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ORIGINAL ARTICLE

Mycofabrication of bioactive silver nanoparticle: Photo catalysed synthesis and characterization to attest its augmented bio-efficacy

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Abstract Photocatalysed mycosynthesis of nanoparticles can be used as alternative for chemical and physical method of nanoparticle synthesis. We report synthesis of silver nanoparticles (SNPs) from two fungal species wherein the reduction in the silver ions might have occurred by nitrate-dependent reductase enzyme/proteins. Reduction in silver ions was an extracellular and made the process rapid coupled with photoreduction; it made us to the develop combination of an easy process for biosynthesis of the SNPs within 20 min. The formation of SNPs was confirmed from the surface plasmon resonance peak in the visible region of the spectrum (410–440 nm). The optimized production process shows Czapek Dox broth in alkaline pH at 60 °C of post culture temperature was found to be finest for maximum yield in both the fungal species. The particles were then characterized by UV-Visible Spectrophotometry, SEM, DLS and XRD analyses which confirm the particles are in nanoscale. The extracellular mycosynthesized SNPs were shown to have antioxidant, anti-arthritic, antiangiogenic and antimicrobial activities. The study confirms SNPs form soft corona with the human serum protein by protein corona studies. Potential of

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fungal-mediated biosynthesis of SNPs is important for development of effective imaging or therapeutic agents applicable for drug targeting.

1. Introduction

Nanotechnology is the field that is vast in making an impact on all fields of human life. Nano-biotechnology can be used as alternative for existing physicochemical method of nanoparticle synthesis and application. Nano science and nanotechnology started to flourish from the past two decades wherein nanoparticles (NPs) attract greater attention due to their various applications in different fields mainly in "Nanomedicine". Nanoparticles are clusters of atoms and their size ranges from 1 to 100 nm. NPs can be broadly grouped into two namely organic nanoparticles which include carbon NPs whereas inorganic NPs include magnetic NPs, noble metal NP (such as gold and silver) and semi-conductor NPs (such as titanium oxide and zinc oxide). Silver, aluminium, gold, zinc, carbon, titanium, palladium, iron and copper have been routinely used as precursor for synthesis of nanoparticles (Varahalarao et al., 2013; González and Noguezm, 2007; Gross et al., 2007; Smith et al., 2006). At nanoscale level, materials have different physical, chemical, optical, magnetic and electrical properties due to their large surface area to volume ratio. Nanoparticles possess more surface atoms than micro particles, which enhance their functional capabilities (Sonal et al., 2013; Rai and Ingle, 2012). NPs are common in nature, for example, life depends on many nanoscaled objects including proteins, enzymes and DNA and nano-sized particles occur naturally in the atmosphere. Nowadays, NPs influence various sectors in the areas of agriculture, environmental pollution cleanup, efficient and safe drug delivery mechanisms with less side effects. In spite of physical and chemical methods, the importance of biological synthesis has been realized globally because of various methods, among which the chemical methods are often energy consuming and intensive, employ toxic chemicals and need a stabilization path. Thus, the need for clean, eco-friendly, cost-effective, and biocompatible synthesis of metal NPs encouraged the researchers to exploit the biological sources such as nanofactories (Garcia-Parajo, 2012; Wei et al., 2012; Seshadri et al., 2011). Recent studies revealed that the metal NP synthesis using plants, microorganisms and algae as source has been unexplored and underexploited. The development of mycosynthesis of NP is evolving into an important branch of myconanotechnology. It has evolved in the presence of natural nanomaterials. However, the probability of microorganism exposure to nanomaterials has increased to a greater extent with the ongoing increasing production and use of engineered nano materials in a variety of instruments and goods. Microbes mediated synthesis of metal NPs is gaining more importance owing to its simplicity, rapid rate of synthesis of NP of attractive and diverse morphologies and elimination of elaborate maintenance of cell cultures and ecofriendliness (Varahalarao et al., 2013). It was found that fungi source more advantages over other biological systems because of their high tolerance towards the heavy metals (Parameswari et al., 2010). Rai and coworkers proposed the term "Myconanotechnology" for the synthesis of NPs by fungi. Fungi have the potential to provide relatively quick and ecologically "clean" metallic nanoparticles. Application of fungi for production of SNPs is potentially exciting because of their ability to secrete large amount of proteins involved in detoxification of materials that are toxic to its metabolic state (Rai et al., 2009; Soni and Prakash, 2012). It has been known for long time that silver ions are highly toxic to a wide range of bacteria and silver based compounds have been used extensively as antibacterial agents (Cho et al., 2005). Silver has an advantage of having broad antimicrobial activity against Gram-negative and Gram-positive bacteria and due to this activity SNPs are extensively used in dental materials, coating stainless steel in medical devices, cosmetics and water treatment (Chladek et al., 2011; Knetsch and Koole, 2011; Kokura et al., 2010; Sheng and Liu, 2011). Microorganisms have shown ability to reduce metal ions to form metallic nanoparticles. The basic principle behind the synthesis is the reduction of silver (Ag^+) of $AgNO_3$ into Ag^0. As the reduction takes place, the biomolecules aggregate with silver metal ions to form nano-sized corona. Silver particles are found to have the property of treating Rheumatoid arthritis (Arumugam, 2013), so we checked the nanoparticles for the anti-arthritic activity and the results are positive. Due to vast emerging applications of SNPs in distinct fields, there is increase in demand for SNPs and to fulfil the demand, there is a pressing need to increase the yield for which optimization of the process is very important step. The synthesis of SNPs at nanoscale is still a challenge. In order to increase the shelf-life of SNPs with minimum investment, it is necessary to optimize the culture conditions and various physical parameters such as pH, temperature, and light intensity. We report the optimization of different media, pH, and temperature for mycosynthesis of SNPs by *Penicillium chrysogenum* and *Rhizopus oryzae*. The synthesised nanoparticles were characterized with UV-Visible spectrometer, X-ray Diffraction (XRD), and scanning electron microscope (SEM). SNPs are also checked for anti-angiogenic activity by open window method. The SNPs are found to have the property of low interaction with the blood protein and it helps in drug targeting which was confirmed by protein corona.

2. Materials and methods

2.1. Fungal cultures

The test fungi viz., *Penicillium chrysogenum* and *Rhizopus oryzae* were procured from Dept. of studies in Microbiology, University of Mysore, and JSS Medical College, Mysuru. They were maintained in Czapek Dox Agar at 28 °C for 5–7 days. Individual fungal colonies were picked and subcultured on Czapek Dox agar and pure cultures were maintained on agar slants.

2.2. Extracellular synthesis of silver nanoparticles

Each mycelium of *Penicillium chrysogenum* and *Rhizopus oryzae* was inoculated, and each contains 150 ml of Czapek Dox broth in 250 ml conical flasks and incubated at 25 ± 2 °C for 6–7 days. Later mycelia were harvested by filtration through Whatman filter paper no. 42 and washed with autoclaved distilled water and the fungal biomass is resuspended in 150 ml of autoclaved distilled water and incubated at 25 ± 2 °C for 1 day. The extracellular protein secretion is stimulated by giving heat shock at 50–60 °C before mycelia were harvested and then filtered. The filtrate was treated with 100 mM silver nitrate solution, mixed well and incubated by exposing each conical flask to intense sun light for 20 min, and reddish brown colour appeared indicating the reduction of Ag^+ of $AgNO_3$ to Ag^0 was successful. A control was maintained without the addition of fungal filtrate. After the synthesis the SNPs were flocculated by centrifugation at 10 × *g* (Arumugam, 2013).

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2.3. Characterization of silver nanoparticles

Characterization of nanoparticles is an important task to understand and control over nanoparticle synthesis and applications. Appearance of dark brown colour of fungal cell filtrate indicates the formation of SNPs. The bio reduction of Ag^+ ions to Ag^0 was monitored by periodic sampling by dual beam UV-Visible spectrophotometer by scanning the absorbance spectra in 200–800 nm range of wavelength. It is well known that, for monodispersed NPs, only one plasma band is obtained and the increase in its intensity is an indication of the advanced degree of reaction with increase in the number of particles (Nayak et al., 2011). Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and X-ray Diffractometry (XRD) were also employed for particle size determination.

2.4. Process optimization for silver nanoparticle synthesis

For the large-scale and stable mycosynthesis of SNPs, it is necessary to optimize the physical and cultural conditions. Several experiments were carried out concerning the rate of synthesis and stability of SNPs. The parameters such as optimization of media, pH, light intensity and temperature were performed wherein for each condition, there was a respective control.

