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Evaluation of enzyme activity inhibition of biogenic silver nanoparticles against microbial extracellular enzymes

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Abstract: In the present study, an attempt has been made to evaluate the enzyme activity inhibition of algal mediated silver nanoparticles against six major fungal mediated extracellular enzymes amylase,protease produced by *Aspergillus niger,* cellulose,lipase by *Tricoderma horzianum,* phytase by *Hypocrea lixii* and xylanase by *Fusarium oxysporum*. through submerged fermentation under optimum condition and the crude enzyme thus obtained was incubated with silver nanoparticles, the enzyme activity was determined after the post treatment with different concentration of nanoparticles .Silver nanoparticles were synthesized by dried biomass of *Spirulina platensis* with uniform spherical nanoparticles of 45-50 nm. Crude enzymes were obtained after the respective incubation period by the respective fungal organism followed by nanoparticles treatment and the enzyme activity was evaluated by suitable enzyme quantification assays. In general, enzyme activity of all the tested enzymes was not inhibited in all the tested concentration.
Keywords; silver nanoparticles, *Spirulina platensis*, enzymes, enzyme activity.

Introduction

Nanoparticles are being viewed as fundamental building blocks of nanotechnology The application of nanoparticles as delivery vehicles for bactericidal agents represents a new paradigm in the design of antibacterial therapeutics[1]. Silver nanoparticles commonly used for nanomedicine production, are reported to be nontoxic to human but most effective against bacteria, viruses, and other eukaryotic microorganisms at very low concentration[2]. They are also effective against tumors with anti-proliferative activity[3].The antimicrobial property allows them to be suitably employed in numerous products such as textiles, food storage containers, home appliances and especially in medical devices[4]. Use of AgNP is in medicine industry as tropical ointments to prevent infection against burn and open wounds is quite effective. Silver nanoparticles (AgNPs) play important role as pesticide filter also[5]. The development of biologically inspired experimental process for synthesis of nanoparticles is evolving into an important branch of nanotechnology[6.] Biologically synthesized silver nanoparticles could have many applications: they might be used as spectrally-selective coatings for solar energy absorption and intercalation material for electrical batteries; they also find use as optical receptors and as catalysts in chemical reactions. Concerning the biological application of nanoparticles it has been emphasized that methods of synthesis through biological systems viz, microorganisms including bacteria, yeasts, fungi and diatoms synthesizing inorganic materials either intra or extracellularly would make the nanoparticles more biocompatible[7].

Biosynthesis of AgNPs are becoming popular day by day using microorganisms[8,9,10].Synthesis of biocompatible potential bioactive silver nnaoparticles from various algae[11,12]. The term “nano(eco-)toxicology”

has been developed as a separate scientific discipline with the purpose of generating data and knowledge about nanomaterials effects on humans and the environment[13]. Introduction of nanoparticles into the environment might have significant impacts as they may be extremely resistant to degradation and have the potential to accumulate in bodies of water or in soil. However, nanoparticles can act on living cells at the nano level resulting in biologically desirable effects. Recently, nanomaterials such as nanotubes, nanowires, fullerene derivatives and quantum dots have received enormous attention in the creation of new types of analytical tools for biotechnology and the life sciences[14]. In the present study,effect of silver nanoparticles against major industrial important enzymes by studying enzyme activity inhibition under laboratory condition.

Materials and Methods

Synthesis of silver nanoparticles

Silver nanoparticles were synthesized from dried biomass of *Spirulina platensis* described in our previous work [15].Synthesized nanoparticles were purified, lyophilized and used for further studies.

Enzyme activity inhibitions study

Effect of synthesized nanoparticles on enzyme activity of amylase, and proteaseextracted from fungal isolate of *Aspergillus niger*, phytase from *Hypocrea lixii* SURT01 strain, cellulose and lipase from *Tricoderma horzianum* and Xylanase from *Fusarium oxysporum* was studied.

