Comparison Between the Nature and Activity of the Silver Nano Particles on Cervical Cancer Cells That Were Produced by the Active and Inactive Fungal Biomass Forms

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Research article

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Abstract

Background Biologic nanoparticles can be produced by the active and passive mechanisms but their properties have not been compared to each other and recent research tries to uncover them.

Methods Biomass of *Fusarium oxysporum* was divided into four groups and pretreated in: room temperature (RT) and refrigerator (for preparation the active biomass forms); autoclave and hot air oven instruments (for preparation the inactive biomass forms). Samples were floated in ddH_2O and SNPs were produced after addition of 1mmol final concentration of silver nitrate solution. Production of SNPs was confirmed using visible spectrophotometer, transmission electron microscopy (TEM) and X-ray diffraction (XRD) analyses. SNPs were washed and their concentration was determined using ICP measurement. Plate well diffusion method was used for antibacterial activity. MTT and Annexin V-FITC/ propidium iodide assays were used for cytotoxicity and apoptosis induction assays, respectively.

Result Results showed that all the four tested samples produced SNPs and had maximum absorbance peaks about 400-450 nm. The produced SNPs had almost same shapes and sizes and XRD results confirmed the presence of SNPs in all the samples. ICP results showed that except the refrigerator incubated sample, the concentration of the SNPs in all the flasks was similar to each other. Due to the differences in the nature of the applied bacterial strains the produced SNPs had some differences in their antibacterial activities. MTT assay results showed that due to lower SNPs concentration of the refrigerator incubated sample they had less toxic effects than the others. Apoptosis induction results revealed that the biologically produced SNPs induced more apoptosis and less necrosis.

Conclusion In conclusion, the active and inactive fungal biomass forms produced SNPs that their nature and activity were not altered.

Background

In the recent years high tendency for different types of nanoparticles production with different applications has been emerged. The nanoparticles have specific properties which are different from their bulk materials[1, 2]. Unlike their bulk materials,they show especial physical, chemical, magnetic, and optical characteristics[2, 3]. Production of nanoparticles is performed using different techniques that are classified into three main types:chemical, physical and biological ones[4]. The first two types are used widely and have their own advantages and disadvantages. Unlike these two techniques, the biological method is known to be safer and more environmental friendly. In this technique, nanoparticles are produced by the aid of the reduction ability of the microorganisms without releasing any harmful by-products in the environment[5]. Unlike the chemical method of nanoparticles production, in the biological technique the produced nanoparticles are clean and the toxic by-products are not precipitate on the surfaces of the nanoparticles[5].

It was reported that the biologic nanoparticles are produced bythe active and passive mechanisms. In the active mechanism, the microbial attached or secreted enzymes such as NADH dehydrogenase and nitrate

reductase reduce the toxic ions into the nanoperticles. In the passive mechanism, the nanoparticles are produced by the aid of the functional groups of the microbial secreted proteins and polysaccharides such as amides, aldehydes, carboxyls, and ketones. Although the presence of both mechanisms is confirmed but the nature and activity of the produced nanoparticles are not compared with each other[5, 6].

It was reported that different types of microorganisms such as some bacteria, fungi and algae can produce the nanoparticles[5]. In order to prevent the risk of the pathogenic microorganisms usage it is important to choose the generally recognized as safe (GRAS) microbial strains for this aim. The alternative method for nanoparticles production is the passive mechanism and the use of the inactive microbial biomass [6]. One GRAS microbial strain that is used widely for nanoparticles production is *Fusarium oxysporum*[7–10].

Due to the lack of enough knowledge about the differences between the nature and activity of the nanoparticles that are produced by the active and passive mechanisms, recent research attempts to analyze the ability of this fungal strain to produce the silver nanoparticles (SNPs) by its active and inactive biomass forms and compared the shapes, size, amount, antibacterial activity, cytotoxicity and apoptosis induction of the produced nanopaticles with each other.

Methods

Fungal cultivation

In order to produce SNPs, *F. oxysporum*(PFCC 238-21-3) was purchased from
Pasteur Institute of Iran and cultured in Sabouraud Dextrose Broth (SDB, Merck, Germany) medium at 30°C for 3 days. The fungal biomass was collected by the aid of the centrifugation at 6000 rpm for 10 min and the obtained biomass was washed three times using ddH_2O. The biomass was weighted and equally divided into four separated flasks and undergone the pretreatments[11].

Biomass pretreatments

Before SNPs production by different active and passive mechanisms, each flask was pretreated separately. For active biomass preparation, one flask was placed in the refrigerator at 4°C for 12h and the other one was placed at the room temperature (RT) for 12 h. For inactive biomass preparation, one flask was placed in the autoclave instrument and sterilized at 15psi; 121°C for 15 min and the other one was placed in the hot air oven instrument and heated at 180°C for 15 min. Finally the biomass in all the flasks was floated in 10ml of ddH_2O and used for further studies.

SNPs production

In order to produce the SNPs, 10 µl of 1 M of silver nitrate solution (Sigma Aldrich, USA) with the final concentration of 1mmol was added to each flask. The flasks were placed in a shaker incubator at 37°C,

200 rpm for 24h. The negative control flask which contained ddH_2O and 1 mmol final concentration of silver nitrate solution was incubated with the others[12, 13].

