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Research article

Biosynthesis of zinc nanoparticles and its effect on enzymes production from *Chaetomium globosum* using different agricultural wastes

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Abstract

Biosynthesis of zinc nanoparticles is one of the environmental eco-friendly. *Chaetomium globosum* isolated from healthy bean plant and identified by 18 S. Different agricultural wastes were used for production of xylanase, pectinase and chitinase in media containing zinc nitrate as nanoparticles. Olive mill wastes were most promising in zinc nanoparticle produce at different pH values (6.0, 8.0 &10). The mean diameter of zinc nanoparticles at pH 8.0 was 18.06nm. Antifungal activity of zinc nanoparticles were most promising on pathogenic fungi at pH 8.0 followed by pH 6.0 &10.0.

Introduction

Agricultural wastes used as carbon source by different fungal strains to produce ethanol, proteins, and microbial enzymes [1]. Biological synthesis for nanoparticles produce from microorganisms by standardization of their circumstances in culture condition [2]. Nanoparticles are the building blocks of nanotechnology which play an important role in pharmaceutical, agricultural biocontrol and biotechnological industrial applications. Nanotechnology is the ability for controlling size, shape at nanometer scale for designing, application of structure and characterization of it [3]. Nanoparticle was prepared for increasing benefit of green synthesis for decreasing cost and safe strategies of environment [4]. Biosynthesis of metal nanoparticles is considered a new technique for nontoxic chemicals, solve environmental problems, production of renewable materials from yeast, bacteria, fungi and plant extract [5, 6]. The biological synthesis of nanoparticles from fungi is a significant branch, due to metal bioaccumulation capability and fungi's tolerance [7]. Filamentous fungi are the best biomimetic process for production of nanoparticles. This is done by secreting high amount of fungal protein in large scale by growing on surface of inorganic vectors [8]. Fungal strains have the ability of secreting different enzymes used in several applications [9]. Enzymes assay indicate the role of enzyme as reduction and shape direction. Applications of nanoparticles are due to their small size and large surface area. Synthesis of metal nanoparticles by the synthesis of physical and chemical process [10]. Fungi able to produce nanoparticles due to their metal bioaccumulation and toleration properties [11]. Synthesize of nanoparticles from silver, titanium, gold, fullerenes, zinc, carbon, palladium, aluminum, iron, and copper salts have been regularly utilized. Silver nanoparticles were used since 16th century, for both medical and staining aims [12]. Nanoparticles produced with high surface area, structures unusual size and crystal which produce an excellent stability or long shelf life with organic antimicrobial agents [13]. The aim of this work was production of zinc nanoparticles from fungi using different agricultural wastes as substrate and its antagonistic effects on pathogenic fungi.

Experimental

Microorganism

Chaetomium globosum was isolated from healthy bean plants and identified by 18S ribosomal RNA gene. Cultures were kept on potato dextrose agar media (DIFCO) PDA slants and at 4 ˚C.

Soil born fungal strains (*Fusarium solani, Rhizoctonia solani and Sclerotium rolfsii)* which are the causative agents of bean damping-off and root-rot diseases, were isolated from bean roots that showed the disease symptoms. The fungi in pure culture were identified after pathogenicity test according to the keys given by [14,15] affiliated to the Plant Pathology Department, National Research Centre, Giza, Egypt.

Identification of microorganism

Isolation of genomic DNA and 18S RNA sequencing

DNA extraction was done by using protocol of Gene Jet genomic DNA purification Kit (Thermo K0721) as following:

Genomic DNA was harvested up to $2x10^9$ fungal spores in a 1.5 or 2 ml micro centrifuge tube by centrifugation for 10 min at 5000 x g. This discard the supernatant was re-

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suspend the pellet in 180µl of digestion solution then 20µl of proteinase k solution and mixed thoroughly by vortex or pipetting to obtain a uniform suspension. Sample was incubated at 56°C while vortexes occasionally until the cells are completely lysed (~30 min). Add 20µl of RNase to the solution, mix by vortex then incubate the mixture for 10 min at room temperature. Then 200µl of lysis solution was added to the sample mixed thoroughly by vortex for about 15 second until a homogeneous mixture was obtained.