Effect of nanoparticles on amylase activity

Fungal Strain

A-amylase producing strain *Aspergillus niger* was isolated from local soil sample by adopting by soil dilution method [16].The fungi were identified based on the morphological and microscopic examination spore by standard method and by the pure culture was maintained on sabouraud dextrose agar slant.

Inoculum Preparation

The spores were obtained from 10 days old SDA slant culture of the fungi by scrapping of the slant surface with sterile distilled water containing few drops of tween 20. The slurry obtained was filtered through muscline cloth to remove the mycelial debris and it was used as a source of inoculum. The spore count was done by the heamocytometer.

Crude Enzyme Preparation

50 ml of the fermentation medium containing soluble starch 15 gL-1, potato starch 15gL-1, lactose 10gL-1, $(NH_4)2SO4$ 5g L-1, CaCl2 2gL-1, NaCl 2gL-1 in 1000 ml of 0.05 M citrate buffer (pH4.5) was transferred to 250 ml of cotton plugged conical flasks. The flasks were sterilized in an autoclave and cooled at room temperature. 1 ml of inoculum was transferred to each flask. The flasks were placed then in the rotary shaker (Remi, India) at 200 rpm and 30±20C upto 72 hours. All the experimental sets were run in triplicate. At the interavel of 12 hrs the fermented broth was centrifuged at 5000 rpm for 20 mins. The supernatant was used as crude enzyme for the estimation of α- amylase activity.

Enzyme assay

Amylase activity was studied by the method of Ertan et al[17] using a reaction mixture comprising of 1 ml of crude enzyme, 1 ml of 1%(w/v) soluble starch solution in 0.05 M citrate buffer solution (pH 4.5).Different concentration of silver nanoparticles (100,200,300,400 and 500 µg/ml) was added separately to the reaction mixture The reducing sugars liberated were estimated by the 3, 5 Dinitrosalicylic acid (DNS) method. The reaction mixture was incubated at 35°C for 20 min and the reaction was terminated by adding 2 ml of DNS in the reaction tube and then immersing the tube in boiling water bath (100°C) for 5 min. The absorbance was measured at 540 nm One unit of amylase activity was defined as the amount of enzyme causing the release of 1 µmole of reducing sugars in 1 min under the assay conditions.

Effect of nanoparticles on protease activity

Fungal strain and inocula preparation for protease production was described earlier. *Aspergillus niger* used in the amylase production was also studied for protease production and the inocula was derived slant culture as described earlier

Crude enzyme preparation

100 ml of the fermentation medium containing casein 200mg, dipotasium hydrogen phosphate 75mg, dextrose 100mg, (pH4.5) was transferred to 250 ml of cotton plugged conical flasks. The flasks were sterilized in an autoclave and cooled at room temperature. 1 ml of inoculum was transferred to each flask. The flasks were placed then in the rotary shaker at 200 rpm and 30±20C upto 72 hours. All the experimental sets were run in triplicate. At the interavel of 12 hrs the fermented broth was centrifuged at 5000 rpm for 20 mins. The supernatant was used for the estimation of protease activity.

Enzyme assay

The activity of protease was assessed in triplicates by measuring the release of trichloroacetic acid soluble peptides from 0.25 casein in 0.1%M Tris-Hcl buffer (pH8.0) at 60 C for 10 minutes. Respective concentration of nanoparticles was added to the reaction mixture.The reaction was terminated by the addition of 0.5ml of 15% trichloroacetic acid and then centrifuged at 20,000rpm for 5 minutes, after cooling. One unit of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 420nm equal to 1.0 in 60 minutes.

Effect of nanoparticles on phytase activity

Fungal strain

Phytase producing *Hypocrea lixii* SURT01 strain was isolated from poultry soil sample adopting soil dilution method[18].

Crude enzyme production

Submerged Fermentation medium for phytase production was prepared according to Soni and Khire [19] and Shieh and Ware [20].After the fermentation, the media was filtered through muslin cloth to remove mycelia debris and the collected filtrate was centrifuged at 10000 RPM for 10 minutes. The supernatant was collected and used as crude enzyme source.

