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A Mechanistic Approach for Biological Fabrication of Crystalline Gold Nanoparticles Using Marine Algae, *Sargassum wightii*

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ABSTRACT

In this paper use of marine macro alga Sargassum wightii in Green orchestration of Gold nanoparticles is reported. In this work, the optimum parameters were encountered to be 30ºC at pH 10 and 100 ppm aurochlorate salt. The contribution of nitrate reductase was studied using biochemical assays. The nitrate reductase activity was found to be reduced from 0.6245 µmole/min/gram to 0.4244 µmole/min/gram after fabrication of gold nanoparticles. The morphology was studied using electron microscopy which showed presence of isotropic spheres along with anisotropic nanoparticles and the size was found to between 30-100nm . The crystalline nature of gold nanoparticles was examined using XRD and was found to be face centered cubic.

Keywords: *Sargassum wightii*, Biosynthesis, Gold nanoparticles, Surface plasmon resonance (SPR), Nitrate Reductase.

INTRODUCTION

Nano-materials, especially bio-inorganic materials can be very complex and intricate in structure, composition and function. They can have various potential applications due to their small size and large surface area; few of the significant ones in the field of biology are: Hyperthermia treatment for malignant cells, Magnetic resonance imaging enhancement, Cell labeling, Cell tracking, *In vivo* imaging, DNA detection , Diagnostics, Gene sequencing, Drug delivery systems, Biomedical sciences etc. These features and applications of bio-inorganic materials synthesized by living systems are almost impossible to mimic using chemical and physical synthesis techniques in the lab.

The bio-based protocols for synthesis of nanometals are both environmentally and economically green as they are based on green chemistry principles and are simple and relatively inexpensive. The chemical methods available are often expensive, utilize toxic chemicals and are comparatively complicated. Certainly such methods are not eco-friendly and hence cleaner, cheaper, green processes that do not employ toxic chemicals for the synthesis of nanoparticles have to be devised. The nanoparticles synthesized by such chemical methods are also unstable and tend to clump or agglomerate quickly and are rendered useless. [1] The nanoparticles synthesized by various living systems have been shown to be coated with peptides or proteins. This leads to a similar charge distribution all over the surface of nanometal which results in repulsion between them. These inter particle repulsive forces prevent aggregation and so, nano metal solutions synthesized by microbes and algae have been shown to be extremely stable

even after a period of six months. This green chemistry approach for nanoparticle biosynthesis is simple, scaling up is possible and it is environment friendly. They also studied the impact of ionic strength of the surrounding medium on synthesis of gold nanoparticles. Pandey et al exploited the reducing potential of *A.vasica* [2] 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 *A.racemosus* [3], *M. charantia* [4] 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. A detailed account of living system used for synthesis of plethora of metal nanoparticles can be understood by referring author's exhaustive review [5]

Thus, biological systems fulfill the above objectives and the required conditions as well. Hence, using bio-based protocols for synthesis of Nano materials with desired properties is contemplated. Mechanism or the modus operandi involved in size regulation of nano particles have been an intriguing complexity. One of the most justifiable mechanisms appears to be the role of glutathione, phytochelatins (PCs) and metallothioneins (MTs).

In this paper, there will be detailed discussion of parametric study of marine water algae *Sargassum wightii* which is a brown macro alga (seaweed). Numerous species are distributed throughout the temperate and tropical oceans of the world, where they generally inhabit shallow water and coral reefs.Their reducing properties are exploited for nanoparticle synthesis.