Add 400µl of 50% ethanol was added and mixed then transferred the prepared lysate to a Gene JET™ Genomic DNA Purification Column inserted in a collection tube, centrifuge the column for 1 min at 6000x g. Discard the collection tube containing the flow through solution. Place the Gene JET™ Genomic DNA Purification Column into a new 2 ml collection tube (included), then add 500µl of wash buffer I. Centrifuge for 1 min at 8000x g. Discard the flow through and place the purification column back into the collection tube. Add 500µl of wash buffer II to the Gene JET™ Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 x g). Discard the collection tube containing the flow through solution and transfer the Gene JET ™ Genomic DNA Purification Column to a sterile 1.5 ml micro centrifuge tube. Then add 80µl of elution buffer to the center of the Gene JET™ Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g.

PCR amplification of 18S *rRNA* gene

PCR amplification of 18S rRNA gene from purified DNA was done by using Maxima Hot start PCR Master Mix (Thermo) K0211using Primer set:

ITS1(5´TCC GTA GGT GAA CCT GCG G3´)
ITS4 (5´TCC TCC GCT TAT TGA TAT GC3´)

The reaction condition was as follows:

Initial denaturation 95◦C for 10 minute then 95◦C for 30 second, Annealing 55◦C for 1 minute and primer extension 72◦C for 1 minute followed by a final extension 72◦C for 15minute.

No. of cycles =35

PCR product purification

The unpurified DNA was purified using PCR clean up to PCR product using Gene JET PCR Purification Kit (Thermo K0701) by adding 45µl of binding buffer to complete PCR mixture. The mix was transferred from step one to the Gene JET Purification Column, centrifuged for 30-60 second at 12000xg then add 100µl of wash buffer to the Gene JET Purification Column for 1 min. Transfer the Gene JET Purification

Column to a clean 1.5 ml micro centrifuge tube then add 25 µl of elution buffer to the center of the Gene JET purification column membrane for 1 min. Discard the Gene JET Purification Column and store the purified DNA at -20◦C.

Sequencing

Sequencing to the PCR product on GATC company by use ABI 3730xl DNA sequenced by using forward and reverse primers.

Phylogenetic analysis

A phylogenetic analysis of the isolate was performed to determine how the 18S rRNA sequence of the isolate and related strain might have been derived during evolution. The evolutionary relationships among the sequences were depicted by placing them as outer branches on a phylogenetic tree. The branching relationships on the inner part of the tree reflect the degree to which different sequences are related.

Combining the traditional sanger technology with the new 454 technology, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. The analysis of nucleotide sequence was done in Blast alignment (www.thermoscientificbio.com).

Substrates

Different agricultural wastes : carrot peel, potato peel, apple peel and olive mill waste were used as sole carbon source at concentration 20 g/l. All wastes peel were washed, dried at 70 ˚C in oven and cut into small pieces before use.

Fermentation condition

Fermentation was carried out in 250 ml Erlenmeyer flask each containing 50 ml of fermentation media consist of (g/l): different agricultural waste (20.0), $NaNO_3$ (2.0), K_2HPO_4 (1.0), $MgSO_4$ (0.5) KCl (0.5), $FeSO_4$ (0.01 and autoclaved at 121˚C for 15 minutes. One ml of 10^6 spore suspension of the selected fungal strain was inoculated in each flask and incubated at 28-30 ˚C for 7 days at 200 rpm.

Preparation of zinc nitrate nano particles

The same fermentation media was carried out by replacing sodium nitrate 2.0 g/l by the same weight of zinc nitrate, under the same condition. pH of both media was adjusted at 6.5, 8.0 and 10.