Enzyme assay

Phytase activity was measured in an assay mixture containing 44.1 mM phytic acid and 200 mM glycine buffers (pH 2.8) and suitably diluted enzyme followed by the addition of respective concentration of nanoparticles. Reaction mixture is incubated at 37°C for 30 minutes, colour reagent was added and the developed colour was read colorimetrically at 400 nm. One enzyme unit was defined as the amount of enzyme liberating 1 µmol of inorganic phosphate in 1 min under the assay conditions.

Effect of nanoparticles on cellulase activity

Fungal strain

Tricoderma horzianum soil isolate was used in the study.Inocula was obtained from slant culture as described earlier.

Crude enzyme preparation

100 ml of the fermentation medium containing carboxy methyl cellulase 1g, peptone 500mg, yeast extract 500mg, Dipottassium hydrogen phosphate 50mg, (pH4.5) was transferred to 250 ml of cotton plugged conical flasks. The flasks were sterilized in an autoclave and cooled at room temperature. 1 ml of inoculum was transferred to each flask. The flasks were placed then in the rotary shaker at 200 rpm and 30±20C upto 46

hours. All the experimental sets were run in triplicate. At the interavel of 12 hrs the fermented broth was centrifuged at 5000 rpm for 20 mins. The supernatant was used for the estimation of cellulase activity.

Enzyme Assay

Cellulase activity was studied by estimation of the reducing sugar liberated by the action of endoglucanase (CMC-ase) on carboxymethyl cellulose (CMC)[21].The reaction mixture (1.0 ml) consists of 0.1 ml crude enzyme solution, 0.5 ml reagent and 0.4 ml of citrate phosphate buffer (CP, pH 0.5) containing 1% of carboxy methyl cellulose followed by the addition of nanoparticles. The reaction mixture was left to stand at room temperature for 15 min. and the optical density was measured at 505 nm.One unit of the enzyme was defined as one micromole of glucose equivalent released per minute under the assay conditions.

Effect of nanoparticles on lipase activity

Fungal strain and inocula preparation

Tricoderma horzianum soil isolate used for cellulose production was also employed in lipase production and inocula was derived from the slant culture as mentioned earlier.

Crude enzyme preparation

100 ml of the fermentation medium containing Tween20 1ml, calcium chloride 100 mg, peptone 500mg, yeast extract 500mg, (pH4.5) was transferred to 250 ml of cotton plugged conical flasks. The flasks were sterilized in an autoclave and cooled at room temperature. 1 ml of inoculum was transferred to each flask. The flasks were placed then in the rotary shaker at 200 rpm and 30±20C upto 72 hours. All the experimental sets were run in triplicate. At the interavel of 12 hrs the fermented broth was centrifuged at 5000 rpm for 20 mins. The supernatant was used for the estimation of lipase activity.

Enzyme activity

Lipase activity was determined according to the method described by Parry *et al* [22] using an emulsion of 10% olive oil in 10% gum Arabic. The emulsion was produced by treating the mixture of olive oil and gum Arabic solution in a top drive homogenizer for 10 min. The reaction mixture contained 3 ml of substrate 2.5 ml of deionized water, 1 ml of 0.2 M Tris-HCL buffer (pH 7.5) and 1.0 ml of crude enzyme and respective concentration of nanoparticles. The reaction was carried out at 37oC for 2 h in a shaking water bath, the reaction mixture was then supplemented with 10 ml ethanol. The amount of oleic was determined by titrating the hydrolysis products with 0.05N NaOH using thymolphthalein indicator. The amount of enzyme catalyzing the formation of one microequivalent (micromole) of oleic acid in 2 hat 37oC and pH 7.5 was taken as one lipase activity unit.

Effect of nanoparticles on xylanase activity

Fungal strain and inocula preparation

Xylanase was extracted from *Fusarium oxysporum* isolated from agriculture field soil sample [16] and the slant culture derived spore suspension was used as source of inocula

Crude enzyme preparation

100 ml of mineral salt medium supplemented with 0.5 % of soluble oat spelt xylan (Sigma) in 250 ml of conical flask at the spore concentration of 108 spores/ml.The seeded flasks were incubated at 30oC with 100 rpm for four days in orbital shaker (Scigenics).After the incubation the culture contents were filtered through a 0.45um pore size filter (HA type; Millipore) and the collected filtrate was used as crude enzyme for further study.