MATERIALS AND METHODS

Sargassum wightii were procured from Bhavnagar and grown in Artificial Sea Water medium (ASW medium): containing 2 M (8%) NaCl, 50 mM $NaHCO_3$, 5 mM $MgSO_4$, 5 mM KNO_3, 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2$, 7 µM $MnCl_2$, 5 µM EDTA, 2 µM $FeCl_3$, 1 µM $CuCl_2$, 1 µM $CoCl_2$, 1 µM $(NH_4)_6Mo_7O_{24}$, 1 µM $ZnCl_2$. The growth *was* measured by dry weight and optical density (O.D) method . The cells at stationary-phase of culture were harvested at 4°C by centrifugation at 6000 x g for 15 min and washed with deionized water. The cells were then dried at room temperature. The dried algae was washed several times with dilute HCl and deionized water to remove adsorbed impurities that might interfere with the formation of gold nanoparticles. The purified algae were ground to a fine powder of approx. 0.5-1 mm particle size.

Chemicals and Glassware - Chemical used for the synthesis of gold nanoparticles were chloroauric acid (HAuCl4) (Sigma-Aldrich). 100mL of 1mM aqueous HAuCl4 solution in 500mL of Erlenmeyer flask was taken for gold nanoparticle synthesis

Fabrication of Gold nanoparticle - 1.0 g algae powder was added to 100 ml deionized water and the mixture was aged for 1-2 days. It was then added to HAuCl4 to make its concentration to 100 ppm. The desired pH of the reaction medium was adjusted by adding 1 M NaOH solution or 1 M HCl solution. The reaction for the synthesis of gold nanoparticle was carried out under vigorous stirring at room temperatures for 0 - 48 h.

In order to optimize the nanoparticle formation, the impact of different temperatures (4, 20, RT, 37 and 100^0C) was assessed. The best temperature (RT= 28 ± 2^0C) was used for studying the effect of pH (3, 4, 6, 10 & inherent) on synthesis of GNPs. The parameters obtained from the above two experiment were kept constant to comprehend the impact of temperature on the optical as well as morphological features of GNPs.

Nitrate Reductase Assay: For extraction of Nitrate Reductase from *S. wightii*, 100 mg alga; powder 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.

Nitrate Reductase activity was measured by Vega and Cardenas method [6].The standard graph was calibrated using 50 µM working standard of Sodium nitrite. To 0.1 ml supernatant known amount of 0.1 M KNO_3 was added and incubated for 24 hours. 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 Nanoparticles –included

UV-Vis Measurements: was carried out on a dual beam spectroscopy Lambda 25 Perkin Elmer, USA using deionized water as the reference. The colloidal solution was then added into a quartz cuvette cell followed by immediate spectral measurements. The SPR peaks were assessed for size and distribution of gold nanoparticles.

Field emission gun-scanning electron microscopy (FEG-SEM): Examination of the nanoparticle morphology by FEG-SEM was performed on a Carl Zeiss Microimaging, GmbH, Germany. For sample preparation, 2-3 drops of the colloidal gold solution were dispensed onto a silicon wafer and dried under ambient condition before examination.

XRD Measurements: Crystallographic information about the samples was obtained from X-ray diffraction (XRD). XRD patterns were recorded by a (PANalytical, Philips PW 1830, The Netherlands) operating at 40 kV and a current of 30 mA with Cu Kα radiation (λ = 1.5404 Å) and the 2θ scanning range was of 30-80° at 2° min^{-1}. The colloidal suspension containing metal nanoparticles was dried on a small glass slab.

RESULTS AND DISCUSSION

Impact of different pH on formation of gold nano particles at 30ºC fabricated using *Sargassum wightii* 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).

Table 1: Impact of pH on the Biosynthesis of gold nano particles at 30^0C using100 ppm Aurochlorate and Sargassum wightii extract and TEM

pH	Temperature
2	Change in color in 24 h Broad plateau seen UV-Visible spectrum XRD - Crystalline structure
3	Change in color in 24 h Weak UV-Vis peak at 650 nm XRD Crystalline structure
4	Change in color in 24 h Intense Peak at 585 nm and weak hump at 780nm XRD Crystalline structure HR-TEM shows spherical gold nanoparticles of size 20nm
8	TSPR at 575nm and LSPR at 725nm Change in color in 24 h Peak of medium intensity at 550 nm HR-TEM shows 25-30nm both Isotropic & anisotropic nanoparticles XRD Crystalline structure
9	Change in color in 24 h peak at 585 nm XRD Crystalline structure TEM showing star-shaped nanoparticles of size 25nm
10	**Change in color in 24 h peak at 585 nm** **XRD Crystalline structure** **TEM showing star-shaped nanoparticles of size 25nm**

