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The possible involvement of Nitrate Reductase from *Asparagus racemosus* in Biosynthesis of Gold Nanoparticles

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Abstract

The green nanotechnological approach for the biosynthesis of gold nanoparticles by exploiting the reducing and thermodynamically efficient molecular mechanisms of medicinal plant Asparagus racemosus is reported in the present work. The most influential parameter was found to be temperature. At lower temperature (30ºC) the other optimum parameters were pH 8 and 100 ppm of aurochlorate salt; whereas at higher temperature (100ºC) the optimum parameters were pH 6 , 100 ppm of aurochlorate salt. The involvement of Glutathione as one of the capping proteins and nitrate reductases as possible reducing agent was confirmed by FTIR and biochemical assays respectively. The nitrate reductase activity was found to be reduced from 0.8293 µmole/min/gram to 0.6654 µmole/min/gram after bio fabrication of gold nanoparticles. Similarly, capping protein assay of Asparagus racemosus showed that total protein concentration in leaf extract was 17.14 µg/ml and its concentration was reduced after biofabrication of GNPs. The morphology and the crystal structure of the gold nanoparticles (GNP) were studied using electron microscopy and XRD respectively.

Keywords: *Asparagus racemosus*, Biosynthesis, Gold-nanoparticle, Nitrate reductase, Glutathione.

INTRODUCTION

Bio-synthesis of metal nanoparticles, exploiting biological systems as an efficient sink has grabbed exceptional attention. Due to their novel optical [1], chemical [2], photoelectrochemical [3], and electronic [4] properties, there has been many chemical protocols developed by material scientists. Plethora of physical, chemical and biological processes results in the synthesis of nanoparticles, some of these are novel and others are quite common. Unlike chemical protocols which demands expensive instruments and results in release of inimical chemicals, biological method is more facile, eco-friendly and results in more monodispersed nanoparticles [5]. Nature has developed variety of processes for the biosynthesis of nano-scaled inorganic materials which are cardinal additions to the development of relatively new and largely un-charted area of research based on the biosynthesis of nanomaterials [6]. The synthesis and assembly of nanoparticles would benefit from the development of clean, harmless and environmentally acceptable ***''green chemistry''*** protocols, perhaps involving organisms ranging from bacteria to fungi and even plants [6]. Hence, both unicellular and multi-cellular organisms are known to fabricate inorganic materials either intra-or extracellularly [7]. Many plants are known to produce nanostructured mineral crystals and metallic nanoparticles with properties similar to chemically synthesized materials, while exercising strict control over size, shape and composition of the particles.

Pandey and co-workers exploited the reducing potential of *A.vasica* [8] for tuning the parameters for GNPs formation. They also quantified the activity of nitrate reductase involved in catalyzing the nanoparticle biosynthesis.

Same group also used *M. charantia* [9] for catalyzing the formation of extremely stable gold nanoparticles.The GNPs were extremely stable than chemically synthesized gold nanoparticles. Such stable GNPs can be used as an ideal vessel for ferrying therapeutic moieties inside the living system. Marine algae were also explored for their potential for synthesis of GNPs. Oza et al used *Sargassum wightii* [10] for bio-fabrication of GNPs. They also studied the impact of ionic strength of the surrounding medium on synthesis of gold nanoparticles. A detailed account of living system used for synthesis of plethora of metal nanoparticles can be understood by referring author's exhaustive review [11]

To the best of our knowledge, we are presenting a first report of use of *Asparagus racemosus* leaf extract for the synthesis of gold nano particles that has not been reported to be used for biosynthesis of gold nano particle. *Asparagus racemosus* is commonly known as Shatavari contain steroids (Saponins and Sarsapogenins) as secondary metabolite.

MATERIALS AND METHODS

Materials

Fresh *Asparagus racemosus* leaves were collected from the plants growing in the premises of nsnRc in Ambernath, as and when required. Gold aurochlorate were procured from Sigma Aldrich, USA. The experiments were performed using double distilled water. The glasswares were washed with aqua regia to remove the traces of metal contaminant. The pipettes were pre-calibrated before using for the measurements. In order to record the temperature, local made thermocouple was used.

Procurement of rest of the material, preparation of aqueous extract of leaves, methodology for biosynthesis of the gold nanoparticles and their characterization was done as per details given earlier [9]

Nitrite Reductase [NiR EC 1.6.6.4] Assay - For extraction of Nitrite Reductase from leaves of A. racemosus, 100 mg plant material was homogenized with Tris-HCl buffer (pH 8.0) and then centrifuged at 0^0C at 2000 rpm for 15 min. The supernatant was used as enzyme source.