Characterization of the produced SNPs

In order to prove the formation of SNPs, the content of each flask was separately analyzed using the following methods:

Spectrophotometer analysis

If the SNPs are produced, the color of the reaction mixture is turned from yellow to dark brown due to the surface plasmon resonance (SPR) of the produced nanoparticles. Moreover the color changed reaction mixtures have the maximum absorbance peak around 400-450 nm. Therefore each sample was analyzed using spectrophotometer instrument in the presence of ddH_2O as the blank. The used wavelengths were from 350-600 nm[14, 15].

Transmission electron microscopy (TEM)

The size and shape of the produced SNPs for each sample was analyzed using TEM. In this test the shape and size of the SNPs were revealed. Ten µl of each sample was placed on the carbon coated cooper grid and after 20 seconds excess of the sample was removed and all the grids were put to dry. Finally, the photographs were obtained using Philips 420T TEM[14, 16].

X□ray diffraction analysis (XRD)

In order to prove the presence of the SNPs in each sample, XRD was performed. The freeze-dried powder of each sample was obtained and analyzed by the aid of XRD-6100X-ray Diffractometer. The measurements were from10°–80° at 2°θ[17, 18].

SNPs purification

The produced SNPs in all four flasks contained some microbial culture impurities and undergone washing process. For this aim the obtained colloidal SNPs were washed using ddH_2O and centrifuged at 14500 rpm for 30 min. Each pellet was suspended in ddH_2O and the process was done trice. Finally, each sample was freeze-dried and 1mg of each was suspended in 1ml of phosphate buffered saline (PBS) and used for inductively coupled plasma (ICP) measurement[19].

Determination the SNPs concentration

Each colloidal SNPs sample (1mg/ml) was used for ICP analysis using ICP-OES 730-ES instrument in the presence of the standard graph of silver and the amount of the SNPs in each suspension was determined[19].

Antibacterial activity test

In order to analyze the differences in the antibacterial properties of four different SNPs samples (1mg/ml) that were produced by the active and passive mechanisms, plate well diffusion method was conducted. Three bacterial strains were purchased from Pasteur Institute of Iran and used for this analysis. The bacterial strains were *Staphylococcus aureus*(ATCC 29213) as a Gram positive bacterium, *Pseudomonas aeruginosa*(ATCC 27853) and *Escherichia coli* (ATCC 25922) as Gram negative bacteria. A single colony of each bacterial strain was dissolved in 1mL of normal saline and its turbidity was adjusted to 0.5 McFarland standards. By the aid of a sterile swab each bacterial strain was cultured on the surface of the Mueller–Hinton Agar (Oxid,UK) entirely and five wells (6 mm in diameter) in each plate was made. Each well was loaded with 50 µl of the produced SNPs that were pretreated in different conditions. The fifth well was loaded with 50 µl of the 1mmol final concentration of the silver nitrate solution. Plates were incubated at 37°C for 24 h and the tests were performed thrice. Finally, the diameters of the obtained inhibition zones were measured and data was analyzed by the aid of one way analysis of variance (ANOVA) in SPSS software version 22[20, 21].

MTT assay

HeLa cell line(the cervical cancer cell line),was purchased from Pasteur Institute of Iran and used for MTT assay. Dulbecco's Modified Eagle's medium (DMEM) which was enriched by 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin was used for culturing the cells in a 96 wells microtiter plate. The cell layer was washed with PBS and all the wells were loaded by 100 µl of the cell culture medium. The first well was field with 100 µl of the sterilized SNPs (1mg/ml) and after pipetting, 100 µl of it was transferred to the second one. Thiscontinued until 11^{th} well. 100 µl of this well after pipetting was discarded. 12^{th} well was a control and only loaded with 100 µl of the culture medium. The plate was incubated at 37℃ for 24 h in the presence of 5% CO_2. Ten µl of 3-(4,5-dimethylthiazol-2-yr)2,2,5-diphenyl tetrazolium bromidedye solution (5mg/ml) was added to all the wells and the plate was incubated in the cell culture condition for 4h. Then the dye was discarded and 200 µl of dimethyl sulfoxide (DMSO) was added and the plate shacked for 20 min in the dark condition. The optical densities (ODs) of the wells were achieved using ELISA reader instrument at the wavelength of 570 nm, the half maximal inhibitory concentration (IC_{50})of the SNPs was achieved and the cell viability percentage was determined[17].

Apoptosis induction assay

In order to analyze the apoptosis induction, the cells were cultured in the six wells plate (500000 cells/well) and Annexin V-FITC/propidium iodide assay kit was used. The cell streated with the determined IC_{50}doses of the SNPs solutions for 6 hours. One of the wells remained as a control and no SNPs were added. The cells were treated by the reagents of the kit according to its instruction and were analyzed using flow cytometry instrument and FlowJo™ V10.6.1 software. Viable (Annexin V− PI−), necrotic (Annexin V− PI+), and apoptotic (Annexin V+ PI+) cells percentages were obtained[22].

Results

After incubation, the fungal biomass was obtained and collected. Fig 1 shows the obtained fungal biomass in the culture medium.

