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Fractal Dimension Analysis of Swarms of Gold Nano/Micro Particles Produced By Wild *Rhizobium* Cultures Using A Novel Slide Based System

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Abstract: **There is a lot of interest in autonomous movement and collective behavior of synthetic nanomaterials which have important applications in nanomedicine, nanobiotechnology and nanosensors. Present work using viable cultures and cell free extracts of *Rhizobium* sp. to produce and analyze swarms of monodisperse Au Nanoparticles (Au NPs) and polydisperse Au microparticles (Au MPs) was inspired by previously reported work on chemically triggered swarming of commercially available Au MPs. We developed a simple, glass slide based technique for rapid production, microscopic visualization, morphological analysis, study of swarming behavior and monitoring of effects of heat on Au NPs and Au MPs forms and assemblages. We designed cell based and cell free microbial system such as *Rhizobium* sp. to test the hypothesis that unidentified proteinaceous factors could be involved in producing simple monodisperse and complex polydisperse geometric forms of Au NPs and Au MPs. We used pure and wild type *Rhizobium* cell suspensions prepared from surface sterilized root nodules of *Mimosa pudica*. Cell free extracts from wild type and pure cultures were prepared by warming cell suspensions for 15, 30, and 45 sec. Cultures and extracts were separately mixed with $HAuCl_4$ in equal proportions in each treatment on slides and monitored microscopically. For studying effect of heat slides were warmed for 15, 30, 45 seconds. Mixed, dense swarms of monodisperse Au NPs were obtained with polydisperse Au MPs using wild type and pure cultures as well as cell free extracts. Heat treatment yielded interesting forms and complex assemblages exhibiting fractal properties. We postulate that *Rhizobium* sp. specific unidentified cell bound heat responsive proteins may trigger monodisperse Au NPs swarms and simple and complex assemblages or acidic conditions may release amino acids from protein hydrolysis which may act as capping and linking agents. Some of these assemblages or acidic conditions may release amino acids from protein hydrolysis which may act as capping and linking agents. Some of these assemblages such as open and closed rings, interlocked rings are unique. Using our simple slide based technique a lot of scope exists for applying a combination of microbial cell free extracts and timed heat treatment to obtain defined monodisperse GNP swarms. This study showed the potential of fractal dimension analysis to study biotechnologically useful forms of Au NPs and polydisperse Au MPs.**

***Keywords*: *Mimosa pudica, Rhizobium,* cell free extracts, Au Nanoparticles, Au microparticles, Au NPs swarms**

I. INTRODUCTION

Gold nanoparticles (GNPs) are of special interest due to their remarkable optical properties such as biocompatibility and ease of surface functionalization. Development of novel profitable and environmentally-friendly methods of obtaining nanoparticles are of high interest and bacteria, fungi, plants and algae have been used for their production [1] [2] [3]. Bacterial cultures such as *Rhizobium* are heavy metal tolerant [4] [5] [6], show swarming migration behavior and produce extracellular polymeric substances (EPS) which contain polysaccharides [7] and proteinaceous factors [8]. Present work using viable cultures and cell free extracts of *Rhizobium* sp. to produce and analyze swarms of monodisperse Au NPs and

polydisperse Au MPs was inspired by previously reported work [9] on chemically triggered swarming of commercially available Au MPs. This work was aimed at developing a simple, glass slide based technique for rapid production, microscopic visualization, morphological analysis, study of swarming behavior and monitoring of effect of heat on AuNPs and AuMPs forms and assemblages using cell free microbial system such as *Rhizobium* sp. to test the hypothesis that unidentified proteinaceous factors could be involved in producing simple monodisperse and complex polydisperse geometric forms of Au NPs and Au MPs at different heat flux. Metal nanoparticle aggregates formed under different conditions show a symmetry that can be described by fractal analysis [10]. The properties of nanoparticles and its aggregation as well as convective heat transfer of nano fluids have received great attentions over the last few decades. It is well certified that nanoparticles and its aggregation can be successfully described by fractal theory and technology [11]. Fractal analysis of nanoparticles can provide extra information about primary particle size distributions. The fractal theory developed by Mandelbrot proposed new approach to define the geometry of those systems which have no definite geometry. The most important numerical parameter to calculate the fractal of any mass is fractal dimension. Fractal dimension is defined simply as the number of independent quantity needed to specify the position or arrangement of point on the object [12]. Many concepts have been proposed for the calculation of fractal dimension of any fractal mass. Low fractal dimension shows formation of aggregates due to cluster to cluster collision. Fractal dimension of nanoparticles have been determined using two-dimensional images and particle size distributions from either electron microscopy or dynamic light scattering measurements [13]. There are reports on fractal analysis of inter-particle interaction forces in gold nanoparticle aggregates [14]. Recently fractal analysis has been employed for characterizing the membrane protein aggregation using fluorescence resonance energy transfer [15] as well as applied to illustrate various behaviors of biological molecules by using data extracted from plentiful techniques, such as light scattering, small angle X-ray scattering, and conductivity measurements [16] [17] [18]. The surface-enhanced Raman scattering (SERS) activity of multi-branched gold nanostars with fractal structure has been investigated for trace detection of pesticide thiram [19].

