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Biosynthesis of Highly Stable Gold nanoparticles Using *Citrus limone*

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ABSTRACT

The work exploits use of eco-friendly method to synthesis GNPs and tuning the optimum parameters using juice of Citrus lemon. The optimum yield of gold nanoparticles (GNPs) was obtained when 100 ppm aurochlorate was treated with lemon extract at pH 2.5 and heated it to 100^{0}C. The results were verified using UV-Vis spectroscopy, XRD and Transmission electron microscopy. The GNPSs were monodisperse and found to be 10-100 nm in size. The stability of the GNPs synthesised using biological protocols was found to be extremely high then the chemically synthesised GNPs when tested with 5M NaCl solution. The reason for high stability can be partly attributed to the encapping of GNPs with citrate.

Keywords: Biosynthesis, GNPs, Citrus limone, ascorbic acid, citric acid.

INTRODUCTION

There are many reports of plant extracts being used for biosynthesis of metal nano particles. Extra-cellular synthesized Au and Ag nanoparticles by using root and stem extracts of Geranium has been reported [1]. Moreover, triangular GNPs synthesis has also been reported using Lemongrass. It was a single step, room temperature reduction of aurochlorate ions that suggested that these nano-triangles were formed by assemblies of spherical nanoparticles that seem to be" liquid-like". The fluidity arises due to nanoparticles surface complexation of aldehydes / ketones present in lemongrass extract **[2]** Extra cellular synthesis of gold and silver nano particles using *Emblica officinalis* fruit extract as a reducing agent has also been reported [3]. Treatment of aqueous solution of chloroauric acid and silver sulfate with *Emblica officinalis* extract caused rapid reduction of silver and chloroaurate ions, leading to formation of highly stable silver and gold nano particles of dimensions 10 – 20 nm respectively. Pandey et al exploited the reducing potential of *A.vasica* [4] 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* [5], *M. charantia* [6] 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* [7] 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 [8]

In this work we report the biogenic synthesis of highly stable GNPs using the aqueous extract of *Citrus limone*. The bio-fabricated GNPs were found to be much more stable than those fabricated using chemical method. The stability was checked by recording the optical response of the GNPs by addition of different values of 5M salt solution. Such GNPs can be orchestrated with linkers and drug for synaphic drug delivery.

MATERIALS AND METHODS

Materials: Fresh fruits of *Citrus limone* were brought from the local market in Ambernath, as and when required. Gold aurochlorate and silver nitrate were procured from Sigma Aldrich, USA. The experiments were performed using double distilled water. The glassware was 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.

Preparation of aqueous extract of plants: 10 ml of juice extracted from freshly collected fruits of *C. limone* was made up to 50 ml with distilled water. The extract obtained was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used as reducing agent for synthesis of gold nanoparticles. In order to retain the activity of the enzymes in the extract it was kept in clean bottle and was refrigerated until further use.

Biosynthesis of the GNPs: Clear extract was used for the biosynthesis of gold nano particles. In a boiling solution of the plant extract, gold salt was immediately added to make the concentration 100ppm. After addition of the gold salt, the solution was agitated till the colour becomes wine red.

The parameters and their variables considered for biosynthesis were (a) pH 3, 4, 6, 8, 10 and inherent pH of plant extract (b) *Concentration of the gold aurochlorate* – after few initial trials it was decided to keep it 50 ppm and (c) 4, 30, 60 and 100^0C temperature.

Chemical synthesis using citric acid as Reducing agent- Turkevich's method was followed to make gold nanoparticle. [9]

Stability of GNPs: The bio fabricated GNPs were synthesised by Citrus lemon at optimum parameter (pH 2.5 at 100^0C). The stabilised GNPs were then diluted (1:2) using distilled water and then the spectra was recorded after the addition of salt ranging from 10µl to 5000µl.

Characterization of the Biosynthesized GNPs

UV- Vis Spectroscopy of the GNPs- The UV-Vis spectra of the GNPS formed were recorded using dual beam spectroscopy Lambda 25 Perkin Elmer, USA. High quality quartz cuvette (Perkin Elmer optics, USA) was used as a vessel to record the spectra.