Table 2 : Impact of temperature at pH 10 using100 ppm aurochlorate on synthesis of Gold nanoparticles using *Sargassum wightii*

Temperature	Observations			
	Visual	**UV-Vis Peak**	**XRD**	**TEM**
20ºC	Change in color in > 24 h	Broad feeble peak at 590 nm	Crystalline	
RT (28 ± 2ºC)	**Change in color in < 24 h**	**Good peak at 555 nm**	**Crystalline**	**Both isotropic & anisotropic gold nanoparticles**
37 ºC	Change in color within 20 min	Intense peak at 567 nm	Crystalline	Both isotropic & anisotropic gold nanoparticles
100 ºC	Change in color in < 5 sec	Flat absorption spectrum	Crystalline	

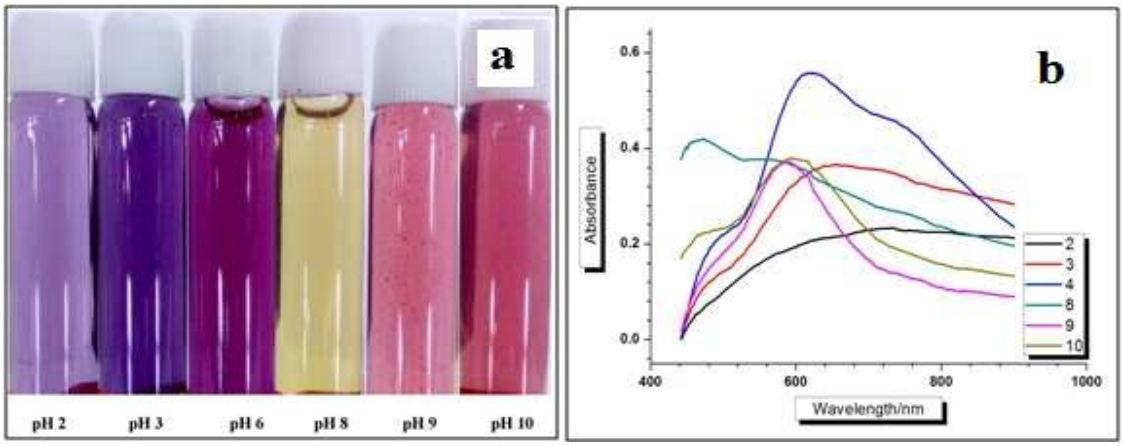

Figure *1: (a)* Visual observation of Effect of pH on changes in color of aurochlorate solution in presence of *Sargassum wightii extract (b)* Impact of pH on bio-fabrication of gold nanoparticles using *Sargassum wightii,* as shown by UV-Vis spectra.

Visual observation

On addition of aurochlorate salt to the intracellular extracts of Sargassum wightii, the reducing agents as well as the capping agents led to the orchestration of gold nanoparticles; in agreement with the vivid range of colours at various pH as observed in fig 1 a. This shows that these gold nanoparticles are pH dependent. Since this organism is a halophile, it possesses a large amount of glycerol which acts as a stabilizer thus the solution is stable even after one year. At lower pH (pH 2,3,6) there is mild activity of the reducing agents .This consequently led to the formation of larger sized nanoparticles as observed in the colours in fig 1a (light purple, purple and violet)[7],but the wine red colour is observed at pH 10. This may be due to enhanced activity of the reducing agent at this pH.