2.4.1. Optimization of culture media

Effect of six different media, namely malt glucose yeast peptone broth (malt extract-0.15 g, yeast extract-0.15 g, peptone-0.25, glucose-0.5 g, pH-7), potato dextrose broth (potato-10 g, dextrose-1 g, pH-6.4), protease production media (glucose-0.1 g, casein-0.025 g, peptone-0.025 g, yeast extract-0.025 g, KH_2PO_4-0.5 g, $MgSO_4$-0.25 g, $FeSO_4$-0.005 g, pH-8.4), lipase assay medium (peptone-1.5 g, NaH_2PO_4-0.6 g, KH_2PO_4-0.1 g, $CaCl_2$-0.0125 g, olive oil-0.5 g, pH-6.5), sucrose peptone yeast broth (sucrose-2.5 g, peptone-0.05 g, yeast-0.05 g, pH-6.5), and Sabouraud broth (dextrose-2 g, peptone-0.5 g) was studied. All the media were screened for the optimum and stable nanoparticle synthesis. The fungal mycelia were grown for 7 days in 100 ml conical flask, each containing 50 ml of test medium. Each mycelium of, *Penicillium chrysogenum* and *Rhizopus oryzae* was inoculated in 100 ml conical flasks, and each contains 50 ml of test media and was incubated at 25 ± 2 °C for 6–7 days. Later mycelia were harvested and mycosynthesis protocol was followed as mentioned previously.

2.4.2. Optimization of post induction pH

For large-scale synthesis and stability of SNPs were studied by suspending the fungal biomass in distilled water having different pH, viz., pH-3, pH-5, pH-7, pH-9, pH-11 and pH of the filtrates were maintained by buffering system.

2.4.3. Optimization of post induction temperature

Effect of temperature on the rate of synthesis of SNPs was studied by transferring fungal biomass into distilled water and incubated at its optimum temperature over night and later they were exposed to different temperatures ranging between 0 °C, 20 °C, 40 °C, 60 °C, 80 °C, and 100 °C for overnight to increase the extracellular protein secretion by giving cold/heat shock which favours the protein secretion and synthesis of SNPs.

2.5. Antioxidant activity of silver nanoparticles

In vitro antioxidant activity has been investigated by different assays namely DPPH radical scavenging assay, ferrous ion chelating assay, hydroxyl radical scavenging assay and nitric oxide radical scavenging assays for synthesized silver nanoparticles.

2.5.1. DPPH (1,1-diphenyl-2-picryl hydroxyl) radical scavenging assay

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. The DPPH radical scavenging activity of SNPs synthesized by fungi was studied. 1 ml of DPPH (0.1 mM) solution was mixed with different concentrations (0, 20, 40, 60, 80 and 100 μM/ml) of synthesized AgNPs shaken well and incubated at room temperature for 20 min. Then the absorbance was taken at 517 nm. The control was prepared as above without adding sample (Fatima et al., 2013). The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect } (\%) = \frac{(\text{Control absorbance} - \text{Sample absorbance}) \times 100}{\text{Control absorbance}}$$

2.5.2. Hydroxyl radical scavenging assay

Hydroxyl radical inhibition of nanoparticle was quantified by the method of Halliwell and Gutteridge (1999), with some modifications, and the hydroxyl radicals were generated using sodium phosphate buffer replaced H_2O_2. The reaction mixture contains ferric chloride (100 μM), ascorbate (100 μM), EDTA (104 μM), hydrogen peroxide (1 mM), and 2-deoxy D-ribose (2.8 mM), mixed with different concentrations of NPs (100–800 μM/ml); blank contains 1 ml of buffer; and 100% oxidation tube contains phosphate buffer, 2-deoxy-D-ribose and all test tubes are incubated for 60 min at 37 °C and then 1 ml of 1% TBA is added again to incubate the reaction mixtures in boiling water bath for 20 min followed by addition of 1 ml acetone. The presence of the hydroxyl radical was detected by monitoring absorbance at 535 nm and percentage of scavenging was calculated using the following formula.

$$\text{Hydroxyl radical scavenging activity } (\%) = \frac{(\text{Control aborbance} - \text{Sample absorbance}) \times 100}{\text{Control absorbance}}$$

2.5.3. Nitric oxide scavenging activity

The scavenging activity of the nanoparticles against nitric oxide was detected by its ability to inhibit the formation of nitrite through direct competition with oxygen and oxides of nitrogen in the reacting mixture (Rozina et al., 2012). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. 1 ml of sodium

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nitroprusside (5 mM) in phosphate buffer saline (0.1 M, pH-7.4) was mixed with different concentrations of SNPs (100–800 μM/ml) and incubated at room temperature for 2 h. For the control same reaction mixture without the sample (SNPs) was maintained. Later 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% N-1-naphthyl ethylenediamine dihydrochloride) was added. The absorbance of the chromophore (pink colour) formed was read at 546 nm and the percentage of scavenging activity was calculated using the following formula:

$$\text{Nitric oxide scavenging activity } (\%) = \frac{\text{Control } A_{546} - \text{Sample } A_{546}}{\text{Control } A_{546}} \times 100$$

2.6. In vitro anti-arthritic activity

SNPs are found to have the property of treating Rheumatoid arthritis (RA). The *in vitro* prevention of protein denaturation was studied according to the method described earlier by Arumugam (2013).

2.7. Antimicrobial activity

The compounds were tested against Gram-positive bacteria viz., *Staphylococcus aureus*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Bacillus subtilus*. Gram-negative bacteria viz., *Escherichia coli*, *Salmonella typhi*, *Pseudomonas fleurescence*, and *Klebsiella pneumonia* were maintained, fresh cultures were spread on Muller Hilten agar plates and antimicrobial efficiency of SNPsat varied concentrations (10 μM, 50 μM, 100 μM and 500 μM) was tested by disc diffusion method wherein gentamycin was taken as positive control.

2.8. Protein corona

In order to study the interaction between the synthesized nanoparticles and the serum proteins protein corona studies were carried out according to the method described previously (39); briefly, the nanoparticles (40 μM and 60 μM concentration) were treated with human serum (500 μl) and add incubated for 2 h at 37 °C and contents were flocculated by spinning at 8500 rpm for 15 min; the pellet was washed in phosphate buffer saline, incubated for 1 h at 37 °C, and then spun at 8500 rpm for 15 min, and the supernatant was separate and stored in a vial; the pellets were again resuspended in 3 M urea and incubated for 1 h at 37 °C and again spun at 8500 rpm for 15 min and supernatant was again stored in a vial; all the aliquots were resolved in SDS-PAGE which was carried out by the method described by Lammeli (1970).

2.9. Anti-angiogenic activity

The *in-vivo* chorioallantoic membrane (CAM) assay for anti-angiogenesis was performed to study anti-angiogenic effect of silver nanoparticles. Fertilized chicken eggs after 11 days of incubation were challenged with silver nanoparticles on the blood capillaries followed by incubation and observed for retarded blood vessel growth.

Figure 1 Cultures of *Rhizophus oryzae* and *Penicillium chrysogenum* in autoclaved Millipore water.

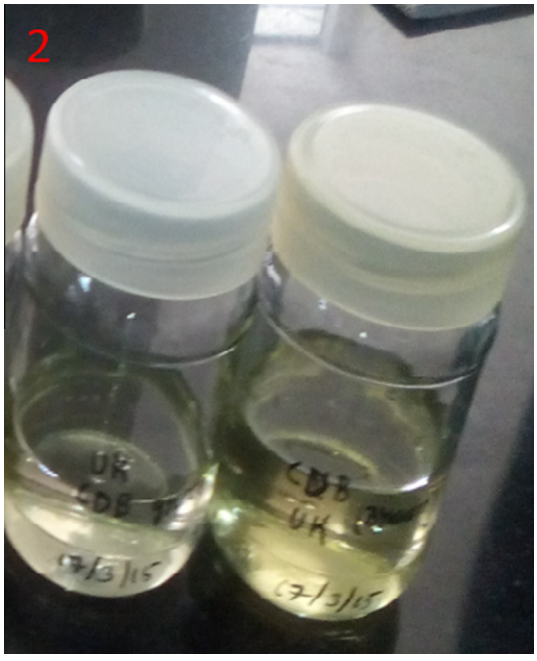

Figure 2 Filtrate of *Rhizophus oryzae* and *Penicillium chrysogenum* obtained after post induction (heat shock).

3. Results

Fungal cultures after obtaining its maximum growth were separated and introduced to sterile Millipore water and were subjected to different temperatures and different pH in order to facilitate the protein/enzyme secretion (Fig. 1).

Fungal filtrate was obtained after exposing them to different post induction temperatures and post induction pH

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Figure 3 Each flask containing individual fungal filtrate and $AgNO_3$, (3A) fungal filtrate with 100 Mm $AgNO_3$ 10 min after exposed to sunlight (3B) fungal filtrate with 100 Mm $AgNO_3$ 20 min after exposed to sunlight.

