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Facile biosynthesis of gold nanoparticles exploiting optimum pH and temperature of fresh water algae *Chlorella pyrenoidusa*

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ABSTRACT

In this paper biosynthesis of stable gold nanoparticles (GNPs) using Chlorella pyrenoidusa extract is demonstrated. The most influential parameters for the synthesis of GNPs were pH 8, 100^{0}C and 100 ppm aurochlorate salt. The results were verified using UV-Vis spectroscopy, XRD and High Resolution Transmission electron microscopy. HRTEM micrographs as well as the SPR peaks of UV-Vis spectra showed that the size of the GNPs ranged from 25-30 nm. The HRTEM demonstrated that at alkaline pH (8, 10) spherical nanoparticles were formed whereas pH 4 showed formation of anisotropic nanostructures. The Nitrate reductase activity was found to be 0.7245 µmole/min/gram in algal extract, which got reduced to 0.5244 µmole/min/gram after the formation of GNPs.

Keywords: Biosynthesis, *Chlorella pyrenoidusa*, Gold nanoparticles, XRD, Nitrate reductase.

INTRODUCTION

Material scientists are constantly striving hard for synthesizing plethora of different methods for synthesis of gold nanoparticles of uniform size, shape, composition and monodispersity. There is overwhelming need for green synthesis of environmental friendly method of nanoparticle synthesis, thus generating negligible amounts of toxic chemicals. Thus, to refrain from catastrophic toxic chemical synthetic strategies, material scientists have turned to organisms for inspiration. Apart from physical and chemical methods, biological system are found to be efficient nano-factories for gold nanoparticle synthesis since they possess reducing agents such as enzymes, which can reduce metals at room temperature and is thermodynamically stable for months together. Gold nanoparticles have their applications in almost all the disciplines of science which necessitated scientists to find various means of their production. Biosynthesis of gold nanoparticles was first reported by using *Bacillus subtilis* [1]. It was noticed that Gold ions accumulated in the bacteria clumping together to form a precipitate. This precipitated complex was then dumped inside the cell-wall. Further insights into this work reported when Lactic acid bacteria was inoculated into gold ion solution [2]. Sulfate reducing bacteria were also found to synthesize gold nano particles intracellularly [3] using gold thiosulfate complex. These nano particles were spherical aggregates of octahedral gold. Mesophilic anaerobic bacterium *Shewanella algae have also been used for synthesis of nanoparticles;* where H_2 acted as an electron donor for intracellular precipitation of gold at 25^{0}C and pH 7[4]. The reductive precipitation was a fast process, producing insoluble nanoparticles of 10 - 20 nm size within 30 min.

Apart from prokaryotes, eukaryotic systems have also been found to possess many biomolecules such as glutathione, phytochelatins and enzymes such as reductases which have got the potential to reduce Gold ions to form gold nanoparticles. Marine alga *Sargassum wightii* [5] as well as brown marine algae *Fucus vesiculosus* [6] has been

exploited for extracellular synthesis of gold nanoparticles. Pandey and co-workers exploited the reducing potential of *A.vasica* [7] 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* [8], *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]

In the present work, we have used a fresh water alga, *Chlorella pyrenoidusa* for synthesis of gold nanoparticles and studied different parameters such as temperature and pH; at which monodispersed nanoparticles with controlled size and shape could be synthesized. Efforts were also directed towards comprehending the modus operandi for synthesis of nanoparticles.

MATERIALS AND METHODS

Chlorella pyrenoidusa was procured from NCIM, Pune; and maintained in Fog's Medium (for Algae) containing MgSO4.7H2O 0.2 g, K2HPO4 0.2 g, Micronutrient 1.0 ml solution (H3BO3 286.0 mg, MnCl2.4H2O 181.0 mg, ZnSO4.7H2O 22.0 mg, Na2MoO4.2H2O 39.0 mg, CuSO4.5H2O 8.0 mg), CaCl2.H2O 0.1 g, Fe-EDTA solution 5.0 ml, Agar (Difco) 12.0 g, 0.2% KNO3 in 1 L Distilled water.

