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An investigation of chemogenic zinc oxide nanoparticles mediated enzyme activity inhibition under *in vitro* condition

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ABSTRACT

Nanotechnology is currently exploited in the various fields of science and technology due to its unique effectiveness. Increased usage of nanotechnology principles, it is necessary to study the non target effect. In the present study, the effect of chemically synthesized zinc oxide nanoparticles on the enzyme activity of amylase, and protease extracted from fungal isolate of *Aspergillus niger*, phytase from *Hypocrea lixii* SURT01 strain, cellulose and lipase from *Trichoderma horzianum* and Xylanase from *Fusarium oxysporum* under laboratory condition. All the tested enzymes were obtained from culture free supernatant and the crude enzyme thus obtained was evaluated for nanoparticles mediated enzyme inhibition by studying enzyme activity after the nanoparticles treatment. Notable changes in enzyme activity was not recorded in the enzyme activity of the tested enzymes except amylase which revealed increased concentration of nanoparticles inhibited enzyme activity. Distinct inhibition was not observed in the other tested enzymes.

Keywords: Zinc oxide nanoparticles, enzyme activity, inhibition, *in vitro*

INTRODUCTION

The rapid expansion of scientific, technological and commercial interests in sub-micron materials, assembly, and properties unique at this size scale has spawned the fields of nanoscience and nanotechnology. Over 500 consumer products currently on the market claim to contain elements of nanoscience and nanotechnology with new entries coming daily [1]. Nanoscience is the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scale where properties differ significantly from those at large scales. Nanotechnology, shortened to "nanotech", is the study of the controlling of matter on an atomic and molecular scale. Generally nanotechnology deals with structures of the size 100 nanometers or smaller in at least one dimension, and involves developing materials or devices within that size [2]. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale to investigating whether we can directly control matter on the atomic scale [3].

There has been much debate on the future implications of nanotechnology. Nanotechnology has the potential to create many new materials and devices with a vast range of applications, such as in medicine, electronics and energy production. On the other hand, nanotechnology raises many of the same issues as with any introduction of new technology, including concerns about the toxicity and environmental impact of nanomaterials, and their potential effects on global economics, as well as speculation about various doomsday scenarios. These concerns have led to a debate among advocacy groups and governments on whether special regulation of nanotechnology is warranted [4,5]. In the present study, inhibitory effect of zinc oxide nanoparticles on enzyme activity of extra cellular enzymes has been reported.

MATERIALS AND METHODS

Synthesis of zinc oxide nanoparticles

Zinc oxide nanoparticles were prepared by wet chemical method [6] using zinc sulphate and sodium hydroxide as precursors and soluble starch as stabilizing agent. Synthesized nanoparticles were purified, lyophilized and used for further studies.

Enzyme activity inhibitions study

Effect of synthesized nanoparticles on enzyme activity of amylase, and protease extracted from fungal isolate of *Aspergillus niger*, phytase from *Hypocrea lixii* SURT01 strain, cellulose and lipase from *Trichoderma 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 [7,8]. 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 hemocytometer.

Crude Enzyme Preparation

50 ml of the fermentation medium containing soluble starch 15 gL⁻¹, potato starch 15gL⁻¹, lactose 10gL⁻¹, (NH₄)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 [9] 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 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, dipotassium 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 [10].

Crude enzyme production

Submerged Fermentation medium for phytase production was prepared according to Soni and Khire [11] and Shieh and Ware [12]/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 cellulose 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 cellulose 1g, peptone 500mg, yeast extract 500mg, Dipotassium 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 interval of 12 hrs the fermented broth was centrifuged at 5000 rpm for 20 mins. The supernatant was used for the estimation of cellulose activity.