Biomass pretreatments

As it was indicated in the materials and methods, the biomass was divided into four separated flasks and each flask was pretreated separately; two of them were used for active (i.e. one placed at the RT and the other one was placed in the refrigerator) and two were used for passive mechanisms (i.e. one was placed in the autoclave and the other one was placed in the hot air oven) of SNPs production. Fig 2 shows the biomass in the four different tested flasks.

SNPs production

After addition of 1mmol final concentration of silver nitrate solution to each flask and incubation, the color of all the tested four flasks was turned from yellow to brown. Different intensities of the color were obtained which indicated the differences in the amounts, shapes or other characteristics of the SNPs. All the solutions were stable in the environment for at least 6 months. Fig 3, shows the four different tested flasks after SNPs production.

Characterization of the produced SNPs

Spectrophotometer analysis

All the color changed flasks were analyzed using visible spectrophotometer. Before the analysis all the samples were diluted 1:10. Results showed that although all the flasks had maximum absorbance peaks about 400-450 nm but amount of the optical density (OD) and the position of the maximum absorbance peak were different. The maximum absorbance peaks for the SNPs that were pretreated at RT, and refrigerator, autoclave and hot air oven instruments were at 425 nm, 449 nm, 400 nm and 445 nm, respectively with different ODs. The maximum and minimum ODs were for the SNPs which were pretreated using hot air oven and refrigerator instruments, respectively. The negative control flask remained unchanged. Fig 4, shows the obtained spectra for all the four tested samples.

Transmission electron microscopy (TEM)

The sizes and shapes of the produced SNPs for the samples were analyzed using TEM. The average sizes of the SNPs were different which indicated the differences in the production methods. The average sizes of the SNPs that were pretreated at RT and refrigerator, autoclave and hot air oven instruments were about 35 nm, 39 nm, 34 nm and 36 nm, respectively.

Totally, the polygonal, round, oval and triangular shapes of the produced SNPs were seen. Polygonal and triangular shapes of the SNPs were seen in the flask which before SNPs production was incubated in the refrigerator and round and oval shapes of the SNPs were seen in the flask that was incubated at RT. Fig 5 shows the obtained TEM photographs in four tested samples.

X-ray difraction analysis (XRD)

XRD results for all the four flasks revealed the presence of four distinct peaks corresponded to the cubical structure of the elemental silver. Fig 6 shows the achieved XRD spectrum for the sample that was pretreated at RT.

Determination the SNPs concentration

Before further experiments, the SNPs concentration for each freeze-dried sample was determined using ICP-OES 730-ES instrument. Table 1 shows the obtained amounts of the samples. Briefly, the highest concentration of the SNPs was calculated in the sample that was pretreated in the hot air oven and the lowest one was for the sample that was pretreated in the refrigerator.

Table1 The obtained SNPs concentration for each sample using ICP-OES 730-ES instrument.

(Wavelength (nm): 328.068, Element: Ag (1mg/ml	Sample
(Concentration (ppm	
0.00	(Blank (ppm
3.20	Pretreated in the hot air oven
2.63	Pretreated in the refrigerator
3.52	Pretreated in the RT
3.58	Pretreated in the autoclave

Antibacterial activity test

In order to analyze the differences in the antibacterial properties of the four different SNPs samples that were produced by the active and inactive biomass samples, three bacterial strains, *S.aureus*, *P.aeruginosa*, and *E.coli,* were used. The tests performed *thrice* and the diameter of the obtained inhibition zones measured and data analyzed by the aid of ANOVA program using SPSS software version 22.The p value< 0.05 was considered as significance. Table 2 shows the obtained results.

Table2 The antibacterial activity results of the four test samples and silver nitrate solution as a control. The tests were performed *thrice*.

Inhibition zones (mm) of the SNPs against the tested (bacterial strains (Mean±SD			The produced SNPs after different pretreatments
	S. aureus	P.*aeruginosa*	*E.coli*
10.50±0.94	11.00±1.00	8.00±0.47	**Hot air oven**
10.50±0.25	7.00±0.62	10.00±0.75	**Refrigerator**
11.00±0.33	11.00±0.20	11.00±0.86	**RT**
11.00±0.62	11.00±0.60	10.00±0.75	**Autoclave**
11.00±0.67	12.00±0.64	12.50±0.62	**Silver nitrate**

The ANOVA results showed that in the tested samples that were used against *E.coli*, there was no significance difference between the SNPs which their flasks before production were placed in the refrigerator and the autoclave (p value > 0.05) but significance differences were seen between the other tested samples (p value < 0.05).

In the tested samples that were used against *P. aeruginosa*, there was no significance difference between the SNPs which their flasks before production were placed in the hot air oven, autoclave and RT (p value > 0.05) but significance difference was seen between these groups and the sample which was pretreated in the refrigerator (p value < 0.05).

In the tested samples that were used against *S. aureus*, no significant differences were seen between the tested samples (p value > 0.05).

