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Biosynthesis of ZnONPs and experimental investigation of the activation effect on the alanine aminotransferase isolated from serum of Ketogenic Diet people

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Abstract

Green synthesis of Zinc oxide nanoparticles has gained popularity as a viable alternative to traditional physical and chemical methods. Using a $ZnSO_4.7H_2O$ solution with extract of sesame seeds extract, a fast and environmentally friendly synthesis process was developed. The resulting ZnO nanoparticles are characterized using the following techniques: UV-Vis, FTIR, XRD, AFM and SEM. Surprisingly, the results prove that silver sizes ranged from 25 to 30 nanometers with spherical shape. Alanine aminotransferase isolated by gel filtration technique from serum of Ketogenic Diet people and characterize with study its kinetics properties, Km and Vmax(172.4 mM and 10.52 IU/L) respectively, also optimum pH 7.5, optimal temperature was 36°C . Also in vitro assays of the effect of prepared ZnO NPs on ALT activity found an increase in enzyme activity due to the influence of manufactured ZnONPs particles and give high percent of activation (26.6 %).

Keywords: ZnONPs, sesame seeds, Ketogenic diet, ALT, Km, Vmax, Activation of enzyme.

1. Introduction

Each physical and chemical process has its own set of disadvantages, such as huge environmental contamination and hazardous compounds formed during synthesis. As a result, "green chemistry" is required to ensure that nanoparticles are produced in a safe, nontoxic, and environmentally acceptable manner.[1],[2]. Zinc oxide nanoparticles (ZnO NPs) are widely used in a variety of sectors due to their unique physical and chemical properties[3], [4].Zinc is widely recognized as an essential trace element that can be found in all bodily tissues, including the brain, muscle, bone, and skin. Zinc participates in the body's metabolism and performs critical roles in protein and nucleic acid synthesis, hematopoiesis, and neurogenesis as a key component of many enzyme systems[4], [5]. Because of the small particle size of Nano-ZnO, zinc is more easily absorbed by the body. As a result, nano-ZnO is frequently utilized in food. Furthermore, the US Food and Drug Administration (FDA) has classified ZnO as a "GRAS" (generally regarded as safe) chemical [6].ZnO NPs have gotten a lot of attention in biomedical applications because of these features. These include anticancer, drug delivery, antimicrobial, and diabetic treatment; anti- inflammation; wound healing; and bioimaging.[7], [8]. The purpose of this work is to look into the synthesis and characterization of ZnO nanoparticles made with green chemistry. We chose sesame seeds, a plant that is abundant in most geographical locations in country, for the synthesis of nanoparticles utilizing biological approaches, due to its availability and the fact that the species has been extensively investigated in terms of chemical composition[5], [6] Al-Dhabi [9]paper characterization measures of ZnONPs an average particle size of 31 ± 2 nm and Miri[8] paper found 40–80nm of ZnOPs particale size. The ketogenic diet, often known as the low-carbohydrate, high-fat, and suitable protein diet, has several health advantages. It necessitates a significant reduction in carbohydrate intake and a replacement with fat. The body enters a metabolic condition known as ketosis as a result of eliminating carbs and following a diet program that has been shown to be helpful for quick weight loss..[10], [11], Ketogenic diets produce ketone bodies, which are formed inside hepatocytes and can be used as a secondary source of energy when glucose is insufficient during fasting periods. [12], [13].In the study found ketogenic diet effect on some biochemical parameters such as alanine aminotransferase which present in the study, Alanine transaminase (ALT) is an enzyme E.C 2.6.2 its m.wt 56 KDa and located in the liver and other tissues such as heart and muscle. ALT catalyzes an equilibrium transfer process of the amino group from alanine to alpha-ketoglutarate.[14], [15].There were a significantly increase in ALT levels in the serum of ketogenic diet people, therefore this enzyme purified from those people by chromatographic methods and study its kinetics properties then applied ZnO NPs as activator of it[16], [17]. As a result, this is the first study of ALT enzyme kinetic evolutions for ZnO NPs generated by sesame seeds extract in an environmentally benign procedure.