II. MATERIALS AND METHODS

A. Sample collection

Mimosa pudica roots were collected from different parts of mining and non-mining areas of Goa (table 1 and 2) from which root nodules were obtained. The root nodules were further used for isolation of bacteria.

TABLE I: COLLECTION OF *MIMOSA PUDICA* ROOTS WITH NODULES FROM MINING AREAS

Sr.no	location	Designation	Latitude	Longitude	Number of samples
1.	Shirgao	RNM-01	15’36’19.54”N	73’53’39.45”E	4
2.	Bicholim	RNM-02	15’35’11.15”N	73’56’35.96”E	5
3.	Pissurlem	RNM-03	15’31’57.39”N	74’03’56.31”E	5
4.	Velgeum	RNM-04	15’30’04.26”N	73’03’22.28”E	10

TABLE II: COLLECTION OF *MIMOSA PUDICA* ROOTS FROM NON-MINING AREAS

Sr.no	Location	Designation	Latitude	Longitude	Number of samples
1.	Taleigao	RNNM-01	15’27’33.36”N	73’49’55.59”E	4
2.	Taleigao	RNNM-02	15’27’42.03”N	73’49’53.34”E	5
3.	Taleigao	RNNM-03	15’27’26.47”N	73’50’05.58”E	5
4.	Chapora	RNNM-04	15’36’08.77”N	73’44’14.08”E	10
5.	Moira	RNNM-05	15’36’05.87”N	73’50’07.70”E	3
6.	Siolim	RNNM-06	15’37’12.89”N	73’46’02.85”E	2
7.	Sancoale	RNNM-07	15’23’47.01”N	73’52’16.39”E	4

B. Isolation of cultures of Rhizobium sp.

Rhizobium, *Cupriavidus metallidurans, necator* are some bacteria found associated with the root nodules of leguminous species such as *Mimosa pudica*. *C. metallidurans* metalogenic bacteria forms biofilms on gold and is involved in the gold biomineralization [20]. Standard technique [21] was used to isolate *Rhizobium* on Congo red Yeast Extract Mannitol (YEM) agar (HIMEDIA). Morphological studies and preparation of cell suspension. The culture obtained were further

sub cultured on plates of YEM agar and maintained on slants of same medium at Goa University Fungal Culture Collection (GUFCC 3050-3078) unit. The isolates were assigned numbers GUFCC 3050 to 3078. The cell morphology was recorded using monochrome staining, were checked for Gram's nature, and also checked for motility using cavity slide.

C. Preparation of extracts and detection of proteinaceous factor

To test the possibility of a proteinaceous factor involved in swarm formation, preparation of hot water extract [22] was carried out using the *Rhizobium* cell suspension. The extracts (10ml) were passed through membrane filter with pore size of 0.22 µm to prepare cell free extracts (CFE). The absorption of CFE at 280nm was measured using UV-1800 spectrophotometer (Shimadzu corp) to check for presence of proteinaceous factor using sterile D/W as a control.

D. Trial Gold nanoparticle (GNP) synthesis and Effect of thermal treatment of GNP assemblies

Extracts and $HAuCl_4$ (0.01275,0.0255,0.051,0.102,0.254,0.508,1.27,2.54,5.08,10.15,20.3,25.375mM) were mixed in equal proportion (50:50 v/v) on a clean microscope slide with plain ground edges (76x26x1.25mm-Borosil). Slides were heated for 15sec, 30sec, and 45sec on a spirit lamp (using methylated Spirit and methanol). The slide were mounted with DPX mountant, and further microscopic characterization was done using Nikon Eclipse E200 microscope. Images were captured at different magnifications using NIS elements microscope imaging software.

