Optimization of silver nanoparticle biosynthesis process using cell-free filtrate of *Aspergillus niger*.

Adnan Hamad Uobeed Al-Hamadani [1] Balsam Miri Mizher Al-Muhna[2]

[1] Department of Microbiology, College of Medicine, AL-Qadissyia University Iraq
[2] Department of Microbiology, College of Vet. Medicine, AL-Qadissyia University Iraq
E.mail:Tamarafirass@gmail.com

Tel:07802656115

الخلاصة

ازداد في الاونة الاخيرة اهتمام الباحثيين بتقنية النانو و المواد النانوية. وانصب الاهتمام بشكل واضح على ايجاد طرق اكثر فعالية لتخليقها. و يعد التصنيع الحيوي من افضل الطرق البديلة والواعدة لانتاج جزيئات النانو. اثبتت الدراسة الحالية انتاج جسيمات الفضة النانوية بواسطة تقنية فعالة من حيث التكلفة و الامان و صديقة للبيئة وذلك باستخدام راشح فطر رشاشيات النيجر(*Aspergillus niger*) كعامل مختزل لنترات الفضة. و قد تم اختبار الظروف المثلى لعملية التصنيع الحيوي و التي شملت الوسط الزرعي، تركيز ايون الهيدروجين، درجة الحرارة، تركيز نترات الفضة و عامل الوقت من اجل الحصول على افضل النتائج من حيث الحجم والشكل والثباتية اضافة الى زيادة كمية الجسيمات المنتجة. اظهرت نتائج التحليل الطيفي للاشعة فوق البنفسجية ان اعلى ذروة للامتصاص كانت عند الطول الموجي 420 نانومتر. كما تم قياس وجود البروتينات كعوامل مختزلة مسؤولة عن عملية التصنيع الحيوي و ثباتية الجسيمات المصنعة وذلك باستخدام التحليل الطيفي للاشعة تحت الحمراء. وبينت نتائج الفحص باستخدام المجهر الالكتروني الماسح وجود جسيمات نانوية كروية متناثرة بشكل جيد وبقطر يتراوح بين 15-50 نانومتر. كما تم قياس العناصر المتواجدة كميا ونوعيا وذلك باستخدام تحليل طاقة التشتت للاشعة السينية. وقد سجل اعلى انتاج لجسيمات الفضة النانوية بواسطة فطر رشاشيات النيجر في وسط مرق البطاطا و الدكستروز وبدرجة حامضية 9 لمدة 120 ساعة في 30 °م مع وجود نترات الفضة بتركيز 1 ملي مول .

Abstract

Nanotechnology and nanoparticle (NPs) research has attracted a lot of interest in recent decades, and there is growing attention to find more effective ways for their synthesis. The use of biological organism as bionanofactories provides a clean and promising alternative process for the fabrication of silver nanoparticles. This study confirmed the production of silver nanoparticles (SNPs) by a cost effective, safe and environment-friendly technique using silver nitrate and cell free filtrate of the fungus *Aspergillus niger* as the reducing agent. The optimization of different parameters, including the culture media, pH, reaction temperature, concentration of silver nitrate solution and reduction time, were carried out to achieve better control of size, shape,

stability, and to increase the yield of SNPs production. The UV-visible spectrophotometric analysis of the biosynthesized silver nanoparticles by *Aspergillus niger* cell-free filtrate showed characteristic surface plasmon absorption peak at 420 nm. The presence of proteins as viable reducing agents for the formation and stability of SNPs was recorded using Fourier transform infrared spectroscopic analysis (FTIR) .Further scanning electron microscopy (SEM) micrograph showed the formation of spherical, well-dispersed nanoparticles with size ranging between 15 and 50 nm in diameter. The element composition of the mixture sample was obtained from the Energy Dispersive Analysis of X-ray (EDX). It concluded that the optimum condition for biosynthesis of SNPs were the use of Potato dextrose broth medium at pH 9, 30°C for 120 h with 1mM silver nitrate.

Key Words: Silver nanoparticles, *Aspergillus niger*, Optimization, extracellular biosynthesis.

