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# Urease Activity Protection With EDTA Against Nanoparticles ($Fe_2O_3$ and $Fe_3O_4$) Inactivation

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## Abstract

In this study the effects of $Fe_2O_3$ and $Fe_3O_4$ magnetic nanoparticles and EDTA on urease activity was investigated. The effect of nano-Fe2O3 and nano-$Fe_3O_4$ on urease activity were investigated. Urease activity was studies by UV-Vis spectrophotometry at 40 °C at pH = 7.2 using sodium phosphate as buffer. Measurements were carried out using 0.075 mg/ml of urease and a range of nano-Fe2O3 and nano-$Fe_3O_4$ concentrations between 0.002-0.006 mg/ml. It was found that by increasing the concentration of nano-Fe2O3 and nano-$Fe_3O_4$, urease activity will be decreased. On the other hand, nano-$Fe_2O_3$ and nano-$Fe_3O_4$ act as non competitive inhibitor for urease. Urease protection studies were corried out by using different concentration of EDTA (0.004-0.008 mg/ml). It was shown by increasing the concentration of EDTA, the activity of enzyme increased.



## 1. Introduction

Over the past few years, the safety of nanoparticles has attracted much attention due to the quick advancement of nanotechnology .Applications are moving from research benches to the manufacturing lines, and come into industrial production and daily life [1-3]. For instance, some of them were used in sunscreens to absorb UV light or in toothpaste as an additive. In addition nanoparticles were applied in paints
and in electronics industry. Moreover, there are many applications in medicine, sporting

equipment, cosmetics, coatings, fuel cells and other industries [4-7]. In these times, some nanoparticles are being tried as drug delivery products and they are used to diagnose disease as quantum dots that can allow the visualization of cancer cells in the body. However, nanoparticles have been indicated to enter human body via inhalation, ingestion, dermal permeation or injection [8, 9]. The small size of nanoparticles (NPs) caused to particles uptake into cells and transfer into blood and lymph circulation to arrive sensitive target sites and they produce physical damage or make harmful inflammatory responses [10-12]. The majority of commercial nanoparticles use in medicine. This application in medicine include: Bio detection of pathogens [13], Detection of proteins [14], tumor destruction via heating (hyperthermia) [15], Separation and purification of biological molecules and cells [16]. Data from some pulmonary toxicity studies in rats demonstrate that exposures to ultrafine/nanoparticles may produce enhanced toxicity when compared to fine sized (bulk) particle-types of similar chemical composition [17]. Metal oxide nanoparticles are often used as industrial catalysts and elevated levels of these particles have been clearly demonstrated at sites surrounding factories [18]. In the recent years, nanosized iron oxide particles have been paid considerable attention in the fields of biological applications [19–23]. Applications of these magnetic iron oxide nanoparticles not only cover traditional electrical, optical, magnetic areas but also expand applications in biotechnologies. Magnetic nanoparticles have been widely used in the immobilization of enzymes [24], immunoassay [25], bioseparation [26], biosensor [27], targeted drug delivery [28], and environmental analysis [29]. Irrespective of the origin, whether bacterial, fungal, algal, plant or soil, ureases (urea amidohydrolases, EC 3.5.1.5) exert one catalytic function that is the hydrolysis of urea, its final products being ammonia and carbonic acid [30-32]. Urease present in many plants, bacteria and in soil, catalyses hydrolysis of urea to ammonia and carbon dioxide at a rate approximately $10^{14}$ times the rate of uncatalysed reaction. Most of the studies have utilized urease obtained from jack bean[33]. In the former, bacterial ureases may serve as a virulence factor, giving rise to pathological conditions, such as peptic ulcer disease, gastric cancer and hepatic coma resulting from the infection of the gastrointestinal tracts (primarily with Helicobacter pylori), and to kidney stone formation and pyelonephritis, resulting from the infection of the urinary tracts (chiefly with Proteus mirabilis and Ureaplasma urealyticum). In agriculture by contrast, a hydrolysis of fertilizer urea by soil urease, if too rapid may lead to unproductive volatilization of nitrogen and may cause ammonia toxicity and alkaline-induced plant damage .arious strategies have been. utilized to counteract these complications, one of them being to incapacitate urease with use of inhibitors [34]. Free urease and immobilized urease has been widely used in biosensors for diagnostic purposes, in the determination of