E. Fractal Dimension analysis

The obtained images were subjected to digital image analysis using two software Mountains Map Premium 7.2 which is surface imaging, analysis and metrology software (Digital surf) (http://cme.msu.edu/cmeias/fracAnalysis.shtml). For fractal dimension analysis JFrad (Center For Microbial Ecology Image Analysis System-CMEIAS) was used (http://cme.msu.edu/cmeias/fracAnalysis.shtml). We obtain fractal dimension which is a measure of fractality or a shape being 'fractal' using different techniques, at least 11 are used by the software JFRAD. Most of these computational techniques like JFRAD produce scores with fractions with several decimal places. Instead of this we simplify the fractal dimension by multiplying by 1000 so we get a whole number. We designate it as Fractality Index (FI) which we defined as the four digit number obtained by multiplying the score produced by the fractal analysis software like JFRAD by 1000 with the last number being rounded up.

III. RESULTS AND DISCUSSION

A. Isolation of bacteria from root nodules of Mimosa pudica plants

The isolation efforts resulted in 25 different *Rhizobium* cultures (table 3) among these 15 isolates were from mining areas (GUFCC 3050 to GUFCC 3064) and 10 were from non-mining areas (GUFCC 3065 to GUFCC 3074). Root nodules separated from roots of *Mimosa pudica* as shown in fig 1a. Viable bacterial cultures were isolated from 90% of root nodules, representative pure culture of *Rhizobium* obtained on Congo red YEM agar is shown in fig 1c. Mining areas of Goa were found to be most fertile for obtaining *Rhizobium* from root nodules. *Rhizobium* cultures were Gram negative, rod shaped, and motile(fig1d).

TABLE III: DESIGNATION OF STRAINS

Sr.no	Designation of strain
1.	GUFCC 3050
2.	GUFCC 3051
3.	GUFCC 3052
4.	GUFCC 3053
5.	GUFCC 3054
6.	GUFCC 3055
7.	GUFCC 3056
8.	GUFCC 3057
9.	GUFCC 3058
10.	GUFCC 3059
11.	GUFCC 3060

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12.	GUFCC 3061
13.	GUFCC 3062
14.	GUFCC 3063
15.	GUFCC 3064
16.	GUFCC 3065
17.	GUFCC 3066
18.	GUFCC 3067
19.	GUFCC 3068
20.	GUFCC 3069
21.	GUFCC 3070
22.	GUFCC 3071
23.	GUFCC 3072
24.	GUFCC 3073
25.	GUFCC 3074

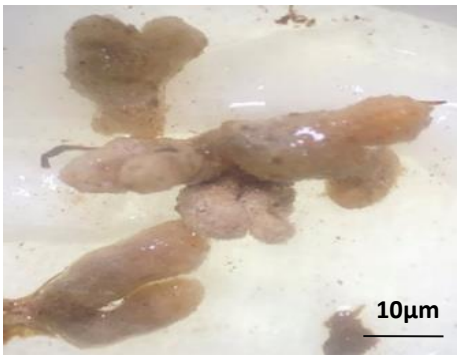

Fig. 1a

Fig. 1b

Fig. 1c

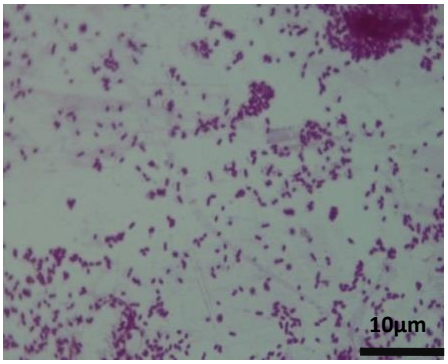

Fig. 1d

Fig. 1(a-d) a-Root nodules separated from roots of *Mimosa pudica;* b-Suspension of Rhizobium cells in Congo red YEM agar; c-Pure cultures of *Rhizobium* obtained on Congo red YEM agar; d-Rhizobium cell morphology in crystal violet stain

B. GNP synthesis and Effect of thermal treatment of GNP assemblies

CFE showed absorption at 280nm indicating presence of unidentified proteinaceous factor having optical density 2.3. After testing all cultures with Only one promising culture (GUFCC 3064) showing more swarm formation was used for the study of AuNPs. 45 sec thermal treatment showed most promising results with 20.3 and 25.375mM concentration of Gold. Cell Free Extract (CFE) showed good results compared to cell suspension. The variation in the formation of AuNPs complexes was noticed, at lowest concentration (0.01275mM) the formation of monodispersed particles swarming occurred. As the concentration increased, dense monodisperse building blocks, globus particles transiting into assemblages was observed (Fig 2a-b). At concentration of 0.102 mM and 0.254mM, formation of dendritic pattern occurred, and further binding of the patterns to form assemblages (fig 2d-f). Floret formation was noticed at the concentration of 2.54mM (Fig 2h). Complex floret forms were seen at concentration 5.08 mM and 10.15mM (Fig 2i-j).