Enzymes assay

Xylanase

Determination of enzyme activity was carried out according to the method of [16]. One ml of 1% birch wood xylan (Sigma) in acetate buffer (pH 4.6) in test tubes was added to one ml of the culture filtrate and mixed by shaking. The mixture was incubated in a water bath at 50˚C for 30 minutes, then cooled and centrifuged before assaying. The amount of reducing sugar was determined with 1 ml of 3, 5-

dinitrosalicylic acid (DNS). The colour was read at 540 nm using a spectrophotometer.
Unit of enzyme: one unit of enzyme activity was taken of the catalyst convert one micromole of substrate in one minute under specific condition.

Pectinase

Pectinase activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; pH 5.5) and suitably diluted crude enzyme, was incubated at 50°C in water bath for 30 min. The reaction was stopped with 1.0 ml dinitrosalycyclic acid solution [17] after which the mixture was boiled for 10 min and cooled. The colour was read at 540 nm using a spectrophotometer.
A standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µm glucose per min.

Chitinase

Colloidal chitin was prepared from chitin powder (Sigma Co.). Determination of enzyme activity was carried out according to the method of [18]. Take one ml of 1 % colloidal chitin in citrate phosphate buffer (pH 6.6) in test tubes. One ml of culture filtrate was added and mixed by shaking. Tubes were incubated in a water bath at 37°C for 60 minutes, then cooled and centrifuged before assaying. Reducing sugars were determined in 1 ml of the supernatant by 3,5-dinitrosalysilic acid (DNS). Optical density was measured at 540 nm. The colloidal chitin suspension was adjusted to pH 7.0 with 1 N NaOH and re-centrifuged. The pelleted colloidal chitin was stored at 4°C until used.

Characterization of zinc nanoparticles

UV visible spectral analysis

The bio reduction of zinc in suspension was observed by ultraviolet-visible spectroscopy (UV-Vis) of the solution between 200 and 500nm by using Perkin-Elmer LAMBDA 35 UV-Vis spectrophotometer (USA).

Transmission electron microscopy (TEM)

For the confirmation of size and shape, (TEM) measurements were carried out using drop coating method in which a drop of solution containing nanoparticles was placed on the carbon-coated copper grids and kept under vacuum desiccation till dryness. TEM and high-resolution (HR)-TEM micrographs of the sample were taken using the JEM-2100F TEM instrument. The instrument was operated at an accelerating voltage of 200 kV.

Comparison between antifungal activity of free and nano-zinc of *Chaetomium globosum* formulated on olive mill waste

Agar well diffusion method

Nano formulations of *Chaetomium globosum* grown on olive mill waste at different pH screened for antifungal activity by agar well diffusion method with sterile cork borer of size 5.0mm according to [19]. Five days old cultures grown on potato dextrose agar (PDA) were used for inoculation of fungal strain on PDA plates. An aliquot (0.02 ml) of inoculums was introduced to molten PDA and poured in to a petri dish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method 500 µl of free and nano particles solution, filled in deep blocks. Incubation period of 5 days at 28°C was maintained for observation of antifungal activity. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the solution and linear growth of pathogens. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates.

Results and Discussion

Morphological identification of the fungal isolate

The fungal isolate*Chaetomium globosum* was purified by making sub-culturing on the potato dextrose agar medium at regular intervals and incubating at 28◦C. The fungus was identified according to molecular identification, microscopic observation ,and colony morphology [20].

Identification and phylogenetic position of fungal isolate

Identification was done according to 18S rRNA gene sequencing and it was compared with the data present in NCBI. The BLASTn of the isolate was showing 100% similarity with *Chaetomium* sp. Figure (1) and Photo (1) showed the phylogenetic relation between the isolate and related fungi. The homology assay result proved that the isolate was in the phylogenetic branch of *Chaetomium globosum*.