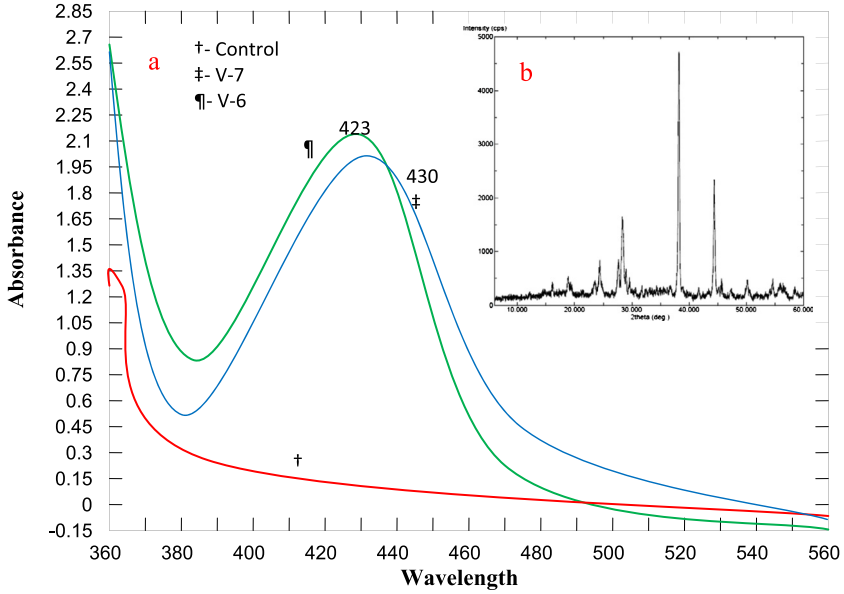

Figure 4 (a) UV-Visible extinction spectra of mycosynthesized spherical SNPs, and (b) X-ray Diffraction studies of SNPs.

favouring the protein/enzyme secretion and is ready to use to catalyse the synthesis of SNPs (Fig. 2).

In order to increase the collision efficiency of reactants the reaction mixture was exposed to sunlight wherein maximum product formation could be seen after 20 min of exposure to natural light (Fig. 3A and B).

3.1. Characterization of silver nanoparticles by UV-visible spectrophotometer

Synthesised SNPs were named as, V-6 and V-7 and they are synthesised from *Rhizopus oryzae* and *Penicillium chrysogenum* respectively. SNPs exhibit strong absorption in the visible range due to the local surface plasmon resonance. UV-vis spectra of the samples were recorded. There was a broad peak with absorption maximum between 420 and 430 nm (Fig. 4a) indicating synthesis of spherical nanoparticles which is specific for the SNPs and X-ray Diffraction studies also showed that the particles are silver nanoparticles (Fig. 4b). It is well known that there is a very close relationship between the UV-vis absorbance spectrum and size and shape of SNPs. With the increase in the particle size, the optical absorption spectra of metal nanoparticles that are dominated by surface Plasmon resonances (SPR) shift towards longer wavelengths (red shift) (Shafeev et al., 2004). Small blue shift or red shift in the wavelength of the absorbance peak could be related to obtaining SNPs in different shapes and sizes.

3.2. SEM analysis

SEM analysis shows that particles are in nanoscale wherein SNPs synthesized from *Rhizopus oryzae* (V-6) were found to be 60 nm (Fig. 5A) and *Penicillium chrysogenum* (V-7) was found to be 70 nm (Fig. 5B) in size based on the DLS analysis (Fig. 5C and D) coupled with SEM analysis.

3.3. Effect of different media

The fungus was cultured in different media in order to evaluate the effect of different media for enzyme secretion and their

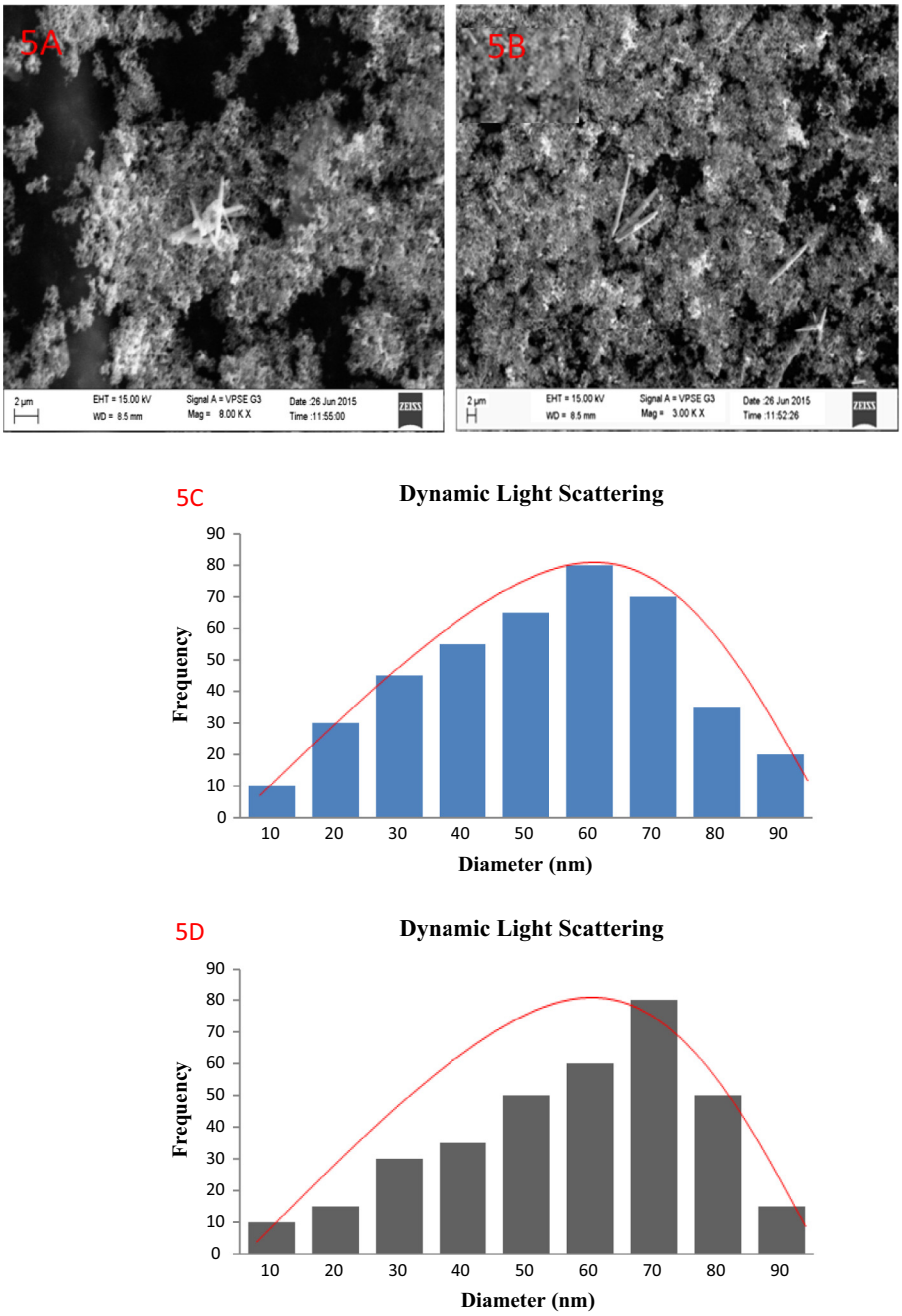

Figure 5 SEM and DLS analysis of SNPs synthesized from *Rhizopus oryzae* (5A and 5C) and *Penicillium chrysogenum* (5B and 5C) under optimized conditions respectively.

effect on synthesis of SNPs. The cell filtrate was used for protein estimation and synthesis of SNPs (Fig. 6). The highest protein concentration was recorded in Czapek Dox broth followed by malt glucose yeast peptone broth (MGYPB) in *Rhizopus oryzae* culture filtrate (Fig. 6) and in *Penicillium chrysogenum* culture filtrate CDB followed by SB (Fig. 7).

3.4. Effect of different pH

The alkaline pH 9 and pH 11 showed the maximum synthesis of nanoparticles while in acidic pH 3 and pH 5 aggregates were observed. At pH 7, there was less synthesis of SNPs as compared to alkaline in both *Penicillium chrysogenum* (Figs. 8 and 9) and *Rhizopus oryzae* (Figs. 10 and 11).