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At concentration of 20.3mM Complexity increased forming 3D stacking [23] and production of thick crust, or plate of gold nanoparticle occurred on the surface of the slide at concentration of 25.375mM (Fig 2k-l).

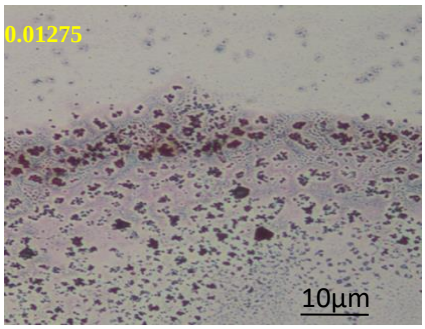
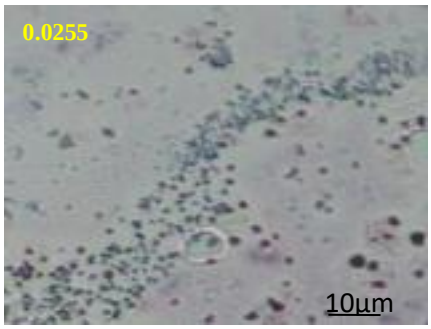


Fig. 2a



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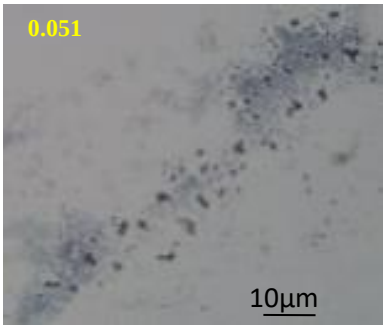
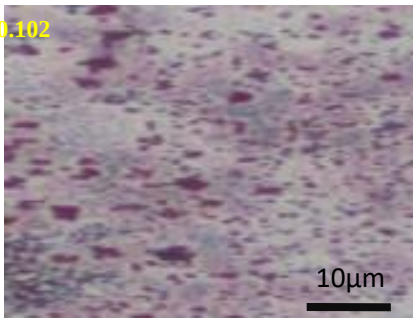


Fig. 2c



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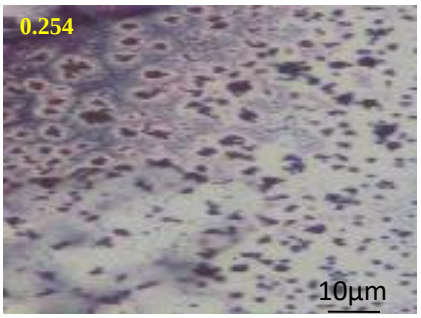