MTT assay

In order to analyze the toxic effects of the SNPs samples, MTT assay performed and the cell viability percentage determined. IC_{50} for each sample was obtained and results indicated that all the four tested samples induced dose dependent toxic effects. IC_{50s} for the samples of the flasks which before SNPs production were pretreated with the hot air oven, autoclave and at RT were in the third well (0.125 mg/ml of SNPs), and for the sample of the flask which before SNPs production was pretreated with the refrigerator was in the second well (0.5 mg/ml of SNPs). Hence the amounts of the SNPs in theirIC_{50} dose based on the ICP obtained results were 0.40, 0.45 and 0.66 and0.44ppm for the samples were pretreated with hot air oven, autoclave, refrigerator and at RT, respectively. Fig 7 indicated the cell viability percentages of the cells based on the MTT assay results.

Apoptosis induction assay

Annexin V-FITC/ propidium iodide assay kit was used for apoptosis induction assay and the cells were incubated with four different types of SNPs at their IC_{50} doses for 6 h. More than 96% of the cells in the control well were alive and in contrast to the control, the biologically produced SNPs induced more apoptosis and less necrosis in the cells after 6 h of incubation. Fig 8 indicated the obtained results.

Discussion

Nowadays there is an interest in the use of thenontoxic method of nanoparticles production which does not produce any harmful by-products in the environment and on the surface of the produced nanoparticles[23]. As it was mentioned previously, there are many different microorganisms that can produce different types of nanoparticles such as gold, silver, titanium, iron and etc. The used microorganisms are usually chosen from the non-pathogenic ones but in some studies it was reported that the pathogenic microorganisms have been used in the reduction process[5].

Some of the studies deal with the intracellular but many of them deal with the extracellular production of nanoparticles. The extraction of nanoparticles that were produced by the second method is easier hence many of the researches have used the extracellular production method[5]. In the extracellular production technique there are two different active and passive mechanisms for nanoparticles production. The first one is performed by the act of the microbial secreted enzymes and the second one is done by the act of the functional groups of different types of the proteins, polysaccharides and etc., that are present in the microbial culture[6].

Based on this introduction recent research has analyzedthe production of SNPs by the aid of the active and inactive biomass of the microbial strain, *F.oxysporum*, to answer these questions: can the inactive biomass instead of the active one be used in nanoparticles production? And if the nanoparticles produce, will their nature and activity be different from the ones that are produced from the active biomass form?

If the inactive biomass like the active one can produce the nanoparticles, in order to decrease the risk of the live pathogenic microorganism usage that may have an impact on human health the utilization of the inactive biomass will be preferred. Furthermore the industrial application of this method will be preferred due to the use of the inactive biomass without the requirement of any sterile condition [4].

In the first phase of the current study, different pretreatment conditions were used, SNPs were produced and their production was confirmed. For this aim *F.oxysporum*was purchased and used for nanoparticles production. This type of fungal strain is known to be safe and due to its strong secretary systems which are responsible for secreting different types of enzymes, polysaccharides, proteins and etc., to the microbial external environment it has been used for the nanoparticles production aim[5]. Before the SNPs production the biomass was treated by different treatment procedures to obtain the active and inactive forms of the fungal biomass. For obtaining the inactive biomass forms harsh conditions such as the application of high temperatures that inactivated the biomass (i.e. the autoclave and hot air oven instruments) which are used for sterilization process were used. For active biomass pretreatment low temperature such as using refrigerator and RT were utilized.

The samples were subjected to SNPs production and after incubation with the silver nitrate ions all of them had a capacity of SNPs production. The obtained SNPs were studied through visible spectrophotometer, TEM and XRD analyses. The visible spectra obtained results from all the four tested samples revealed that the SNPs were produced. The obtained peaks were between 400-450 nm. For the

sample that was pretreated in the refrigerator it was 400 nm and for the one that was pretreated in the autoclave instrument it was 445 nm. The TEM results showed that the shapes and sizes of the produced SNPs were alike and XRD analysis showed the presence of the elemental silver in all of the four tested samples. TEM images revealed that SNPs due to the presence of capping proteins on the surfaces of the produced SNPs were well separated from each other[4].

Therefore all the used analyses showed that the active and inactive forms of the fungal biomass had the ability of SNPs production. In order to compare the concentrations of the produced SNPs that were obtained by those two mechanisms, ICP measurement was applied. Results showed that although all the samples contained SNPs but the sample which was pretreated in the refrigerator contained lower amounts of SNPs than the others which indicate that for nanoparticles production the use of the active biomass is not always show acceptable results.

In the second phase of the experiment we have tried to answer that do all the SNPs that were produced by both active and passive mechanisms of bio-production have the same nature and activity?

Although there are some studies about the biologic method of nanoparticles production but there are not any studies about the comparison between the characteristics, nature, activity and the effects of the produced nanoparticles by active and passive mechanisms of bio-production. For example Binupriya et al in 2010 used inactive cell filtrate of *Rhizopusstolonifer* for production of silver and gold nanoparticles. They have used autoclave for inactivation the biomass and the obtained biomass produced both types of the above mentioned nanoparticles[4]. Also they have used the inactive biomass of *Aspergillusoryzae* and produced silver and gold nanoparticles successfully[24].