3.5. Effect of different post induction temperatures

In order to study the effect of temperature on extracellular protein secretion by both the fungi, biomass was exposed to different temperatures and the total protein concentration in the culture filtrate is estimated separately for both the cultures. The result indicates that protein secretion was maximum when

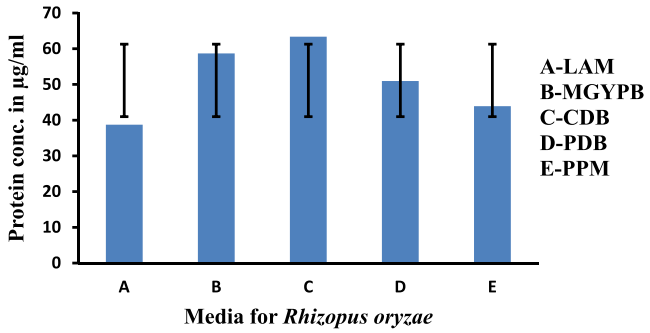

Figure 6 Histogram of protein concentration in different media in *Rhizopus oryzae* culture. (A) Lipase assay media, (B) malt glucose yeast peptone broth, (C) Czapek Dox broth, (D) potato dextrose broth, (E) protease production media. Highest protein concentration is found in CDB (C) and MGYPB (B) in *Rhizopus oryzae* culture filtrate. Experiment was carried out in triplicates and values obtained represent mean values (±SD).

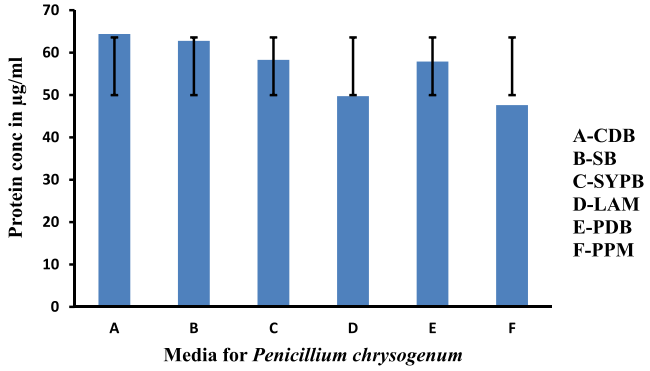

Figure 7 Histogram of protein concentration in different media in *Penicillium chrysogenum* culture. (A) Czapek Dox broth, (B) Sabouraud broth, (C) sucrose yeast peptone broth, (D) lipase assay media, (E) potato dextrose broth, (F) protease production media. Highest protein concentration is found in CDB (A) and SB (B) in *Penicillium chrysogenum* culture filtrate. Experiment was carried out in triplicates and values obtained represent mean values (±SD).

the cultures were exposed to heat treatment at 60 °C favouring the extracellular release of enzymes/proteins in the culture filtrate of *Rhizopus oryzae* (Figs. 12–14). In *Penicillium chrysogenum* culture filtrate maximum protein secretion was found at 60–80 °C (Fig. 15), which may be due to the heat shock. The temperature is one of the important factors in any chemical and biological reaction as it affects the rate of reaction (Fig. 16). The increase in the absorbance indicates the increase in the number of nanoparticles or increase in size of individual SNPs. For 60 °C and 80 °C, the surface plasmon resonance band showed high intensity with dark brown colour of the filtrate (Fig. 17). Experiment was carried out in triplicates and values (Figs. 12 and 15) obtained represent mean values (±SD).

3.6. Antioxidant activity

The DPPH radical scavenging activity of SNPs synthesized by *Rhizopus oryzae* and *Penicillium chrysogenum* was studied. The decolourization from purple to yellow is due to the scavenging of DPPH in a dose dependent manner (Fig. 18), experiment was carried out in triplicates and the values represent mean values (±SE). IC_{50} values of SNP's were found to be less than butylated hydroxyl toluene (BHT) indicating better antioxidant activity (Table 1).

The significant decrease in the concentration of nitric oxide radical was similar to the standard BHT (Fig. 19). Experiment was carried out in triplicates and the values represent mean values (±SE). It indicates that the SNPs showed equivalent scavenging activity to those of positive control based on IC_{50} values (Table 2).

The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage in adjacent biomolecules. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (Halliwell and Gutteridge, 1999). In the present study, the hydroxyl radical-scavenging effect of SNPs shows activity in a dose dependent manner (Fig. 20). Experiment was carried out in triplicates and the values represent mean values (±SE). Hence, the newly synthesized SNPs can be considered as a good scavenger of hydroxyl radicals with similar effect to those of control (Table 3).

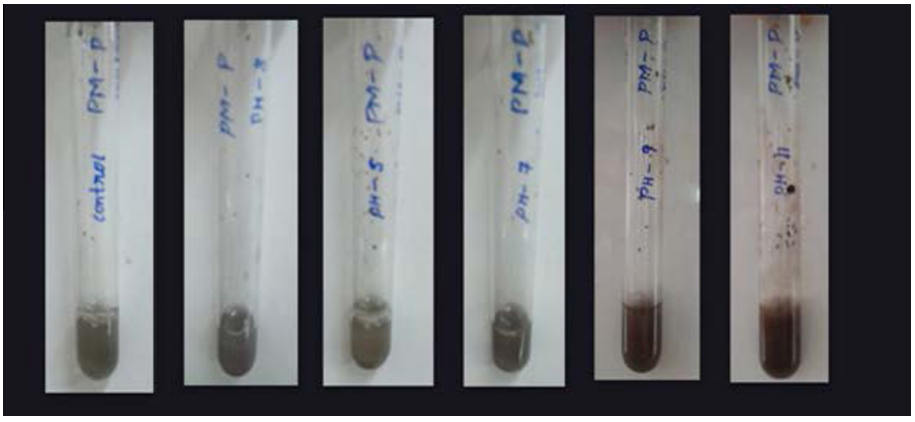

Figure 8 Effect of pH on synthesis of SNPs by *Penicillium chrysogenum* culture filtrate obtained from optimized media (CDB).

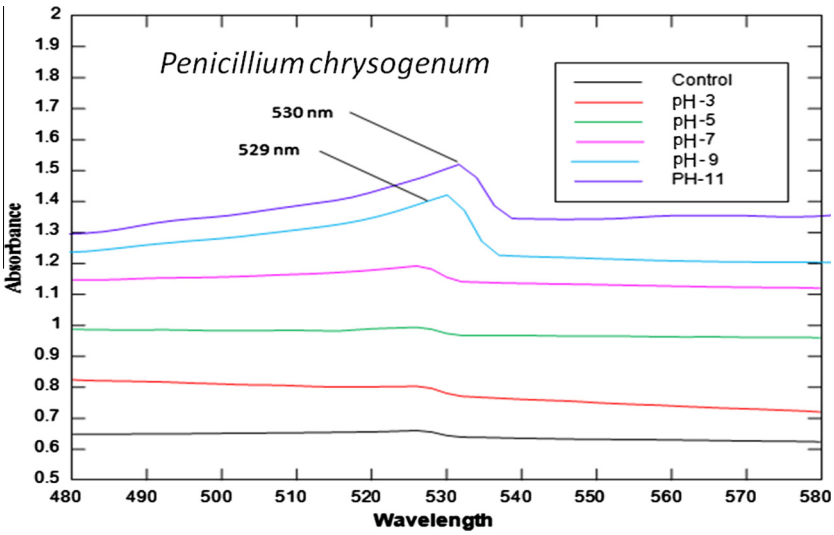

Figure 9 UV-Visible extinction spectroscopy of SNPs with the effect of pH on synthesis of SNPs by *Penicillium chrysogenum*. Results obtained show alkaline pH (9 and 11) favouring the synthesis of SNPs.

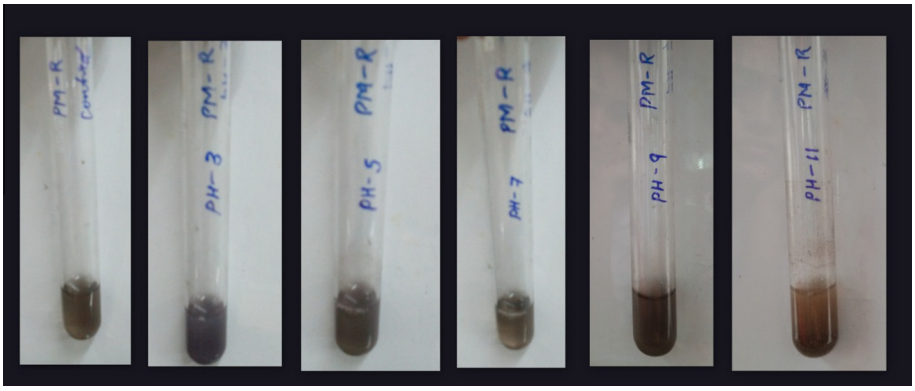

Figure 10 Effect of pH on synthesis of SNPs by *Rhizopus oryzae* culture filtrate obtained from optimized media (CDB).