Introduction

Currently, the nanotechnology undergoing explosive development and provide an excellent platform for promising applications in various fields of life (1). SNPs have entered into a new arena, opening new possibilities in medicine, biological product development, drug delivery system, combating cancer and antimicrobial activity (2). The major methods used for conventional synthesis of SNPs are the physical and chemical methods. The problem with these methods is that the synthesis is energy and capital intensive and often employ toxic chemicals and can hinder their usage in medical applications(3). Moreover, chemical synthesis of silver colloids mostly leads to aggregation as the period of storage increases (4). The biological methods regard the best choice to obtain these advantages. There are three major sources of biological synthesis of SNPs: plant extracts, bacteria and fungi (5). Fungi have some distinct advantages, in comparison with bacteria, it can produce larger amounts of NPs because they can secret larger amounts of proteins which directly translate to higher productivity of NPs (6). Moreover, fungi have an additional advantage that handling of the fungal biomass would be much simpler and fungal broths can be easily filtered by simple equipment (7). The present study was focused on the biosynthesis of SNPs using *A. niger* and characterization of synthesized NPs. Biosynthesis of SNPs by using a fungi (8,9,10 and 11) has been reported. In case of extracellular synthesis of SNPs by *A. niger*, the production of enzyme is highly affected by the condition in

which the fungus is cultivated. Therefore, the first aim of this work is to find a cost effective and environment-friendly technique for synthesis of SNPs , and the second is to optimize the physioculture conditions to improve the yield of SNPs production.

Materials and methods:

Fungal strains and their maintenance:

The fungal strain of *A. niger* was obtained from Agriculture College / Al-Kufa university (kindly provided by prof. Dr. Majeed M. Dewan) and it previously isolated from soil sample and diagnosed by PCR technique.

Fungal biomass production

The preparation of biomass was performed according to Vigneshwaran *et. al.*, method. (12).

Biosynthesis of nanoparticles

For biosynthesis of SNPs, 50 ml of cell –free filtrate was mixed with 50 ml of 1mM $AgNO_3$ in 250 ml Erlenmeyer flask and kept in shaking incubator at 150 rpm at 28°C for 24 hours. Simultaneously, a positive control of cell filtrate without metal salts and a negative control containing only metal salts solutions were run along with the experimental flasks (13). All reaction mixtures were kept in dark to avoid any photochemical reactions during the experiment.

Characterization of biosynthesized SNPs:

UV-Vis spectroscopy analysis

The detection of SNPs was primarily carried out by visual observation of color change of the fungal filtrate after treatment with silver nitrate. Appearance of dark brown color of fungal cell filtrate indicates the formation of SNPs. Further, the formation of SNPs were confirmed with the help of dual beam UV-Visible spectrophotometer (SPEKOL1300, Germany)

Fourier Transform Infrared Spectroscopy (FTIR)

The interaction between the biosynthesized SNPs and biomolecules was analyzed using FTIR spectrophotometer (Bruker Tensor 27, Germany) in the range of 500 - 4000 cm-1.

Scanning Electron Microscopy (SEM) studies.

The biosynthesized SNPs were also subjected to SEM analysis (Inspect S50, Netherland) to evaluate their size and morphological characteristics. The elemental structure of powdered specimen was evaluated using SEM equipped with an EDS detector.

Optimization of SNPs biosynthesis

Effect of Different culture Media

Five different media ,namely, PDB (, containing potatoes infusion 200g, dextrose 20g and distilled water 1000mL), GPYB (glucose 50 g, peptone 10 g, yeast extract 10 g PH 7), Czapek broth (sucrose 30g, NaNO3 3 g, K2HPO4 1 g, MgSO4 0.5 g, KCl 0. 5 g, and FeSO4 0. 01 g /1000 mL, pH 7.3) , MYPG (malt extract 3g , yeast extract 3g, peptone 5g and glucose 20g ,PH 7) and SB (peptone 10g and dextrose 40g ,PH 5.6) were tested . The fungal mycelia were grown for 7 days in 500 mL Erlenmeyer flasks, each containing 250 mL of test medium. The synthesis of SNPs was carried out as mentioned above.

