SYNTHESIS OF GOLD NANOPARTICLES USING BACILLUS ALCALOPHILLUS AND ITS APPLICATION

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Abstract

The bacterial infections, fungal type of diseases and cancer can be cured with the help of synthesis of gold nanoparticle using microbes. The gold nanoparticles with microorganisms haveexcellent medical applications. Gold nanoparticles were synthesized using *Bacillus alcalophillus*. This synthesis of gold nanoparticles are characterized by visual analysis, UV-Visible Spectroscopy, Fouriertransform infrared Spectroscopy, Scanning Electron Microscopy. The gold nanoparticleshave antimicrobial activity, antifungal activity, and anticancer activity. The aim of the study is that by using some of pathogen such as *E.coli*,*P.aeroginosa*, *S.aureus* and the fungal *Aspergillusniger*, the gold nanoparticles with *Bacillus alcalophillus*act against these bacteria. The study establishes the fact that biogenic gold nano particles synthesized in a facial manner can be well used in various fields as these are appropriately stabilized by *Bacillus alcalophillus*.The marine bacterial strain of *Bacillus alcalophillus* is useful to achieve a fastest rate of nanoparticles synthesis which may be of high in interest for future determination in development of AuNPs,theseisolated gold nanoparticles can be further studied for medical and clinical purposes to cure bacterial diseases.

KEYWORDS: Synthesis, Gold nanoparticles, *Bacillus alcalophillus*, Characterization**.**

INTRODUCTION

One of most fascinating branch of nanotechnology is Nano biotechnology. The term nano is tailored from the Greek word sense, dwarf. A nanoparticle is a microscopic particle with at least one dimension less than 100 nm [Kaushik et al., 2010]. Nanoparticles are cluster of atoms within the size range of 1-100nm. Scientists develops reliable, eco-friendly techniques for atom-by-atom construction of objects in fields like medicine, electronics, catalysis [Mourato et al., 2011], photonics, optoelectronics, information technology, environmental monitoring and remediation, military equipment and weapons and other fields [Hussein et al., 2007].Nanotechnology are often defined as a search for the planning, synthesis, and

manipulation of structure of particles with dimension smaller than 100nm. Nanotechnology is enabling technology with the ability to work at the atomic, molecular and sub molecular levels in order to understand, create and use material structures, devices and systems with fundamentally new properties and functions resulting from their small structure [Thirumurugan et al., 2009]. The methods of synthesis can be divided on intra cellular and extracellular. One of the most important criteria of nanotechnology is that of the development of clean, non-toxic and eco-friendly green chemistry procedures Nanotechnology has dynamically developed as an important field of modern research with potential effects in electronic and medicine. Microorganisms such as bacteria, fungi, and yeast play an important role in the remediation of toxic metals through reduction of metal ions and act as interesting Nano factories. These microbes are extremely good candidates in the synthesis of gold nanoparticles. Themicroorganisms have extraordinary bactericidal effect on gold nanoparticles that depends on the dimensions and shape of the particle. The microorganisms, prokaryotic bacteria have received the most attention in the area of biosynthesis of nanoparticles. Moreover, bacteria are easy to handle and may be manipulated genetically. Considering these advantages, a bacterial system could convince be a superb alternative of chemical methods for the synthesis of gold nanoparticles.Nanoparticles can act as antimicrobial and antifungal agents, because of their ability to interact with microorganisms. Vital cell Biosynthetic methods can employ either microorganism cells or plant extract for nanoparticles production.

MATERIALS AND METHODS

Collection of sample

The *Bacillus alcalophilus* (MTCC 4668) marine bacteria isolated. Preparation of Gold stock-1mM ($HauCl_4$)

M = Required volume x required concentration x Molecular weight/1000

mM M = M/1000

Gold nanoparticles synthesis by *Bacillus alcalophilus*

The culture filtrate was centrifuged at 5000 rpm for 5 minutes. After centrifugation the supernatant were used as a culture filtrate and the entire culture taken as culture sample. In both sample, 500 µl HAuCl4 were added and incubate at room temperature in dark shade. The change in color from pale yellow to a purple appearance was found.