Table – 1: Impact of pH and Temperature on the Biosynthesis of gold nano particles using 100 ppm Aurochlorate and *C.limone pulp extract*

pH	Temperature	
	30^0C	**100^0C**
2	Change in color in 30mins Sharp peak at 418nm and a broad hump at 761nm XRD - Crystalline structure TEM Both iso & anisotropic GNPs could be seen	Change in color in < 5 sec Good peak at 520 nm and broad hump at 720nm XRD Crystalline structure TEM Both iso & anisotropic GNPs could be seen
4	Change in color in 30mins Broad hump at 538nm XRD Crystalline structure	Change in color in < 5 sec Weak hump at 535nm XRD Crystalline structure
6	Change in color in 30mins Broad hump at 544nm XRD Crystalline structure	Change in color in < 5 sec There was a flat peak which was obtained XRD Crystalline structure
8	Change in color in 30mins Broad hump at 542nm XRD Crystalline structure	Change in color in < 5 sec Weak peak at at 540 nm XRD Crystalline structure
10	Change in color in 30mins A flat spectrum was observed XRD Crystalline structure	Change in color in < 5 sec There was a flat peak which was observed XRD Crystalline structure
Inherent pH of plant extract 2.5	Change in color in 30mins Intense peak at 418nm XRD Crystalline structure	Change in color in < 5 sec There was a intense peak which was found to be at 533nm XRD Crystalline structure

Transmission electron micrographic Analysis-To elucidate the morphology of the GNPS biosynthesised using *Citrus lemon* plant extract high resolution transmission electron microscope (HRTEM), Carl Zeiss Micro imaging, GmbH, Germany, was used. Sample was ultrasonicated for 15 minutes and then coated on ultraclean carbon coated copper grid for analysis. The SAED pattern of the GNPs indicates presence of crystalline GNPS as deciphered using the diffraction pattern using X-rays.

X-Ray diffraction studies (XRD)-To peep into the crystallinity and the lattice properties of the GNPs, XRD (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) was used. The colloidal suspension containing metal nanoparticles was dirtied on a small glass cover slip.

RESULTS AND DISCUSSION

Visual Observation: Time taken for change in color as observed visually is presented in table 1 and 2.Which shows that pH 2 has yielded the best results at both the tried temperatures.

As the inherent pH of 2.5 showed the best Plasmon peak the further experiments were carried out at pH 2.5 of the pulp extract of *Citrus limone.*

Table – 2: Impact of different temperatures on the Biosynthesis of gold nano particles using 100 ppm Aurochlorate and *Citrus limone* pulp extract at pH 2.5

Temperature	Observations			
	Visual	UV-Vis Peak	XRD	TEM
4 ºC	Change in color in > 30mins	Broad peak at 545 nm	Crystalline	Both isotropic & anisotropic GNPs
RT (28 ± 2ºC)	Change in color in < 30mins	Broad peak at 545 nm	Crystalline	-
60 ºC	Change in color within 20mins	Broad peak at 545 nm	Crystalline	-
80 ºC	Change in color in <30 sec	Intense peak at 545 nm	Crystalline	-
100 ºC	Change in color in <5 sec	Intense peak at 545 nm	Crystalline	Both isotropic & anisotropic GNPs

Visual studies:
The appearance of the wine red colour in the solution confirmed the formation of nanoparticles. However the tenure for the reduction of gold ions and hence the appearance of wine red colour was found to be temperature dependent. Due to high ascorbic acid and citric acid content of *C. limone*, the colour change took place less than 5 mins at 30°C whereas it took few seconds at 100°C for the appearance of the colour. The stability of the GNPs were found to be very high when synthesised using acidic environment (pH2.5) whereas at alkaline pH (pH 6 to 10), the colour changed from wine red to purple indicating the increase in the size of the nanoparticles.

Uv-Vis Spectroscopy studies:
Figure1a displays the formation of the GNPs synthesized at 30°C. At pH 2 and the inherent pH (2.5), there was an intense SPR peak at 418nm. This indicates the formation of monodispersed nanoparticles. The wine red color of the GNPs is due a size dependent quantum mechanical phenomenon when the electrons of the matter get caged to nano-boxes. The phenomenon is dominant when the materials enter from a bulk to a nano regime (10-50nm). The wine red color becomes more intense when the De-Broglie wavelength of the valence electrons becomes equal to or less than the size of the particle. Due to this phenomenon the freely mobile electrons are caged in GNPs and exhibit a characteristic collective coherent oscillation of plasmon resonance giving rise to *surface plasmon resonance (SPR)* [10]. At pH 4, 6 and 8 there was a broad SPR hump which was seen which may be due to the incomplete reduction of gold, thus resulting in formation of polydisperesed GNPs. But, at pH 10 there was no SPR peak due to non-favorable environment for the reduction of the gold ions into nanoparticles.