Nitrite Reductase activity was measured by Vega and Cardenas method [12] with few variations. The reactions were conducted in uncapped test tubes to maintain aerobic condition. 0.3ml of 0,5M Tris HCl buffer (pH 8,0) + 0.2 ml of 20 mM $NaNO_2$ + 0.2 ml of 5.0 M Ferredoxin + 0.1 ml diluted enzyme and 0.3 ml of Sodium dithionite solution (freshly prepared was taken in a test tube and incubated in dark for 10 min at 30^0C. During incubation some amount of $NaNO_2$ gets converted to NH_3 with catalytic action of nitrite reductase and Ferredoxin. After incubation the reaction was stopped by vigorously shaking the test tube on cyclomixer until dithionite was completely oxidized and the dye became colorless. Then 1ml of diazo coupling reagent (1% Sulphanilamide in 3 ml HCl and 0.02% N-(1-naphthyl) ethylenediamine hydrochloride) was added to 3 ml reaction mixture and diluted 10 folds to detect the remaining NO_2. After 30 min of incubation in dark at 30^0C for development of color; O.D. was recorded at 540 nm. The result was calculated against the standard graph of nitrite.

Characterization of Biosynthesized Gold Nanoparticles: The characterization methods used included: *UV- Vis Spectroscopy, High Resolution Transmission Electron Micrographic Analysis, X-Ray diffraction studies (XRD)* and *FTIR*.

RESULTS AND DISCUSSION

Impact of different pH on formation of gold nano particles at 30 and 100^0C fabricated using *Asparagus racemosus* are presented in table -1, which shows that pH 6 has yielded the best results at both the tried temperatures. Therefore, further trials were done using pH 6 and a range of variable temperatures (Table-2). At same pH, the impact of gold salt concentration was also studied (Table-3).

Visual observation:

Gold nanoparticle formation using the plant extract was confined due to the change of color from colourless to wine red, pink and blue color depending on the size of the nanoparticles [13] . The time taken for the reduction of gold ions to gold nanoparticles was dependent on the temperature of the reactant as well as the concentration of gold salt. At lower temperature such as 30°C, complete reduction of gold ions and hence, the formation of nanoparticles took place more than 24 hrs. This may be accounted due to the less activation energy and slow reduction activity of reducing agents present in the plant extract. Moreover, due to the optimal activity of capping agents as well as enzymes involved in reduction, GNPs formed were highly stable at room temperature. The stability of the GNPs was tested using 5M NaCl. In contrast to GNPs fabricated at 30°C, the nanoparticles were formed in less than 5 seconds.

However, the GNPs were less stable than those fabricated at 30°C. This may be due the destruction of capping proteins as well as non-optimal activity enzymes.

Table – 1: Impact of pH and Temperature on the Biosynthesis of gold nano particles using 100 ppm Aurochlorate and *Asparagus racemosus leaf extract*

pH	Temperatures	
	30^0C	**100^0C**
2	Change in color in 24 h Very weak broad hump UV-Vis peak at 587 nm XRD - Crystalline structure	Change in color in < 5 sec Dual peak at 559 nm and 709 nm XRD Crystalline structure
4	Change in color in 24 h Intense UV-Vis peak at 554 XRD Crystalline structure	Change in color in < 5 sec Intense Peak at 574 nm XRD Crystalline structure
6	Change in color in 24 h Intense Peak at 550 nm XRD Crystalline structure	**Change in color in < 5 sec** **Intense Peak at 543 nm** **XRD Crystalline structure** **TEM of polydispersed isotropic gold nanoparticles could be seen**
8	**Change in color in 24 h** **Peak of medium intensity at 546 nm** **XRD Crystalline structure** **TEM of polydispersed isotropic gold nanoparticles could be seen**	Change in color in < 5 sec Good peak at 536 nm XRD Crystalline structure
10	Change in color in 24 h Good peak with broad hump at 553 nm XRD Crystalline structure	Change in color in < 5 sec Weak broad peak at 587 nm XRD Crystalline structure
pH of plant extract 4.7	Change in color in 24 h Intense peak at 540 nm XRD Crystalline structure	Change in color in < 5 sec Intense peak at 571 nm XRD Crystalline structure

Table – 2: Impact of different temperatures on the Biosynthesis of gold nano particles using 100 ppm Aurochlorate and *Asparagus racemosus* leaf extract at pH 8

Temperature	Observations			
	Visual	**UV-Vis Peak**	**XRD**	**TEM**
4 ºC	Change in color in > 24 h	Broad peak at 544 nm	Crystalline	Both isotropic & anisotropic gold nanoparticles
RT (28 ± 2ºC)	Change in color in < 24 h	Good peak at 551 nm	Crystalline	
37 ºC	Change in color within 10 min	Intense peak at 543 nm	Crystalline	
60 ºC	Change in color in < 5 sec	Feeble peak at 544 nm	Crystalline	
100 ºC	Change in color in < 5 sec	Feeble peak at 559 nm	Crystalline	Both isotropic & anisotropic gold nanoparticles