2. Experimental Part.

2.1. Chemicals and instrumentation

Except for the sesame seeds extract, all of the chemicals needed for the study were purchased from Sigma Aldrich.

The UV–Vis spectra of the various solutions were measured to monitor the generation of ZnO NPs. From

200 to 800 nm, the spectra were monitored on a Shimadzu double beam spectrophotometer. A Shimadzu8400S spectrometer was used to monitor the IR spectrum in the 400–4000 cm1 wavenumber region. The XRD Diffractometer model was used to record the PXRD measurements of ZnO NPs powders (XRD-6000, Shimadzu x-ray Diffractometer). The morphology of the produced ZnO NPs was characterized using AFM and SEM (JEOL 2010 working at 200 kV).

LDH activity measured by kits supplied from AGAPPE-Switzerland.

2.2. Sesame Seeds extraction Preparation.

Sesame seeds were washed utilizing distilled water, dried and then grinded. The seed powder (20 g) was coupled with 100 mL of distilled water and boiled for 45 minutes at 70°C. The extract was collected and kept at 4 ° C..

2.3. Synthesis of Zinc Oxide nanoparticles

Zinc nanoparticles were prepared by adding 20 ml of sesame seed extract in 80 ml of the 1 mM ZnSO4.7H2O solution. For 1 hour at 60 ℃, sonicate the solution. A start changing in the color to yellow indicated the creation of ZnONPs

2.4. Purification of ALT from serum of Ketogenic Diet.

ALT was purified from the serum of of Ketogenic Diet using the following steps: 1- Addition ammonium sulphate (75%) 2- Dialysis 3- Gel Filtration Chromatography(using Sephadex G100)

2.5. Kinetics study of ALT

The kinetics of ALT were studied after its separation and purified from serum Ketogenic Diet by gel filtration. These included:

1- This effect has been studied using different concentrations of substrate : Alanine on ALT(110,55,27.5,13.75,6.87,3.43mM),

2- Effect of pH: The pH effect of ALT reaction. Solutions of various pH (4.5, 5.5, 6.5, 7.5, 8.5, 9.5) at 37 C° and concentration : Alanine (110Mm).

3- Effect of temperature: using to measure the effectiveness of ALT. The reaction was conducted at various temperatures (6, 16, 26, 36, 46, and 56 °C).

2.5. Effect of ZnO NPs on enzymes activity

After purification of enzyme,the effect of ZnO NPs that made by different concentration has been studied upon the activities of ALT, specially that which has activation effect if following concentrations are used (25, 12.5, 6.25, 3.12, 1.56, 0.781) mM of ZnO NPs and interaction was made with the buffer solution (sodium carbonate –Bicarbonate) pH7.2 .Enzyme activity was measured as mentioned method described in kit from AGAPPE company, then calculation of percentage of activation.

3. Results and Discussion

3.1 Characterization of silver nanoparticles.

3.1.1. UV-Visible absorption study of ZnO NPs

UV-Visible absorption were initially characterized of a prepared Zinc nanoparticles in deionized water was measured by recording the absorption spectra within a wavelength range range of 200–905 nm. A change in extract color with ZnSO4 solution showed reduction in zinc ions which confirmed the formation of ZnO NPs. Formation of the Zn NPs was revealed by yellow colour appearance and act as indicating the production of ZnONPs [18]. The broad-single surface plasmon resonance (SPR) peak found at 205.5-298.5 nm in the UV-Vis spectrum for Figure 1, conforms to the formation of stable ZnONPs and the shape and wavelength of the resultant peak of ZnONPs are strongly dependent on particle size [19].

