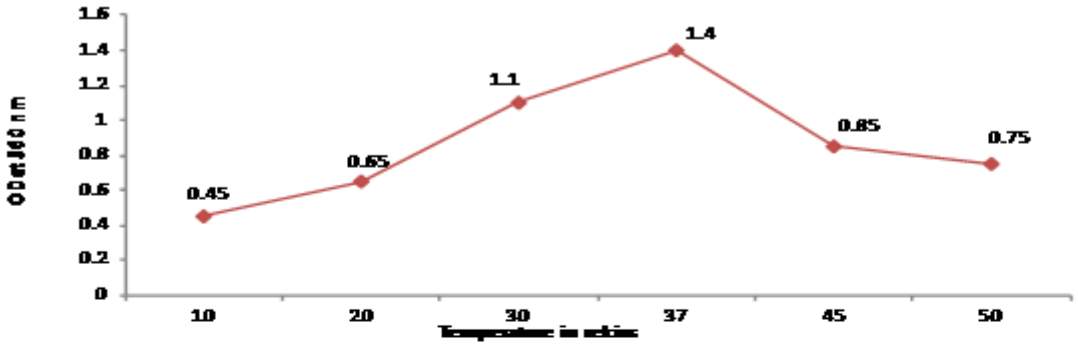


Figure 5c: Optimization of temperature for maximum gold nanoparticles synthesis

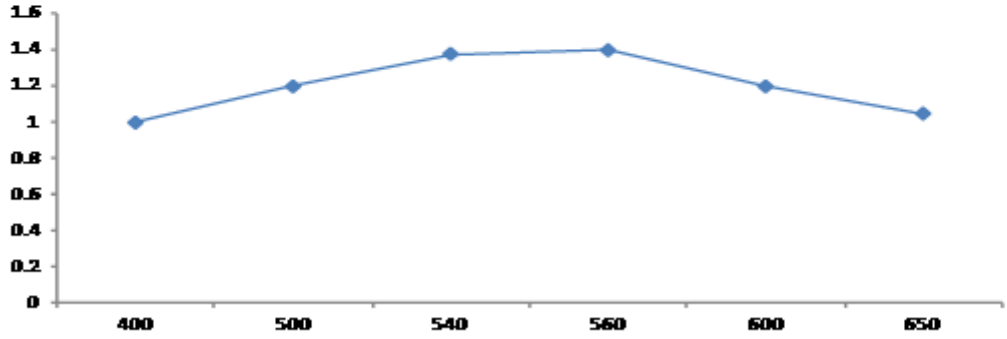


Fig 5d: Optimization of wavelength on gold nanoparticles synthesis

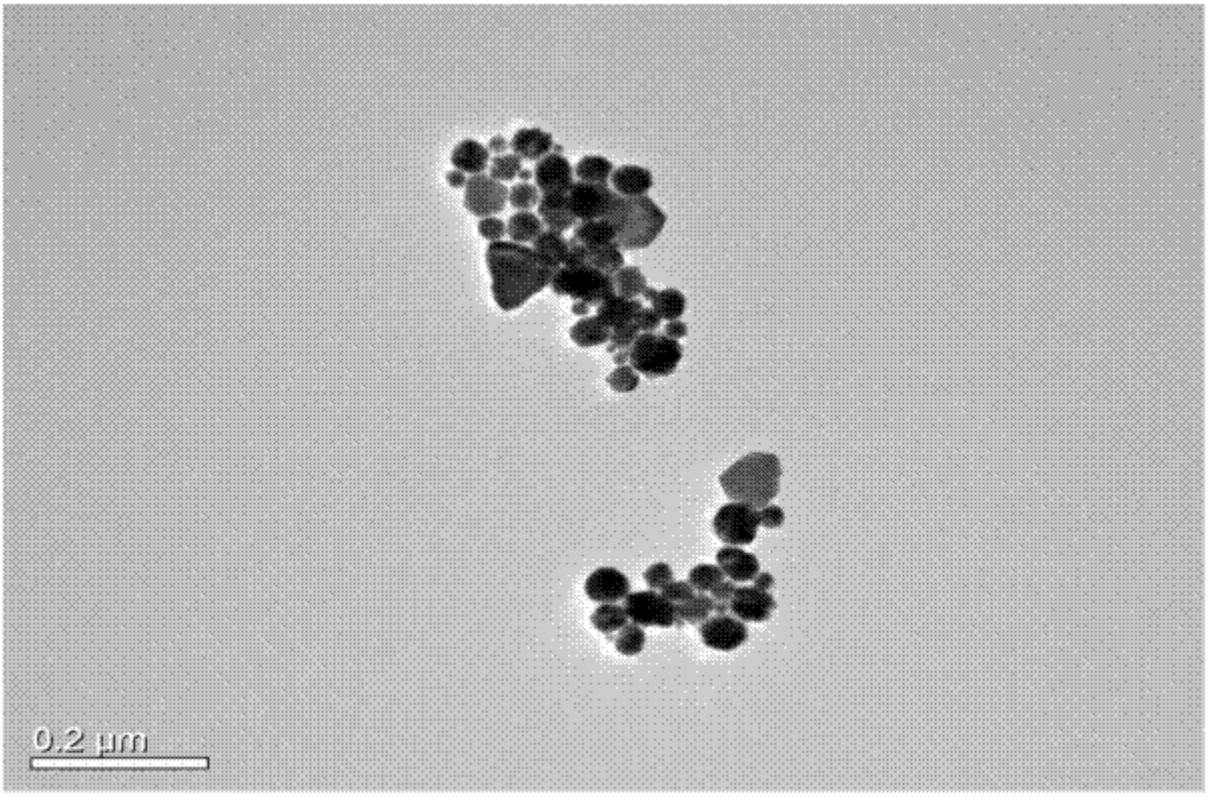


Figure 7a: Characterization of gold nanoparticles through transmission electron microscope showing the different morphology of gold nanopartides.