Table 3: Impact of different concentrations on the Biosynthesis of gold nano particles at 100ºC and *Asparagus racemosus* leaf extract at pH 8

Concentrations	Observation	
	Visual	**UV-Vis Peak**
50 ppm	Change in color in < 5 sec	Broad peak at 550 nm
100 ppm	Change in color in < 5 sec	Good peak at 558 nm
150 ppm	Change in color < 5 sec	Intense peak at 552 nm
200 ppm	Change in color in < 5 sec	Feeble peak at 563 nm
250 ppm	Change in color in < 5 sec	Feeble peak at 533 nm

Fig 1 represents the change in colour of gold nanoparticles bio-fabricated at different parameters .There were 3 dominant colours observed during the synthesis. The wine red colour accounts for smallest nanoparticle [14], whereas; the blue and purple colour indicated the increment in size and hence peculiar interactions with light. As per Fig 1 a , pH 8 and at 30^0C exhibited exceptional stability at monodispersity which can be verified from the colour as well as the spectroscopic properties . At lower pH values (pH 2, 4 & 6) mild reduction occurred as witnessed by the light pink colour as shown in the Fig . Inherent pH was found to be considerably influential for synthesizing nanoparticles ranging from 10-50 nm which can be speculated due to the clear pink colour of Gold nanoparticles. Surprisingly, pH 10 was inefficient in reducing gold ions to gold nanoparticles .

At higher temperature(100°C) , the influential pH values were found to be 6 & 8 as shown in the colours of GNPs exhibited due to SPR (Fig 1b) . In a stark contrast to previous result, lower pH values (pH 2 & 4) were found to be capable of synthesizing gold nanoparticles of very large size. This resulted in agglomeration of the nanoparticles in

the solution (Fig 1 b)[12]. pH 10 was also found to be efficient but agglomeration was observed in the solution .In agreement with the earlier results, inherent pH was also found to be efficient.

Fig 1c represents the colour exhibited by GNPs synthesized at various temperatures. The nanoparticles synthesized at 100ºC exhibited a remarkable wine red colour (Fig 1 c).The nanoparticles were stable for more than 3 months (data not shown). At lower temperatures (4, 28±2, 37 & 60 º C) mild reduction occurred as witnessed by the colours in the Fig 1 c.

Fig 1 d depicts the impact of concentration of aurochlorate at pre-optimized parameters. The nanoparticles synthesized using 100 ppm gold was found to be more stable. At higher concentrations (150, 200, 250 ppm) increment in the size of nanoparticles was observed which was supported by the gradual change in colour from red to purple (Fig 1 d).