CHARACTERIZATION OF GOLD NANOPARTICLES

UV-Visible spectrophotometer

Using UV-VIS spectrum the gold ions reduction was monitored at 24 hours' time interval by drawing 1cm3 of the samples and their absorbance was recorded at a resolution of 0.5m at 300-600nm using UV-VIS spectrophotometer (Elico, UV-VIS SL 159). Formation of Plasmon peak was observed and noted**.**

Fourier Transform Infra-red Spectroscopy

Spectroscopy chemical bonds present within the analyzed chemicals are often interpreted by FTIR spectrum, by using the KBr pellets with prominent resonance spectra. The filtrate containing the extra cellular proteins secreted by the bacteria in the presence of Au was salted out overnight at 4^0C using ammonium sulphate precipitate followed by centrifugation at 5000 rpm for 10 minutes. The protein obtained was dissolved in the minimal volume of deionized water and dialyzed using 12kDa to cut off dialysis membrane.

Scanning Electron Microscope

Thin films of the sample were prepared on a carbon coated copper grid by dropping the sample on the grid. Extra solution was removed using blotter. The film on the SEM grid was allowed to dry by putting it under a mercury lamp for five minutes for emitting characteristic X-rays. These characteristic X-rays identify the composition and measure the abundance of elements within the sample.

Antibacterial activity

The antibacterial activity of gold nanoparticles synthesized by *Bacillus alcalophilus* were determined by well diffusion method. Nutrient agar were prepared and sterilized at 121^0 C for 15 minutes, after sterilization the medium was poured into sterile petri plate and allowed to solidify. After solidification the plates were swabbed with 50µl of *Escherichia coli, Bacillussubtilis, Staphylococcus aureus, and Pseudomonas aeruginosa.*Cork borer were used to make well, and to the each well 20µl of culture filtrate, culture of synthesized sample and HAuCl4 (control) 30mcg of methicillin disc were placed respectively. Then the plates were incubated at 37ºc for 24hrs.

Antifungal activity

The antifungal activity of gold nanoparticles synthesized by *Bacillus alcalophilus*were determined by well diffusion method. Malt agar were prepared and sterilized at 121^0C for 15 minutes, after sterilization the medium were poured into sterilized petri plate and allowed to solidify. After solidification the plate were swabbed with 50µl of *Aspergillusniger*. By using

cork borer wells are punched. And to the each well 20µl of culture filtrate, culture, $HauCl_4$ (control) were added. Then the plates were incubated at 37^0c for 24hrs.

Antimicrobial activity of gold nanoparticle by MIC method

Minimum inhibitory concentration (MIC) of gold nanoparticles were calculated by antimicrobial activity of synthesized gold nanoparticles using *Bacillus alcalophilus* against bacterial strains of 50µl of *Escherichia coli*, in different concentration of gold nanoparticles at 10µl, 20µl, 30µl, 40µl, 50µl respectively.

Turbidity method

Nutrient broth (20ml) were prepared, and dispersed into 4test tubes with 5ml of each. To that 60 µl of *Escherichia coli and Staphylococcus aureus* were inoculated. After inoculation 40 µlof culture filtrate and culture samples were added and incubated at 37^0C for 24 hrs. After 24hrs, the growth was measured at 600nm against control. The cell death was calculated using by formula, % of cell death = Control-test/ Control x 100

Food Borne Pathogen

The nutrient agar were prepared and sterilized with 121^0C for 15mins and the medium were poured into sterilized petri plate and allowed for solidification, after solidification the plate were swabbed with *E.coli, Staphylococcus aureus* organism. By using cork borer to make well and to each well add 20µl of unpurified, purified, gold synthesized sample and (ampicillin) drug sample poured respectively. Then the plates were incubated at 37^0c for 24hrs. After incubation the result were observe.