Enzyme Assay

Cellulose activity was studied by estimation of the reducing sugar liberated by the action of endoglucanase (CMC-ase) on carboxymethyl cellulose (CMC) [13].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 interval 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 [14] 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 37°C 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 37°C 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 [15] 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 30°C with 100 rpm for four days in orbital shaker (Scigenics). After the incubation the culture contents were filtered through a 0.45µm pore size filter (HA type; Millipore) and the collected filtrate was used as crude enzyme for further study.

Figure 1. Effect of zinc oxide nanoparticles on amylase activity (U/ml)

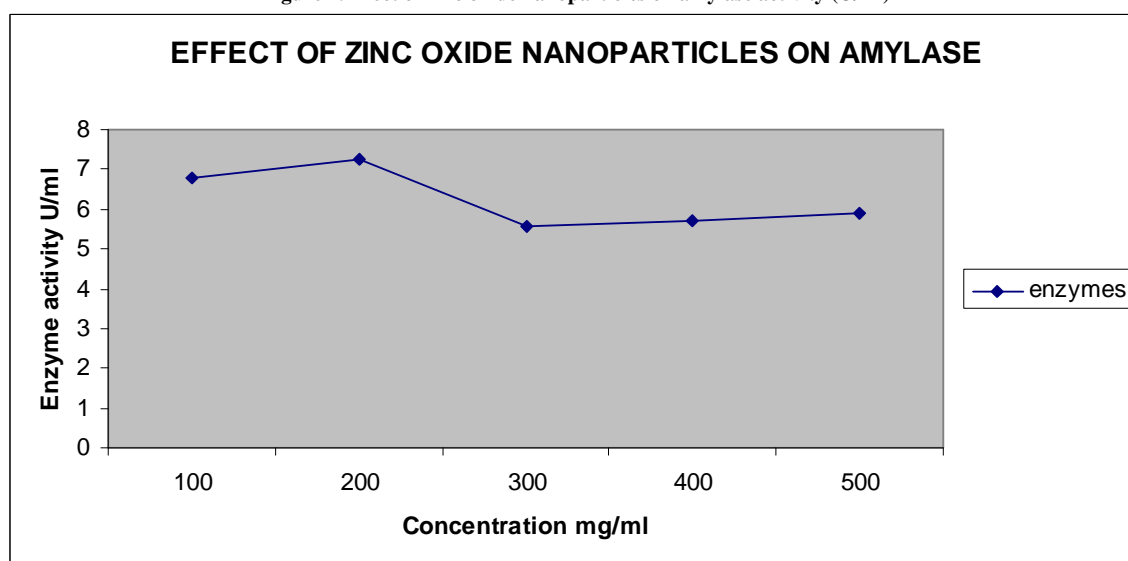
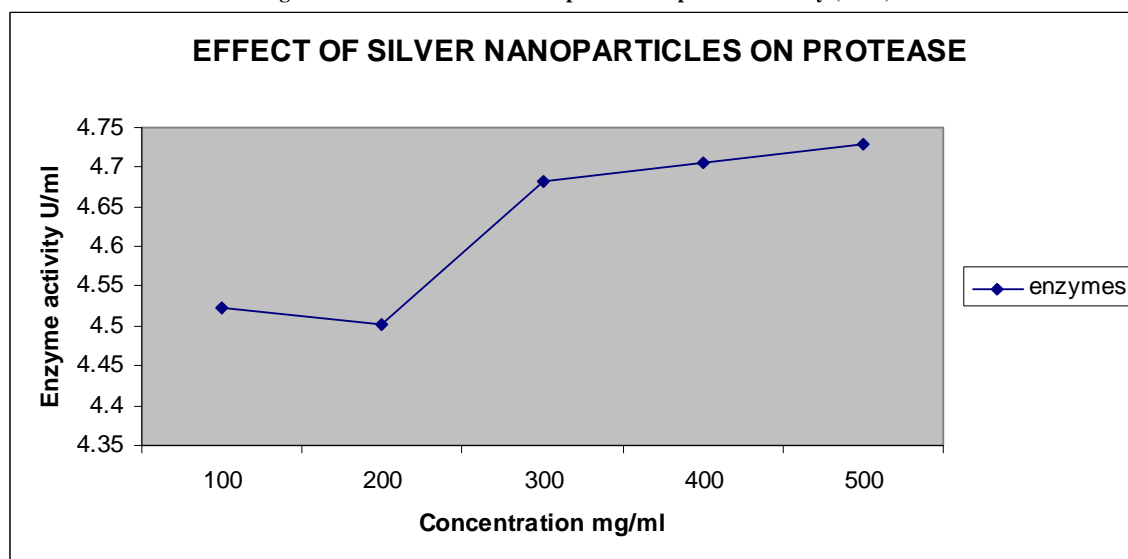