UV-Visible Spectroscopy

Impact of pH for biosynthesis of nanoparticles- The biofabrication of Gold nanoparticles was done at varying pH, room temperature (30ºC) and optimum concentration of 100ppm. Fig 1 b depicts that at pH 2, there is a very broad plateau which clearly indicates that the size of the nanoparticles are too large since at this pH due to large proton concentration, all the functional charges possess positive charge which have got less reduction potential. Gold ions also possess positive charge which leads to less interaction of gold ions with the positively charged functional groups, thus forming very large sized nanoparticles of anisotropic structures. Further such nanoparticles also unstable and start agglomerating due to instability in the double layer formed. At pH 3, there is broad hump with a SPR band centered at 650nm (Fig 1 b). This is also due to large size of the nanoparticles. They are also unstable since they lose the stability within 24 hrs. Due to stearic destabilization which leads to Ostwald ripening, thus allowing smaller particles to agglomerate [8].This is a clear indication that in acidic pH, reducing property of the algal extracts are exhibited but the capping mechanism is inactive or very weak. Since the solution possess very high ionic strength, stearic destabilization of the electric double layer leads to agglomeration of the particles. As pH rises to 4, there is more stearically stable nanoparticles, but with little larger size as can be seen from the broad TSPR peak at 585nm (Fig 1b). The larger size can be due to the coalescence of smaller nuclei since there is presence of smaller nanoparticles which can be seen in TEM. At this pH, there is multipole oscillation since it shows a peak at 585, 625 as well as at 725nm (Fig 1 b). This indicates that there is introduction of anisotropy in the nanostructures. Two broad SPR bands at 625 and 725 nm are clearly visible, which could be attributed to the in-plane dipole resonance and the out-of-plane quadrupole resonance, respectively. It has been reported that the in-plane dipole plasmon absorption peak is highly sensitive to the sharpness of the triangle vertexes. As pH is further raised to 8, which is also the inherent pH of the algal extract, there is formation of yellow coloured nanoparticles which can be depicted by a broad hump existing at 580nm indicating negligible reducing capacity of the reducing agents. (Fig 1 b). At pH 9 and 10, there is a sharp peak at 585nm due to catastrophic deterioration of the capping agent which leads to vulnerable deposition of gold atoms on all the facets forming thermodynamically favorable spherical or ellipsoidal nanoparticles. (Fig 1 b) Due to alkalinity, hydroxides get deposited on the gold nanoparticles. It is hypothesized that at this inherent pH both reducing as well as capping agents are dominant which efficiently reduce the particles and further cap them at specific facets [9]. Due to strong capping agent, even at high ionic strength caused by sodium chloride, it does not lead to destabilization of the electric double layer and forms spherical

nanoparticles which can be indexed due to single TSPR peak. The reducing power of capping agents at lower pH is less, but as the pH increases to alkalinity range, the reduction potentials of all these functional groups are enhanced, thus allowing the formation of thermodynamically favorable structures.

Impact of Temperature on GNP synthesis - Gold nanoparticles were fabricated using *Sargassum wightii* at various temperatures viz. 20,30,37& 100ºC.Nanoparticles fabrication was speculated at room temperature (30º C) with alkaline pH (pH 10) wherein the solution gives sharp peak at 555nm.(Fig 2) . At lower temperature (20ºC) with a SPR band centered at 590 nm polydispersed nanoparticles were speculated; as the area under UV-Vis spectrum is broad as seen in Fig 2. This indicates that at lower temperatures, the electron transfer rate of the reducing or nucleating agent, which is a rate determining step, is negligible.[10] On fabrication of GNPs at 37ºC there is formation of larger sized gold nanoparticle which is in agreement with the SPR band in UV-Vis spectrum at 567 nm.(Fig 2) As the temperature is raised further(100ºC), there is no nanoparticle synthesis which symbolizes a decrement in the refractive index of the solvent which also affects its dielectric constant. Further, high ionic strength coupled with high temperature also might be instrumental in creating a barrier for synthesis of nanoparticles.