Fig. 2e

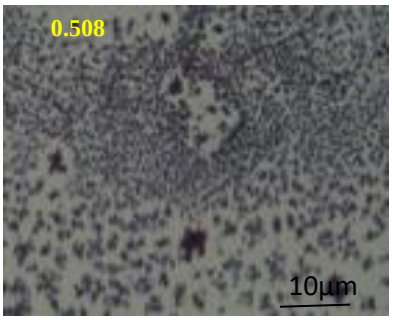

Fig. 2f

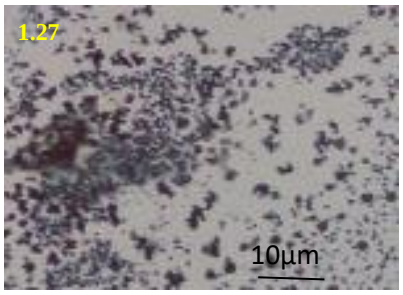

Fig. 2g

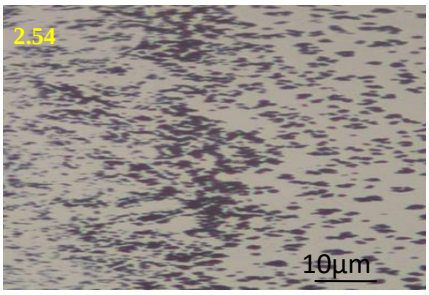

Fig. 2h

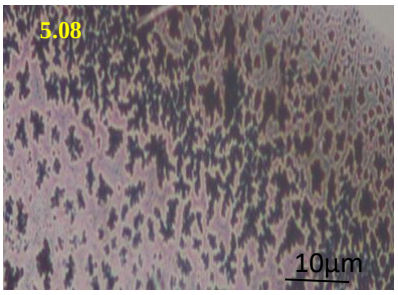

Fig. 2i

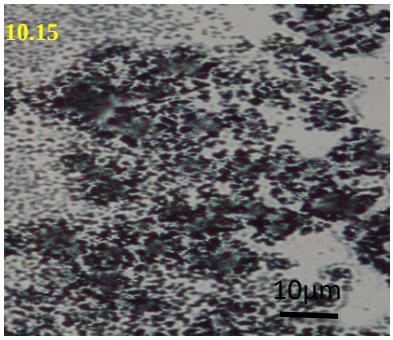

Fig. 2j

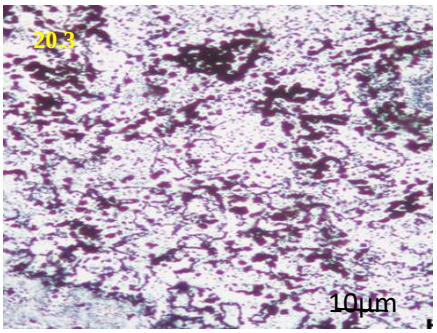

Fig. 2k

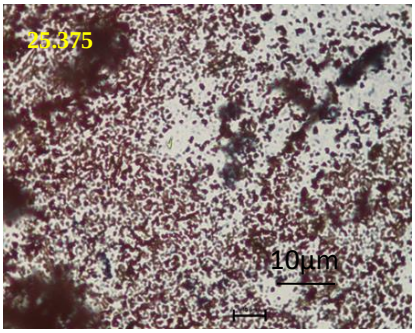

Fig. 2l

Fig. 2a-l Indicate the fractal nature of the GNP swarms and their complexity with increasing concentration of Gold (yellow colour denotes the concentration of Gold in mM)

C. ***Fractal Dimension analysis***

The variation in the fractal Index (FI) values by Euclidean distance methods is shown in Fig 3. As the concentration of Gold in mM increased, the fractality Index also increased from 1201 to 1550. CFE produces unidentified proteinaceous

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factor which could be involved in producing simple monodisperse and complex polydisperse geometric forms of Au NPs and Au MPs at different heat flux. 1100deg Celsius methanol flame gives (over 400mm2 surface) 3780 Joules of energy in 45 sec heat treatments [24]. The complex fractal forms of swamps are shown in fig 4a and 4d. Fig 4b and 4e shows the 3D images of the swarms and 4c and 4f shows the results of fractal analysis having fractal dimension value of 2.42 and 2.53.

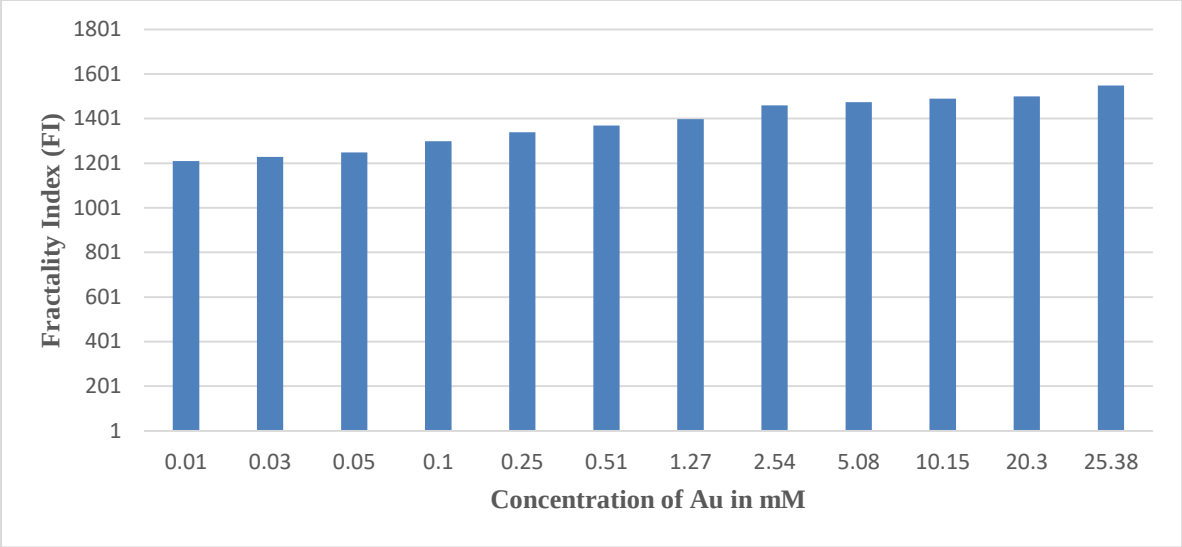
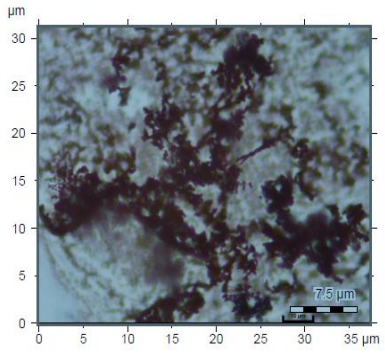
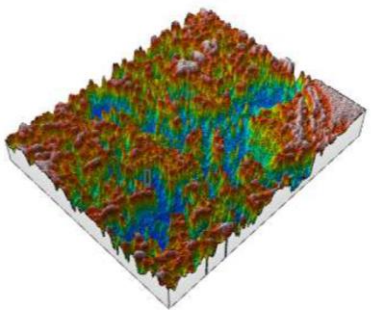