Sneha et al in 2010 have used both of the active and inactive forms of *Corynebacterium glutamicum* biomass for production of SNPs and they have shown that in contrast to the active form, the inactive biomass could produce much more amounts of SNPs. The inactive form of the biomass was prepared using autoclaving. The color intensities and the ODs of the SNPs that were produced using inactive biomass were higher than the active one which was due to higher SNPs content of the reaction mixture[3].

Salvadori et al in 2013 discovered that the dead biomass of *Hypocrealixii* produced copper nanoparticles and it was used for bioremediation of copper. They concluded that the dead biomass had a good capacity in nanoparticles production[25]. Again in 2014, Salvadori et al have showed that the dead biomass of fungus *Aspergillus aculeatus* could produce nickel oxide nanoparticles. They showed that the fungal reducing agents were responsible for this bio-reduction process[26]. All these studies confirm the passive mechanism of bio-production could produce the acceptable amounts of nanoparticles but what about the nature and activity of the nanoparticles? It is not fully searched. Hence we have analyzed the cyotoxocity, apoptosis induction and antibacterial assays of the SNPs that were produced by both active and passive mechanisms of bio-production.

Results obtained from antibacterial assay showed that the antibacterial activity of the SNPs would be different based on the used microbial strain. Therefore antibacterial activity does not depend on the nanoparticles bio-production method. Moreover in the antibacterial testing we must consider the amounts of the SNPs that were produced by each sample. The ICP measurement results showed that the amounts of the SNPs that were produced by the active biomass in one situation (i.e., the sample that was pretreated in the refrigerator) were lower than the ones that were produced by the inactive biomass. This sample induced the lowest cytotoxicity and apoptosis induction. Incubation of the sample in the refrigerator before the SNPs production caused less release of enzymes and other reducing agents from the biomass that may lead to lowering the SNPs production ability. In contrast to the refrigerator pretreatment, heating the biomass with the sterilizing instruments caused better release of the reducing agents from the fungal biomass which led to higher ability of SNPs production.

Spectrophotometer results showed that the SNPs that were produced by the inactive biomass forms showed higher ODs than the active ones. These results confirmed that the application of the SNPs which are produced by the inactive biomass forms will have the same capacity in their antibacterial activity with the ones which are produced by the active biomass forms. Because there were minor differences in the amounts of the produced SNPs by the samples they induced the same antibacterial activity. In this test the silver nitrate in contrast to the SNPs group had the same and higher activity against all the tested bacterial strains. It means that the bio-reduction of the harmful silver nitrate ions to the SNPs leads to production of less toxic SNPs which is the major key aim of this reduction in the nature.

These results aligned with the cytotoxicity test results which showed that all the four tested samples induced dose dependent toxic effects and except of the sample which was before SNPs production pretreated in the refrigerator, all the samples had the same IC_{50s}. When the samples were analyzed for the apoptosis assay at their IC_{50s} all of them induced more apoptosis and less necrosis in the cells after 6 h of incubation which were largely the same for all the samples. It was previously showed that the cytotoxicity effect of the non-biologically produced SNPs was due to the induction of reactive oxygen species (ROS) which was associated with apoptosis and necrosis[27]. Foldbjerg et al in 2009 showed that the incubation of the non-biologically produced SNPs with the human lung carcinoma cell line (A549) induced the above mentioned responses and showed that these effects depend on the time of exposure and dose of the used SNPs. They showed that more amount of necrosis yielded after application of more incubation time and higher doses of SNPs [27]. Lee et al in 2014 showed that the non-biologically produced SNPs induced ROS and apoptosis in the NIH 3T3 cell line. They showed that SNPs reduced the viability of the cells in a dose dependent manner and in the first 12 h more apoptosis in contrast to the necrosis was seen. But similar result was seen after 24 h of incubation the cells with the SNPs [28]. Kumaret al in 2015 showed that not only the time of exposure and dose of the non-biologically produced SNPs but also the size of the SNPs effected on the balance between amount of the necrosis and apoptosis. They showed that for 10nm non-biologically produced SNPs with the dose of 50µg/ml more apoptosis and less necrosis was seen after 4h and this balance inverted after 24h of incubation [29]. Hence altogether the biologically produced SNPs with the sizes about 35 nm induced more

apoptosis and less necrosis after 6h of incubation with HeLa cell line that is similar to the behavior of the non-biologically produced ones which confirmed induction of ROS after their usage *in vitro*. It is recommended to use different doses (not only the IC_{50} one), different sizes and different time of exposure to analyze their exact behavior in future. Our results showed that the SNPs that were produced by both of the inactive and active biomass forms induced the same apoptotic and necrosis effects *in vitro*.

Recent study conducted to confirm that the nanoparticles can be produced by the active and inactive microbial biomass forms without any alternations in their activity. Hence for SNPs production when the pathogenic microbial strain is utilized, the inactive biomass form can be replaced and used for nanoparticles production without any doubts in the reduction of the nanoparticles activity. It is recommended to use the other microbial strains and the other pretreatments to confirm the recent results.

Conclusions

In conclusion, the active and inactive biomass forms of *F.oxysporum* could successfully produce SNPs that the nature and activity of the produced nanoparticles were not altered. Therefore using the inactive microbial biomass form is recommended to ensure its safety.