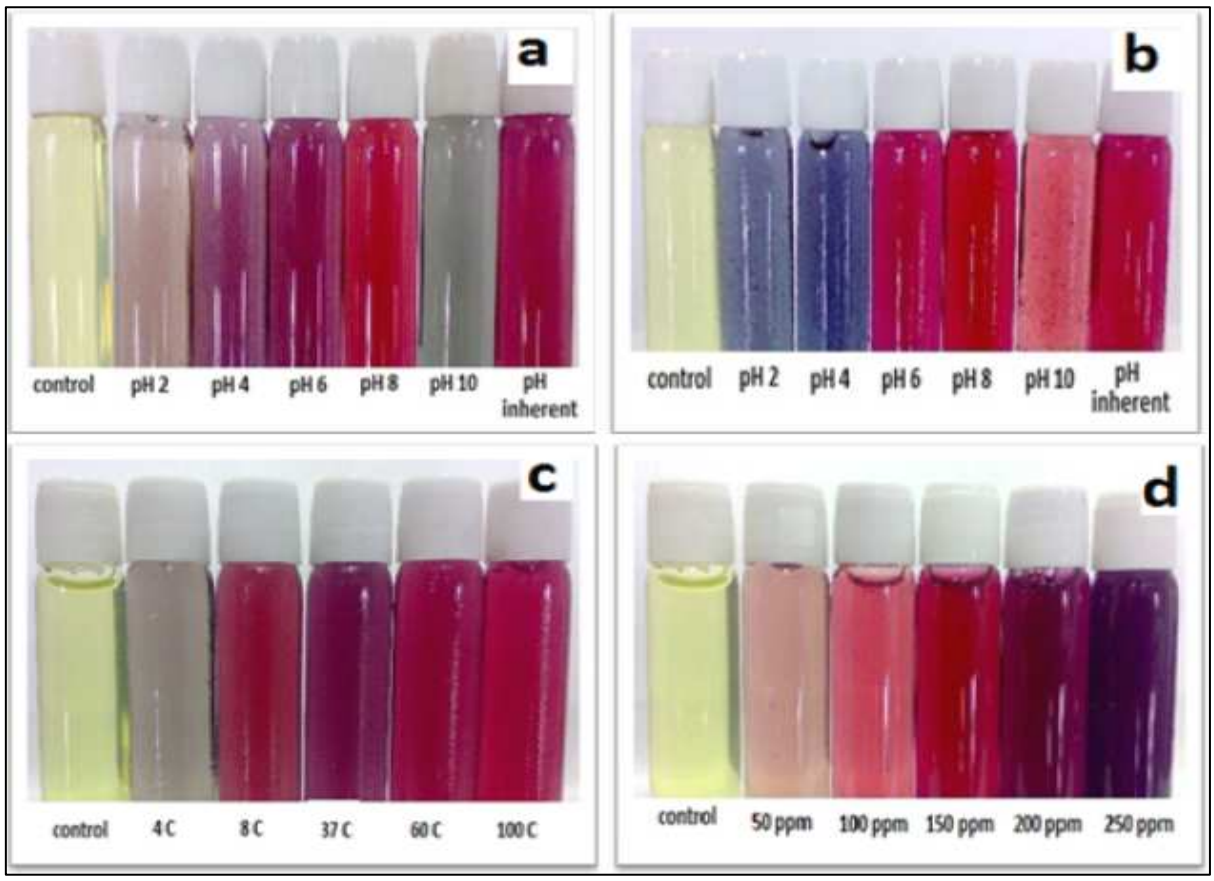

Figure-1: Visual observation of temperature, pH and concentration on bio-fabrication of gold nanoparticles using *A. racemosus,* (a) impact of 30°C & different pH values (b) Impact of 100°C & different pH values. (c) Impact at pH 8 at various temperatures (d) impact at 100ºC and pH 8 at various temperatures

UV-Visible Spectroscopy

Surface plasmon band (SPR) centered between 500-600nm as shown in Figure2a, confirms the formation of gold nanoparticles in the solution. Inception of the vivid color and SPR in gold nanoparticles is due to the confinement of electrons when in the size of the particles enters in the realms of nano-scale (10-100nm). The freely mobile electrons are caged in gold nanoparticles and exhibit a characteristic collective coherent oscillation of plasmon resonance giving rise to surface plasmon resonance (SPR). The consequence of SPR is a sharp peak observed between 500-600nm.

The SPR band of GNPs varied with change in the parameters used for biosynthesis. At highly acidic pH value (pH 2), mild reduction of gold ions occurred as seen in SPR pattern (Figure 2b). This may be due to excess protonation of the capping proteins which in turn change the surface charge and its association with the gold nanoparticles. This

can also be accounted due to decreased activity of the nitrate reductase at acidic pH. Unlike acidic pH, alkaline pH (pH 8) was found to highly influential in bio-fabrication of GNPs.

When gold nanoparticles were fabricated at inherent pH at 30^0C, a sharp peak was observed at 540 nm. The less area under the peak signifies the presence of uniform sized nanoparticles. The uniformity found in the nanoparticles is a result of synergistic action of capping proteins as well as biological macromolecules such as nitrate reductases which thermodynamically stabilizes the nanoparticles in the colloidal solution.

At pH 2, a mild peak was observed approximately at 561 nm which may be due to excess protonation which results in change of charge of the capping protein (Fig 2a). A broad hump observed at 554 nm in the nanoparticles fabricated at pH 4 indicates the formation of gold nanoparticle of the size below 50 nm .there was a red shift of 14 nm with respect to SPR band observed at 540 nm incase of GNPs fabricated at inherent pH.This indicates the minor increase in the size this is in visual agreement with Fig 1 a .Further red shift in SPR band (from 540 nm to 550 nm) of the GNPs formed at pH 6 signify the increase in the size which changes the colour as presented in Fig 1 a.

A sharp SPR band at 546 nm as well as vivid red colour of GNPs fabricated at pH 8 declares it the most optimum parameter for the biosynthesis of uniform sizes and thermodynamically stable gold nanoparticles. Poly-dispersed nanoparticles were seen with a broad peak at pH 10. The area under the peak depicts agglomeration of the nanoparticles.

As displayed in Fig 2b, the inherent pH exhibited a broad hump at 571 nm indicating the presence of polydispersed nanoparticles .This may be due to non favourable conditions for the optimum functioning of capping proteins as well as enzymes. Particularly arresting results were shown in case of GNPs fabricated at pH 2. A dual peak observed at 559 nm and 709 nm depicts the formation of non-spherical nanoparticles and/or agglomeration of nanoparticles [15] .There was a minor shift (from 571 nm to 574 nm) and a flat spectrum observed in GNPs synthesized at pH 4 and pH 8 respectively . The most influential parameter at high temperature was found to be pH 6 which can be inferred due to a sharp peak at 543 nm and bright red colour shown in Fig 1 b.