Effect of PH

In order to study the effect of pH concentration, the biomass was transferred to distilled water with different pH, namely, pH 5, pH 7and pH 9. To get the required pH, the distil water adjusted by 0.1 N NaCl and 0.1 M NaOH and the final pH measured by using electrical pH meter before incubation for 72 hours at 28 ºC.

Effect of temperature

The effect of different temperatures is carried out by suspending the fungal biomass in de-ionized water and incubated at different temperature 20, 30, 40, 50 and 60º C for 3 days.

Effect of different concentrations of AgNO3

Different concentrations of silver nitrate (1mM, 2mM, 3mM, 4mM and 5mM) in the reaction solutions were investigated in order to obtain the optimum concentration of the substrate for SNPs production.

Effect of time

The fungal filtrate was obtained and treating with 1mM $AgNO_3$ for preparation of SNPs as prescribed. Then it monitored at different duration by UV.visible spectroscopy for synthesis of SNPs (after 1, 24, 48, 72, 120 hours and 2 months).

Results

Characterization of biosynthesized SNPs:

After the end of growth period of *A. niger*, the cell-free filtrate was used for biosynthesis of SNPs. The filtrate was initially pale yellow in color. When the filtrate challenged with AgNO3, the color of the mixture was turned to yellowish brown at first and then the intensity of the color was increased with the period of incubation, so the color was changed to dark brown on completion of the reaction with Ag+ ions. Color change was noticed only in the test flask and it a clear indication for the formation of SNPs in the reaction mixture. The remaining two control flasks i.e., aqueous solution of AgNO3 and fungal filtrate without AgNO3 showed no change in color when incubated in the same condition .

The formation and stability of the reduced SNPs in colloidal solution was detected and monitored by using UV-visible absorption spectrum .The λ max 420 nm was observed only in the test flask which confirmed the production and indicating the specific surface Plasmon resonance of SNPs.

Fourier Transform Infrared Spectroscopy spectrum of biosynthesized SNPs revealed the presence of different distinct peakslocatedat3421,2962,2926,2854,1638,1554,1428,1410,1333,1276,1256,1239,1073,1048,782,467 cm-1 . The peak at 3421cm^{-1} is ascribed to the N-H stretch vibration of primary amides of protein. The peaks at 2926 cm-1 and 2961 cm-1could be due to the C-H stretch of the methylene groups of protein and to N-H stretching of amine salt. The absorption peak at 2854 cm-1may be assigned to the C-H symmetrical

stretch vibration of alkenes. The peak at 1638 cm-1 corresponds to the presence of amide I and amide II, which arises due to the carbonyl stretch and N-H stretch vibration while the band at 1554 cm-1 refers to C=C stretch corresponding to an aromatic ring. Peaks located at 1410 cm-1 and 1428 cm-1 may be related to COO^- symmetrical stretch from carboxyl groups of the amino acids residues. The peak at 1333 cm-1 corresponds to carbon hydrogen (CH3) bending vibration. The peak located at 1276, 1256 and 1239 cm-1represent C-O stretching of primary alcohol and P=O stretching respectively. The bands at 1073cm-1 and 1048 cm-1 refer to C-O bonds of aliphatic amines while the peaks at 782cm-1 and 467 cm-1 can be assigned to the aromatic C-H out of plane bending vibration of aromatic primary amines.

Scanning electron microscopy has been employed to determine the shape and morphology of biosynthesized SNPs. Figure (4) reveals SEM micrograph of SNPs obtained by the reduction of AgNO3 solution with cell-free filtrate of *A.niger* . The morphology of NPs was spherical in shape, uniformly (monodispersed) without significant aggregation. The particle size was ranged from 15-50 nm.