***Harvesting*:** The algal culture was harvested at stationary-phase at 4°C by centrifugation at 6000 x g for 15 min and washed with deionized water. The cells were then dried and with the help of mortar and pestle and ground to a fine powder 0.5-1 mm in particle size. Prior to the experiments, the dried powder was washed several times with dilute HCl and deionized water to remove adsorbed impurities that might interfere with the formation of gold nanoparticles.

***Procedure* for GNP synthesis:** About 1.0-2.0 g of thoroughly washed powder under all experimental conditions was added to 100 ml of deionized water and the mixture was aged for 1-2 days. In a typical experiment, algal extract was mixed with deionized water, followed by the immediate addition of $HAuCl_4$ to make its concentration to 100 ppm. The pH of the reaction medium was adjusted by adding 1 M NaOH solution or 1 M HCl solution. The reaction was carried out under vigorous stirring at room temperatures (vide infra) for 0-48h.

Chemicals and Glass wares: Chemical used for the synthesis of gold nanoparticles was Chloroauric acid ($HAuCl_4$) (Sigma-Aldrich). 100mL of 1mM aqueous HAuCl4 solution in 500mL of Erlenmeyer flask was taken for gold nanoparticle synthesis.

***Experimental conditions*:** After harvesting the cultures, they were used for gold nanoparticle synthesis. Initially the impact of different temperature (4, 37, 60 and 100ºC) on synthesis of GNPs was studied. When the most suitable temperature was found to be 100^0C; then the impact of different pH (2, 4, 6,8,10 & inherent pH of algae) on synthesis of GNPs were studied. The most influential concentration of aurochlorate was fond to be 100 ppm; hence for all the experiments this concentration was used.

Characterization of Nanoparticles: UV-Vis Measurements- UV-visible spectroscopy 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. A UV-Vis spectrum is an indication of Surface Plasmon Resonance (SPR) that depicts the size and distribution of nanoparticles.

Transmission electron microscope- Examination of the nanoparticle morphology by high-resolution analytical transmission electron microscopy (TEM) was performed on a Carl Zeiss Micro imaging, GmbH, Germany with an electron kinetic energy of 200 kV. For sample preparation, 2-3 drops of the colloidal gold solution were dispensed onto a carbon-coated 200-mesh copper grid 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 (P Analytical, 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.

Nitrate Reductase Assay-For extraction of Nitrate Reductase from C. pyrenoidusa, 100 mg alga; powder was homogenized with Tris-HCl buffer (pH 8) and then centrifuged at 0°C at 2000 rpm for 15 minutes. The supernatant was used as enzyme source.

Nitrate Reductase activity was measured by Vega and Cardenas method [12].The standard graph for nitrite was calibrated using 50 µM working standard of Sodium Nitrite. To 0.1ml supernatant known amount of KNO_3 was added and incubated for 24 hours. Then 1 ml of diazo coupling reagent (1% sulfanilamide 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 remainingNO_2.After 30 minutes of incubation in dark at 30°C for development of colour;O.D. was recorded at 540 nm. The result was calculated against the standard graph of nitrite.

RESULTS AND DISCUSSION

When Gold salt solution was added to chlorella extracts and then was heated to 100^0C, it resulted in change of colour from yellow to wine red. Wine red is known to be associated with the formation of gold nano particles since the days of Faraday [13]. At 100°C, the influential pH values were found to be 6, 8, 10 & inherent pH of extract (8.3) as shown in the colors of GNPs exhibited due to SPR (Fig 1. a).At lower pH (pH 2 & 4) very large gold nanoparticles were synthesized that had a tende4ncy to get agglomerated (Fig 1.a), impact of pH on agglomeration has been reported earlier also [14]. Interestingly even very high (alkaline) pH 10 was also found to be efficient in producing nanoparticles, but they agglomerated within few days.