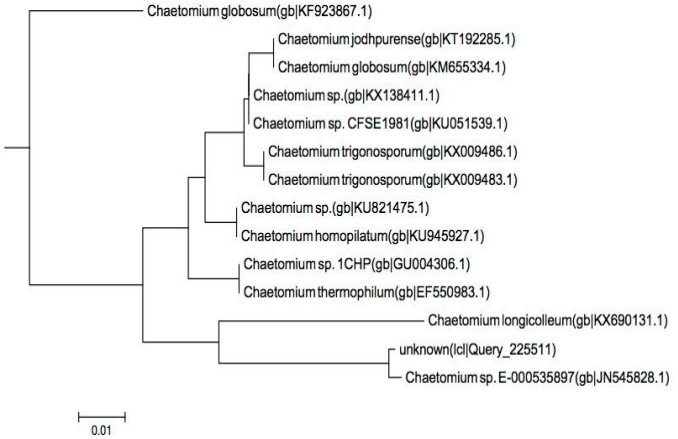


Figure 1. Phylogenetic tree of *Chaetomium globosum* based on 18S ribosomal RNA gene sequencing showing homology with *Chaetomium sp.*

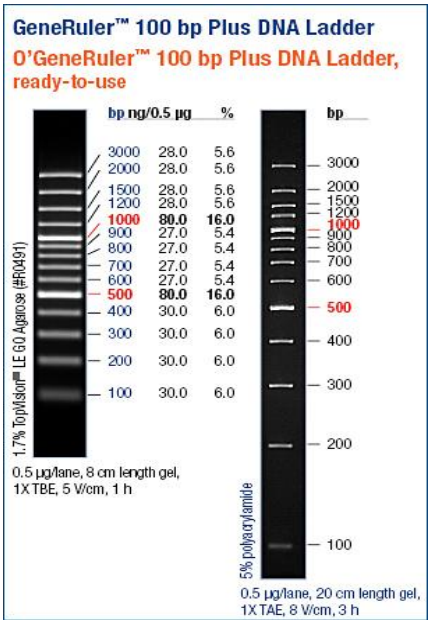


Photo 1. Gene ruler of the fungal strain *Chaetomium globosum*

Comparison between production of xylanase, pectinase and chitinase free and nano- media by using different agricultural wastes

Chaetomium globosum was tested for its ability for production of xylanase, pectinase and chitinase in Czapek, s Dox fermentation media one contain $NaNO_3$ as nitrogen source while the other contain $ZnNO_3$ instead of $NaNO_3$ for nanoparticle production. Results in Table (1) showed that enzymes production were high in media containing $ZnNO_3$ for nanoparticle production. Olive mill waste in nano-media produce higher enzymatic activity for all enzymes, 96.98 U/ml for xylanase, 54.28 U/ml for pectinase then 28.50 U/ml for chitinase, followed by apple peel for xylanase and pectinase while potato peel for chitinase. Other wastes showed moderate to low activity. Fungi are tolerant and have the ability to make metal bioaccumulation, thus they are more attracted on biological production of metallic nanoparticles [21]. The effective secretors of extracellular Fungi have the ability to produce metal nanoparticles and nanostructure by the reduction of enzyme extracellularly or intracellularly and the production of biomimetic mineralization [23,24]. The biosynthesis of metal nanoparticle by the fungal strain *Aspergillus fumigatus* TFR-8 extracellular enzymes production which convert the metal salt of macro and micro scale to nano-scale diameter by catalytic effect [25]. The biological syntheses of zinc nanoparticles are safe, cheap because they are capping agent of naturally fungal protein [26]. Synthesis of extracellular enzymes by nanoparticles from *F. oxysporum* has been conducted production of different forms and size variety [24].

UV-Vis spectroscopy

Results in Figure (2, 3, 4 & 5) showed that different wave length was determined with different agricultural wastes. The strong surface plasmon resonance occur at λ max 238 nm in media containing olive mill waste which is considered an ideal wavelength for nano-zinc at pH 6.0 nanoparticles colloidal solution.