Fig. 3 Effect of Gold Concentration on fractal dimension of GNP





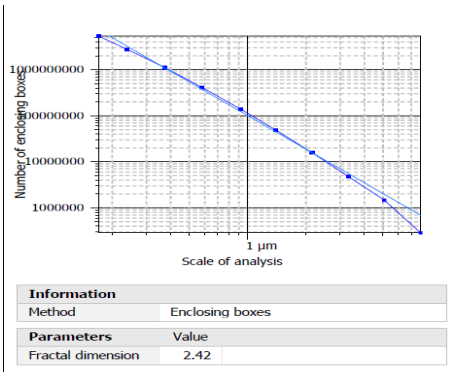
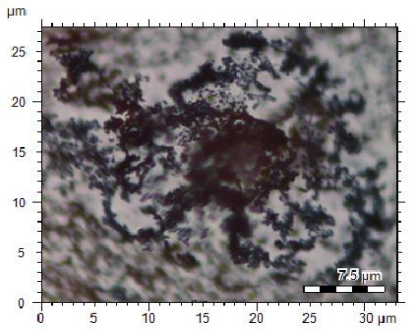
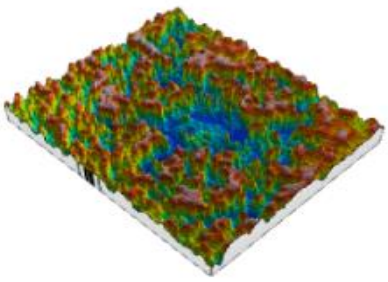


Fig 4 a **Fig 4b** **Fig 4c**

Fig. 4(a-c) a-Complex fractal forms produced at concentration of 25.375mM; b-3D images of the swarms; c-Fractal analysis showing fractal dimension value of 2.42





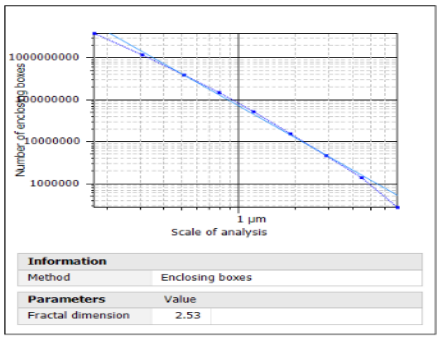


Fig 4d **Fig 4e** **Fig 4f**

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Fig. 4(d-f) d-Complex fractal forms produced at concentration of 25.375mM; e- 3D images of the swarms; f- Fractal analysis showing fractal dimension value of 2.53

It was found that when concentration at (25.375mM) leading to steady concentration and variation of thermal treatment was from 60 to 300 sec produced complexed 3D stacking and thick crustose assemblies indicating that heat resistant proteins may be implicated, which can withstand prolonged heat (Fig 5).