Anticancer activity

Cell viability and Cytotoxicity assays were performed to find the cytotoxicity of samples. The Dulbecco Modified Eagle Medium(DMEM) 0.195g, Glucose 0.045g, Sodium carbonate 0.037g was prepared in 10 ml T Flask. HeLa cell lines ($1X10^4$ cells) were inoculated to this medium. The media was incubated at 37˚C/24 hrs. The test dilution 500 µl and 250 µl were added along with Cell line 500 µl and MTT Dye 500 µl. After incubation at 37^0 C for 1 to 2hrs, the UV reading was taken by UV-Visible Spectrophotometry at 450nm and 540nm and the taken reading were calculated by using formula, % of cell death = Control-test/ Control X 100

RESULT AND DISCUSSION

Visual observation

The formation of gold nanoparticle in the solution of 1mM HAuCl4 and sample of *Bacillus alcalophillus*was confirmed by color change from pale yellow to purple (plate-1)

Characterization of gold nanoparticles

UV-Visible Spectrophotometer

The gold nanoparticles were confirmed by UV-Visible Spectrophotometer. The absorbance was measured at 300-600nm of the AuNPs. In the present study the UV-Vis spectrum of the culture filtrate gold nanoparticles obtained a clear distinct Plasmon peak at 540nm as like as the culture gold nanoparticles obtained Plasmon peak at 560nm. (Figure 1) showed UV-Visible of gold nanoparticles. The surface Plasmon bands for the AuNPs usually ranges between 510 and 560 nm in aqueous solution depending upon the function of their morphology, dielectric constant of surrounding media. Since Plasmon bands are very sensitive to the length and sharpness of the tips of nanometer. It was started earlier that spherical nanoparticles have strong absorption at 520 nm with almost no absorption after 600 nm with only a single SPR (Surface Plasmon Resonance) band in the absorption spectra. The single peak obtained observed at 536nm which indicates the spherical nature of AuNPs by UV-Visible Spectrophotometer according to the nanoparticles had a range of 20-40 nm with spherical in shape. In the same way by using *Helianthus annulus* (Sun flower) synthesis of gold nanoparticles performed and shows a strong Plasmon resonance that is located at 550nm [Liny *et al*., 2012].

Fourier Transform Infra-red Spectrophotometer

FTIR analysis was carried out to understand the possible biomolecules involved in the synthesis of nanoparticles. During the selection of environment the specialty of FTP over other technique are that spectra are obtain from protein. Requiring less time, sample, and direct correlations between the IR amide I band frequencies and the secondary structure components can be found [Kong *et al*., 2007]. The gold nanoparticles synthesized from *Bacillus alcalophillus* exhibit a lot of biomolecules which were involved in the synthesis of AuNPs process. FTIR shows the presence of various functional groups, which produce to the well-known signatures within the IR region of spectrum. The strong and broad band observed at 3512.76, 1989.65, 1706.46, 1436.69, 1434.76, 1284.65, 986.54, and 699.77. The band of 3512.76 corresponds to O-H (polyphenolic) bond, 1989.65 corresponds to C-N bond, 1706.46 indicates to amide I corresponds to C=O bond, 1436.69 and 1434.76 corresponds to C-C stretching aromatic ring, aliphatic amines C-C stretching assigned at 1284.65, 986.54 corresponds to S-O bond stretching of sulfonates, 699.77 corresponded to alkyl halides.

This study also confirms that the carbonyl group from amino acids or proteins has stronger ability to bind metal so that the proteins or enzymes could most possibly cap the metal

nanoparticles to forbid the cluster of the particles. (Figure 2) showed the FTIR of synthesized gold nanoparticles. This standard peak of FTIR spectrum of both proteins and peptides confirms the presence of extracellular proteins that is responsible for the synthesis of gold nanoparticles. The capping agents (proteins) carbonyl group from the amino acid residues shows stronger ability to bind to metals. The IR spectral data of high polymers are usually interpreted in terms of the vibrations of a structural repeat unit. The polypeptide and protein repeat units produce to nine characteristic IR absorption bands, namely, amide A, B, and I−VII. Of these, the amide I and II bands are the 2 most prominent vibrational bands of the protein backbone. The amide I band (1700-1600 cm-1) is the most sensitive spectral region to the protein secondary structural components, which is almost entirely to the C=O stretch vibrations of the peptide linkages (approximately 80%). Gold nanoparticles can bind to proteins through free amine groups or B carboxylate groups in the protein. The presence of the extreme peak at C=O stretching mode indicates the presence of carboxylic groups within the material sure to gold nanoparticles.