urea in biological fluids, in artificial kidney devices for the removal of urea from blood for extracorporeal detoxification,in enzyme reactor for the conversion of urea present in fertilizer wastewater effluents or in the food industry for removal of urea from beverages and foods[35-37].Though composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi, exhibit similar amino acid sequences and share common catalytic characteristics [38]. A pivotal catalytic characteristic common to all ureases is the presence of nickel(II) ions in the active site, essential for activity. by the structure of plant urease from Canavalia ensiformis [39], the active sites of the enzymes contain a binuclear nickel centre where nickel(II) ions are bridged by a carbamylated lysine and a hydroxide, Ni(1) being further coordinated by two histidine residues, and Ni(2) by two histidine residues and an aspartic acid residue[40-43]. Urease from jack beans (Canavalia ensiformis), a protein characteristic of ureases from various sources. Composed of three identical subunits, each of 90.77 kDa, was proven to contain 90 cysteinyl residues per molecule (15 per subunit) [44]. Fifty four residues are buried in the molecule and are disclosed only in denaturating conditions. Of the 36 residues disclosed in non-denaturating conditions by contrast, 30 are highly reactive, but without major impact on the enzyme activity, the remaining six being less reactive, but importantly, of critical importance for the catalysis. The Cys-592s are located in the mobile flap of the active site, one per each of the six sites in the hexameric molecule Although not essential, the residue is assessed to have a role in positioning other key residues in the active site appropriately for the catalysis, which is why when chemically modified, it restricts the mobility of the flap, hence, the reaction is perturbed and the enzyme activity reduced [44, 45].

## 2. Materials and methods

### 2.1. Instrument and materials

The absorption spectra of all liquids/solutions were recorded with a Model ultrospec 4000, UV-visible equipped with a thermostatic cell holder. Jack bean urease, Sigma type III, was used. containing 0.075 $mg.mL^{-1}$ Urease in 20 mM sodium dy hydrogen-phosphate buffer (pH7.2) and different concentrations(0.002-0.006 mg/ml )of nanoparticler and EDTA(0.004-0.008 mg/ml) were prepared. An electrolyte solution (1.5M NaCl) was prepared to adjust the ionic strength of the solution. A scanning electron microscope (SEM) was used to measure the size and shape of the, nano-$Fe_2O_3$ and nano-$Fe_3O_4$. nano-$Fe_2O_3$ and nano-$Fe_3O_4$ was used without further modification. It was suspended in deionized water and mixed ultrasonically for 3 times in 15 min before use.

### 2.2. Standard urease activity assay

The standard urease assay mixture contained different concentrations (4-30Mm) urea in 20 Mm phosphate buffer , pH 7.2. The reactions

were initiated by the addition of small aliquots of enzyme soulation.The assay was run for 5 min and the anzyme activity was determined by measuring the concentration of the ammonia released. For that samples were withdrown from the reaction mixtures and the ammonia was determined by the colorometric phenol-hypochlorite method [46-48].

### 2.3. Inactivation kinetics of urease

Catalytic activity of enzyme was determined by measuring the decrease in absorbance at 630 nm and 40°C of 0.075 $mg.mL^{-1}$ ureaase suspension in 20 mM sodium-phosphate buffer, pH 7.2 in the absence and presence of different concentrations of each nanoparticle. The rate of the decrease was proportional to the native enzyme concentration.

### 2.4.Urease protection against nanoparticles inactivation

Urease protection studies were carried out at pH7.2, the urease concentration in the incubation mixture always beingn 0.75 mg/ml. For the protection with thiols, urease was preincubated With differentconcentrations of EDTA from the range 0.004 to 0.008 mg/ml and each of Nanoparticles (0.006mg/ml). The pre-incubation lasted 15 min in all cases. The activity of urease was next followed by the colorometric phenol-hypochlorite method.

### 2.5. Calculation of Kinetics parameters on inactivation

Urea was used for substrate and amount of product were recorded at 630nm [30-32]. In this work two important parameter, Km and Vmax, were calculated for analysis of activity of urease . Km is the [S] at 1/2 Vmax. Km is a constant for a given enzyme. Km is an estimate of the equilibrium constant for substrate binding to enzyme. Small Km means tight binding and high Km means weak binding. Km is a measure of [S] required for effective catalysis to occur. Km= k-1+k2/K1. Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation: $v$ = Vmax * [S]/ Km + [S]; Vmax is a constant for a given enzyme and it is the theoretical maximal rate of the reaction. To reach Vmax would require that all enzyme molecules have tightly bound substrate [49].

## 3. Results

### 3.1 Determination of particle size of nano$Fe_2O_3$ and nano-$Fe_3O_4$

In Fig.1 nano-$Fe_2O_3$ was found to be spherical in shape and the average particle diameter was about 11 nm.

In Fig.2 nano- $Fe_3O_4$ was found to be spherical in shape and the average particle diameter was about 12 nm.