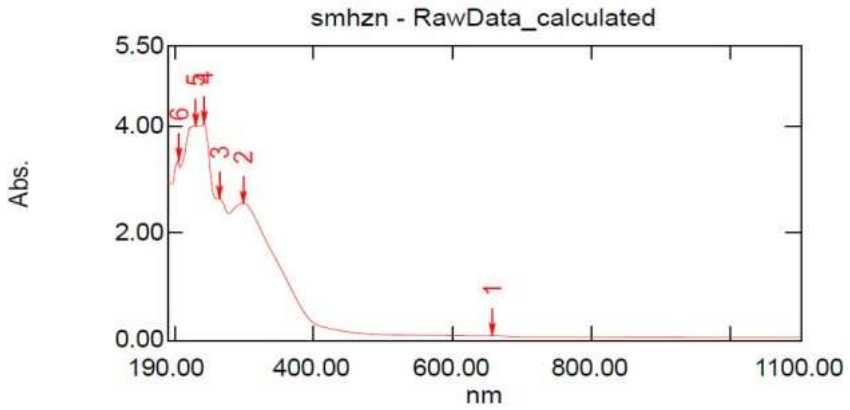

Figure 1. UV-VIS spectra of ZnO NPs

3.1.2. FTIR Spectroscopic study of ZnO NPs

FTIR spectroscopy is an useful technique for determining the major functional groups (reduced bio- molecules) necessary for the green synthesis of ZnO NPs through the reduction and stabilization of Zn ions. A number of absorption bands were discovered in the FTIR spectra and figure 2. shown that. Peaks were observed at 439.78, 462.93, 495.72, 599.88, 667.39, 827.49, 869.92, 949.01, 1004.95, 1051.24,

1105.25, 1354.07, 1388.79, 1626.05, 1764.93, 2335.87, 2360.95, 2397.60, 2428.46, 2740.94, 2771.80,

2935.76 and 3458.48 cm^{-1} .At 869.92, 949.01, 1004.95and 1051.24cm^{-1} absorption band revealed the existence of C–H bending of the aromatic alkanes, alkenes, alkynes and aromatic hydrocarbons. Similarly, the presence of N–H bending (Primary, secondary amines and amides) and (C–H) bending was confirmed by peaks at the 2335.87 to 2428.46 cm^{-1} (-C–H) of aliphatic content from extract. (C-O) appear at 1626.05 cm^{-1} . Existence of C=O functional group of carboxylic acid in addition with ketones and aldehydes was confirmed by peak at the 2935.76cm^{-1} . Presence of the O–H vibration of phenol and alcohols was revealed by the absorption band at the 3458.48 cm^{-1}.The presence of various biomolecules in the stability and reduction of NPs was shown by all of these vibrations, where the main peaks had shown primary and secondary amides and amines, alcohols, proteins, aromatic hydrocarbons, ketones, aldehydes and carboxylic acids as well as stretching and bending vibrations confirmed that all these are connected with ZnO NPs[19]

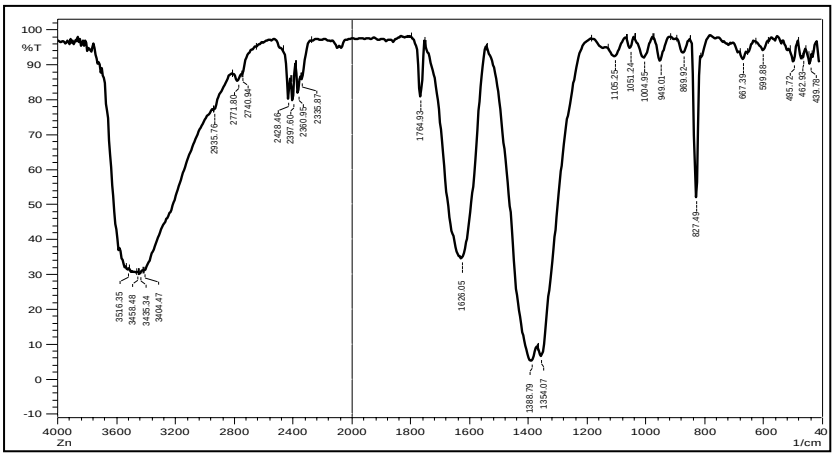

Figure 2. FTIR Spectroscopic of ZnO NPs

3.1.3. XRD study of ZnO NPs

The XRD pattern of the synthesis of ZnO NPs with extract of sesame seeds is shown in Figure 3, main peaks were observed at (2θ) 32.0535°, 34.6640°, 36.4737°, 47.7646°, 56.9123°, 63.0838°, 66.4997°,