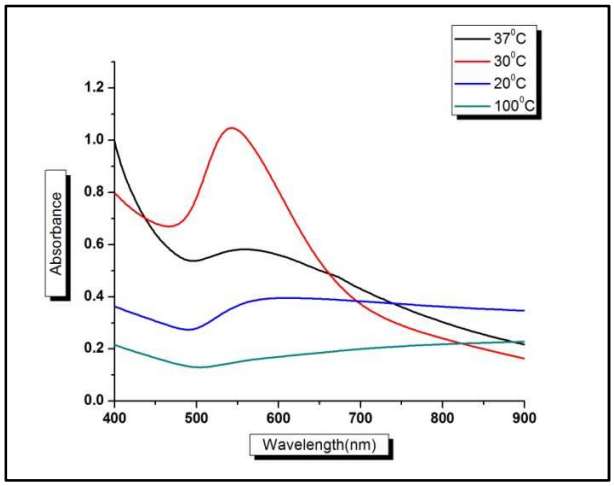

Figure 2: Impact of temperature on Gold nanoparticle synthesis using *Sargassum wightii* using uv-spectroscopy

FEG-SEM

The morphology of gold nanoparticles was studied using field emission gun scanning electron microscope (FEG-SEM).The synthesis of Gold nanoparticles by Sargassum wightii has led to the formation of several different forms of polydispersed small spheres and anisotropic structures (triangular and icosahedrons) at different pH and different temperatures which are as follows:

Impact of pH on size and morphology of the nanoparticles: The FEG-SEM analysis exhibits spherical structures at lower pH. The size of the gold nanoparticles at pH 3 is found to be 100nm while as the pH increases to 6 the size of the nanoparticles starts increasing which can also be speculated by UV-visible spectra due to the red shift of the TSPR peak and confirmed by TEM analysis which gives a measurement of 30-100 nm. At this pH, there is appearance of triangular, hexagonal nanostructures along with spherical ones. Further at pH 10 the morphology is retained to spherical ones.

Effect of Temperature on Gold nanoparticle synthesis: FEG-SEM of gold nanoparticles at different temperatures are depicted in the Fig 4.Formation of mixed spherical as well as anisotropic nanostructures at 30ºC is shown in Fig 4a. The size of the nanoparticles is 100 nm. Anisotropic gold nanoparticles of size 100nm were formed at room temperature and 37ºC .At 20ºC no nanoparticles were synthesized which was confirmed by TEM micrograph shown in fig 4a.

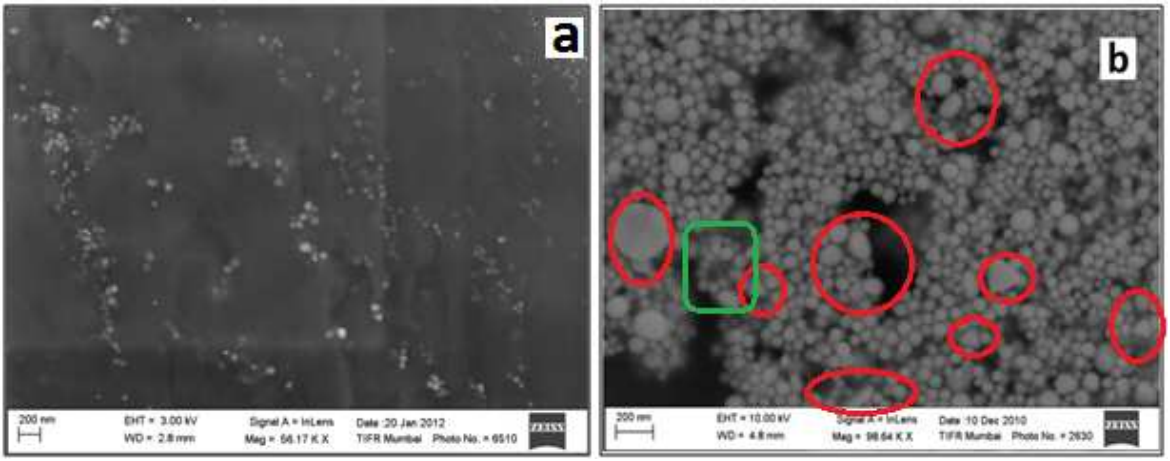

Figure 3: Scanning electron micrographs of gold nanoparticles a) at pH 3 of size 100nm b) at pH 6 of size range 30-100nm