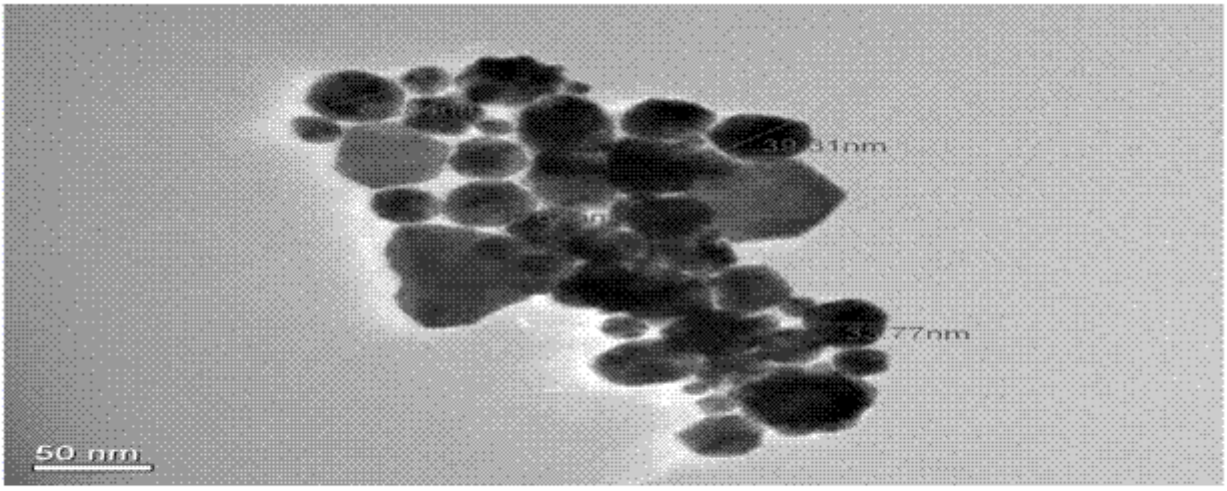
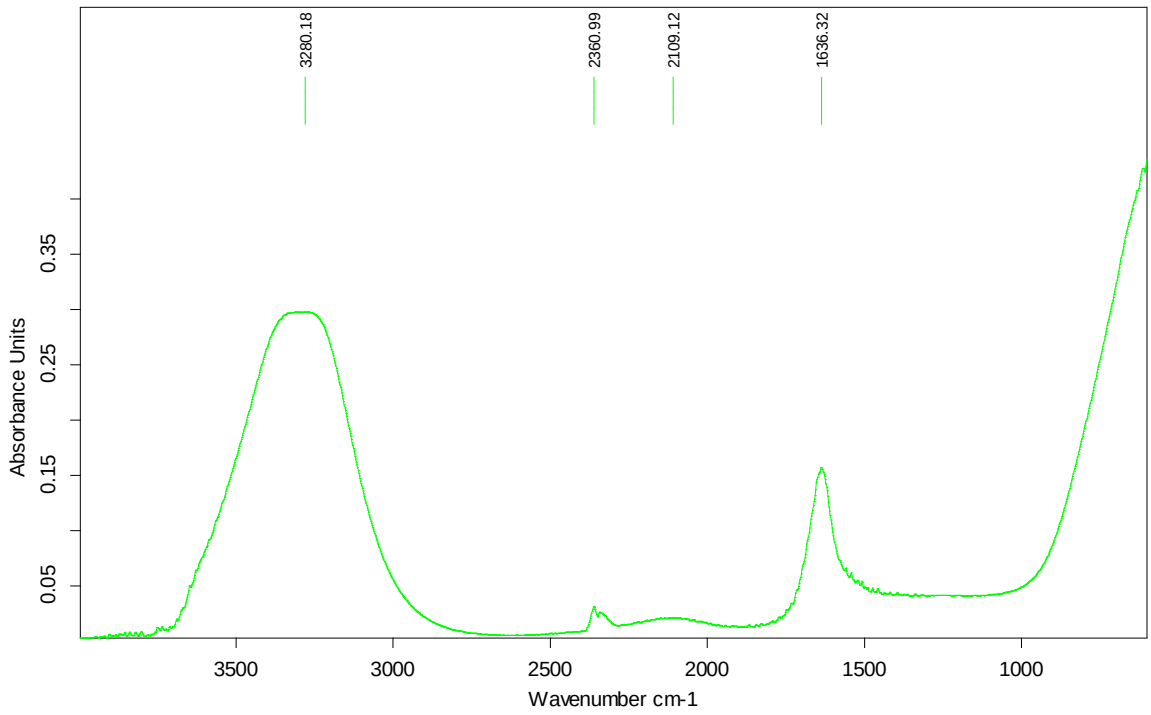