Figure 2. Effect of zinc oxide nanoparticles on protease activity (U/ml)



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 to room 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.

Figure 3.Effect of zinc oxide nanoparticles on cellulose activity (U/ml)

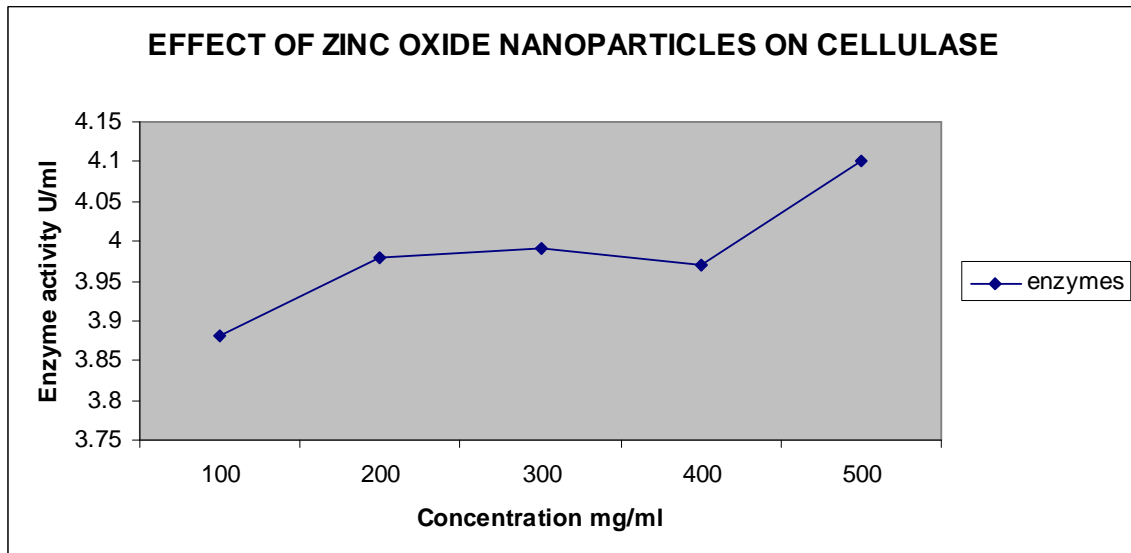


Figure 4.Effect of zinc oxide nanoparticles on lipase activity (U/ml)