Scanning Electron Microscope

Morphology, size and distribution of gold nanoparticles were confirmed by SEM. Gold nanoparticles loaded in the *Bacillus acidophilus* were found to be in the size range of 56-124 nm. As the metal particles are good conductors, they were observed at magnification of 7000x in a voltage of 20KV. The average size of gold nanoparticles determined 2µm in size. The Plate 2 showed size of gold nanoparticles. By the average size of gold nanoparticle is 1µm in size. According to Alexander Bootz*et al*, 2004, the diameter of gold nanoparticles when viewed under scanning electron microscope, it showed 167nm size particle.

ANTIBACTERIAL ACTIVITY

The antibacterial activity was carried out using four different strains. Zone of Inhibition in the plate showed that gold nanoparticles synthesized using Bacillus *alcalophilus*have the antibacterial activity against test pathogens namely E.*Coli, P.aeruginosa, B.subtilis, S.aureus.*For this the antibacterial activity of culture filtrate gold nanoparticles showed higher zone of inhibition against the test pathogens *S.aurues, P.aeruginosa, B.subtilis* and *E. coli* respectively. On comparison with the antibiotics and $HAuCl_4$, Synthesized culture filtrate gold nanoparticles have bactericidal effect. Plate 3- (A) *P.aeruginosa*, (B) *E.coli*(C) *S.aureus* (D) *B.subtilis* showed antibacterial activity of gold nanoparticles. The zone of inhibition was showed in Table 1. InLifeng Qi*et al*, 2004, study shows about the chotosan nanoparticle that act against *E. coli, S. choleraesuis, S. typhimurium,* and *S. aureus* and shows the zone of inhibition.

Table 1: zone of inhibition of antibacterial activity of gold nanoparticles

Bacteria	**Culture filtrate**	**Culture**	**Disc**	**HAuCl4**
E.coli	4 mm	Nil	Nil	Nil
B.subtilis	6 mm	Nil	Nil	3 mm
S. aureus	3 mm	Nil	Nil	Nil
P. aeruginosa	3 mm	Nil	Nil	2mm

ANTIFUNGAL ACTIVITY

The antifungal properties of gold nanoparticles against fungal gave zone of inhibition in the plate. The gold nanoparticles synthesized using of *Bacillus alcalophilus*have the antifungal activity against test pathogen namely *Aspergillusniger*. On comparison the gold nanoparticles shows higher zone of inhibition against the test pathogen *Aspergillusniger* respectively. The result was showed in Plate 4. The zone of inhibition was shows in table 2.

Table 2: zone of inhibition of antifungal activity of gold nanoparticles

Organism	**Culture filtrate of gold nanoparticles**	**Entire culture of gold nanoparticles**
Aspergillusniger	8 mm	5 mm

Gold nanoparticles are lethal to fungi. The lack of capping polymers on the surface of gold could contribute to the enhancement the nanoparticles lethality. The mechanism of the antibacterial effects of gold ions Au+ involves their absorption and accumulation by the bacterial cells that would lead to shrinkage of the cytoplasm membrane or its detachment form the cell wall. The gold nanoparticles effect on fungi have attributed to genome islands encoding a lot of toxins. The interaction of the surface modified nanoparticles with the peptide glycol layer of the cells has a remarkable effect on the inhibition of growth of microorganisms [Li *etal*., 2010].

Minimum Inhibitory Incubation

Minimum inhibitory concentration of gold nanoparticles was conducted to investigate the antimicrobial activity of gold nanoparticles synthesized using *Bacillus alcalophillus* againstbacterial strains of *E.coli* in various concentrations of gold nanoparticles such as 10 µl, 20 µl, 30 µl, 40 µl, 50 µl respectively. In this study the result were determined by using spectrophotometer reading. The result was shown in -table 3. In the present study MIC shows better result in high concentration of 40 µl and again growth of organism were obtained in high concentration of 50 µl. The Plate -5 shows MIC of gold nanoparticles. In related with

[Y.Li*et al*, 2006] the minimum inhibitory concentration of the nanoparticle against *Escherichia coli* and *Staphylococcus aureus*were 1/128 and 1/512 respectively.