3.7. Anti-arthritic activity

Anti-arthritic effect of SNPs synthesised from fungus were studied significantly by using *in vitro* inhibition of protein denaturation model wherein V-6 could inhibit denaturation of protein in a concentration dependent manner compared to positive control Voveron wherein the required concentration is more for V-6 (Fig. 21). V-7 could inhibit denaturation of protein in a concentration dependent manner compared to positive control Voveron wherein V-7 shows higher percentage of protection in 0.4 and 0.6 mg/ml over the V-6 (Fig. 22). From results obtained it shows that V-6 and V-7 at different concentrations provided significant protection against denaturation of proteins when compared with standard diclofenac sodium. Obtained data reveal that SNPs synthesised by *Rhizophus oryzae* (Fig. 21) and *Penicillium chrysogenum* (Fig. 22) could be used for designing anti-arthritic drugs.

3.7.1. Anti-microbial activity

The antimicrobial activity of biosynthesised SNPs was carried out on pathogens such as Gram-negative bacteria and Gram-positive bacteria (Table 4), wherein Gentamycin was used as positive control. It is reported that SNPs attach to the surface of the cell membrane, disturb its function, penetrate directly with the bacterial outer membrane and releases Ag^{+} ions.

3.8. Protein corona

Nanoparticles are targeted to specific tissues by attaching ligands on its surface for receptor-mediated mechanism. However, the nanoparticles are immediately covered by plasma proteins and molecules when administered in vivo known as "protein corona" and obstructs the ligand-receptor recognition. For targeted drug delivery, protein corona effect is a formidable challenge for targeting nanoparticles to a specific cell type and influences phagocytosis and removal of nanoparticles from bloodstream (Monopoli et al., 2011). SDS-PAGE analysis of the human serum proteins interacted with the surface of the nanoparticles demonstrated that the majority of the serum proteins after incubation for 1 h were subjected to PBS wash and subjected to reducing SDS PAGE which indicates that the proteins were bound by reversible electrostatic interactions

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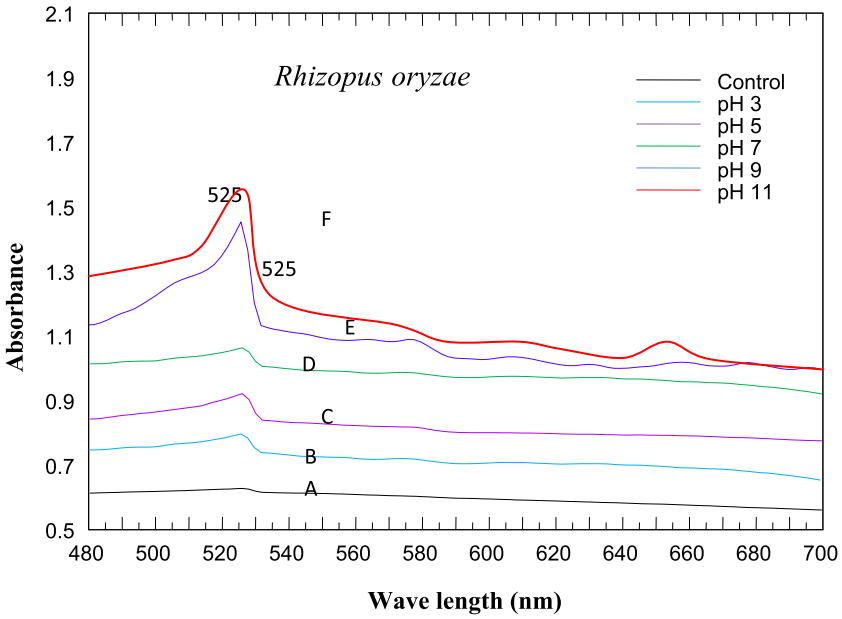

Figure 11 UV-Visible extinction spectroscopy of SNPs with the effect of pH on synthesis of SNPs by *Rhizopus oryzae*. Results obtained show alkaline pH (9 and 11) favouring the synthesis of SNPs.

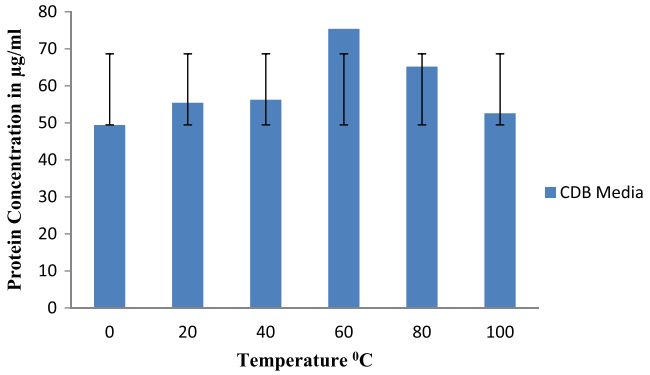

Figure 12 Effect of temperature on Protein concentration in CDB media in *Rhizopus oryzae* culture.

forming soft corona (Fig. 23). A subsequent wash with 3 M urea was used to remove the permanently bound proteins which were found to comprise of 28% and 34% of the total adsorbed proteins for nanoparticle V-6 and V-7 respectively based on band intensity.

3.9. Anti-angiogenic activity

Nanotechnology in cancer therapy includes a nano-sized material, generally ranging in dimensions from 1 nm to a few hundred nanometres in at least one dimension (Satchi-Fainaro et al., 2005). These nanoparticles are designed to carry therapeutic drugs and imaging agents, which are loaded on or within the nanocarriers by chemical conjugation or simply by encapsulation. Nanoparticle based chemotherapeutic agents are designed such that they can passively or actively target cancer cells. Shell less CAM assay was performed to know the possible action of SNPs on angiogenesis wherein both V-6 and V-7 at the concentration of 0.1 µg were used to saturate the disc and therein it could inhibit the growth of blood vessel in the region where drug was loaded (indicated by arrows) compared to unloaded spots conveying that it prevents angiogenesis (Fig. 24).

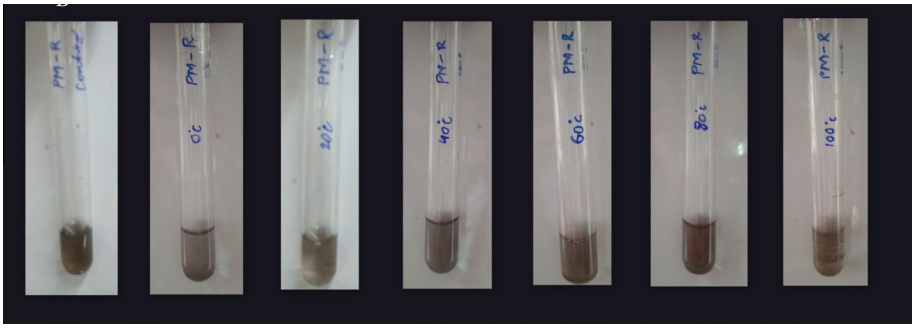

Figure 13 Effect of temperature during post induction on synthesis of SNPs by *Rhizopus oryzae* culture filtrate.

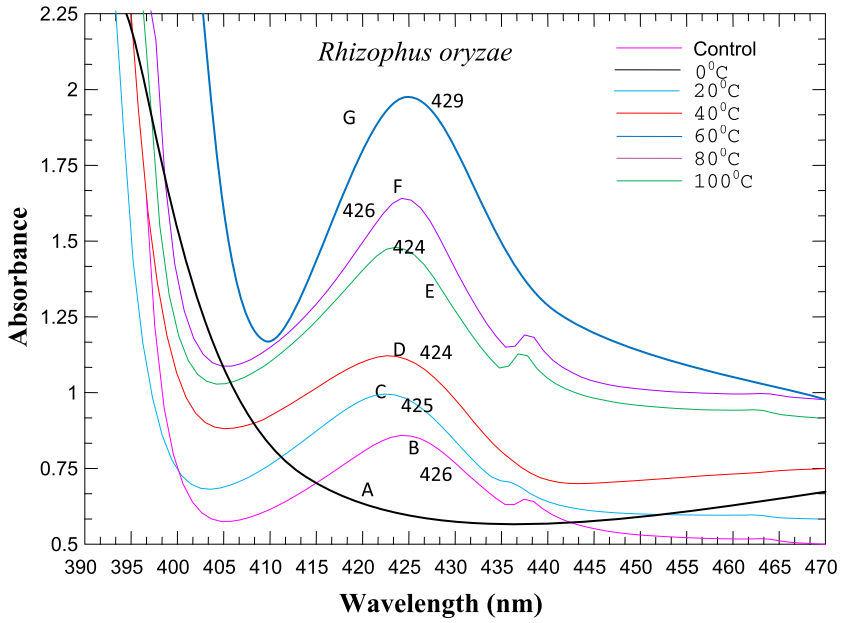

Figure 14 Effect of temperature on size of SNPs synthesis in CDB media by *Rhizopus oryzae*. In UV-Visible wavelength scan the spectrum F: 80 °C and spectrum G: 60 °C have the blue shift which indicate the small size of synthesised SNPs. Spectrum E: 100 °C asymmetric spectra indicate the aggregation of particles at high temperature.