Enzyme assay

Assays for crude xylanase were performed using 0.5% soluble oat spelt xylan (Sigma) in 50 mM sodium phosphate buffer, pH 7.0.The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude

enzyme and nanoparticles concentration.. The mixture was incubated in a water bath at 60oC for 15 min. The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method in which the reaction was stopped by adding 3 ml of DNSA acid. A reddish brown colour developed after placing the reaction tubes ina boiling water bath for 5 min. After cooling the reaction tubes toroom temperature, the O.D. was measured at 575 nm with xyloseas the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 _mol xylose/min/ml under the above mentioned conditions.

Result and Discussion

Enzyme activity inhibition of algal mediated silver nanoparticles against microbial extra cellular enzymes has been studied. Synthesis of silver nanoparticles by algal biomass was characterized[16] which revealed a strong broad surface plasmon peak located at 430 nm by by UV visible spectrophotometry. Moreover a surface plasmon peak remain in the range of 420-440nm throughout reaction period. That is suggesting the particles are completely dispensed in the aqueous solution. Particle morphology by scanning electron microscopy showed uniform spherical Nanoparticles in the size range of 45-50 nm.The synthesized Nanoparticles thus obtained was extremely stable for several months after the reaction which indicated by no formation of aggregates and turbidity which rose the high stability of silver Nanoparticle in the reaction mixture..Though, inhibitory effect of heavy metals and other chemical inhibitors on enzyme activity of industrial important enzymes produced from various microorganism, nanoparticles mediated enzyme inhibition studies are very few.In this point of view, synthesized silver nanoparticles were evaluated against fungal mediated extra cellular enzymes under laboratory condition.