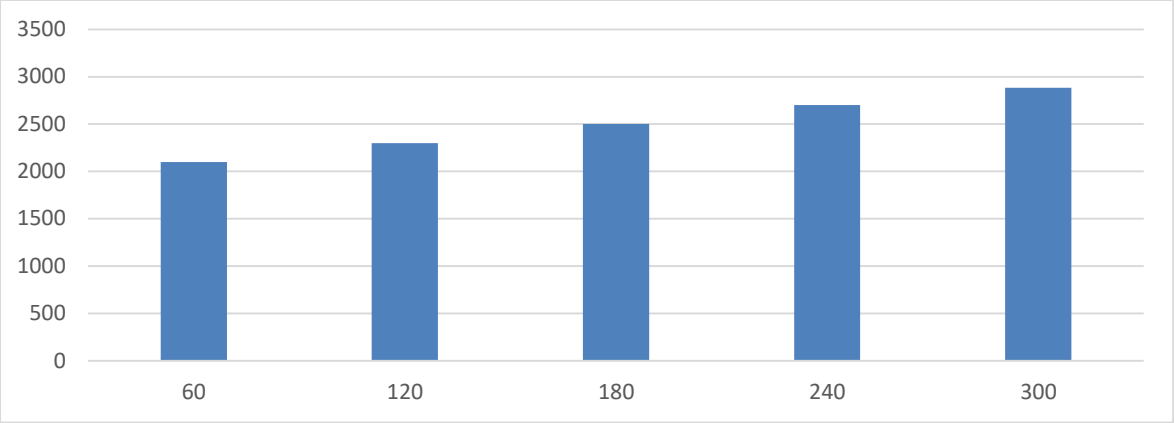

Fig. 5 Effect of heat on fractality of AuNP swarms

It was possible to obtain GNP swarms. The results shows that a simple, glass slide based technique can be successfully used for rapid production, microscopic visualization, morphological analysis, study of swarming behavior and monitoring of effect of heat on Au NPs and Au MPs forms and assemblages using cell free microbial system such as *Rhizobium* sp. In this study we used CFE of *Rhizobium* culture which are reported to be heavy metal tolerant [4] [5] [6]. CFE produces unidentified proteinaceous factor which could be involved in producing simple monodisperse and complex polydisperse geometric forms of Au NPs and Au MPs at different heat flux. Earlier reports were on chemically triggered swarming of Au microparticles [9]. 1100deg Celsius flame gives 2.09 Mega Joule energy but heating slide over 400mm^2 surface produces energy which means 3780 Joules of energy is involved in 45 sec heat treatment [Farrier law of heat conduction heat release rate is 84Joule/second, 1100deg Celsius flame=2.09 Mega Joule energy but heating slide over 400mm^2 surface produces energy which means 3780 Joules of energy is involved in 45 sec heat treatment]. As the concentration increased from 1.21 to 1.51mM the complexity of GNP increased, concentration from 1.21 to 0.051mM produced swarms, as the concentration crosses 0.051mM nanoparticle alignment occurs forming dendritic assemblages, florets forms, finally leading to complex forms, similar finding has been reported where amino acid induced fractal aggregations of AuNPs occurs [25]. Earlier studies were on the synthesis of star-like gold nanoparticles (SGNs) in a temperature-controlled environment which allows for temperature modulation and facilitates the growth of highly branched nanoparticles [26]. There are reports on dendritic structures of AuNPs [27] [28]. It was noticed that swarms were appearing in low concentration treatment ie 0.021mM concentration and this concentration was found to be best for monodispersed GNP production. There are studies on a single large assembly with dynamically fluctuating swarms of gold nanoparticles formed by trapping laser [29]. Previous report shows aggregates formed more quickly at higher concentrations and temperatures. It was found that when concentration was kept (25.375mM) constant and thermal treatment is increased from 60 to 300 sec, the complexity increasing forming 3D stacking and producing thick crust surface indicating that heat resistant proteins are implicated, which can withstand prolonged heat. Scheme has been summarized in the figure 6 and 7. Previous reports has also shown that aggregates formed more quickly at higher concentrations and temperatures [30]. When CFE containing the proteinaceous factor is added to highly acidic solution of $HAuCl_4$ (pH 1.5), hydrolysis of protein occurs giving rise to mixture of amino acid, thus AuNPs which are formed may be amino acid capped AuNPs, thus forming a crossed bridging between AuNPs giving rise to complexity of AuNPs. It is possible to analyze the fractal forms by using Jfrad and mountains Map software. Earlier reports were on fractal analysis of inter-particle interaction forces in gold nanoparticle aggregates [31]. Using our simple slide based technique a lot of scope exists for applying a combination of microbial CFE and timed heat treatment to obtain defined monodisperse GNP swarms and study postulated heat responsive protein mediated genesis and architecture of biotechnologically useful forms of Au NPs and polydisperse Au MPs.