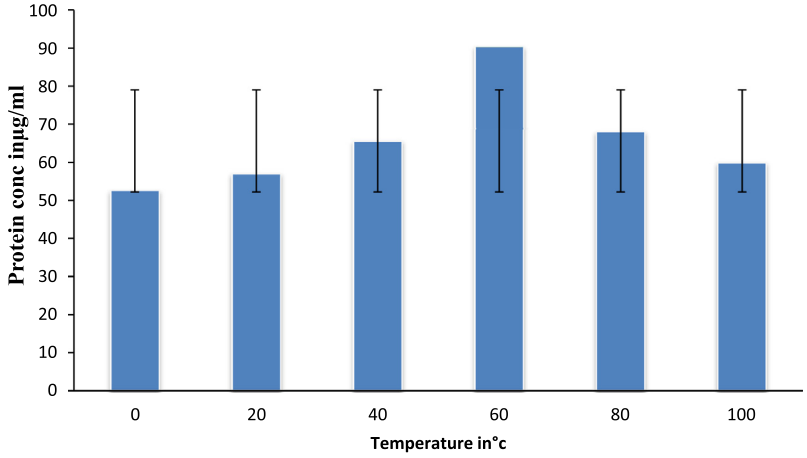

Figure 15 Effect of temperature on protein concentration in CDB media in *Penicillium chrysogenum* culture. The result indicates protein secretion was maximum when the cultures were exposed to heat treatment at 60 °C favouring the extracellular release of enzymes/proteins in the culture filtrate. Experiment was carried out in triplicates and values obtained represent mean values (±SD).

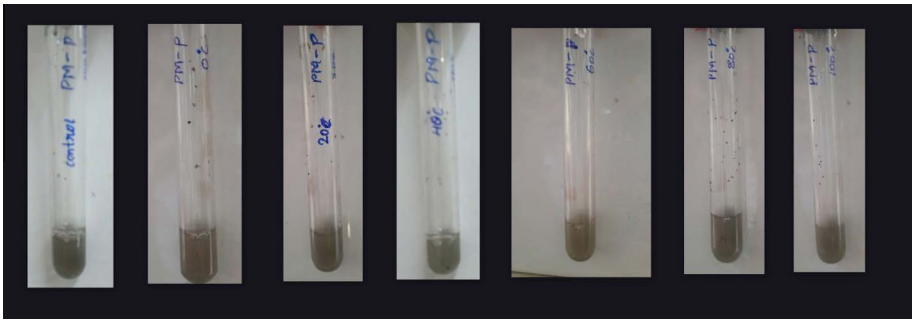

Figure 16 Effect of temperature during post induction on synthesis of SNPs by *Penicillium chrysogenum* culture filtrate.

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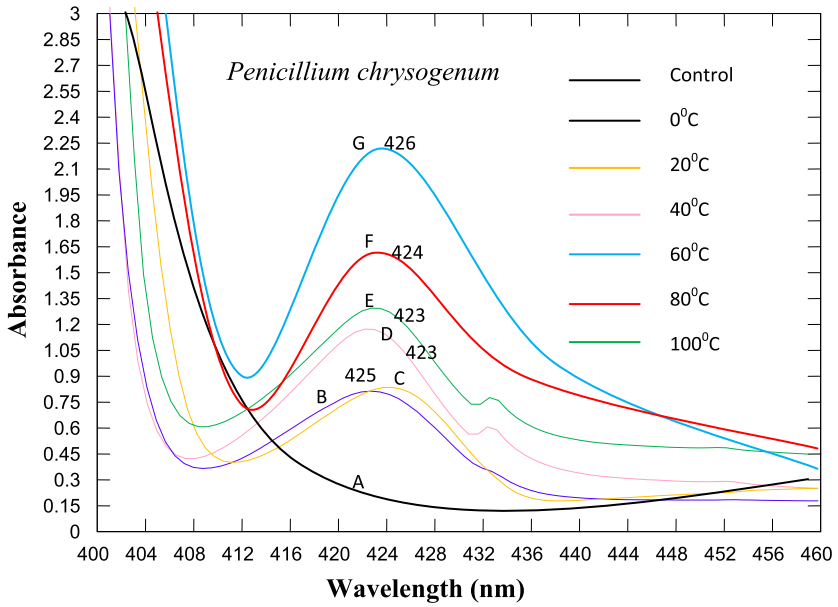

Figure 17 Effect of temperature on size of SNPs synthesis in CDB media by *Penicillium chrysogenum*. Spectrum F: 80 °C and spectrum G: 60 °C have the blue shift which indicates the small size of synthesized SNPs. Spectrum E: 100 °C asymmetric spectra indicate the aggregation of particles at high temperature.

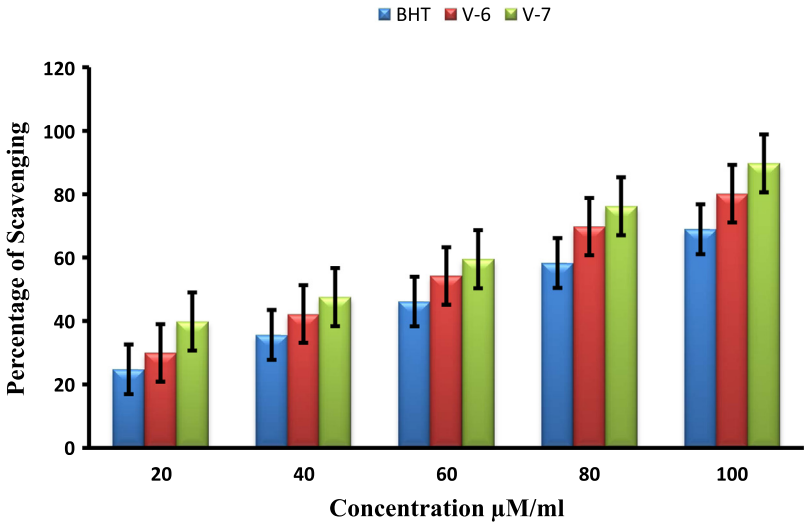

Figure 18 DPPH radical scavenging assay of mycosynthesized SNPs.

Table 1 IC_{50} value of DPPH scavenging activity.

Samples	IC_{50} value (µM/ml)
BHT	64.97
V-6	47.35
V-7	42.06

4. Discussion

Due to easy accessibility, the photocatalysed biological synthesis seems to be convenient than physical and chemical methods to synthesize the SNPs. In fact, in order to increase the yield of SNPs, we studied the effect of different physicocultural parameters, such as effect of different media, temperature, pH, and intensity of light. It is well known that in different culture media conditions and compositions microbial cell responds differently and secretes different metabolites and different kinds of proteins. Also, it is known that the biological synthesis of SNPs is enzyme catalysed reaction (Xie et al., 2007). In case of maximum production of SNPs, fungi should secrete specific enzymes or metabolites (Figs. 1 and 2) which are responsible for reduction in silver ions. In the present study, CDB media (Figs. 6 and 7) have promoted the extracellular nitrate reductase secretion and hence enhance the synthesis of SNPs (Fig. 3). Proteins have multiple effects on the dispersion, including potential screening of the surface charges that

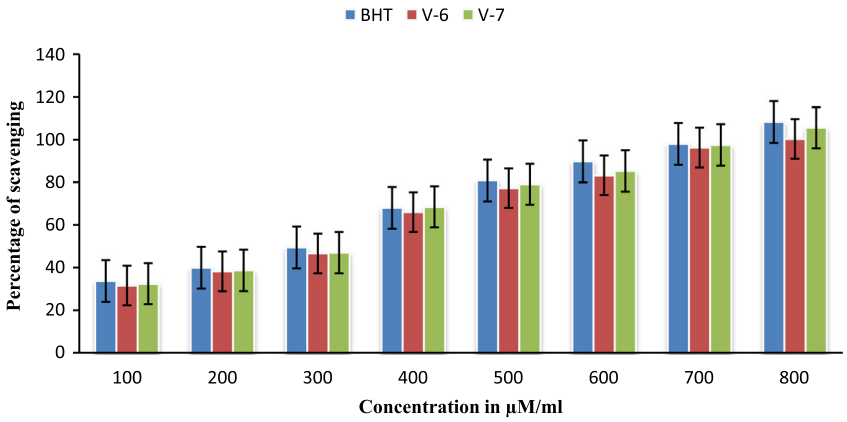

Figure 19 Nitric oxide scavenging assay of mycosynthesized SNPs.