Table 3: UV reading of MIC of gold nanoparticles

Control	**AuNPs (10µl)**	**AuNPs(20µl)**	**AuNPs (30µl)**	**AuNPs (40µl)**	**AuNPs (50µl)**
1.246	0.942	0.816	0.703	0.420	0.614

TURBIDITY METHOD

The turbidity method was used to detect bacterial death concentration. The *S.aureus*was found to be more efficient compare with *E.coli.* The result was showed in (Table 4).

Table 4: Turbidity method of gold nanoparticles

Bacteria	**Culture filtrate**	**Culture**
E.coli	41.5%	0.94%
S.aureus	50.34%	14.6%

Food borne pathogen

Gold nanoparticles showed antibacterial activity against *E.coli* and *Staphylococcusaureus*effectively. The gold nanoparticles were found to be effective against food pathogenic bacteria. The results were given in Plate 6, (a) *E.coli*, (b) *S.aureus* and zone of inhibition were measured. (Table 5)

Table 5: zone of inhibition of food borne pathogen activity of gold nanoparticle

Bacteria	**Culture filtrate**	**Culture**	**Disc**	**Gold**
E.coli	4 mm	Nil	9 mm	2 mm
S.aureus	5 mm	Nil	9 mm	1 mm

ANTI CANCER activity (MTT assay)

After HeLa cell line incubation the color changed from pink to yellow was observed.The basis for numerous in vitro assays of a cell population's response to external factors depends upon the measurement of cell viability and proliferation. The reduction of tetrazolium salts is accepted as a reliable way to examine cell spread. The tetrazoliumMTT (yellow color) is reduced metabolicallyby dehydrogenase enzymes to generate reducing equivalents (NADH and NADPH). The resulting intracellular purple formation is quantified by spectrophotometer.The number of assay steps has been decreased as much as possible to accelerate sample processing. The MTT reagent yields decrease values in cell absence. For each cell type, the relationship between cell number and signal produced, thus allowing an accurate quantification of changes in the rate of cell multiplication. The viable cells were determined by the absorbance at 450nm and 540nm was measured with a UV-

Spectrophotometer without sample containing cells as blanks. The effect of the samples on the proliferation of HeLa was expressed as the % cell viability, using the following formula, the result were showed (Plate 7)and (Table 6). Culture filtrate gold nanoparticles synthesized by using *Bacillus alcalophils*shows 40% of anticancer activity. Gold nanoparticles synthesized from Fruit Extract shows 50% of anticancer activity [Lokina*etal*., 2013].

Table 6: UV reading ofanti-cancer activity of AuNPs

Gold (µl)	**UV-450 nm**	**UV-540 nm**
200µl	23.05 %	13.86 %
400µl	22.27 %	38.58 %

SDS PAGE

In the present study the synthesis of gold nanoparticles using *Bacillus alcalophillus*bioreduction may be protein of molecular weight determined as 60Kda.In some cases, tyrosine can bind to gold surface via amine groups and reduce silver ions at high pH, thereby, producing Au core–Ag shell nanostructures [Selvakannan*et al*., 2004]. Tryptophan produces metal NPs (basic pH). A metal ion forms NPs by accepting an electron from a transient tryptophylradical formed because of conversion of tryptophan residue present in peptide. Itwas also reported that the capping and stabilization of AuNPs are affected by different proteins [Sastry*et al.*, 2003]. Fungi secrete proteins and reducing agents which help in the stabilization of extracellular synthesized NPs [Mukherjee *et al.*, 2002]. Controlled synthesis of AuNPs is achieved by controlling Activities and cellular growth conditions in yeast strains.