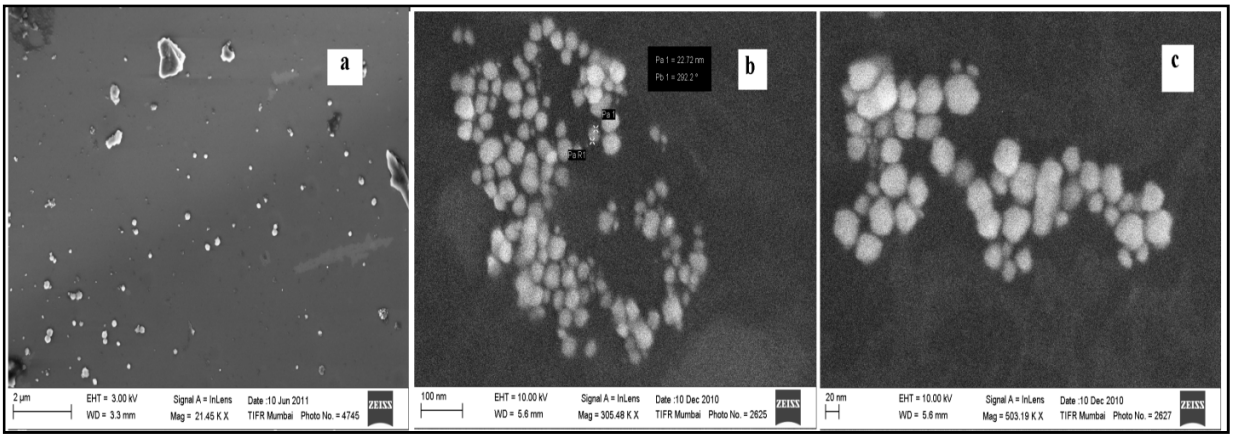

Figure 4: Impact of temperature on biosynthesis of 100nm GNP using *S wightii* at (a) 20°C (b) RT and (c) at 37°C

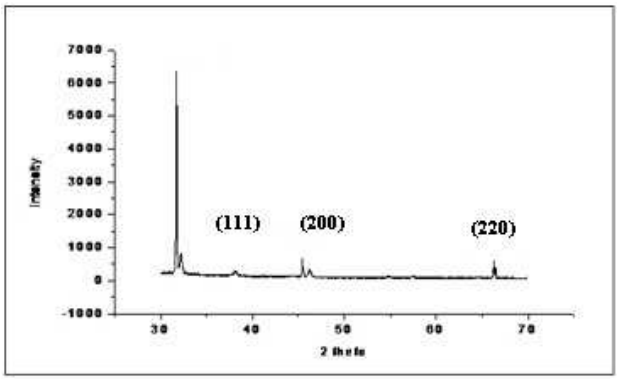

Figure 5: XRD of the gold nanoparticles on silicon wafer, the characteristic Bragg reflections {1 1 1}, {2 0 0}, {2 2 0} sets of lattice planes are observed.

X-ray diffraction -The XRD pattern which corresponds to the gold nanoparticles exhibits Bragg reflections, which could be well manifested on the basis of the face centered cubic (fcc) gold nanostructures. A number of Bragg reflections corresponding to the {1 1 1}, {2 0 0}, {2 2 0} {3 1 1} sets of lattice planes are observed, which had been indexed on the basis of the FCC structures of Au (JCPDS file no.01-1174).The very strong diffraction peak at 38 degrees is considered to be of {1 1 1} facet of the face centered cubic structure, while the diffraction peaks of other gold peaks are found to be much weaker compared to standard gold nanoparticles. It is imperative to note that the ratio of intensity between {2 0 0} and {1 1 1} peaks, {2 2 0} and {1 1 1} peaks as well as {3 1 1} and {1 1 1} peaks are much smaller compared to the intensity ratios of standard gold nanoparticles.[11]

Nitrate Reductase Activity

Sargassum wightii is marine water algae, rich in reductases.[12] Nitrate reductases are the most influential enzyme which are considered to be a scaffold or nucleating agent allowing NADH-dependent reduction of Au^{+3} to Au^{0}. The same enzyme later then acts as a capping agent, thus ensuring complete formation of thermodynamically stable nanostructures [13].