The UV-Visible spectral data was also analyzed along with visual observations and impact of different pH on GNP synthesis was studied. At pH 2, flat absorption spectrum was observed. A broad hump centered at 542 nm was exhibited at pH 4 (Fig.1). This indicates formation of both spherical as well as anisotropic nanostructures (Fig 2.d). At pH 6, a small hump at 535 nm was observed depicting polydispersed nanoparticles. The larger size can be due to the coalescence of smaller nuclei since there is presence of smaller nanoparticles. When gold nanoparticles were synthesized at pH 8, a sharp peak was centered at 530 nm (Fig 1. b). The peak signifies uniformly shaped nanoparticles. A possible explanation for this would be that due to alkalinity, i.e. hydroxides getting deposited on the gold nanoparticles. It is hypothesized that at this pH both reducing as well as capping agents are efficiently reducing the particles and further encaping them at specific facets. This allows growth of spherical nanoparticles due to vulnerable deposition of gold atoms on all the facets forming thermodynamically favorable spherical nanoparticles. The special groups which are actually involved in the nanoparticle formation includes amino, sulphydryl and carboxylic acid [15, 16, 17, 18, 19].Due to very high proton concentration at lower pH, all these functional groups possess positive charge. Thus even if the nanoparticles are formed but then also they are not stable enough to prevent agglomeration. The reducing power of these functional groups 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. The SPR band centered at 540 nm at pH 10 and the solution showed agglomeration (Fig 1. a).

High resolution transmission electron microscopic (HRTEM) studies

HRTEM analysis of GNPs at different pH showed that the gold nanoparticles are in the range of 25-30 nm. At pH 4, the TEM micrograph exhibits both spherical as well as icosahedral nanostructures of size 25-30 nm. At pH 8 as well as at inherent pH (8.3), there is appearance of larger spherical nanoparticles with multi twinned structures of size 20nm. And at pH 10 the GNPs are roughly spherical having 10 nm diameter, which tends to agglomerate due to electric double layer destabilization.

X-ray diffraction

The XRD pattern obtained corresponds to the gold nanoparticles exhibits Bragg reflections, which could be well manifested on the basis of the face centered cubic (fcc) gold nanostructures. The very strong diffraction peak at 38 degrees is considered to be of {1 1 1} facet of the face centered cubic structure (Fig 3), while the diffraction peaks of other gold peaks are found to be much weaker compared to standard GNPs. 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 GNPs [20].

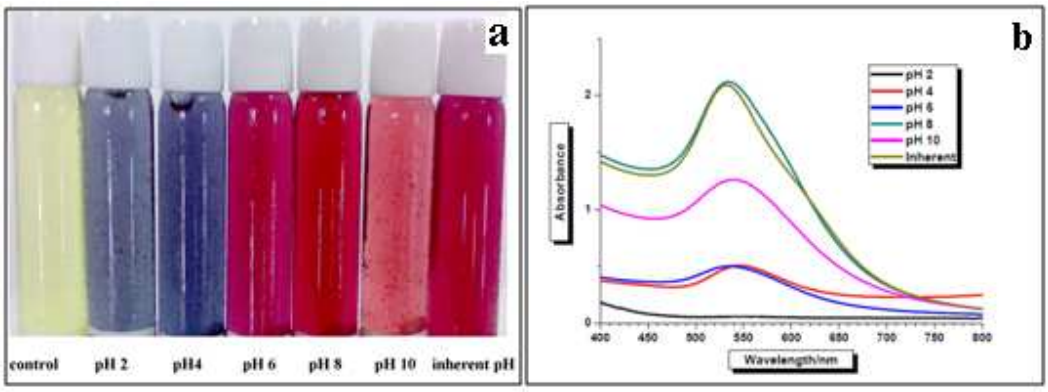

Figure 1: Effect of pH on GNP synthesis using *Chlorella pyrenoidusa*; (a) Change in SPR with respect to different pH (b) UV-Visible spectra