FIGURES

Figure 1:UV –visible of AuNPs

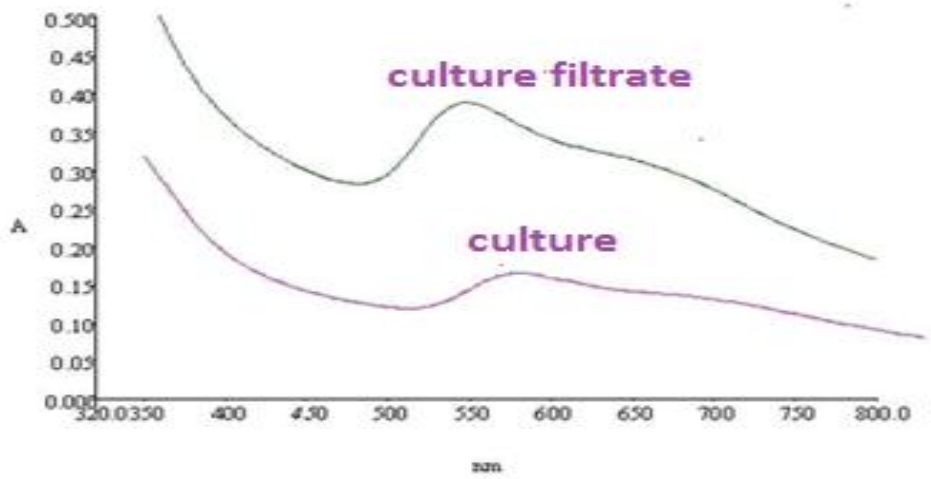
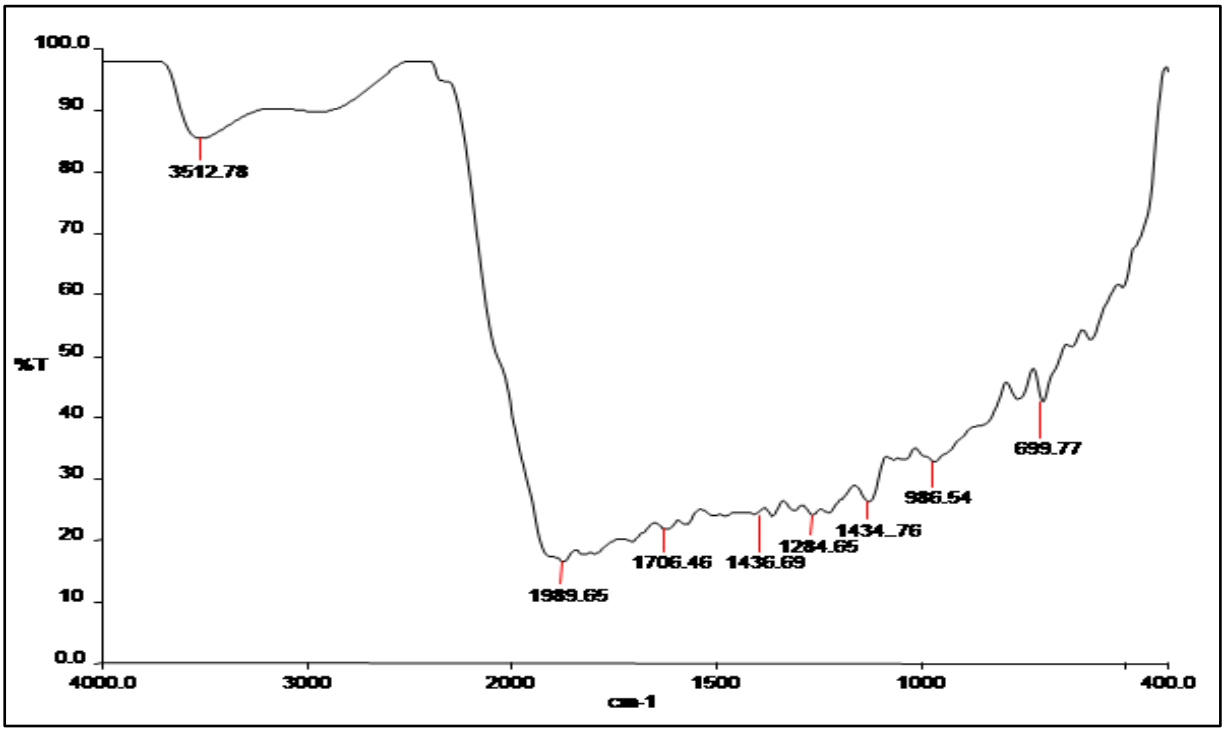