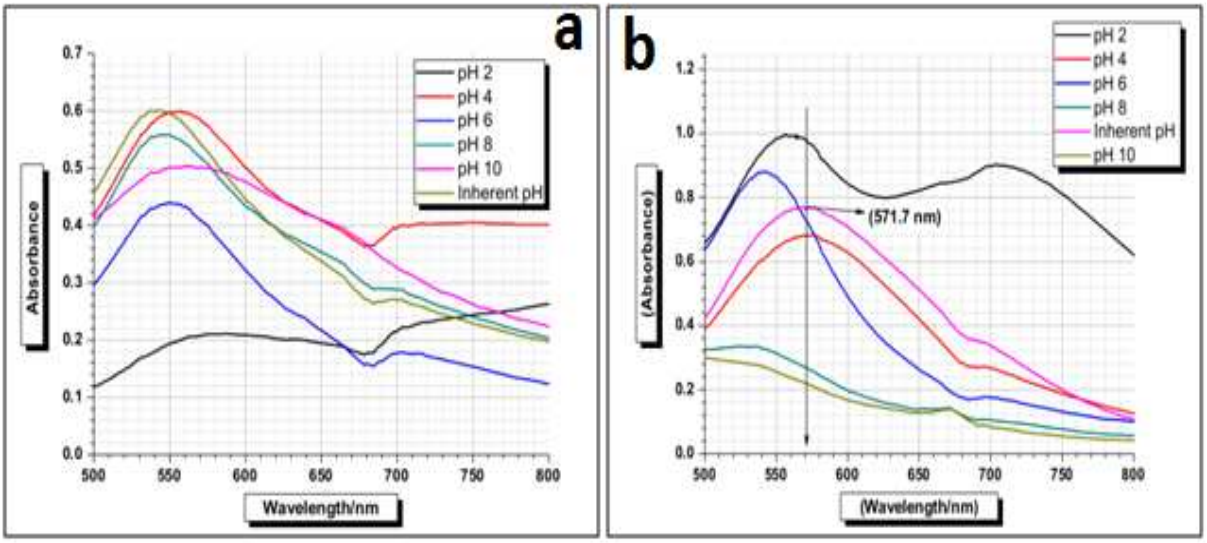

Figure-2: Impact of temperature and pH on bio-fabrication of gold nanoparticles using *A. racemosus,* as shown by UV-Vis Spectroscopy (a) impact of 30°C & different pH values (b) impact of 100°C & different pH values. The inset shows the change in SPR with respect to different parameter

After pre tuning the parameters (100ppm, 100ºC & pH 8) the impact of lower temperatures on the biological fabrication of GNPs were scrutinized (Fig 3 a). At 4ºC, mild reduction was observed as verified due to a flat peak and mild colour observed in Fig 1 c. Unlike 4º C, all other temperature exhibited a sharp peak between 500 to 559 nm. However, the stability of the GNP synthesized at 100ºC was found to be superior .Wine red colour and a sharp peak supports our observation. (Fig 1c)

The most effective concentration for the biosynthesis of nanoparticle was found to be 100 ppm as supported by Fig 1 d and UV-Vis spectrum as seen at 559 nm (Fig 3b).Though , the nanoparticles synthesized at 150 ppm was also optimum , the stability was inferior as compared to GNPs fabricated using 100 ppm aurochlorate salt .At higher

concentration of salt (200 to 250 ppm) , there was a red shift observed (Fig 3 b) .This implies the increase in the size of the GNPs as supported by the violet and purple colour respectively (Fig 1 d).