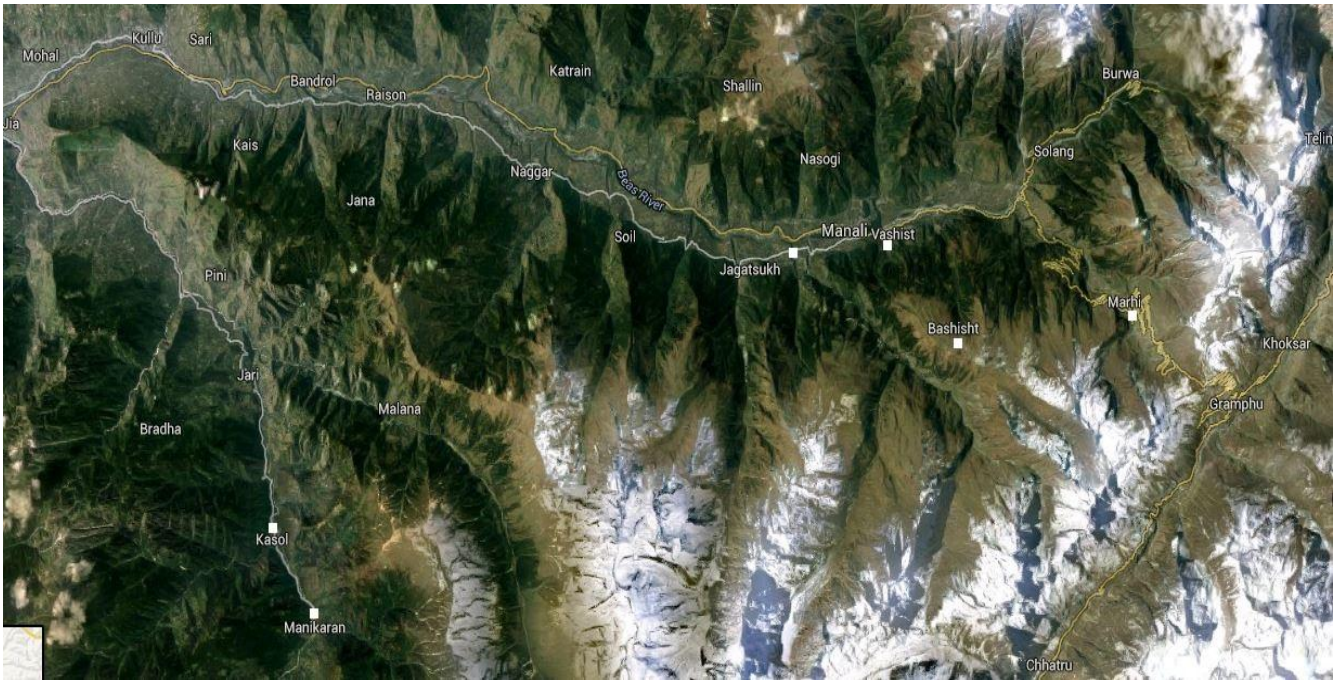