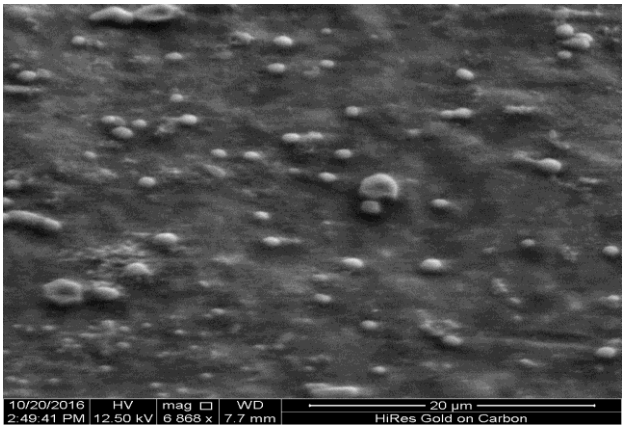

Figure (4): SEM micrograph of biosynthesized SNPs. The image shows size and spherical shape of monodisperse SNPs.

The EDX analysis was established to detect the elements that may be involved in the formation of SNPs. Figure (5) reveals a strong signals for metallic NPs. The optical absorption peak was noticed at approximately 3 KeV, which is typical for the absorption of mineral silver nanocrystalites. In addition, another peak for oxygen was observed on the left part of the spectrum at ~0.5 KeV. It is clear from Table (1) that the weight percentage of silver is 62.49 %.

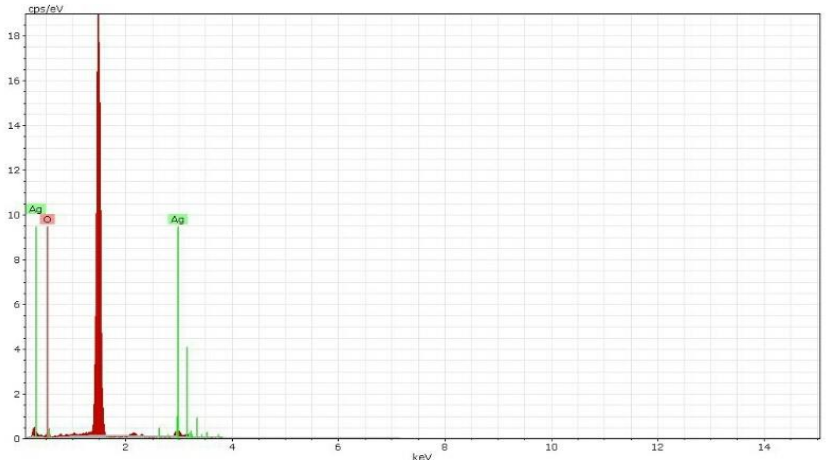

Figure (5): EDX analysis of biosynthesized SNPs. The vertical axis shows the number of X-ray counts, whereas the horizontal axis shows the energy in KeV.

Table (1): The elements composition in biosynthesized SNPs solution of EDX spectra.

Spectrum:Acquisition					
Element	Series	unn.(wt.%)	C norm.(wt.%	C Atom.(at.%)	Sigma(wt.%)
Oxygen	K-series	4.78	37.51	80.19	2.07
Silver	L-Series	7.96	62.49	19.81	0.56
Total		12.75	100.00	100.00	

It is evident from Figure (6) that the highest SNPs production was recorded in PDB medium followed by MYPGB medium. Samples obtained from these culture media have high absorption intensity, with peaks at 435nm, 440 nm respectively; the sharp and smooth curves represent small and uniform size distribution . Symmetry in graph indicates the monodispersity and stability in synthesized NPs. The GPYB medium showed minimum surface plasmon intensity with peak at 445nm, whereas CzapekB and SDB medium showed peaks at 440 nm and 445 nm respectively.

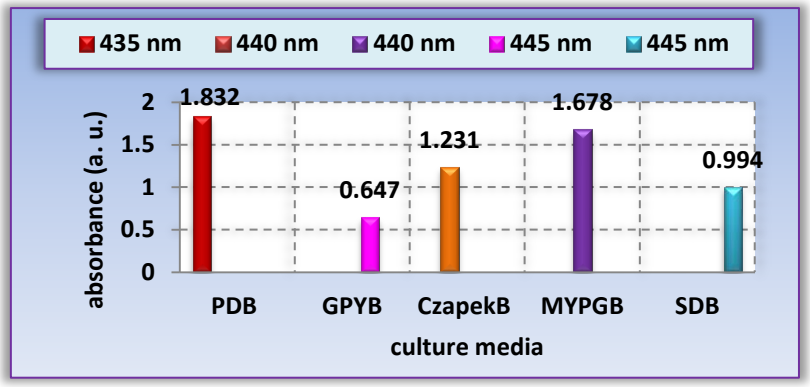

Figure (6): Comparison the absorbance of SNPs obtained with different culture media