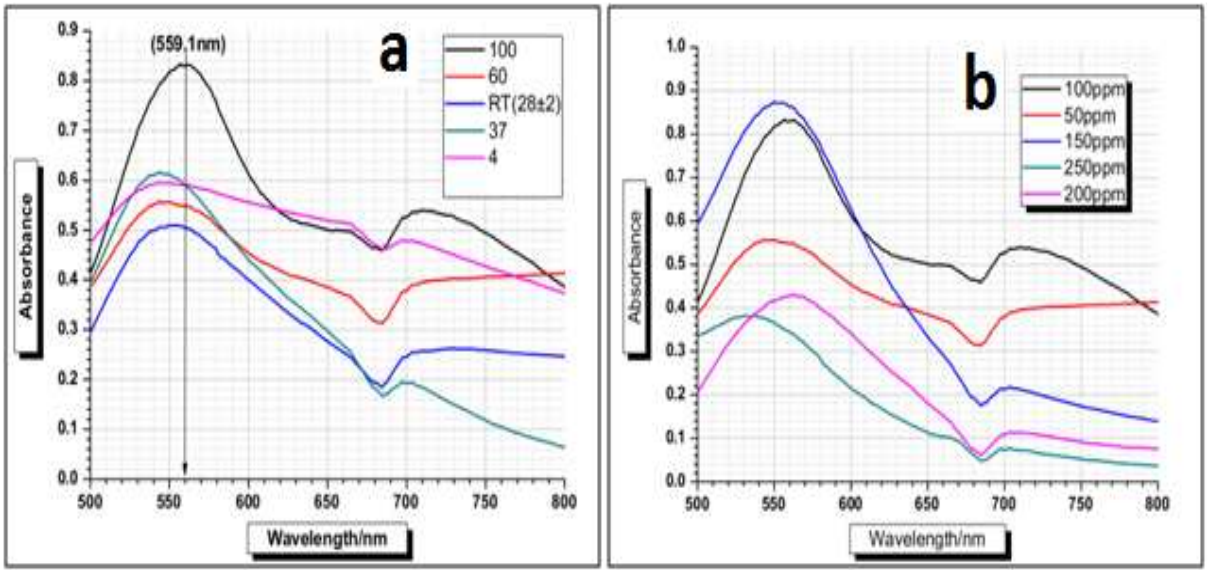

Figure- 3: UV-Vis spectra of gold nano particles biosynthesized at pH 6 using leaf extract of *A. racemosus* at (a) Different temperatures and (b) Different concentrations of aurochlorate

Electron microscopic studies:

The morphology of gold nanoparticles was studied using field emission gun scanning electron microscope (FEG-SEM). SEM image of the gold nanoparticles bio-fabricated at 30°C & pH 8, exhibit the presence of extremely small gold nanoparticles (Fig 4 a). The size of the nanoparticles was found to be 10-50nm. Alkaline pH, like earlier results, was observed to be the most favourable condition for the synthesis of monodisperse and stable nanoparticles. At pH 2 at 100°C, the gold nanoparticles with unique morphological features were observed. As shown in Fig. 4 b, anisotropic gold nanoparticles having triangular, oval and hexagonal shape were formed, as shown in red rings. Moreover, typical ring of gold nanoparticles was observed. This may be: An intermediate step in the formation of non-spherical nanoparticles .Due to less potential at edges, gold nano-spheres must have aligned themselves in such typical morphology. This also helps in overall thermodynamic stability of the structure.

The mechanism for formation of such unique structures by biological system is still in a nutshell. However, it can be speculated that this is due to the enzyme or capping protein assisted nucleation and growth at different facets (111, 200, and 220) of gold nanoparticles.

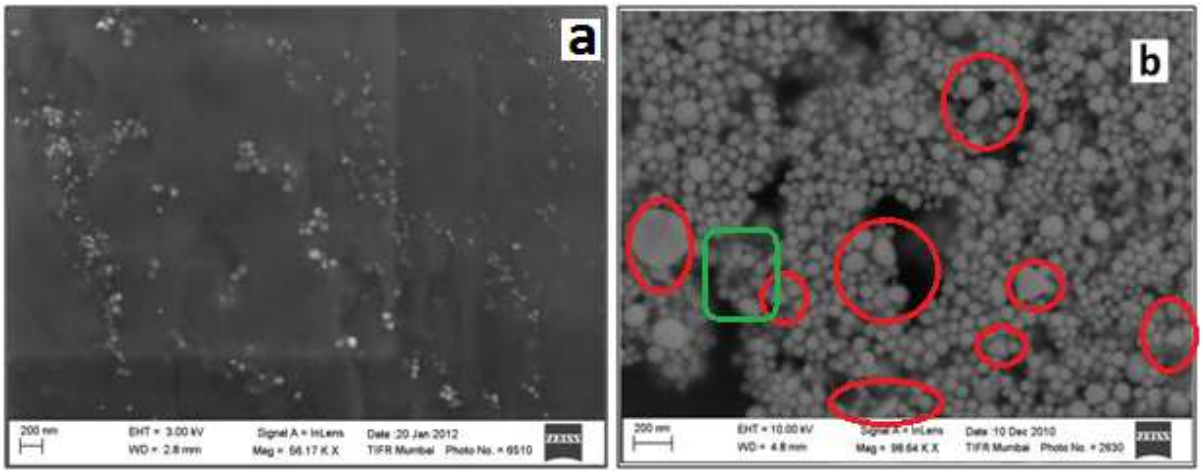

Figure – 4: FEG SEM image exhibiting the impact of temperature and pH on the biosynthesis of gold nanoparticles using *A. racemosus* (a) pH8 at 30°C (b) pH2 at 100°C. Red circles show the presence of non-spherical gold nanoparticles such as triangles, oval and hexagonal shaped. Green box demonstrate the formation of a unique ring of spherical gold nanoparticles.