68.2679°, 69.2806°, 72.7639°and 77.1735°. With inter planar spacing (d calculated) values are 2.79238,

2.58784, 2.46349, 1.90421, 1.61796, 1.47372, 1.40607, 1.37390, 1.35627, 1.29970 and 1.23607Å. The

other peaks could be organic residues left over from the extract of sesame seeds. These planes are indexed so that they face a centered cubic face[20]. From XRD data and depending on Debye-Scherrer equation, ($Dc = K\lambda/\beta\cos\theta$), where β is really the breadth of the experimental diffraction line at half of the maximum intensity, K is indeed the shape factor constant = 0.9, and λ is the wavelength of X-ray source used in XRD instrument, the average crystallite diameter (Dc) of ZnO nanoparticles were found to

really be approximately 25.50 nm. These findings are harmonized to normal zinc oxide Nano particle [21].

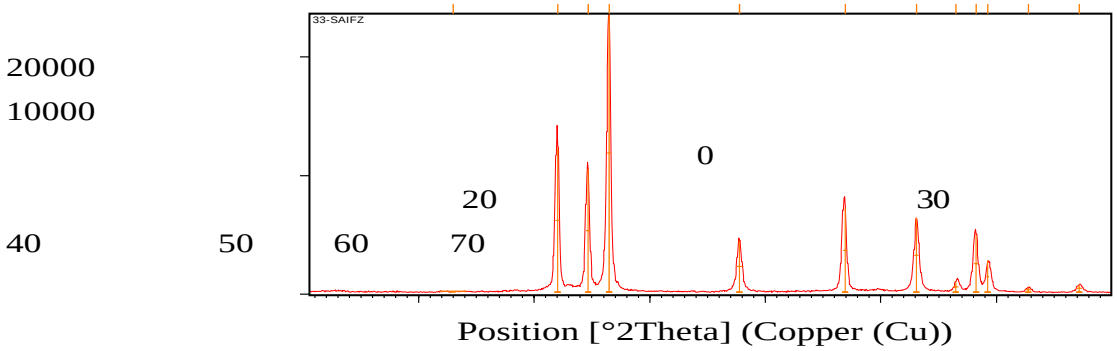

Figure 3. XRD pattern of ZnO NPs

3.1.4. AFM study of ZnONPs

The AFM measurement of the ZnONPs generated via bio reduction was utilized to provide information about the material's surface morphology (2D and 3D) as shown in figure 4, a.2D,b.3D structure. The greatest height was 4.643 nm, which indicates that the material is of nanoscale size The results obtained utilizing all characterization approaches were consistent with the literature [21]

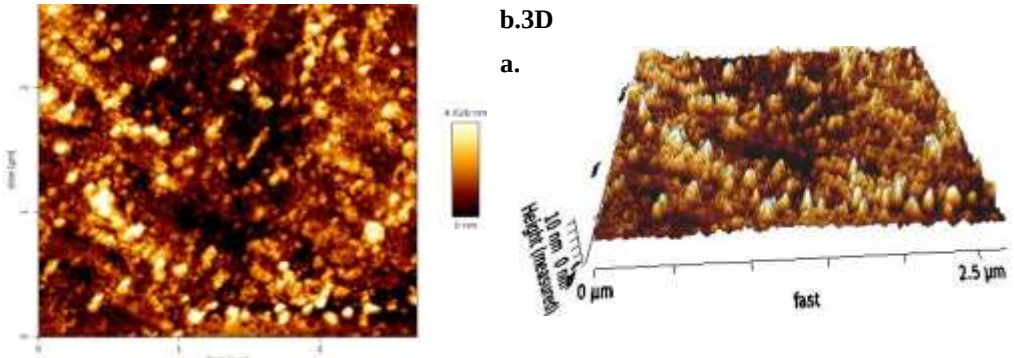

Figure4.a.2D structure , b.3D structure of AFM of ZnO NPs

3.1.5. SEM study of ZnO NPs

The morphology of the prepared ZnONPs was measured using SEM measurements to determination of a particle structure and average size within the nanoscale .Its shape as spherical and the approximate size of these spherical is 25-30 nm, as estimated from the micrograph as shown in Figure 5, where SEM showed that evaporation of the solvent during the preparation phase causes the formation of larger NPs, resulting in particle size variation[21].These findings are in agreement with XRD measurements, which show particles of around approximately near the same size. Our zinc Oxide Nano structure was clearer and more precise than nanostructures created with other parts of other plant extracts[20][22].