When the fungal filtrate was challenged with 1mM $AgNO_3$ at different pH, all the test flasks showed variable change in color of reaction mixture from pale yellow to brown color and the intensity of Plasmon surface resonance bands have been varied as depicted in figure (7) by UV-visible absorption spectra. pH 9 was found to provide optimal conditions for maximal biosynthesis of SNPs with maximum peak at 425 nm. On the other hand, absorbance decreased with decreasing in pH value. Particles aggregation were observed in acidic pH after few days from reaction, while there is no evidence for any aggregation in alkaline pH up to the end of experimental period which indicates the stability and monodispersity of SNPs at alkaline pH .

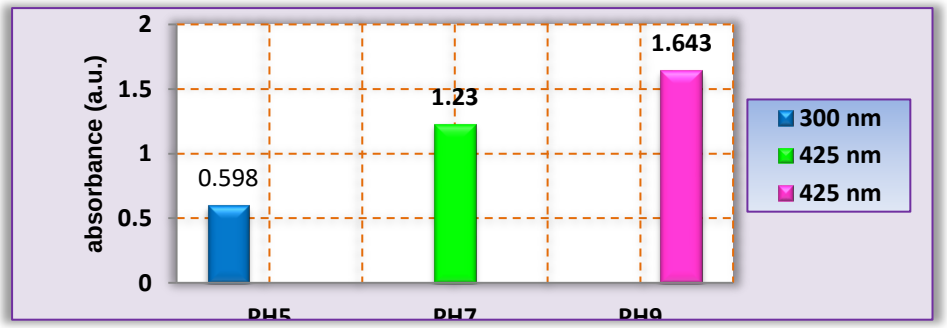

Figure (7): UV-vis spectrum showing maximum SNPs synthesis at PH 9.

Figure (8) detected significant differences in absorbance among different temperature degrees. The optimal temperature for silver bioreduction was 30C° as demonstrated by color change and absorbance measurements by UV-visible spectroscopy with a sharp peak at 420nm, followed by 25C°. As we increased the temperature, the absorbance intensity of surface Plasmon resonance was decreased .

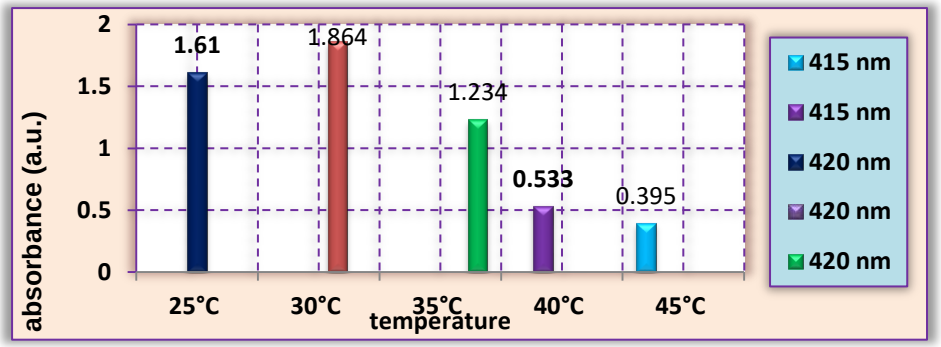

Figure (8): Higher absorption at 30 ºC indicate the optimum temperature for synthesis of SNPs.

As shown in figure (9) the absorbance intensity decreases with increase in concentration as recorded by UV-vis. Spectroscopy. The concentration of 1mM showed maximum absorbance with a characteristic SPR band around 420nm indicating efficient production of SNPs. From the graph, it is obvious that the highest concentration (5mM) showed the least bio-reduction of silver ions to NPs and the reaction mixture appear unstable with aggregation and precipitation at the bottom of flasks.