Declarations

Compliance with Ethical Standards:

Funding

This study did not have a specific fund.

Conflicts of interest

All of the Authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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Figures

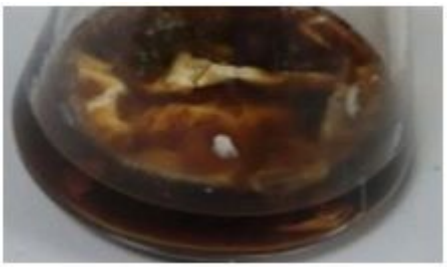

Figure 1

F. oxysporumbiomass in the culture medium

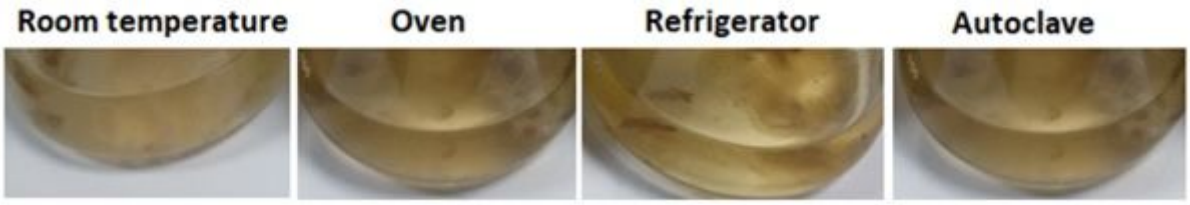

Figure 2

Four tested flasks that were pretreated in different conditions; flasks that were placed in the refrigerator, autoclave, hot air oven instruments and the flask that was placed at the room temperature.

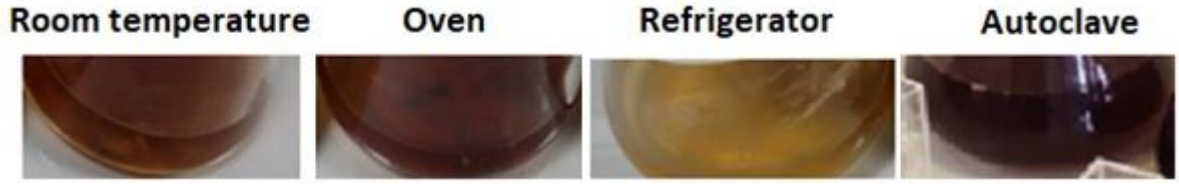

Figure 3

Four tested flasks after SNPs production. Different intensities of the color were seen in the flasks.

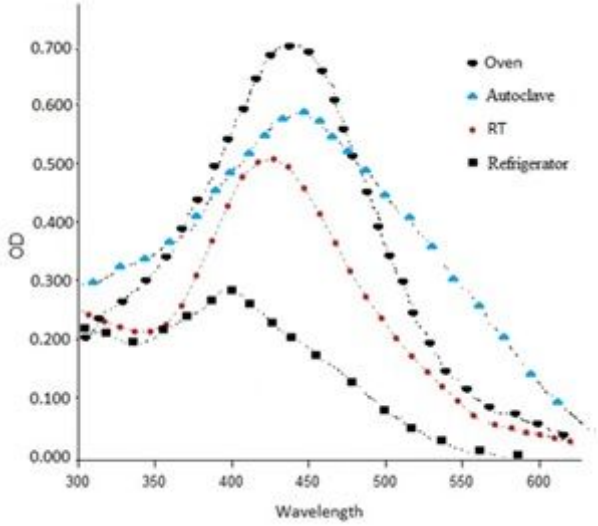

Figure 4

Visible spectra that were obtained from four different tested samples. The maximum absorbance peak for each sample was different and shows in this figure. The maximum and minimum ODs were for the SNPs that were pretreated using hot air oven and refrigerator instruments, respectively. The samples were diluted 1:10.