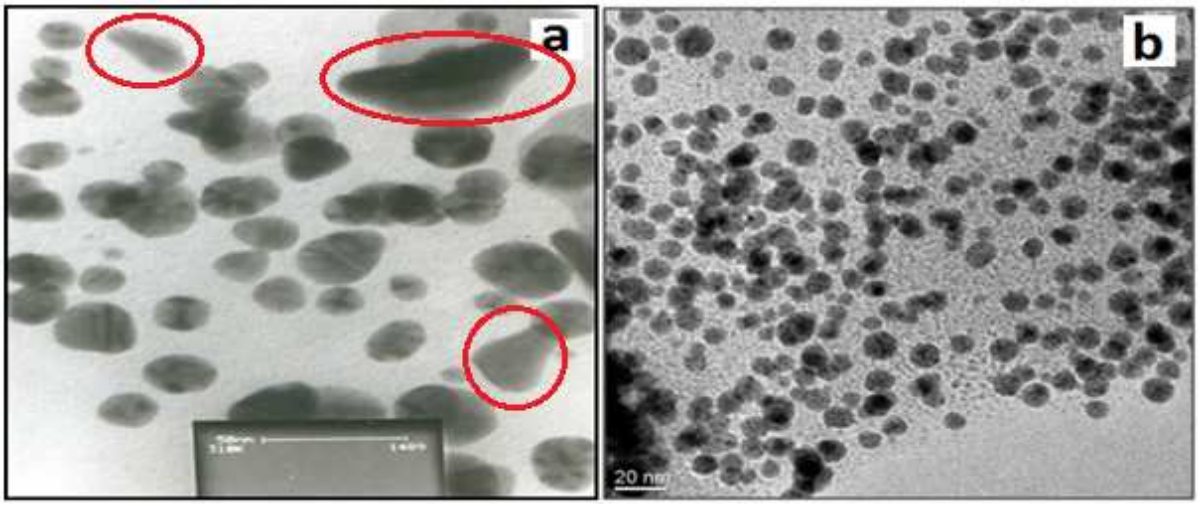

Figure-5: TEM image of gold nanoparticles synthesised using *A.racemosus* showing the impact of concentration of gold salt and temperature (a) 150ppm gold salt at pH 8 at 100 ° C(b) 37°C at pH 8 and 100 ppm gold salt

A typical HRTEM image is presented in Fig 5, shows gold nano particles of a maximum of 20 nm size; having mixed morphology was formed. Typical bone shaped nanoparticles can be seen in Fig. 5 a (as seen in red circle). This may be due to extra deposition of gold ions at typical facets of gold nanoparticles were found to considerably spherical. The microscopic observation is in agreement with the UV-Vis spectroscopic studies. Impact of temperature on morphology of gold nanoparticles is depicted in TEM image (Fig. 5 b). Nanoparticles synthesied were highly uniform in size ranging 10-50nm . Such stable nanoparticles can be used as nano-vehicle for carrying therapeutic moeties inside the cells since they are stable at 37°C.

X-ray diffraction studies: The confirmation of formation of elemental gold nanoparticles is provided by X-ray diffraction (XRD) analysis of the thin film prepared by coating the gold nanoparticle solution on Si (111) substrate. The colloidal gold nanoparticles on a glass cube showed intense peaks at (111), (200), (220) and (311) Bragg reflections in the 2 θ range 30°-80 ° as shown in Figure 6; this is in agreement with the previous data available on gold nanocrystals [16] . The 111 facet is extremely reactive due to high rate of electron transfer. It must be mentioned here that XRD data of many samples were taken, but since they all showed similar results here only one typical XRD is presented.

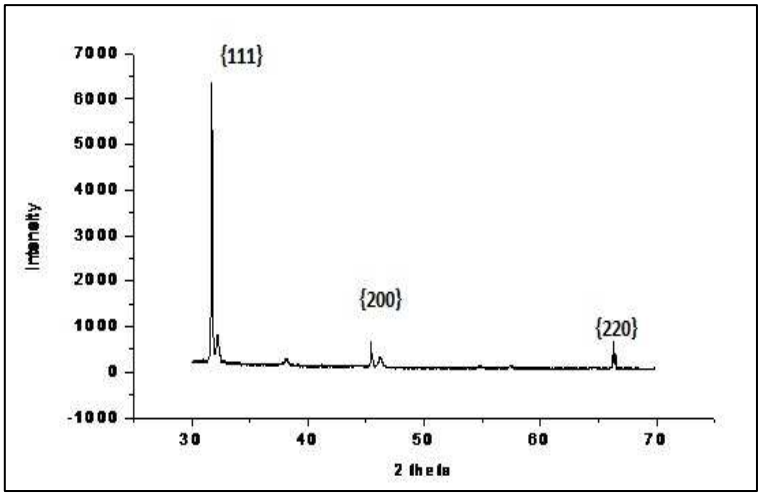

Figure- 6: XRD pattern of gold nano particles synthesized using leaf extract of *Asparagus racemosus*, showing typical Bragg reflections for gold nanoparticles