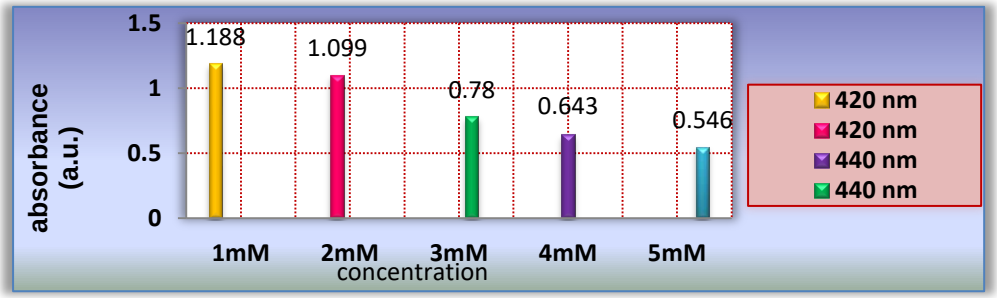

Figure (9):Comparison the absorbance of SNPs obtained with various AgNO3 concentration.

Time consider a major factor in the biosynthesis of NPs. The change in color of reaction solution from pale yellow to dark brown was detected only after 24h of incubation. It was also observed that the biosynthesis rate increased with increase in time (figure 10). The highest absorbance value was recorded after 2 months with broad peak at 440nm. whereas after 120 h of incubation, the peak was recorded at 420 nm.

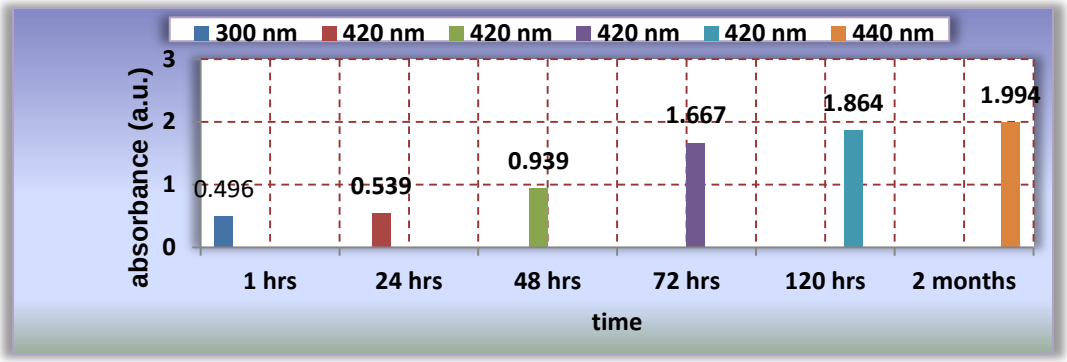

Figure (10): Comparison the absorbance of SNPs obtained at different time intervals

Discussion

This study had successfully demonstrated an easy, rapid and efficient route for extracellular synthesis of SNPs by employing the cell- free filtrate of *A.niger*. It was observed that after addition of the silver ion (1 mM) into the flask containing the cell filtrate, the color of the medium changed from pale yellow to brown, which indicates the formation of colloidal SNPs. The brown color of the medium could be due to the excitation of surface plasmon vibrations, typical of the SNPs (14). The results of UV-Visible spectrophotometer showed strong and characteristic surface plasmon resonance centered at 420 nm. Many studies confirmed that the fungal cell filtrate treated with AgNO3 solution gave a peak around 420 nm (15). FTIR spectrum indicted that *A.niger* cell-free filtrate contain active biomolecules which may be responsible for the biotransformation of silver ions to SNPs. These results confirmed that amino acid residues and peptides of proteins have a stronger ability to bind with metal and capping it to prevent agglomeration of the particles and stabilizing in the medium (13). These finding resembles with the results of Gole et al. (16). The nanostructural studies of SEM micrograph showed SNPs to be spherical in shape and are uniformly distributed (mono dispersed) without significant agglomeration. The monodispersity of NPs attributed to the capping agents which provide stability of NPs and prevent agglomeration of it. These results were compatible with Elgorban *et al* .(17). The energy dispersive X-ray analysis displayed the strong signal at about 3 keV of the Ag regions. Same result of EDS optical absorption peak at 3kev was also reported by Elgorban *et al* (17). Although the exact mechanism of NPs biosynthesis by fungi is not yet clearly defined but several hypothesis have been proposed by many research scientists. It is stated that certain extracellular proteins released into the