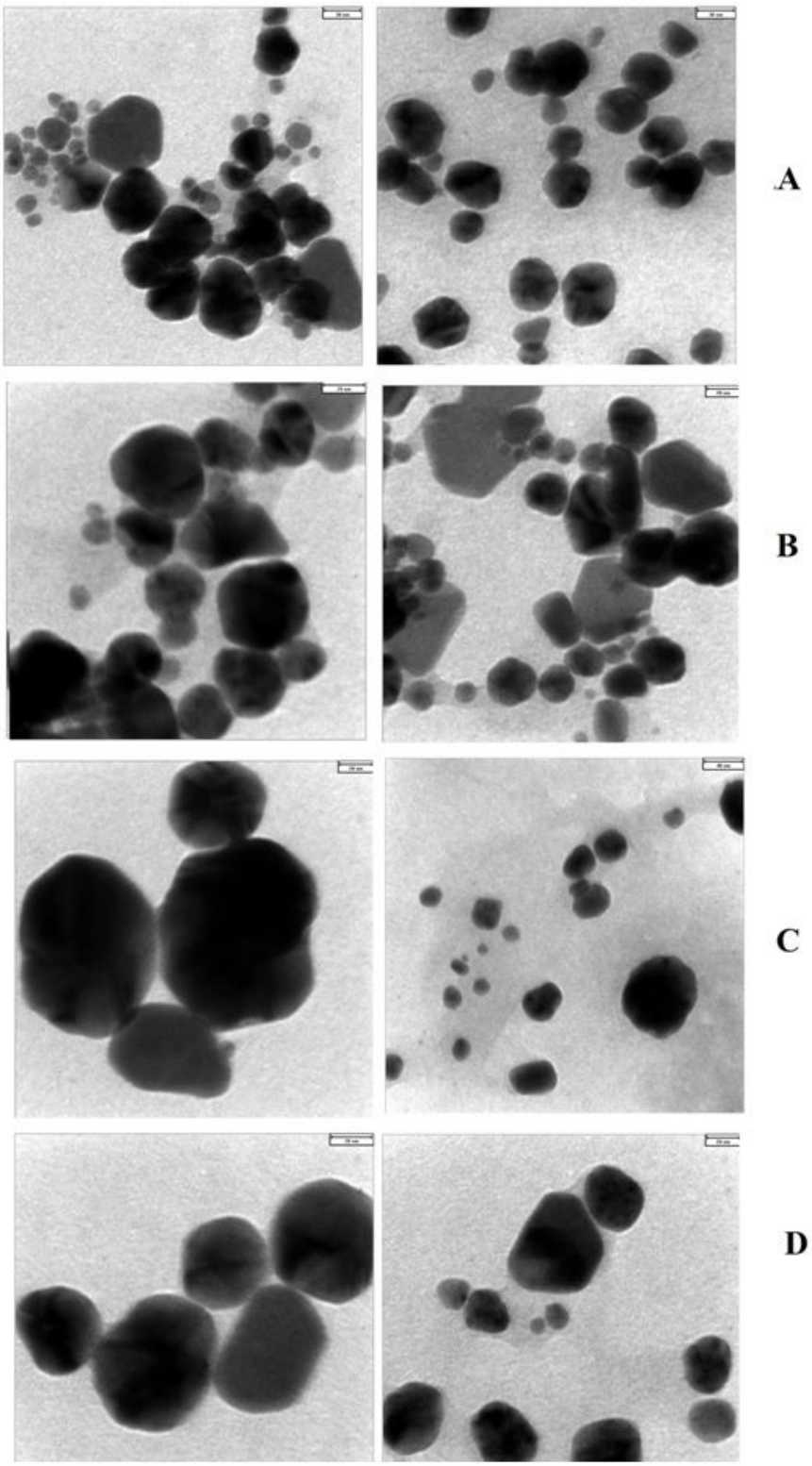

Figure 5

The obtained TEM photographs of four tested samples. A. sample of the flask which before SNPs production was incubated in the hot air oven. B. sample of the flask which before SNPs production was incubated in the refrigerator. C. sample of the flask which before SNPs production was incubated at RT and D. sample of the flask which before SNPs production was incubated in the autoclave.

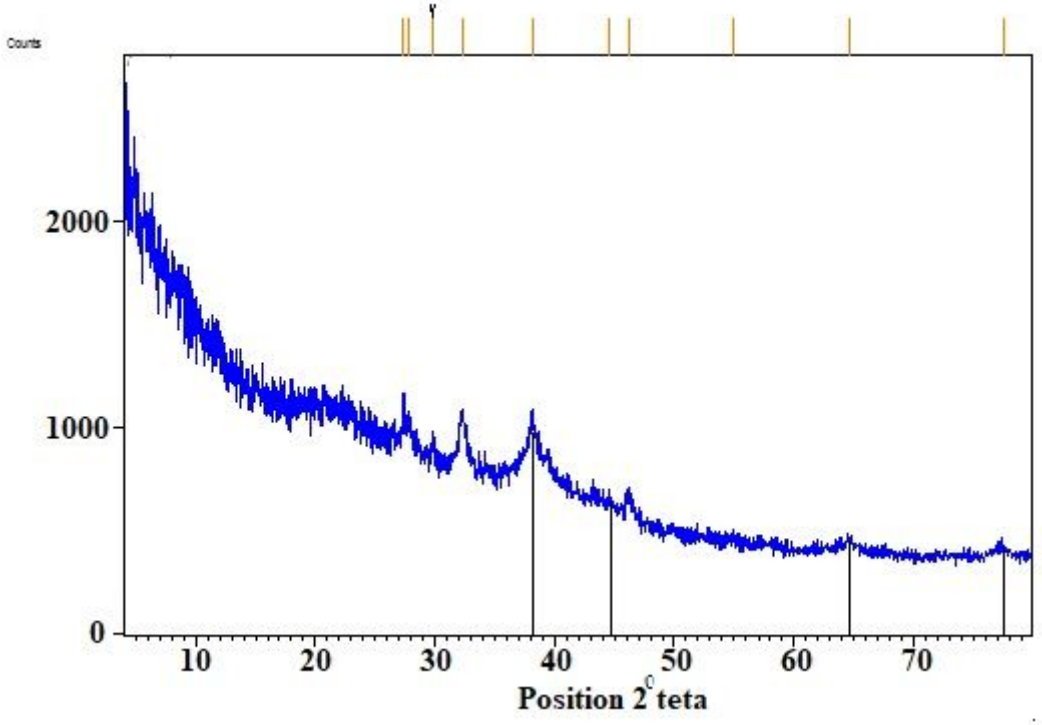

Figure 6

The achieved XRD spectrum that was obtained from the RT pretreated sample.