Figure: 7b TEM image of gold nanoparticles showing different sizes of gold nanoparticles

Fig.6 FTIR spectra recorded after 36 hrs of incubation by using supernatant of Bacillus licheniformis GPI-2 with gold chloride



Map.1 Representing sites from where samples has been collected (Kasol, Vashist, Manikaran, Kalath)



Amide 1 absorption is primarily determined by the backbone conformation and independent of the amino acid region, Its hydrophilic or hydrophobic properties and charge. Average frequency of the main components is about 1629 cm^{-1}. Arginine amino acid role was found at 1636 cm^{-1} in gold nanoparticles synthesis through results of FTIR. The two TEM images (Figure 7a and7b) clearly showed discrete gold nanoparticles in the size range of 40 to 45 nm which were mostly triangular, irregular and hexagonal indicating that it was possible to synthesize gold particles of nanodimensions with satisfactory level of monodispersity.

The present work help us develop green route of simple and economic synthesis of gold nanoparticles of 40 –45 nm. The particles synthesized by *Bacillus licheniformis* GPI-2 were characterized by UV vis spectrophotometer and confirmed by TEM. Extracellular, spherical, monodispersed /small clusters of gold nanoparticles were successfully produced, which were confirmed by Transmission electron. Proteins that serve as biomolecules responsible for the reduction process were confirmed by Fourier Transform Infrared Spectroscopy (FTIR).

The biological function of the gold nanoparticles shown great promise to deliver industry demands, moreover, this process could be easily scaled up for the industrial applications to increase the yield of the nanoparticles significantly, which undoubtedly would establish its commercial viability. Our research was focused on identification and exploration of gold nanoparticles synthesizing bacteria.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PS conceptualized and designed experiments and provided technical support and helped in drafting the manuscript. RKT conducted experiments, isolation of bacteria, morphological and molecular characterization, *in vitro* synthesis of gold nanoparticles and characterization using FTIR and TEM and helped in drafting the manuscript both author read and approved the final manuscript.

Author information

RKT is a PhD research scholar currently and member of six international and national science societies. PS is a professor of Biotechnology with 30 years research experience and has published research papers in the journals of international and national repute.

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