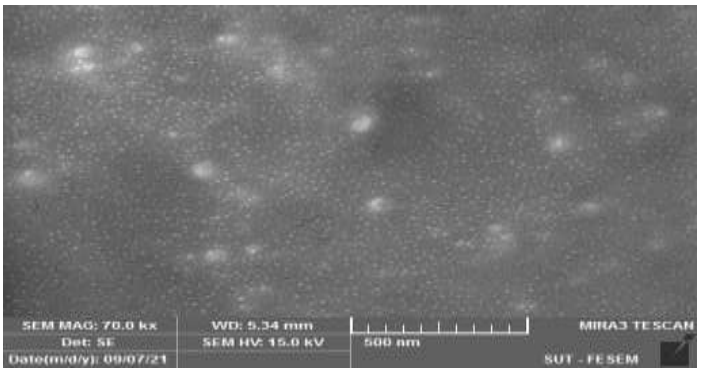

Figure 5. SEM micrograph of ZnONPs

3.2. Purification of ALT from serum of ketogenic diet

The basic principle is to equalize the charges on the surface of the protein(enzyme) and to minimize the degree of watering,

solubility, and sedimentation of the protein by degrading the water layer surrounding the protein[23]. As a result, the separation and purification of ALT from ketogenic diet serum was carried out in stages, with the first stage involving the enzyme being precipitated using ammonium sulphate salts $(NH_4)_2SO_4$ to concentrated enzyme, and the access of the resulting salts being removed by dialysis using buffer solution 1Mm Tris-HCl pH 7.4, with the degree of purification of the enzyme being (1.25) folds and the yield of enzyme being 35.4%, specific activity 0.531 IU/mg showing the result in table (1), the stages of purification were complete by using gel filtration using Sephadex G100 which showed a single peak in fraction four with the degree of purification was (5.79) fold and enzyme yield was(108.2%) while specific activity was 2.45 IU/mg .

Table1. Steps of ALT purification from Serum of ketogenic diet

Steps of purifications	Elute Volume ml	Activity IU/L	Total Activity IU	Protein Conc. mg/ml	Specific activity IU/mg	Yield %	Folds	Total protein mg
Crude serum	12	36	432	85	0.423	100	1	1020
Ammonium sulphate	8	27.5	220	57.5	0.487	50.9	1.13	460
Dialysis	9	17	153	32	0.531	35.4	1.25	288
Gel filtration	6	15	90	6.1	2.45	20.8	5.79	36.6

In this research, this technique was used to isolate and purify ALT. This was achieved through ammonium sulfate precipitation and purification of it using a Sephadex G100 column and Figures 6. below shows our results.