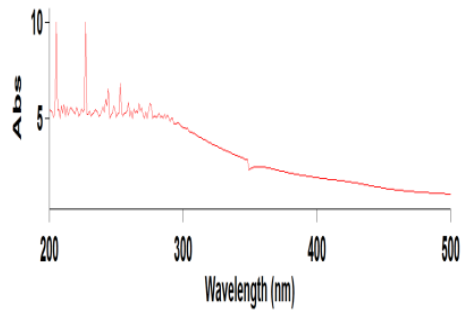


Figure 2. UV-Vis spectra of Apple peel

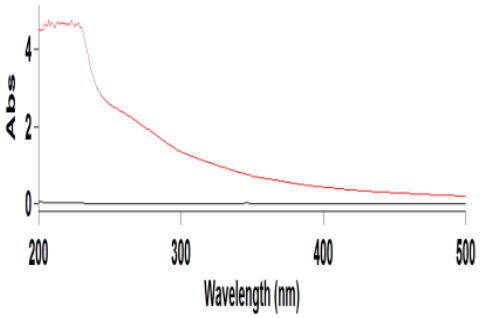


Figure 3. UV-Vis spectra of Carrot peel

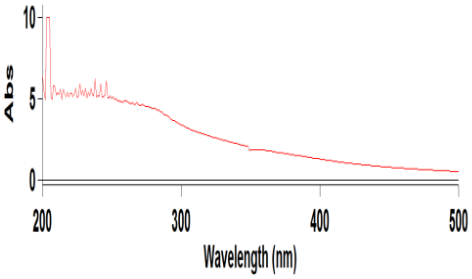


Figure 4. UV-Vis spectra of Olive mill waste

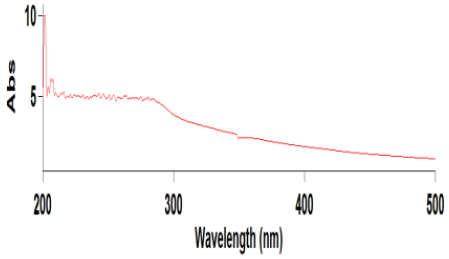


Figure 5. UV-Vis spectra of Potato peel

Table 1. Study the effect of nano-zinc using different agricultural wastes on enzymes production.

Different agricultural wastes	Enzymes activity (U/ml)					
	Xylanase		Pectinase		Chitinase	
	$NaNO_3$ (Free)	$ZnNO_3$ (Nano)	$NaNO_3$ (Free)	$ZnNO_3$ (Nano)	$NaNO_3$ (Free)	$ZnNO_3$ (Nano)
Apple peel	67.32	52.44	25.93	12.83	15.20	6.33
Carrot peel	59.64	44.89	10.70	5.28	8.40	3.75
Olive mill waste	86.55	96.98	37.38	54.28	15.87	28.50
Potato peel	70.10	41.30	31.20	6.40	18.50	8.35

Transmission electron microscope

Transmission electron microscope (TEM) was used for detection of purity and particle size. Different pH values (6.0, 8.0 and 10.0) were adjusted in fermentation media containing olive mill waste as carbon source. Results in figure (6, 7 & 8) showed that spherical and granule morphology of zinc nanoparticles were required specially at pH 8.0 the particle size was 18.06 nm. Zinc nanoparticles produce spherical and unique distribution particles [27].