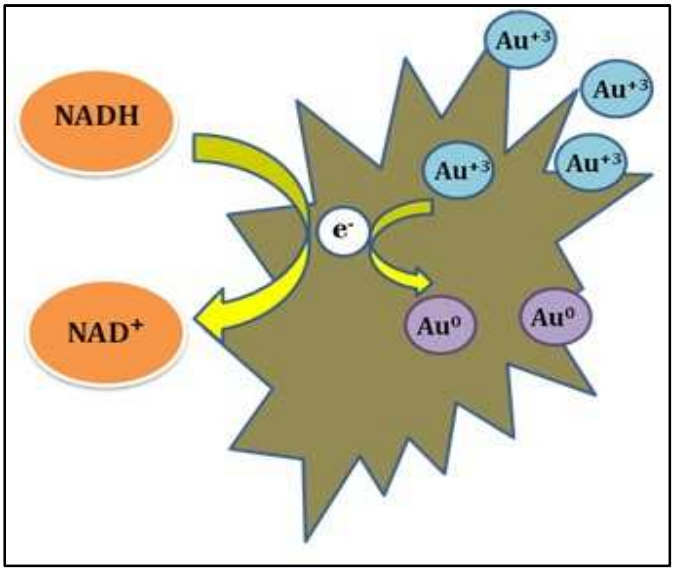

Figure 6 : Schematic Representation Of Nitrate Reductase Activity In Reduction Of Gold Salt And Later Formation Of GNP.

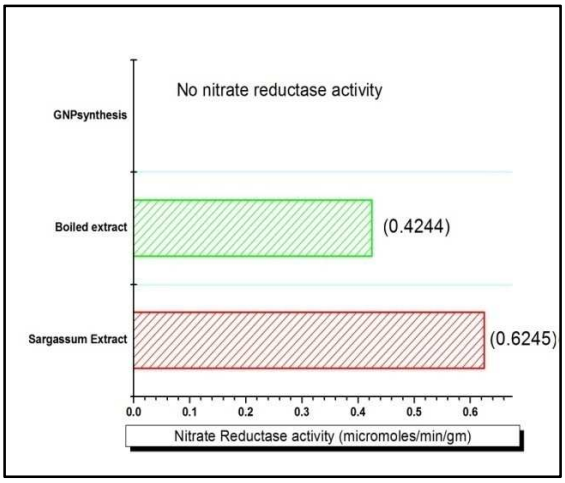

Figure 7: Nitrate reductase activity of *Sargassum wightii* algal extract, Boiled algal extract and gold nanoparticles respectively in µmoles/min/gm.

The molecular activity of nitrate reductase in the extract was found to be 0.6245µmole/min/gram of algal powder which was reduced to 0.4244 µmole/min/gram when subjected to 100^0C (Fig 7). 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 (algal extracts) having gold nanoparticles as compared to nitrate reductase activity in algal extracts without gold nanoparticles. This result confirms the involvement of nitrate reductases in the reduction of gold ion to gold nano particles.

CONCLUSION

Sargassum wightii extract has shown the capability of biosynthesizing gold nano particles from gold salt solution under the influence of nitrate reductase. The optimum conditions for biosynthesis of stable gold nanoparticles were alkaline pH and Room temperature. SEM and TEM Micrographs suggest presence of isotropic and anisotropic nanoparticles. The Activity of nitrate reductase supports 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 *Sargassum wightii* are efficient fabricators for biosynthesis of gold nano particles.

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