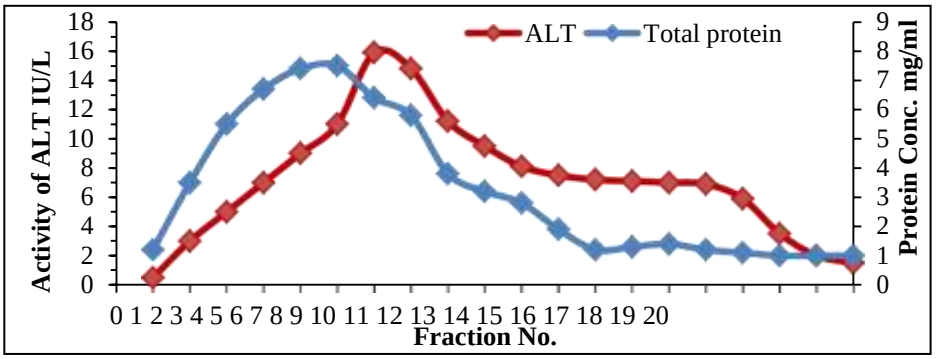

Figure6. Gel filtration chromatography of ALT by Sephadex G100

3.3. Study of kinetic properties of ALT Purified from serum of ketogenic diet

3.3.1. Effect of Effect of Different Substrate Concentrations

The activity of the ALT was measured in the presence of different concentration Alanine on ALT (600, 300, 150, 75, 37.5, 18.75, 9.375 mM),as a substrate. , whereas optimal concentration of substrate was obtained 110 mM of alanine , the figure 7. Shows that, Also study determined Michael's-minten constant (K_m) that mean as the affinity between the enzyme and substrate. A linear relationship was obtained the constant value of the Michael's-Minten of substrate Km was (172.4 mM) and the maximum velocity value V_{max} (10.52 IU/L) as showed in figure 8 .The differences between all these studies were clear and almost natural as a result of the different enzyme sources which one study Maher,F (2019) had referred to the study of the purified ALT from cardiovascular patients value of Km was 1.1 mM and Vmax was 33.9 IU/L[24] and Ahmed,N (2019) referred to the study of the purified ALT from serum of patients with Gallstone, Km was (0.89mM) as well as the V max) at the same substrate equal to (33.33 mM/L)[25].

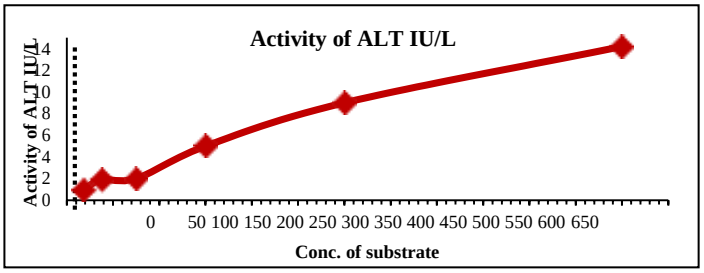

Figure7. Michaelis–Menten plot show effect of substrate concentration on ALT activity

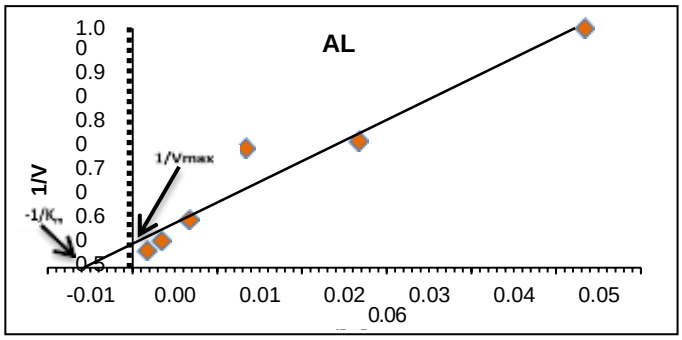

Figure8. Line Weaver–Burk plot for partially purified ALT

3.3.2. Effect of pH on Enzyme Activity

The effect of pH on enzyme activity was investigated, and it was discovered that pH has a substantial impact on the enzyme's ability to manage ionization and ionic aggregates at the active site. An essential property of enzymes is their optimal pH of stability[26] The findings of the enzyme kinetic analysis revealed that ALT optimum pH was (7.5), as shown in figure 9.The results of pH differed with the other studies carried performed, In these study by Maher,F(2019) for ALT purified from cardiovascular patients which pH was 7.3[24]. Ahmed,N (2019) for ALT purified from serum of patients with Gallstone which pH was 6.5-8[25]**.**

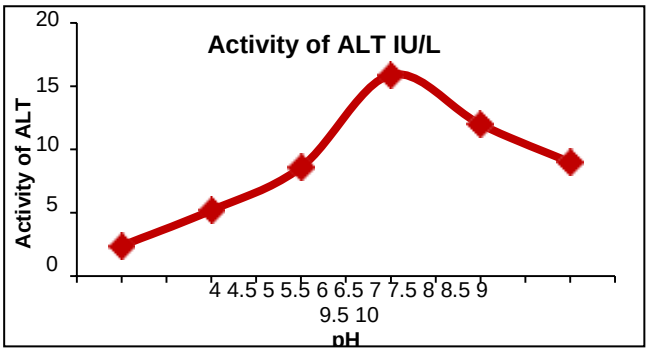

Figure 9. Effect of pH on ALT activity.