filtrate could play a role in the synthesis and stability of the SNPs. The biosynthesis of SNPs may be attributed to the reductase enzyme, one of the most important extracellular enzymes produced by *A.niger*. Zomorodian *et al* (18) showed a reasonable relationship between nitrate reductase activity and the efficiency of *A.niger* in the production of SNps.

In the present study, fungal biomass grown in PDB has shown enhanced SNPs synthesis. This may be due to presence of ingredients in PDB stimulating better growth of fungi and help in producing augmented level of reducing agent (19). Birla *et al*. (19) showed that MGYP medium may promote the extracellular nitrate reductase secretion by *F. oxysporum* and enhance the synthesis of SNPs. Result clearly indicated that absorbance increased with pH, suggesting that an alkaline environment was more suitable for SNPs biosynthesis. The availability of OH- ions in alkaline medium is very important for reduction of metal ions. While studying the effect of temperature, it was found that maximum production of SNPs was recorded at 30°C. This may be due to the 30°C was the optimum temperature for enzymatic activity while high temperature resulting in denaturation or inactivation of enzymes. The results of present study clearly indicate that the relationship between the biosynthesis of SNPs and concentration value of AgNO3is inversely proportional. The maximum synthesis of nanoparticles occurred at 1 mM AgNO3 in the reaction mixture. This can be interpreted on the basis of enzyme-substrate kinetics; i.e. the active site in the key biomolecule responsible for reduction of NPs is already saturated with the silver ions, and no site is available for excess ions to get reduced (20).The synthesis of SNPs by the fungus had been enhanced with the reaction time. The increase in the absorbance with the reaction time means that the concentration of biosynthesized SNPs increases. When the reaction time reached 2 months the absorbance increased slightly, and the λ max value was shifted to toward long wavelength.

Conclusion

The obtained results established the fact that SNPs can be synthesized in ecofriendly, inexpensive and promising technique by fungal strain of *A.niger*, and the qualities of these NPs. can be controlled by monitoring the environmental factors such as culture media, pH, temperature, substrate concentration and contact time.

References

1-Jaidev, L. R. (2010). Fungal mediated biosynthesis of silver nanoparticles, characterization and antimicrobial activity, Colloids. Surf B., 81: 430-433.

2- Krishnaraj, C. ;Ramachandran, R.; Mohan, K.and Kalaichelvan, P.(2012).Optimization for rapid synthesis of silver nanoparticles and its effect on phytopathogenic fungi .Spectrochimica Acta Part A: *Molecular and Biomolecular Spectroscopy*, 93: 95-99.

3- Parashar, U.K.; Saxena , S.P.and Srivastava, A. (2009). Bioinspired synthesis of silver nanoparticles. *Dig. J. Nanomat. Biostruct*., 4: 159–166.

4- Abou El-Nour, K. M. ; Eftaiha, A.; Al-Warthan, A. and Ammar, R. A. A. (2010). Synthesis and applications of silver nanoparticles. *Arabian Journal of Chemistry*, 3(3): 135–140.

5-Yen, S.C. and Mashitah, M.D. (2012).Characterization of Ag Nanoparticles Produced by White –Rot Fungi and Its in Vitro Antimicrobial Activities. *Int. Arab. J. Antimicrob. Agents* 2:1-8

6- Mohanpuria, P.; Rana, K.N.and Yadav, S.K. (2008).Biosynthesis of nanoparticles: technological concepts and future applications. *Journal of Nanoparticle Research*, 10: 507–517.

7- Korbekandi, H.; Ashari, Z.; Iravani, S. and Abbasi, S.(2013). Optimization of Biological Synthesis of Silver Nanoparticles using *Fusarium oxysporum*. *Iran J Pharm Res.*, Summer; 12(3): 289–298.