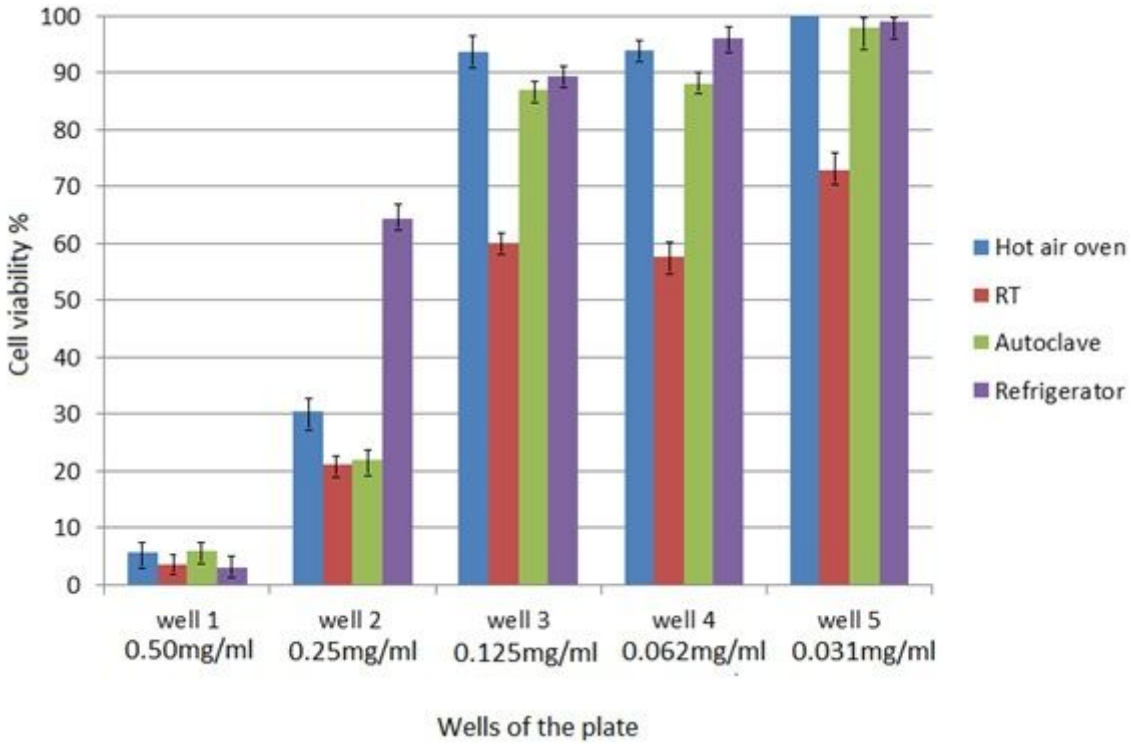

Figure 7

The cell viability percentages of the cells based on the MTT assay results.

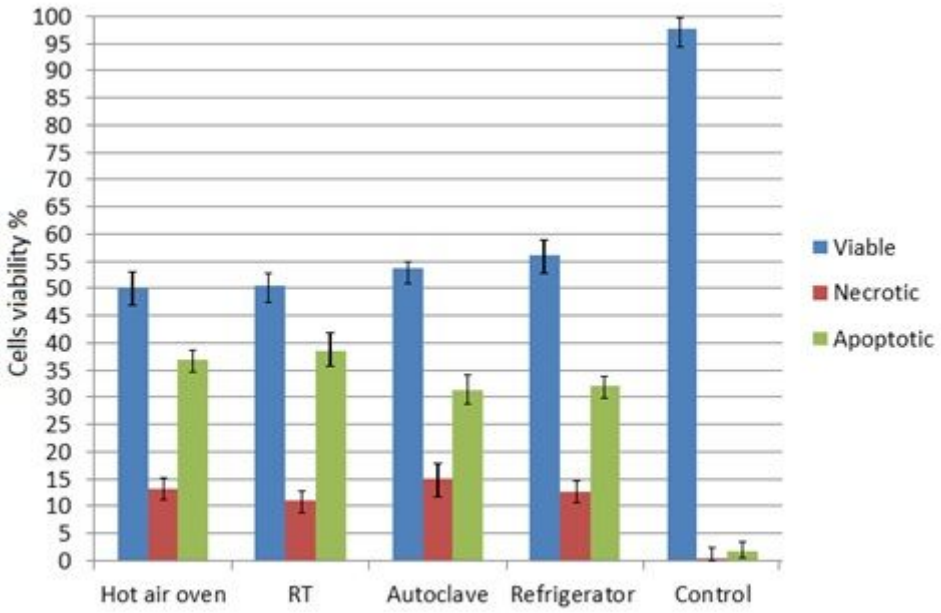

Figure 8

Apoptosis induction assay of the cells and the obtained cell viability percentages for the four SNPs and the control samples.