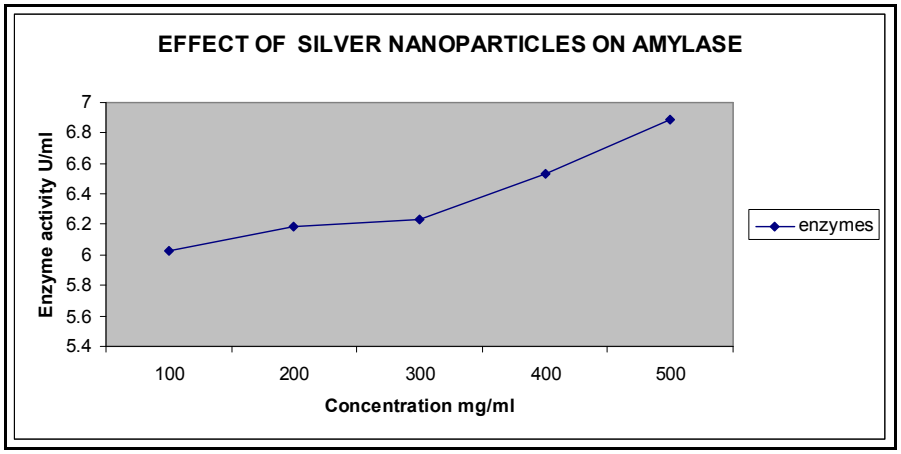

Figure 1.Effect of silver nanoparticles on enzyme activity of amylase

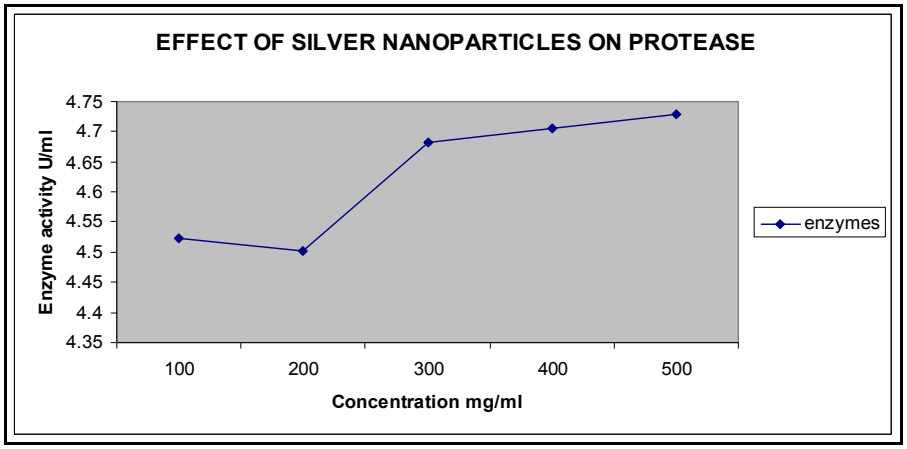

Figure 2. Effect of silver nanoparticles on enzyme activity of protease

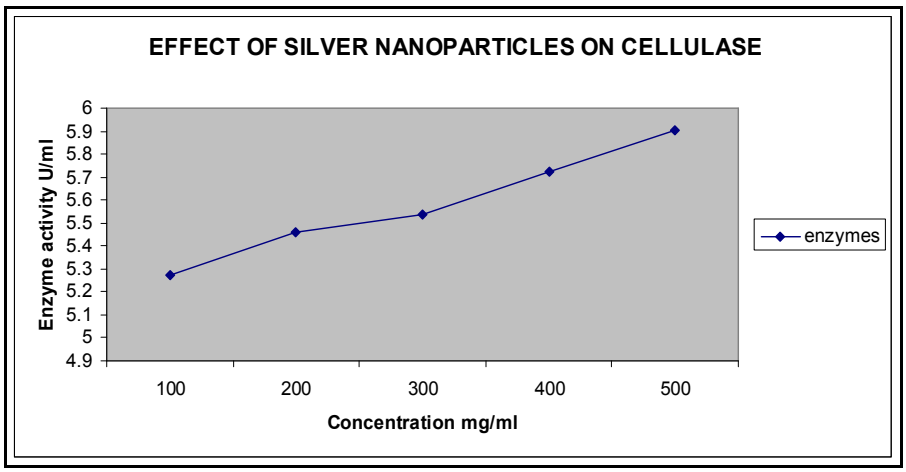

Figure 3.Effect of silver nanoparticles on cellulose activity

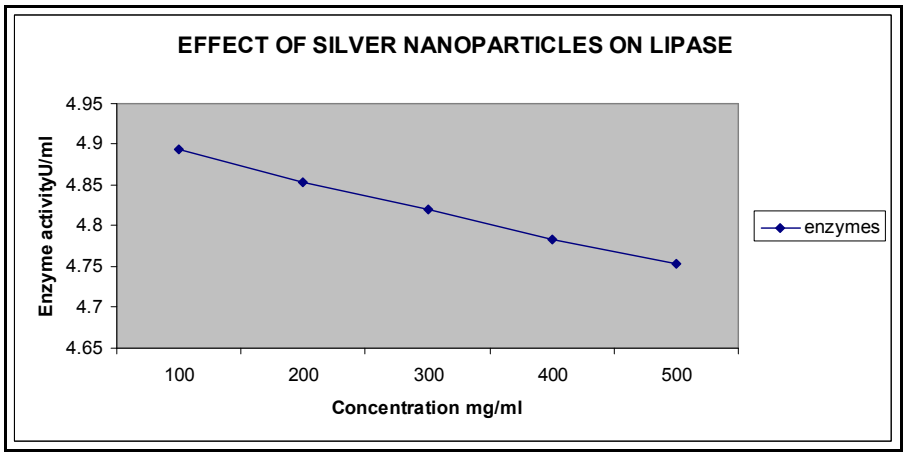

Figure 4. Effect of silver nanoparticles on lipase activity

In the case of amylase and protease which were extracted from *A.niger* by submerged fermentation was observed 36th hour of incubation, maximum production at 72 hour and the enzyme obtained was not inhibited at all the tested concentration. As in control ,there was no significant difference in enzyme activity (P>0.5 % l) which revealed. 6.02, 6.18, 6.23, 6.53, 6.88 µ/ml (figour1) and 4.52,4.50,4.68,4.72 µ/ml respectively(figure 2). Cellulase and lipase obtained from *Tricoderma horzianum* which recorded maximum enzyme production during 48 and 12 hours incubation. Nanoparticles with all the tested concentration treated