8- Biswas, S. and Mulaba-Bafubiandi, A. (2016). Optimization of process variables for the biosynthesis of silver nanoparticles by *Aspergillus wentii* using statistical experimental design. *Adv. Nat. Sci.: Nanosci. Nanotechnol.*, 7 (10pp)

9- Singh, P. and Raja, R. B. (2011). Biological Synthesis and Characterization of Silver Nanoparticles Using the Fungus *Trichoderma harzianum*. *Asian Journal of Experimental Biology and Scien*ce, 2(4): 600-605.

10- Singh,D.; Rathod,V.; Ninganagouda,S.; Hiremath,J.; Singh,K., and Mathew,J. (2014). Optimization and Characterization of Silver Nanoparticle by Endophytic Fungi Penicillium sp. Isolated from Curcuma longa (Turmeric) and Application Studies against MDR *E. coli* and *S. aureus*. *Bioinorganic Chemistry and Applications* Volume 2014 , Article ID 408021, 8 pages

11- Khan, N.T.and Jameel, J. (2016). Optimization of Reaction Parameters for Silver Nanoparticles Synthesis from *Fusarium oxysporum* and Determination of Silver Nanoparticles Concentration. *J Material Sci Eng*., 5:283. doi:10.4172/2169-0022.1000283

12- Vigneshwaran, N.; Ashtaputre, N.M.; Varadarajan, P.V.; Nachane, R.P.; Paralikar, K.M. and Balasubramanya, R.H.(2007).Biological synthesis of silver nanoparticle using the fungus *Aspergillus flavus*, *Materials Letters*, 61: 413– 1418.

13- Basavaraja, S., Balaji, S.D., Arunkumar, L., Avenkataraman, A. (2008). Extracellular biosynthesis of AgNPs using the fungus *Fusarium semitectum* . *Mater.Res. Bull*., 43:1164-1170.

14- Li, G.; Dan, H.E.and Yongqing, Q.(2012). Fungus-mediated green synthesis of silver nanoparticles using *Aspergillus terreus*. *Int J Mol Sci*.,13:466476

15- AbdelRahima, K.; Mahmoudc,S.; Alic,A.; Almaarya,K.S.; Abd El-Zaher M.A., Mustafaa, D.and Husseinye,S.H.(2017).Extracellular biosynthesis of silver nanoparticles using *Rhizopus stolonifer*. *Saudi Journal of Biological Sciences,* 24, 1: 208–216

16- Gole, A.; Dash, C.; Ramakrishnan, V.; Sainkar, S.R.; Mandale, A.B.; Rao, M., (2001).Pepsin-gold colloid conjugates: preparation, characterization and enzymatic. *Langmuir*. 17:1674–1679.

17- Elgorban ,A.M.; Aref, S.M. ; Seham, S.M.; Elhindi, K.M.; Bahkali, A.H.; Sayed, S.R., Manal M.A. (2016).Extracellular synthesis of silver nanoparticles using *Aspergillus versicolor* and evaluation of their activity on plant pathogenic fungi. *Mycosphere,* 7 (6): 844–852 .

18.Zomorodian,K.; Pourshahid,S.;Sadatsharifi,A.; Mehryar,P., Pakshir,K.; Rahimi,M ., and Monfared, A. (2016), Biosynthesis and Characterization of Silver Nanoparticles by Aspergillus Species. *BioMed Research International*., Article ID 5435397, 6 pages

19- Birla ,S.; Gaikwad, S.; Gade, A.and Rai, M. (2013).Rapid synthesis of silver nanoparticles from *fusarium* oxysporum by optimizing physicocultural conditions. *Sci World* J.:1–12.

20-Singh,D.; Rathod,V., Ninganagouda,S.; Hiremath,J., Singh,K., and Mathew,J. (2014). Optimization and Characterization of Silver Nanoparticle by Endophytic Fungi Penicillium sp. Isolated from Curcuma longa (Turmeric) and Application Studies against MDR *E. coli* and *S. aureus*. *Bioinorganic Chemistry and Applications,* Article ID 408021, 8 pages