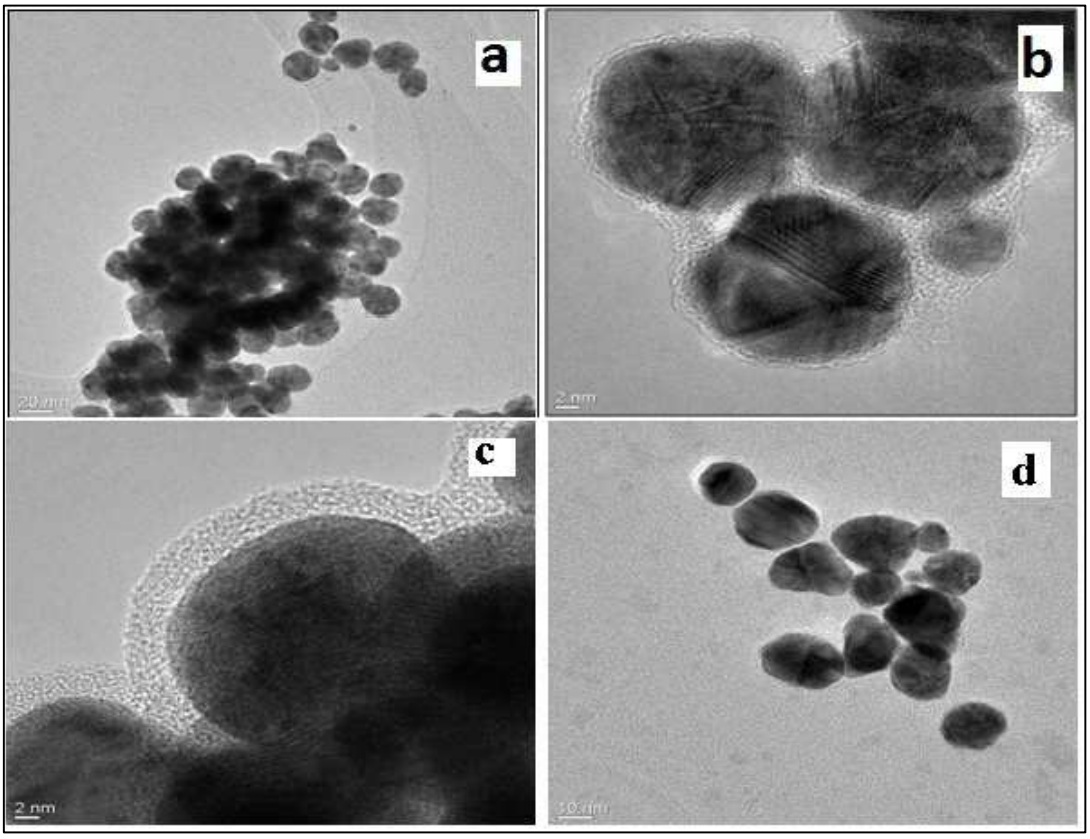

Figure 2: HR-TEM analysis of *Chlorella pyrenoidusa* showing the impact of temperature on biosynthesis of GNPs at 100^{0}C at (a) pH 10 (b) pH 8 (c) inherent pH (d) pH 4

Table 1: Impact of pH on biosynthesis of GNPs at 100°C using100 ppm Aurochlorate ions

pH	Observation
2	Change in colour in < 5 sec Flat absorption spectra
4	Change in colour in < 5 sec Broad hump at 542 nm XRD Crystalline structure **TEM- spherical as well as anisotropic nanoparticles**
6	Change in colour in < 5 sec Small hump at 535 nm XRD Crystalline structure
8	Change in colour in < 5 sec Sharp peak at 530 nm XRD Crystalline structure **TEM- Large Spherical nanoparticles**
10	Change in colour in < 5 sec Broad peak at 540 nm XRD Crystalline structure **TEM- Roughly Spherical nanoparticles**
pH of algal extract (8.3)	Change in colour in < 5 sec Sharp peak at 540 nm XRD Crystalline structure **TEM -Spherical nanoparticles(30-40 nm)**