enzymes retained maximum activity (figour 3,4).Enzyme activity was not affected by all the tested concentration of nanoparticles treated *Hypocrea lixii* SURT01 strain mediated phytase (figour 5) which produced by submerged shake flask culture of the fungal strain. Xylanase was extracted from *Fusarium oxysporum* soil isolate showed maximum enzyme production under shake flask culture at 96 th hour of post inoculation.No inhibitory effect was observed at all the tested concentration (figour 6).The present findings are in agreement with Namasivayam et al[23] who reported non toxic effect of fungal mediated silver nanoparticles against soil enzymes. Further study will helpful to formulate enzymes with nanoparticles for the various biotechnological applications without affecting environment.

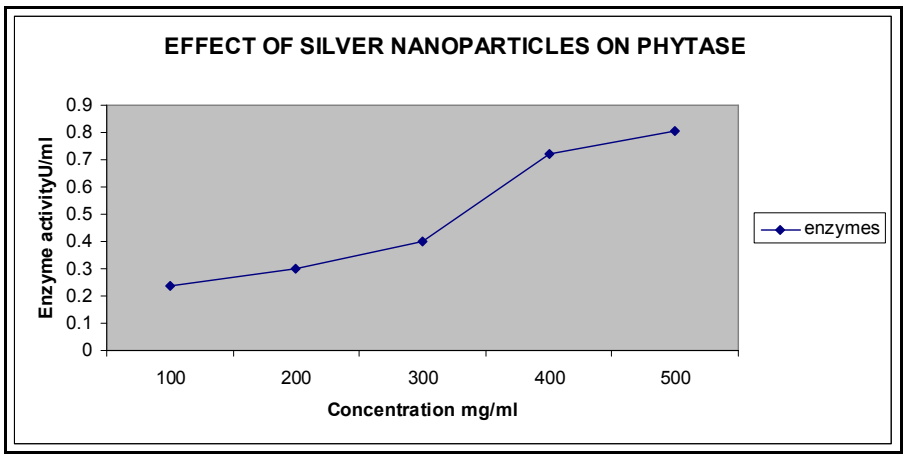

Figure 5. Effect of silver nanoparticles on phytase activity

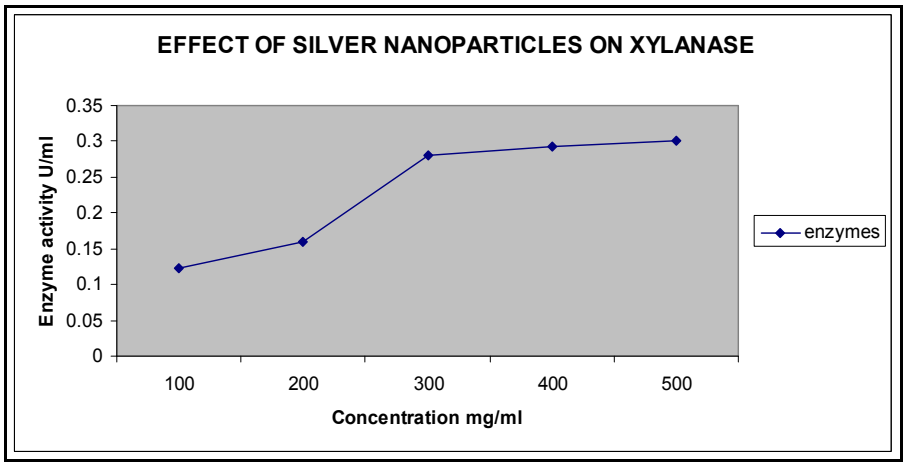

Figure 6. Effect of silver nanoparticles on xylanase activity

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