Protein estimation and Nitrate Reductase Assay:

Asparagus racemosus is a monocot; rich in reductases and capping proteins. These reductases help in bioreduction of Au^{+3} to Au^{0} and subsequent formation of gold nanoparticles. These proteins act as stabilizing agents for gold nanoparticles in the solution. The molecular activity of nitrate reductase in the leaf extract of *A. racemosus* was found to be 0.8293 µmole/min/gram; which got reduced to 0.6654 µmole/min/gram of plant tissue when it was subjected to 100^{0}C (Fig 8). After the formation of gold nanoparticles the nitrate reductase activity was again assayed in the reactant mixture which showed a substantial decrease in the solutions (plant extracts) having gold nanoparticles as compared to nitrate reductase activity in plant extracts without gold nanoparticles. This result confirms the involvement of nitrate reductases in the reduction of gold ion to gold nano particles. The content of protein in *Asparagus racemosus* showed that total protein concentration in plant extract of *Asparagus racemosus is 17.14 µg/ml* and its concentration was reduced when gold was added and boiled. This explained the phenomenon of encapping of these proteins to gold and thus stabilizing it**. *However, the fact that which other capping proteins are involved in stabilizing the particle is yet to be explored.***

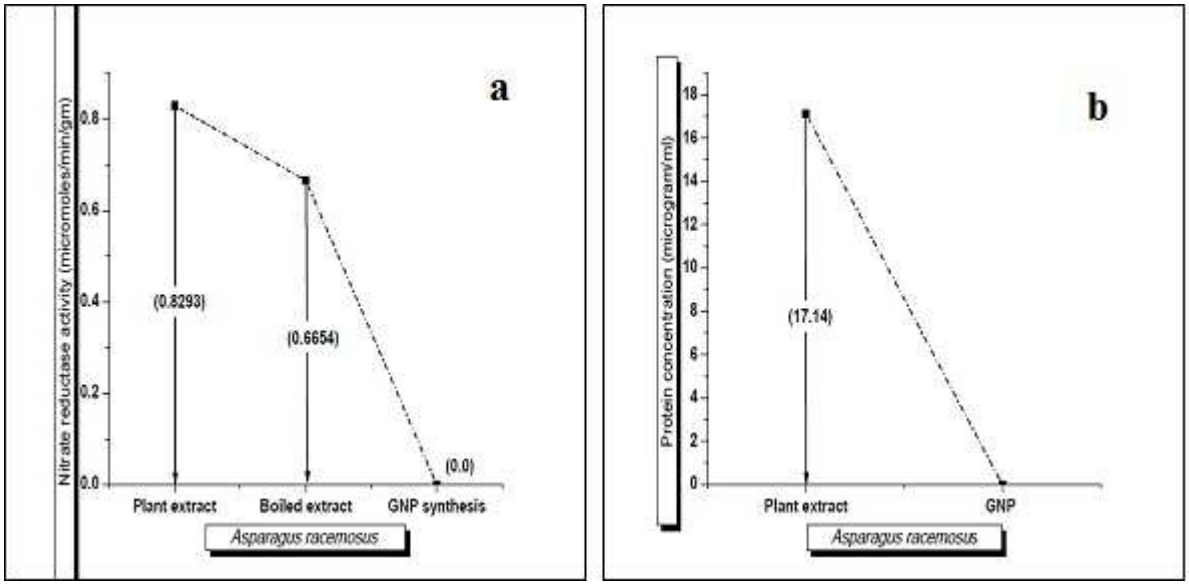

Figure 9: (a) Nitrate reductase activity of *Asparagus racemosus* plant extract, Boiled plant extract and gold nanoparticles respectively in µmoles/min/gm. (b) Total protein concentration in *Asparagus racemosus* plant extract and gold nanoparticles respectively in mg/ml.

CONCLUSION

Asparagus racemosus leaf extract has shown the capability of biosynthesizing gold nano particles from gold salt solution under the influence of nitrate reductase and capping proteins. The optimum conditions for stable gold nanoparticles biosynthesis using *Asparagus racemosus* was observed to be at pH 6, 100^{o}C and 100 ppm aurochlorate and at 30 ^{o}C pH 8 and 100 ppm .Activity of nitrate reductase support the view that they are involved in reducing and stabilizing the gold ions to gold nano particles. It presents a controllable method of tuning the synthesis of thermodynamically stable desired size and shape of gold nanoparticle. It can be concluded that *Asparagus racemosus* is an efficient fabricator for biosynthesis of gold nano particles.

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