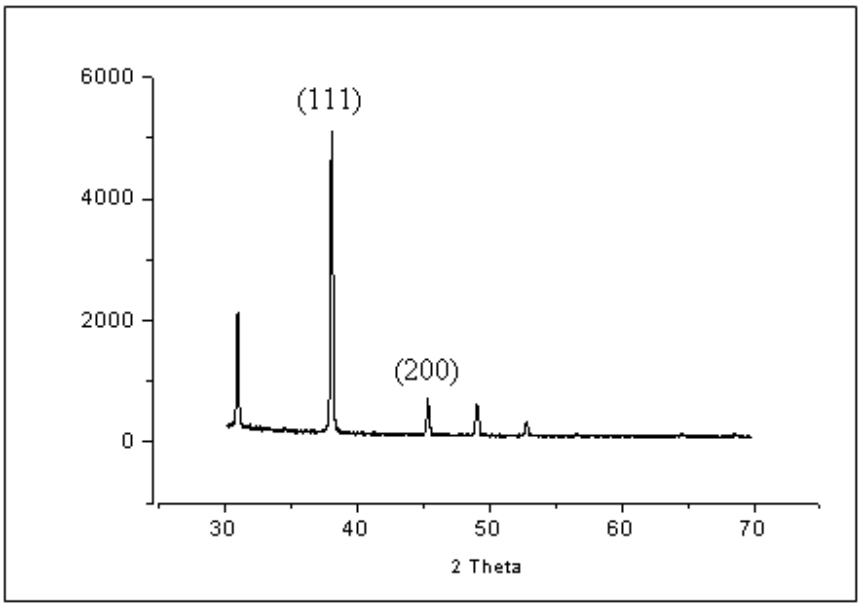

Figure 3: X-ray diffraction pattern of gold nanoparticles biosynthesised using *Chlorella pyrenoidusa*

Nitrate Reductase Activity

Chlorella pyrenoidusa is a fresh water alga rich in reductases and dehydrogenase. These reductases help in NADH dependent extracellular reduction of Au^{+3} to Au^{0} thus leading to the formation of GNPs. Nitrate reductases are considered to be most efficient NADH-dependent enzyme acting as a nucleating as well as capping agent for gold nanoparticle synthesis(Fig. 4).