Figure 2: FTIR of AuNPs



PLATES

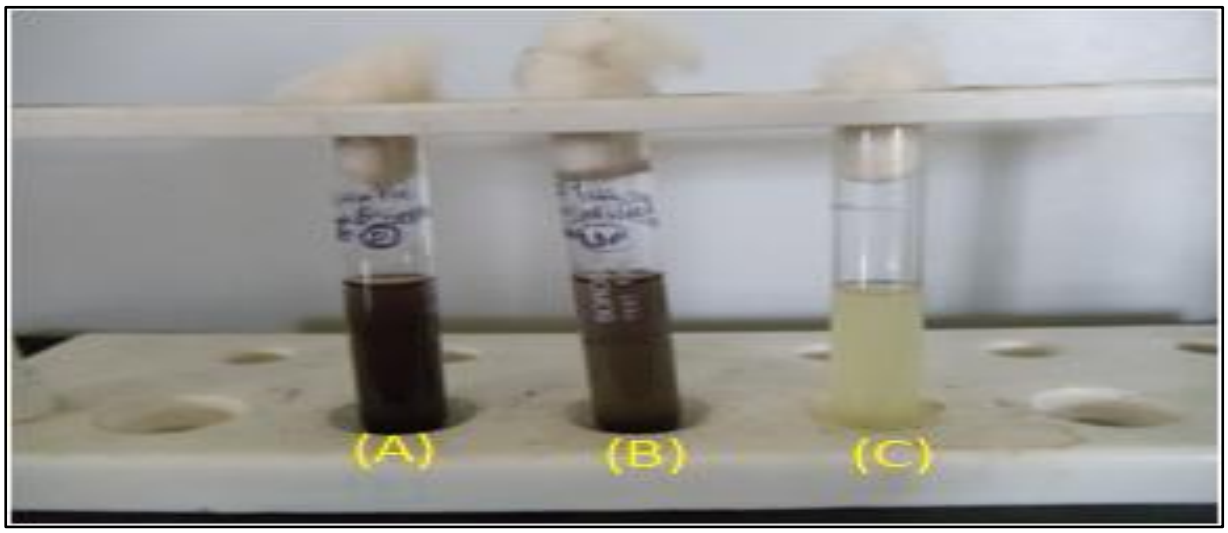


Plate 1: Synthesis of AuNPs- (A-culture,B- Culture filtrate,C- B.alcalophillus

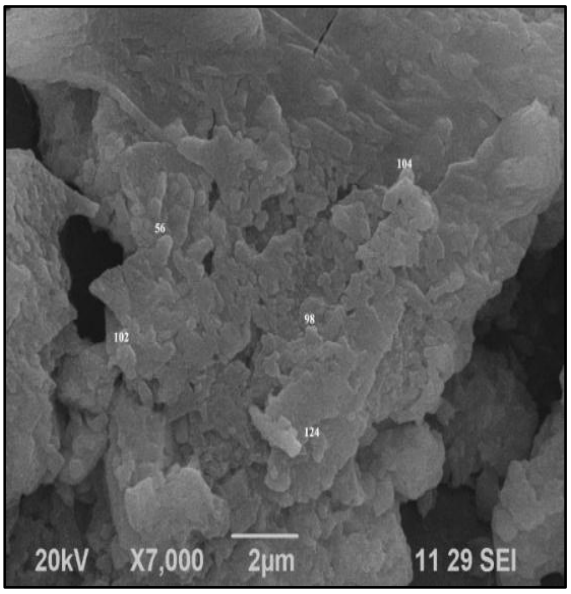


Plate 2:size of gold nanoparticles

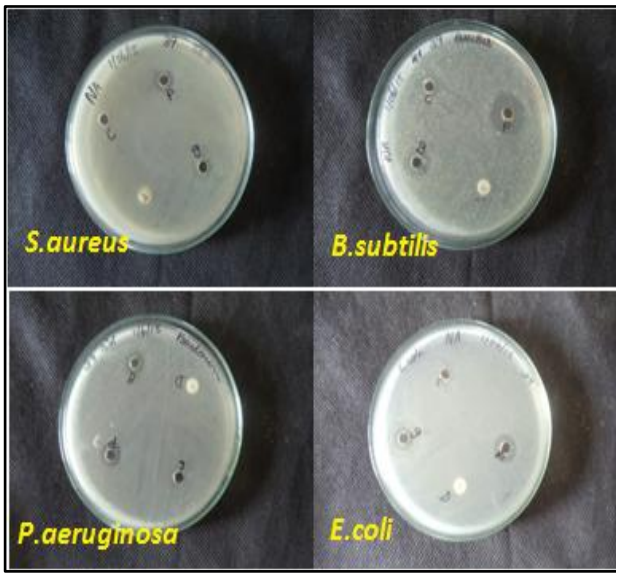


Plate 3:Antimicrobial activity of AuNPs

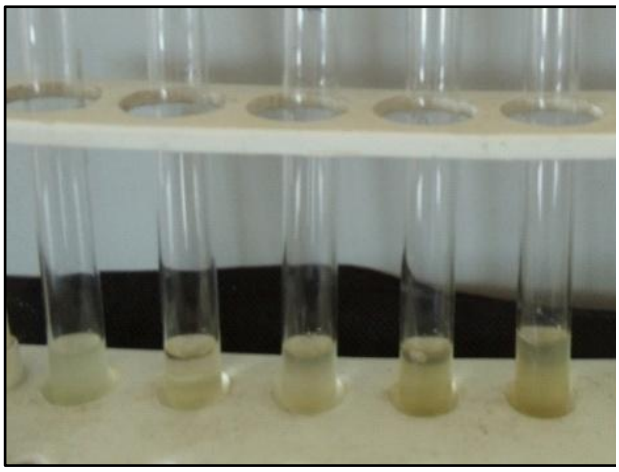


Plate 4:Antifungal activity of gold nanoparticles

Plate 5:MIC of gold nanoparticles

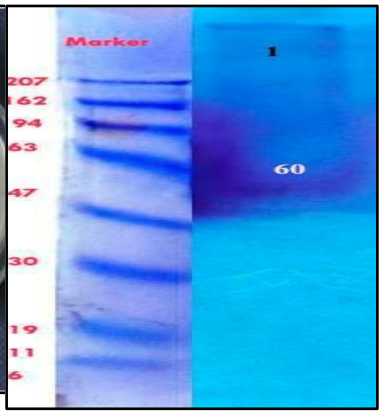


Plate 6: Food borne pathogen activity of AuNPs

Plate 7: SDS of gold nanoparticles

CONCLUSION

In several types of marine microbial strains revealed that the *Bacillus alcalophillus* has the capability to synthesize AuNPs from HAuCl4 solution. The microorganism has been characterized UV-Visible spectroscopy, FTIR, SEM. From the study microorganism was found to haveAuNPssynthesize which are less than 10nm size at varied shape. For future process development of AuNPs which will enable harnessing marine water as a rich source of gold nanoparticles. It is proven that the gold nanoparticles synthesized from *Bacillus alcalophillus* seem to be promising and effective antibacterial agent against the multidrug resistant strains of various bacteria. The gold nanoparticles are effective antifungal activity. Result concludes that isolated gold nanoparticles are further medical and clinical purposes to cure bacterial infectious diseases such as antibacterial and antifungal activity. The gold nanoparticles also have ability to against food borne pathogen and anti-cancer effect on HeLa

cell line. To study about bio molecular weight by SDS PAGE. Synthesis of gold nanopoarticles using *Bacillus alcalophillus*, bio reduction may be protein molecular weight determined as 60kDa. This study establishes the fact that biogenic gold nanoparticles synthesized in a facile manner can be well used in various fields of medicine as these are appropriately stabilized by *Bacillus alcalophillus*. The marine bacterial strain of *Bacillus alcalophillus* is useful to achieve a fastest rate of nanoparticles synthesis which may be of high in interest for future determination in development of AuNPs.

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