Table 2 IC_{50} value of nitric oxide scavenging activity of SNPs.

Samples	IC_{50} value (µM/ml)
BHT	303.70
V-6	321.75
V-7	319.01

help to maintain the repulsion between the particles, or bridging type interactions (Montes-Burgos et al., 2007).

OH– ions are nucleophiles which play crucial role in maintaining the stability of SNPs by adsorbing on it and in synthesis of smaller size SNPs by providing electrons for reduction in silver ions. More nucleation regions are formed due to the availability of OH– ions which helps in preventing the aggregates that are formed through adsorbing on nanocrystals and maintains the smaller size of SNPs (Gurunathan et al., 2009). Our results showed similarity with Chen and Carroll findings that at alkaline pH SNPs are stable and aggregates formed at lower pH (Chen and Carroll, 2004). It indicates that, by controlling the pH of SNPs synthesis, it is easy to control the size of SNPs. Nayak et al. (2011) hypothesized that the proton concentration affects conformational changes in the nitrate reducing enzymes present in the fungal filtrate, which may change the morphology and size of the SNPs. Deepak et al. (2011) stated that when the condition of the SNPs Mycofabrication is alkaline, the synthesis will be faster than in acidic conditions. In other words, synthesis enhances as the pH increases towards alkaline region (Figs. 8 and 10). In alkaline conditions there was no need of agitation of the mixture for the formation of SNPs and all the silver ions supplied were converted to SNPs within 10 min (Figs. 9 and 11). The proteins involved in the synthesis may bind with silver at thiol regions (–SH) forming an S–Ag bond, a clear indication of which aids the conversion of Ag^{+} to Ag^{0}. In addition, the alkaline ion (–OH) is very much required for the reduction in metal ions. Moreover, under alkaline conditions, the ability of the 243 enzymes responsible (not only nitrate reductase) for the synthesis of SNPs increases (Sanghi and Verma, 2009).

While studying temperature effect it was found that as the temperature increases the absorbance (yield) was also increased (Figs. 13 and 16). Complete reduction in silver ions to SNPs is corroborated with the result of Darroudi et al., which shows the increase in SNPs synthesis with increasing temperature (Darroudi et al., 2011). Post induction temperature range of 60 °C and 80 °C is suitable for both extracellular protein/enzyme secretions (Figs. 12 and 15) in both the tested fungi for stable synthesis of SNPs with short time period (Figs. 14 and 17). Sarkar et al. (2007) reported that, with increase in temperature, the kinetic energy of the SNPs in the solution also increases; as a result, the collision frequency between the particles also rises, and this leads to the higher rate

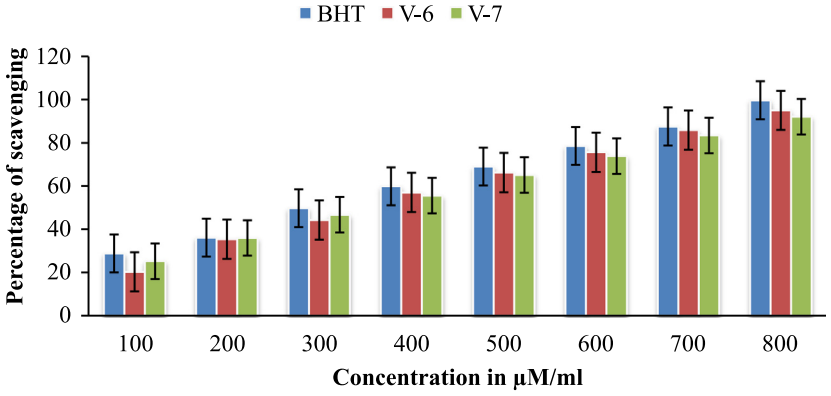

Figure 20 Hydroxyl radical scavenging assay of mycosynthesized SNPs.

Table 3 IC_{50} value of hydroxyl radical scavenging activity.

Samples	IC50 value (μM/ml)
BHT	301.50
V-6	338.60
V-7	320.71

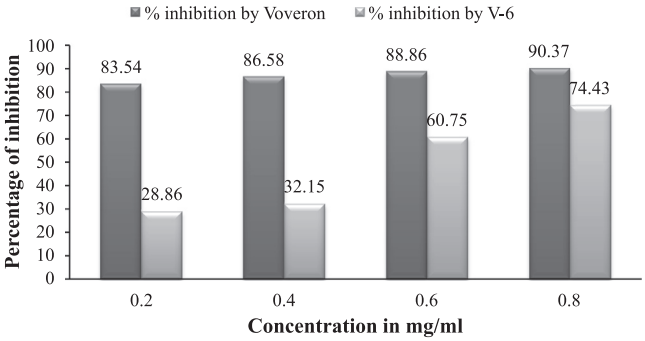

Figure 21 Percentage inhibition of protein denaturation by V-6.

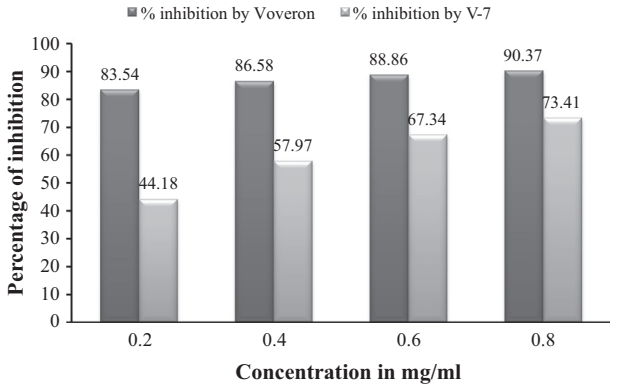

Figure 22 Percentage inhibition of protein denaturation by V-7.

of agglomeration. Surface potential of SNPs is inversely proportional to temperature which leads to the formation of aggregates. They demonstrated the particle growth rate over a range of ionic strengths and reaction temperature in which particles interact via electrostatic and Van der Waals forces (Sarkar et al., 2007; Van Hyning et al., 2001).

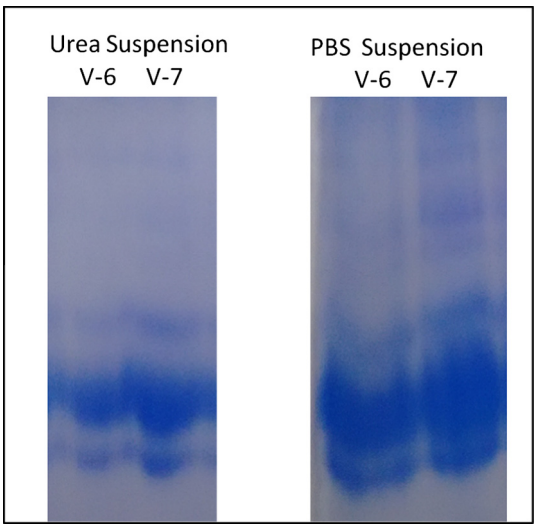

Figure 23 SDS PAGE electrophoresis pattern of human serum protein interaction with SNPs.

There are very few reports on photo mediated biological synthesis of SNPs. It's well known that certain reactions occur by the adsorption of photons of suitable energy. Photo reduction in silver ions to SNPs was carried out successfully in our study. Several studies showed the involvement of nitrate reductase enzyme (49) and different proteins in the synthesis of SNPs (Jain et al., 2011). Here, experimentation also demonstrated that, without fungal cell filtrate synthesis of SNPs did not occur in sunlight. In addition, the rapid synthesis of SNPs was observed in the presence of sunlight (Fig. 3) and at high temperature. However, the exact mechanism of SNPs in light has not been demonstrated. In our photo reduction study, there may be photosensitization of aromatic amino acids (photosensitizer) of filtrate protein that may absorb the light energy and transfer to the reactants being itself doesn't undergo any changes, which may help in reduction in silver ions by providing electrons as well as silver ions to catalyse the amine oxidation. In case of high intensity light, heat generates, which may also accelerate the rate of synthesis of SNPs, or photolysis of silver ions may take place (Fig. 4).

Free radicals are produced in normal and/or pathological cell metabolism. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free

Table 4 Antimicrobial activity of SNPs (V-6 and V-7).