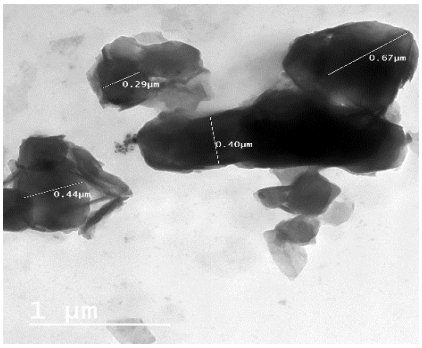


Figure 6. TEM of zinc nanoparticles at pH 6.0

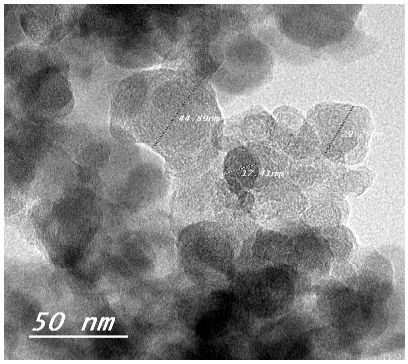


Figure 7. TEM of zinc nanoparticles at pH 8.0

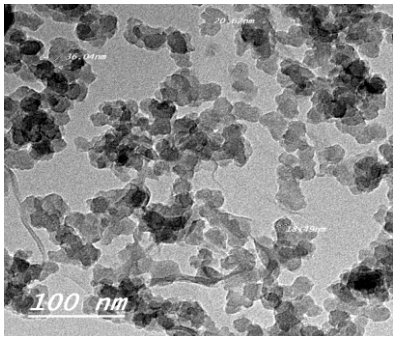


Figure 8. TEM of zinc nanoparticles at pH 10.0

Comparison between antifungal activity of free and nanoparticles of *Chaetomium globosum* formulated on olive mill waste

All fungi tested were inhibited to various extents by different nanoformulations of $ZnNO_3$ with *Chaetomium globosum* grown on olive mill waste. Table (2) OMW formulated as nanoparticles showed highest antifungal activity than all treatments exhibited highest inhibition against *Fusarium solani, Rhizoctonia solani* and *Sclerotium* rolfsii as shown by the zone of inhibition (17.0,15.0 and 13.0 mm) respectively. In cases of nano synthesis by *Chaetomium globosum* at different pH showed the highest effect on reduction on linear growth of all tested fungi with treatment by OMW at pH8 followed by pH 6 and pH 10.

ZnO-nanoparticles showed antifungal activity, ZnO-NPs synthesized by microwave method have antifungal activity against plant fungus *Pythium debarynum* in concentration about 10 mm [28]. The fungicidal from zinc nanoparticles inhibit the growth of *Penicillium expansum* and *Botrytis cinerea*, conduct metric in high concentrations (100 mg mL^{-1}) [29].

Zinc compounds are nontoxic, cheap and stable because they have advantages in comparison to other antifungal compounds. Utilization of adequate amounts, and low concentrations have a great antifungal activity. For those features Zn-compounds being the bests with $ZnSO_4$ and Zn $(ClO_4)_2$ treatments, which had stronger anti mycotoxins properties. The modifications of fungal hyphae were observed. The Zn-compounds applications impeded in the cellular metabolism of the fungi. Morphological alterations, hyphae, conidial and reduction mortality [30, 31].

Conclusion

Chaetomium globosum isolated from healthy bean plant and identified by 18 S. Different agricultural wastes were used for production of xylanase , pectinase and chitinase in media containing zinc nitrate as nanoparticles . Olive mill wastes were most promising in zinc nanoparticle at different pH values. Antifungal activities of zinc nanoparticles were most promising on pathogenic fungi at pH 8.0.

Table 2. Antifungal activity of zinc nanoparticles of *Chaetomium globosum* formulated on olive mill waste.

Treatments	*F. solani*			*R. solani*			*S. rolfsii*		
	L.g	%R.	Z.I	L.g	%R.	Z.I.	L.g	%R.	Z.I.
OMW Free (control)	47.0	47.8	5.0	60.0	33.3	6.0	50.0	44.4	5.0
OMW Nano	45.0	50.0	13.0	58.0	35.6	11.0	42.0	53.3	9.0
OMW Nano pH6	50.0	44.4	9.0	53.0	41.1	8.0	40.0	55.6	7.0
OMW Nano pH8	43.0	52.2	17.0	50.0	44.4	15.0	37.0	58.9	13.0
OMW Nano pH10	50.0	44.4	7.0	55.0	38.9	10.0	50.0	44.4	6.0
Control	90.0	-----	-----	90.0	---	---	90.0	---	---

L. growth: linear growth (mm.) R. reduction Z.I. Zone of inhibition (mm.)

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