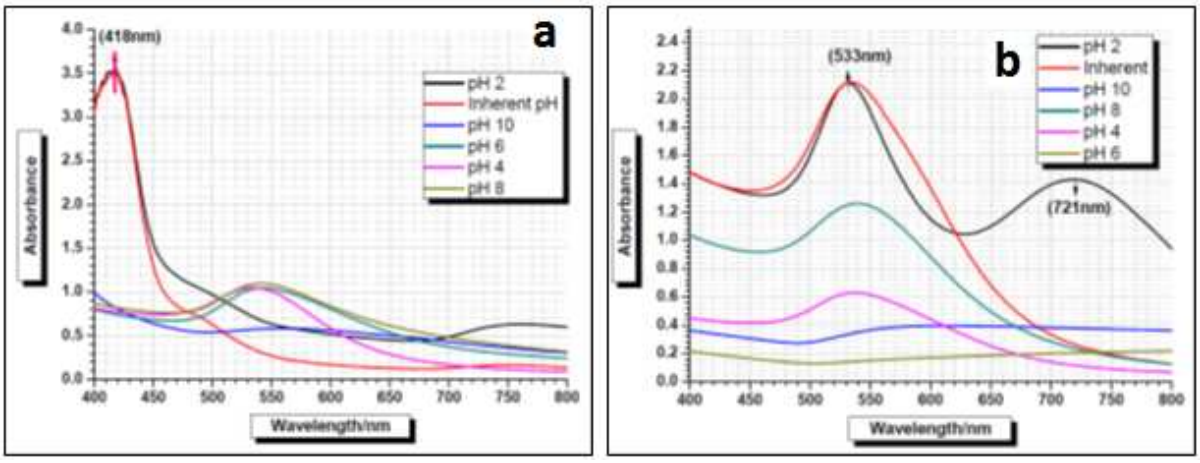

Figure- 1: Impact of temperature and pH on synthesis on GNPs using juice of *C.limone*; as recorded by the UV-Vis spectroscopy of the sample synthesized at (a) 30°C (b) 100°C.

At higher temperature (100°C), the optical spectrum is displayed in figure 1b. At pH 2, there were two prominent SPR peaks at 533nm and 721nm which clearly signifies the formation of anisotropic GNPs. Such dual peaks appear because of formation of non-spherical GNPs and/ or agglomeration [11]. At pH 2.5(inherent pH), SPR band was found to be centered at 533nm indicating the formation of monodispersed gold nanoparticle. At pH 4 and 8, there was a broad SPR hump which may be due to the incomplete reduction of gold which resulted in the formation of polydispersed GNPs. At pH 6 and pH10 there were no nanoparticles formed as there was no SPR peak which was found.

High Resolution Transmission Electron (HRTEM) microscopic studies: The HRTEM of the nanoparticles prepared from the pulp of lemon indicates that most of the particles which are formed are spherical but in some frames polydispressed and mono-dispersed nanoparticles is also formed (fig 2a). The nanoparticles which were formed at 100°C were found to be coffee bean shaped (fig2b). The HRTEM of the nanoparticles also clearly shows the lattice fringes of the crystalline structure of the nanoparticles (fig 2c).

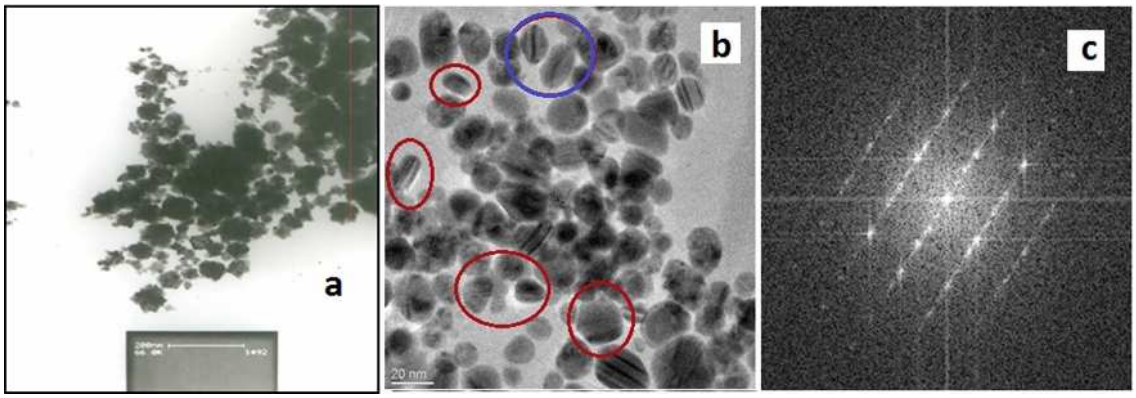

Figure –2: TEM of *C.limone* mediated synthesis of GNPs at (a) at 30°C & pH 10 shows the formation of gold nano-star (b) at 100°C & pH 2.5 (inherent). Red circles depict the formation of rod, triangular and hexagonal GNPs. Blue circle exhibit the formation coffee bean shaped GNPs (c) SAED pattern of the GNPs which proves GNPs to be crystalline