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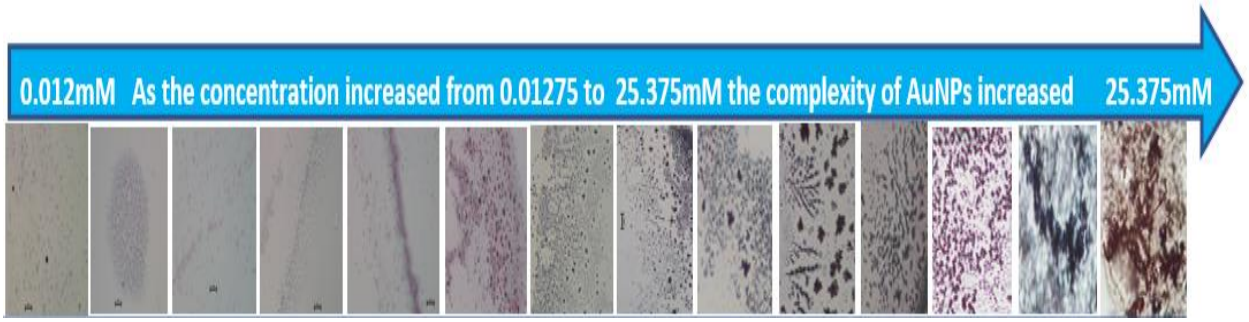

Fig. 6 Change in complexity of AuNPs with increase in the concentration from 0.01275 to 25.375mM

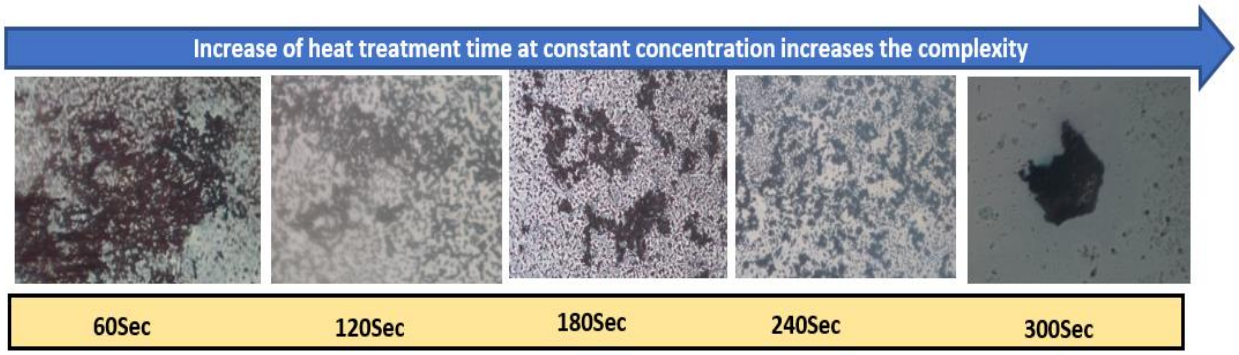

Fig. 7 Increase of complexity with increase in thermal treatment and constant concentration

IV. CONCLUSIONS

Rhizobium sp. cultures were successfully isolated from root nodules of *Mimosa Pudica* plant sp. and used for the production of AuNPs. It was possible to obtain GNP swarms. It was found that CFE showed good results at 45sec thermal treatment forming complex structures at 25.75mM concentration. This is the first study of use of *rhizobium* cultures for the production of AuNPs in Goa and India. This is the one among the few report on study of fractality Index of AuNPs. Using our simple slide based technique a lot of scope exists for applying a combination of CFE and timed heat treatment to obtain defined monodisperse GNP swarms. As the concentration crosses 0.051mM nanoparticle alignment occurs forming dendritic assemblages, florets forms, finally leading to complex forms, similar finding has been reported where amino acid induced fractal aggregations of AuNPs occurs [25]. In our studies we also observed that when concentration is increased to 25.375mM and thermal treatment is increased from 60 to 300 sec, the complexity increases forming 3D stacking and producing thick crust surface. We postulate that *Rhizobium* sp. specific unidentified cell bound heat responsive proteins may trigger monodisperse Au NPs swarms and simple, complex assemblages or acidic conditions may release amino acids from protein hydrolysis which may act as capping and linking agents. Some of these assemblages or acidic conditions may release amino acids from protein hydrolysis which may act as capping and linking agents. Some of these assemblages such as open and closed rings, interlocked rings are unique. This heat responsive protein mediated genesis and architecture of biotechnologically useful forms of Au NPs and polydisperse Au MPs. This indicated that heat resistant proteins are implicated, which can withstand prolonged heat. If it is possible to produce AuNPs swarms with use of *Rhizobium* cultures than it is possible to use other microorganisms to produce NPs. It is possible to analyze the fractal forms by using Jfrad and mountains Map software. There is a lot of interest in autonomous movement and collective behavior of synthetic nanomaterials which have important applications in nanomedicine, nanobiotechnology and nanosensors. Further work is required to study and characterize the proteinaceous factor. Further modified microfluidic technique will be designed and used for AuNPs synthesis.

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