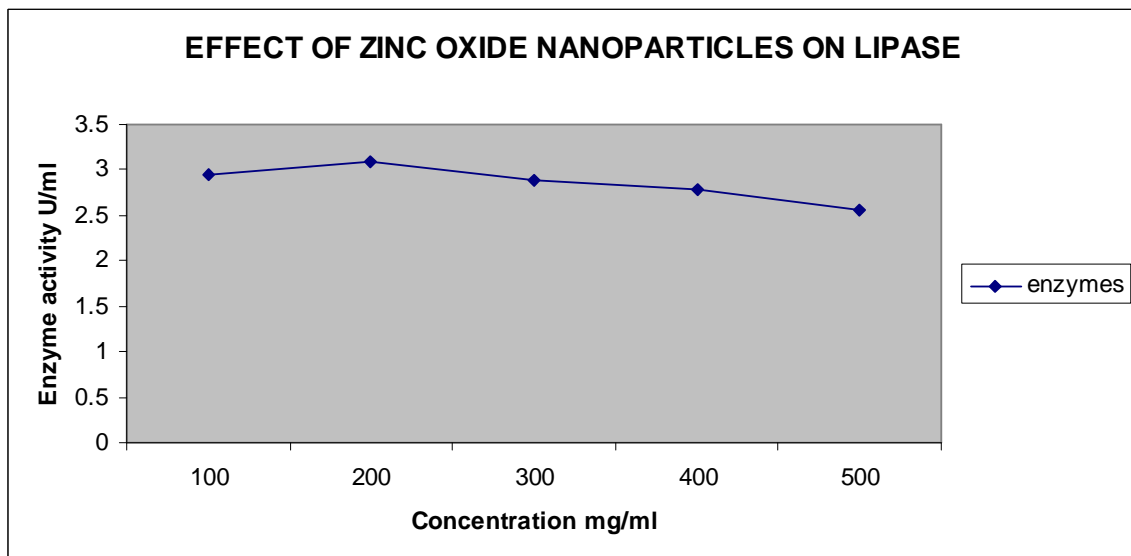


Figure 5.Effect of zinc oxide nanoparticles on phytase activity (U/ml)

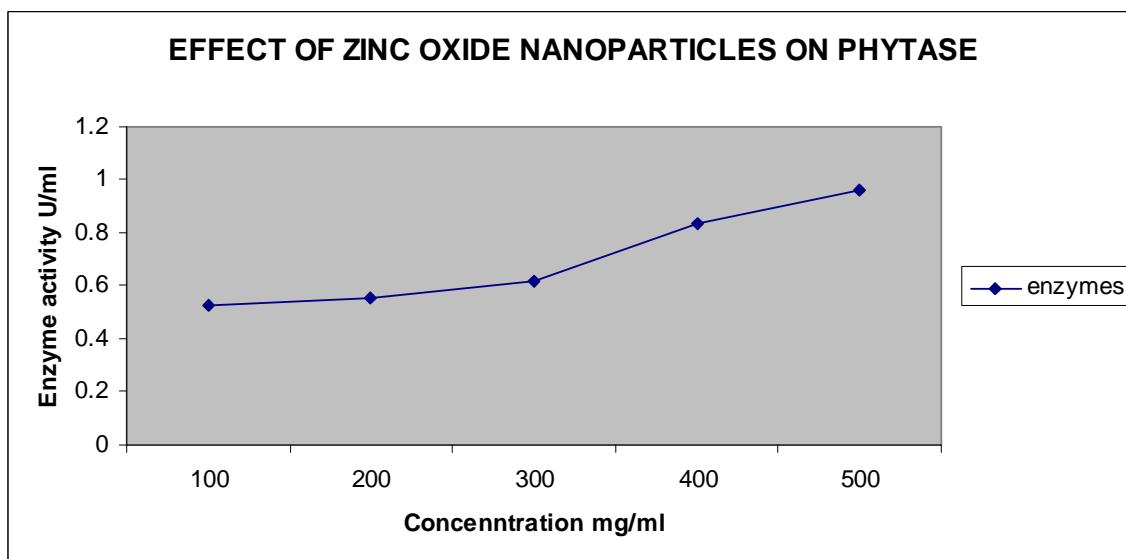
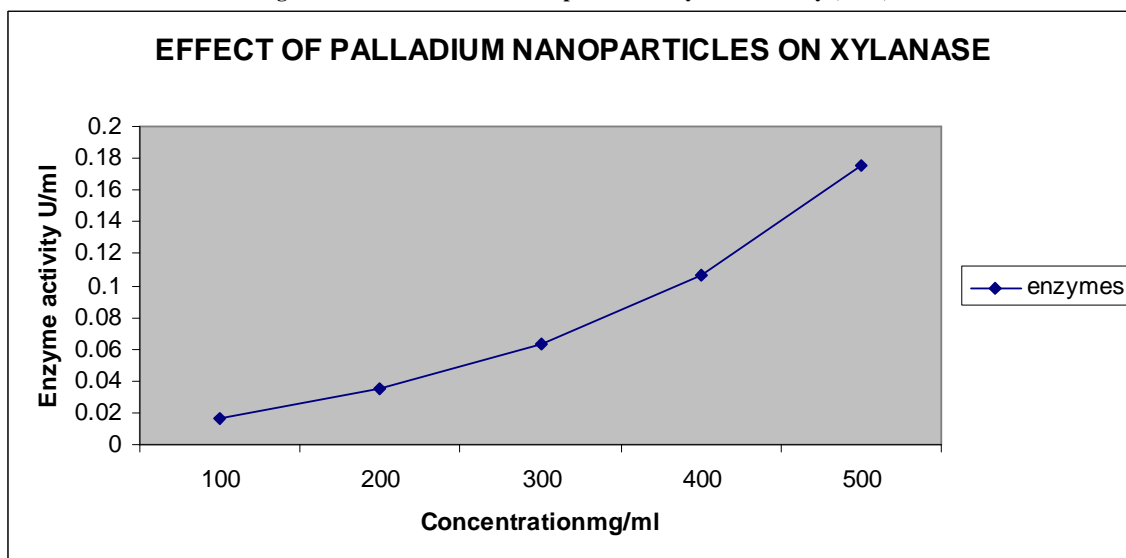