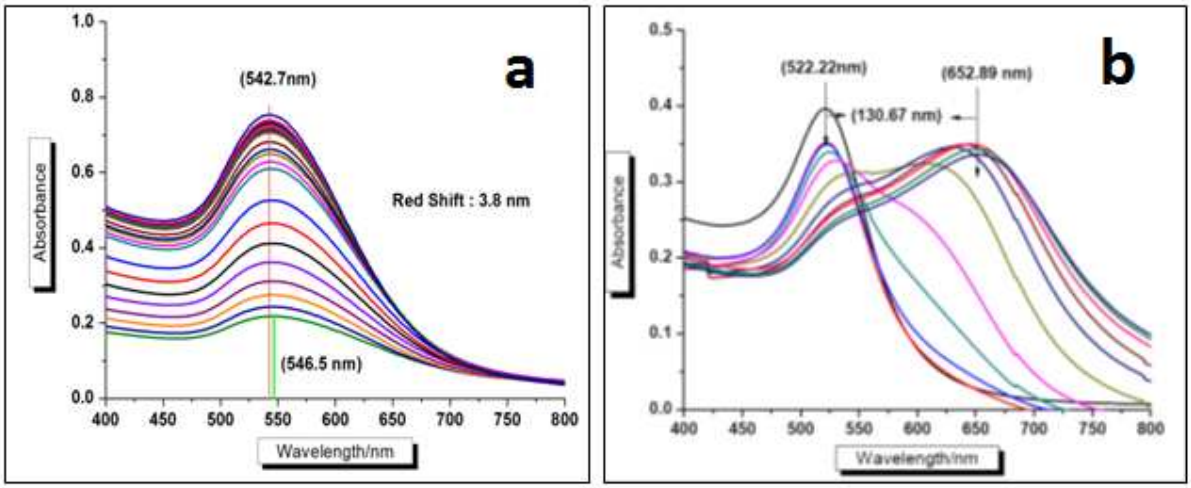

Figure-3: UV-Visible spectra showing the stability of (a) Biogenic nanoparticles using C.limone synthesized at pH 2.5, 100° C & 100 ppm of aurochlorate (b) Chemically synthesized nanoparticles using the same parameters used for biogenic nanoparticles.

Stability of GNPs

To solve the problem of agglomeration of GNPs in solution, particularly when it is suspended in high salt concentration for clinical uses such as drug delivery, the stability of biological nanoparticles was tested against very high salt concentration. As shown in the figure 3 a, there was a red shift of 3.08 nm after addition of approximately 5 ml of 5M NaCl. In stark contrast to this, the shift in chemically synthesized nanoparticles using same parameters was found to be 130.67 nm after addition of merely 100µl of 5 M NaCl. This exceptional stability of biogenic nanoparticles can be attributed to protection of GNPs by intelligent capping proteins. Under optimal ionic strength of the solution these proteins avoid the columbic attraction between the nanoparticles by maintaining suitable surface potentials.

X-ray diffraction studies: The confirmation of formation of elemental GNPs 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 GNPs 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-4; this is in agreement with the previous data available on gold nanocrystals [12]. 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.

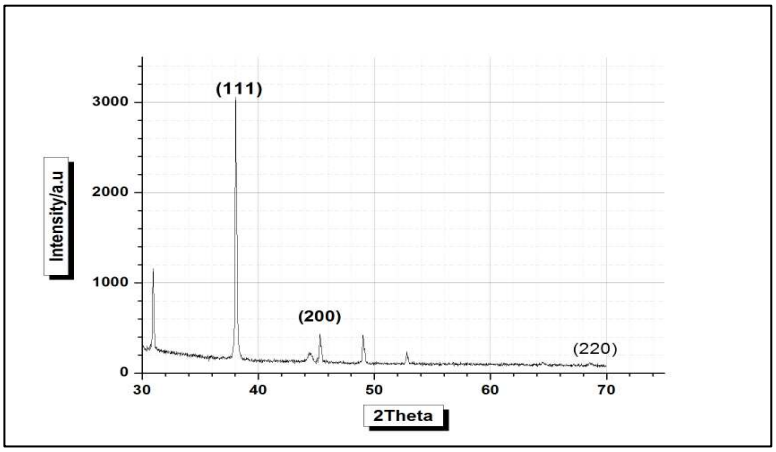

Figure- 4: XRD pattern of gold nano particles synthesized using leaf extract of *Citrus limone*, showing typical Bragg reflections for GNPs

CONCLUSION

Citrus limone pulp extract has shown excellent capability of biosynthesizing gold nano particles from gold salt solution. The optimum conditions for stable GNPs biosynthesis using *Citrus limone* was observed to be at inherent pH (2.5), Temperature 100°C and 100 ppm Au metal concentration**.** The presence of the ascorbic acid content and the citric acid content in the fruit has a large advantage over other plants as it highly reduces aurochlorate to GNPs and the particle size is also very small which makes these nanoparticles favorable for a wide spread application with increased stability and very good shelf life

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