Microorganisms	Type	MIC of V-6 (μM/ml)	MIC of V-7 (μM/ml)	MIC of gentamycin (μM/ml)
Escherichia coli	–	0.5	0.5	0.4
Salmonella typhi	–	0.4	0.8	0.3
Pseudomonas fleurescence	–	0.6	0.6	0.3
Klebsiella pneumonia	–	0.8	0.5	0.3
Staphylococcus aureus	+	0.5	0.6	0.3
Listeria monocytogenes	+	2.5	0.5	0.6
Micrococcus luteus	+	0.7	0.8	0.2
Bacillus subtilus	+	1.0	0.5	0.3
Staphylococcus saprophyticus	+	0.6	0.6	0.3
Staphylococcus epidermidis	+	0.5	0.4	0.3
Pseudomonas aeruginosa	+	0.5	0.4	0.5

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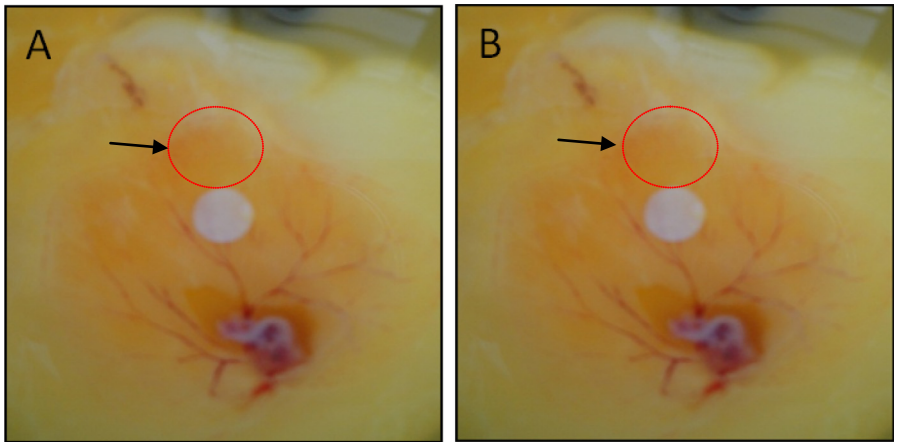

Figure 24 Shell less CAM (chorioallontoic membrane) assay for antiangiogenic property of V-6 (A) and V-7 (B).

radicals is involved in the onset of many diseases such as cancer (Emerit, 1994), Rheumatoid arthritis, cirrhosis and arteriosclerosis (Patel et al., 2000) as well as in degenerative processes associated with ageing (Hipkiss, 2006). Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage. In the recent years, there has been increasing evidence that reactive oxygen species (ROS) are associated with pathological conditions such as atherosclerosis and carcinogenesis, as well as with ageing. The mycosynthesized SNPs were found to possess strong antioxidant activity wherein SNPs could scavenge DPPH radicals (Fig. 18) with lesser IC_{50} values with ~28% (V-6) and ~36% (V-7) more potent than the positive control (Table 1). The antioxidant mechanisms of SNPs may be attributed to their free radical-scavenging ability (Halliwell and Gutteridge, 1999). Similarly SNPs (both V-6 and V-7) could inhibit the formation nitrite ions (Fig. 19) by competing with molecular oxygen with almost equivalent in its percentage of action with that of positive control (Table 2). Free radicals are produced in the body which are detrimental in attacking lipids, DNA and Proteins. To neutralize and scavenge the free radicals, antioxidants play an important role (Lohoues et al., 2014). The present investigation discloses the free radical scavenging activity of mycosynthesized SNPs. Hydroxyl radical scavenging activity of V-6 and V-7 (Fig. 20) was in concentration dependent manner with higher IC50 values (Table 3). Most common radicals are derivatives of oxygen such as superoxide free radical anion (O_2^-), lipid alkoxyl (OO), and lipid peroxide (LO_2^{-2}) as non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) which are collectively known as ROS. These free radicals are produced mainly from two important sources in the biological system, i.e., cellular metabolism such as mitochondrial electron transport chain, endoplasmic reticulum, oxidation, NADPH oxidase, nitric oxide synthetase and environmental sources such as drugs, pesticides, transition metals, tobacco smoke, alcohol, radiations and high temperature (Patel et al., 2000).

Most of the investigators have reported that denaturation of protein is one of the causes of Rheumatoid arthritis. Production of auto antigens in certain rheumatic diseases may be due to in vivo denaturation of proteins (Arumugam, 2013). Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. Such type of studies was carried out i.e. anti-arthritic effect of SNPs synthesised from the fungi was studied significantly by using *in vitro* inhibition of protein denaturation model. The effect of standard drug and SNPs on inhibition of protein denaturation was studied wherein SNPs i.e., V-6 (Fig. 21) and V-7 (Fig. 22) at different concentrations provided significant protection against denaturation of proteins.

Human beings are often infected by microorganisms such as bacteria, moulds, yeasts, and viruses present in their living environments. Because of the emergence and increase in the number of multiple antibiotic-resistant microorganisms and the continuing emphasis on health-care costs, many scientists have researched methods to develop new effective antimicrobial agents that overcome the resistances of these microorganisms and are also cost-effective. Silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities (Berger et al., 1996) which were confirmed in our studies also (Table 4). When bacterial growth was inhibited, silver ions were deposited into the vacuole and cell walls as granules (Brown and Smith, 1976). They inhibited cell division and damaged the cell envelope and cellular contents of the bacteria (Richards et al., 1984) which makes the possible use of silver nanos as microbicidal either alone or as a carrier of antimicrobials for topical applications or via the blood stream (for intravenous) with the prior assessment of protein corona.

The protein corona, which is usually enriched with about 10–50 proteins has highest affinity for the surface and gives information on the outer amino acid sequences (epitopes) that are in contact with living systems. The biological responses to nanoparticles are highly affected by forces at bio-nano interface (hydrodynamic, electro-dynamic, electrostatic or steric forces, and solvent and polymer bridging). Another complementary factor to the protein corona is the safe design of any type of nanoparticles, which is called the cell "vision" (Walczyk et al., 2010). There are about 200 differentiated cell types in the human body, which have different cellular membranes of significant variability. Thus, the interaction of foreign objects viz., biomolecules, drugs, nanoparticles is in contact with these cell types causing variable cellular response

(Laurent et al., 2012). In this study it is confirmed that there was a transient interaction between the serum proteins and the SNPs wherein the appearance of more protein bands in the lane of PBS washes over the Urea wash lane with less number of bands (Fig. 23). Besides the adsorbed corona on the surface of nanoparticles, the proteins on their surface could lead to a significant in vivo response introducing another concept of cell "vision" which is dependent on the cell type. The nanoparticles trigger different cellular responses with various detoxification strategies in different cell types. Thus cell "vision" approach aids in achieving a more effective design and usage of desired nanoparticles depending on the targeted cell types and it is the fingerprint defence mechanism of various cells to the environment (Mahmoudi et al., 2012).

Another vital role for nanoparticles is to serve as therapeutic agents, with the potential to overcome many of the hurdles that conventional therapies face. Various nanoparticle systems have been explored as carriers to overcome the blood-brain barrier (BBB) for targeted treatment of brain tumours. Additionally, nanoparticles respond to external triggers such as an applied magnetic field and light which offer new therapeutic avenues for targeting malignant brain tumour. One way to target is preventing angiogenesis; such type of prevention was observed in our study by CAM assay (Fig. 24). Nanoparticle-based delivery systems for therapeutic agents such as chemotherapy drugs are expected to have a great clinical impact on brain tumour treatment (Madhankumar, 2006; Krauze, 2007; Krol et al., 2013). Many hydrophobic agents that normally cannot cross the BBB can accumulate at a tumour site when incorporated into a nanocarrier (Cui et al., 2013; Yoo et al., 2002; Zhang et al., 2011).

On combining all optimized conditions, ecofriendly and inexpensive method has been developed for the rapid and large-scale synthesis of SNPs. However, we report the optimization of cultural and physical conditions on biological synthesis of SNP. Cultural (culture medium, quantity of biomass, filtrate volume, and salt concentration) and physical conditions (pH, temperature, and light intensity) have been found to affect the maximum yield, rate of synthesis, and size of SNPs. In sunlight, synthesis of SNPs takes place within few minutes indicating the activation of reducing enzymes/proteins in the fungal filtrate. Exploitation of natural energy source for synthesis of SNPs can overcome the problem of energy consumption as well as rate of synthesis. In combination with all desired conditions, SNPs with uniform size distribution of 60 nm (V-6) and 70 nm (V-7) by DLS (Fig. 5C) and SEM analysis (Fig. 5D) with stability and promising increase in yield were obtained. The study revealed that the synthesis of SNPs may take place due to peptides/proteins. The optimization of the parameters would lead to the rapid and large-scale production of SNPs at industrial level, which may be used as novel biocompatible antimicrobials against multidrug resistant microorganisms. Protein corona result indicates that human serum proteins interacted with SNPs forming soft corona confirmed by more number of protein band appearance in PBS wash than compared to urea wash. It indicates that the nanoparticles can be used for drug targeting.

Conflict of interest

We authors declare no conflict of interest.

Acknowledgements

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