3.3.3. Effect of Temperature on Enzyme Activity

The maximum temperature, where the rate of enzymatic reaction rate is maximal, while the enzyme is very effective, was studied on the activity, and is impacted by pH and other factors[27]. When the pH was validated and the substrate concentration was determined, the optimum temperature for enzyme activity was (36 C°). The findings differed from those of other studies such as Ahmed,N (2019) for ALT purified from serum of patients with Gallstone which optimum temperature was 37 °C [25] .While Donald E. *et al*.,(2000) stated that optimum temperature for ALT purified from Hyperthermophilic Archaeon Pyrococcus is 95°C [28], and Results of Blanca LIAN et al.,(1991) have shown that the optimum temperature for the enzyme ALT work in Chlamydomonas reinhardtii is 50C°[29]. The figure 10, showed that**.**

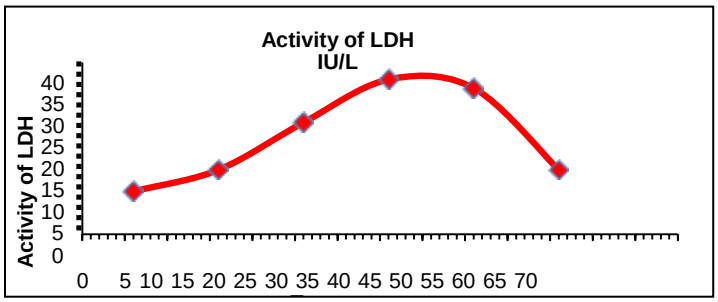

Figure 10. Effect of temperature on ALT activity.

3.4. The Effect of ZnONPs on ALT Purified Enzyme

In vitro assays of the effect of prepared ZnO NPs on ALT activity found an increase in enzyme activity due to the influence of manufactured particles, a study was conducted on the influence of ZnONPs on the activity of purified ALT. Various concentrations of ZnONPs (25, 12.5, 6.25, 3.12, 1.56, 0.781) mM were added to an enzyme interaction combination including 600 from substrate of ALT and and pH 7.4 buffer solution (carbonate-Bicarbonate).On ALT purified enzyme, ZnONPs showed a little activation effect on enzyme activity when above concentration was used. The highest percentage of the activation was at concentration (12.5mM) (26.6%) of ALT as illustrated in table 2**.**

Table 2. Activation percentage of ALT purified using ZnONPs

	Activation %					
ZnONPs Conc. mM	25	12.5	6.2	5	3.12	1.560.781
ALT	13%	26.6 %	10	%	6.6 %	3.3%2%

High level of zinc is essential for cells and zinc is a component of so many enzymes and transcription factors[30] . Proposal was made for strong interaction occurring among metallic ions and the enzyme due to having enzymes some mineral related with active site , ZnONPs activated by the released the zinc ions as cofactors , in some vivo study a good biomarker for cytotoxic effect of ZnONPs with their direct and indirect effects cause serious damages in cellular structure and elevated levels of these enzyme and these particles is a possible anticancer agent[31].

There were a several studies were conducted to determine the enzyme's effectiveness with ZnO nanoparticles, in one of the studies conducted on ALT and LDH enzyme where ZnO nanoparticles were found to increase the activity of these enzymes[30]. In another research the activities of alanine aminotransferase (ALT) increased significantly in the treated 20 animals and did not return to normal after the recovery time[31].

4. Conclusion

This article claims that ZnONPs can be made without the use of a stabilizing agent from sesame seeds extract with a nanoscale size of 25-30 nm. After purification of Alt from serum of ketogenic diet kinetics study of purified enzyme were carried out study, by the Line weaver – Burk relation, also the Km value was (172.4 mM) and Vmax without inhibitor (10.52 IU/L).Optimum pH 7.5 and temperature 36 C°. Then prepared nano particles study on ALT activity and found an increase in enzyme activity due to the influence of ZnONPs and give high percent of activation (26.6 %).

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