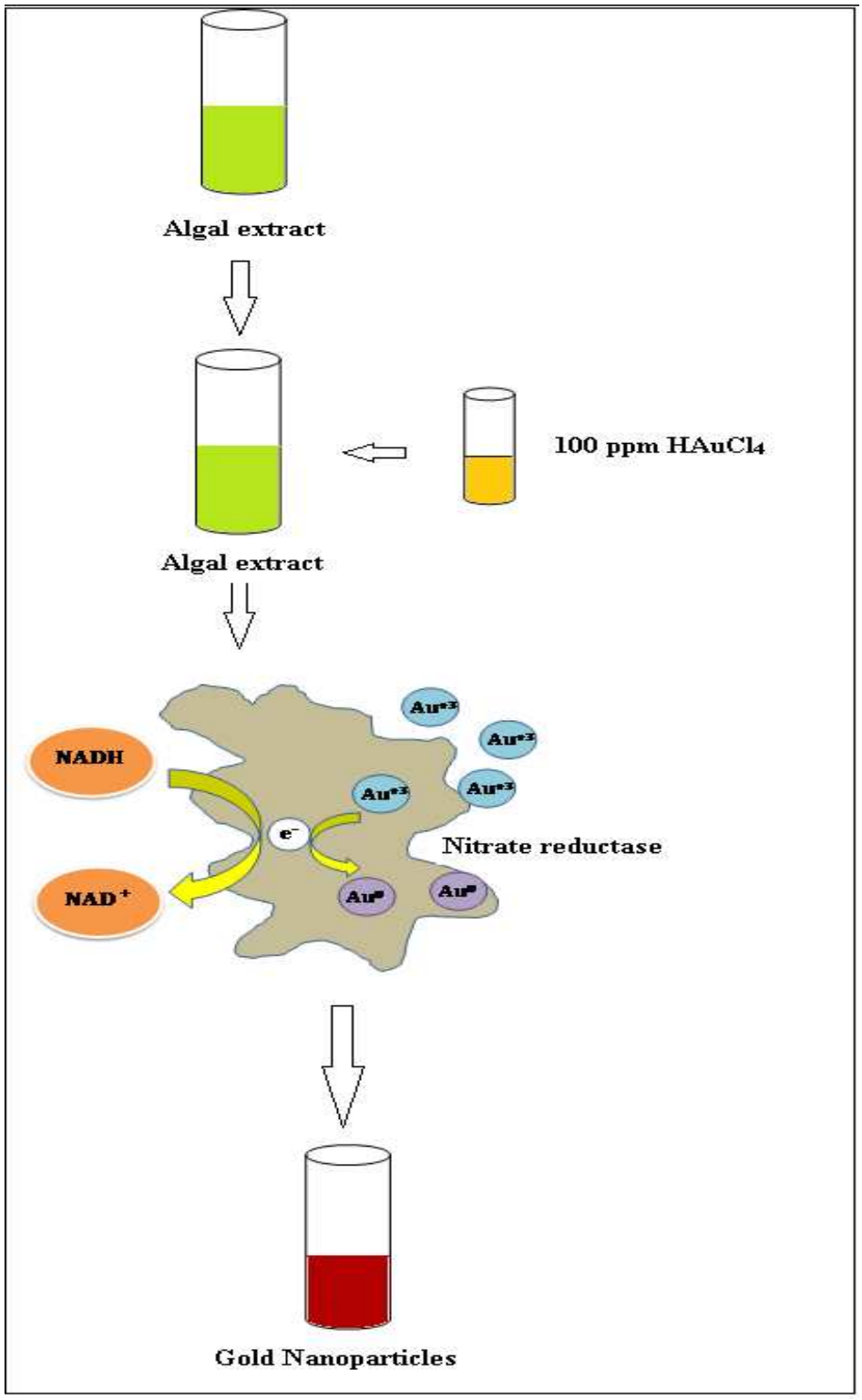

Fig. 4: Schematic representation of nitrate reductase activity in reduction of gold salt and formation of GNP

It was found that *Chlorella pyrenoidusa* extract exhibited the Nitrate reductase activity as 0.7245 µmole/min/gram of extract, which got reduced to 0.5244 µmole/min/gram of extract when it was subjected to 100° C (Fig.5). After the

formation of GNPs, the nitrate reductase activity was again assayed in the reactant mixture which showed a substantial decrease (almost nil) in the solutions having GNPs. This result confirms the involvement of reductases in the reduction of gold ion to GNPs.

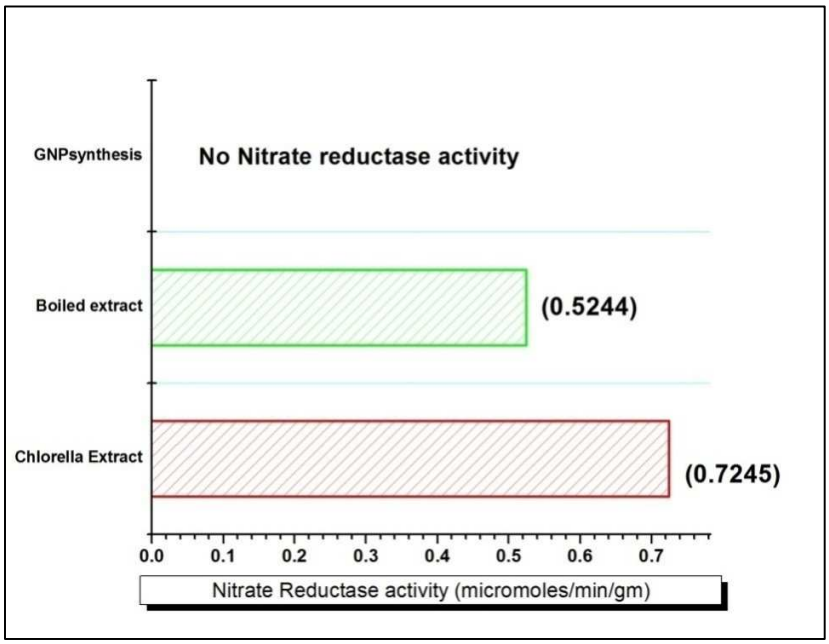

Figure 5: Nitrate reductase activity of *Chlorella pyrenoidusa* extract, Boiled plant extract and gold nanoparticles respectively in µmoles/min/gm.

CONCLUSION

Chlorella pyrenoidusa can be used for rapid synthesis of GNPs. The most influential parameters were found to be alkaline pH and high temperatures. The TEM micrographs of GNPs show the presence of both spherical and icosahedral nanostructures. The substantial decrease in the nitrate reductase activity confirms that reductases are involved in the reduction of gold ions to GNPs. The methodology presented can be used for a controllable tuning of the synthesis of thermodynamically stable gold nanoparticle.

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