Figure 6.Effect of zinc oxide nanoparticles on xylanase activity (U/ml)



RESULTS AND DISCUSSION

The use of nanoparticles of silver and zinc oxide has been seen as a viable solution to stop infectious diseases due to the antimicrobial properties of these nanoparticles. Taken together, this compound as a highly safe compound may be considered for combination therapy against pathogenic microorganism due to its potential synergistic effect with important antibiotics [16]. ZnO nano-particles have some advantages, compared to silver nano-particle, such as lower cost, white appearance [17] and UV-blocking property [18].

In the present study, enzyme activity inhibitory effect of zinc oxide nanoparticles against industrial important extra cellular enzymes has been studied. Amylase enzyme activity was not affected in the least concentration, But, enzyme activity was found to be decreased in high concentration.(Figure 1).Significant reduction on the activity was not recorded in protease activity. 2.05, 2.88, 2.85, 2.45, 2.34 u/ml of enzyme activity was recorded in the respective concentration (Figure 2).In the case of cellulose activity, slight increase in activity has been observed in the maximum concentration (Figure 3).Nanoparticles at the respective concentration showed 3.88,3.97,3.98,3.99 and 4.10 U.ml of activity. Lipase activity was not found to be reduced in the nanoparticles treatment at all the tested concentration (Figure 4). Interestingly, increased activity was recorded at the increasing concentration. 0.08, 0.12, 0.15, 0.17 and 0.17 U/ml was observed in the respective concentration .In the case of phytase activity ,the activity was retained in all the increasing concentration of nanoparticles.0.52 U/ml of activity was increased to 0 .55, 0.62, 0.83, 0.96 U/ml in the respective concentration (figure 5).As in other enzymes, xylanase activity was not affected in nanoparticles treatment (figure 6).Respective tested concentration of nanoparticles revealed 0.08, 0.12, 0.15, 0.17,

0.17u/ml of activity. Previous study supported our present findings. Our recent study showed that best compatibility of biogenic silver nanoparticles on industrial important enzymes under laboratory condition which revealed the enzyme activity of all the tested enzymes was not affected in the nanoparticles treatment [19]. Evaluation of nanoparticles mediated enzyme activity inhibition will be useful in future for the preparation of enzyme- nano conjugate for the enhanced activities.

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