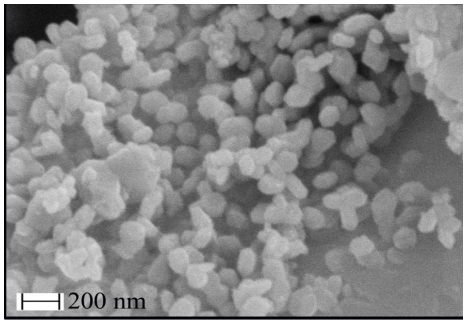


**Fig.1** SEM images of nano-$Fe_2O_3$.

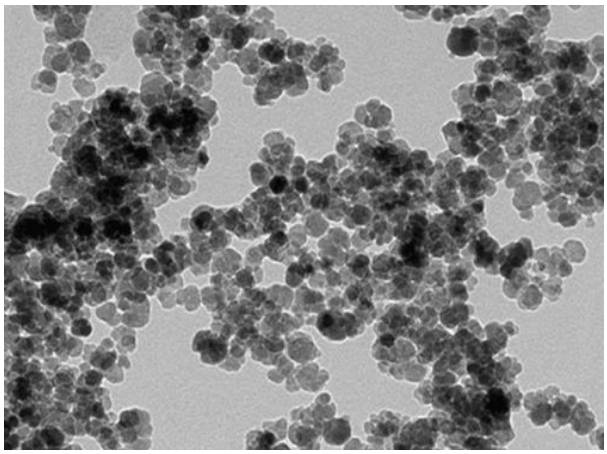

**Fig. 2.** TEM images of nano-$Fe_3O_4$.

### 3.2. Kinetics study of urease in the Presence of nano-$Fe_2O_3$ and $Fe_3O_4$

Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation. The best way to analyze enzyme kinetic data is to fit the data directly to the Michaelis-Menten equation using nonlinear regression. Before nonlinear regression was available, investigators had to transform curved data into straight lines so they could analyze with linear regression. One way to do this is with a Lineweaver-Burk plot. Ignoring experimental error, a plot of 1/V vs. 1/S will be linear, with a Y-intercept of 1/Vmax and a slope equal to Km/Vmax. The X-intercept equals -1/Km [50]. We measure enzyme velocity at many different concentrations of substrate. Urea was used as substrate and ammonia and $CO_2$ is product that amount of its measure at 630 nm. Result show urease had better activity on 40°C and in 30mM of substrate, enzyme arrived to Vmax. In Fig. 3 and 4 effect of different concentration of nanoparticles $Fe_2O_3$ and $Fe_3O_4$ was shown

As see in this figure in presence of nanoparticles $Fe_2O_3$ and $Fe_3O_4$, Vmax was decreased but Km was fix that indicate nanoparticles were inhibited enzyme by non-competitive mechanism. kinetics data are tabulated on Table 1 and 2.

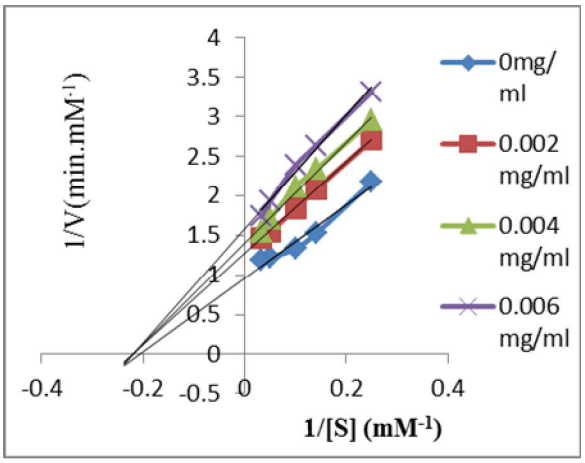


**Fig.3**. Lineweaver-Burk plot of urease at various nano-$Fe_2O_3$ concentrations at 40°C, pH =7.2.

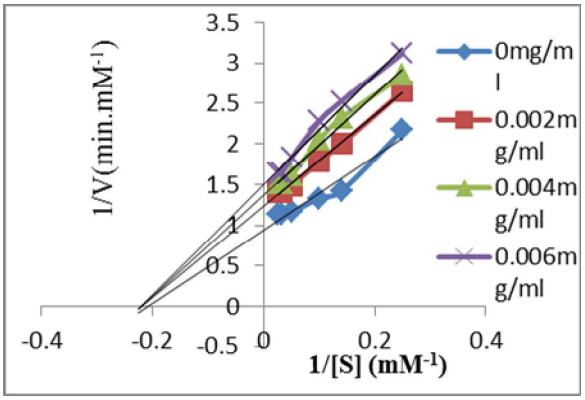


**Fig.4**. Lineweaver-Burk plot of urease at various nano-$Fe_3O_4$ concentrations at 40°C, pH =7.2.

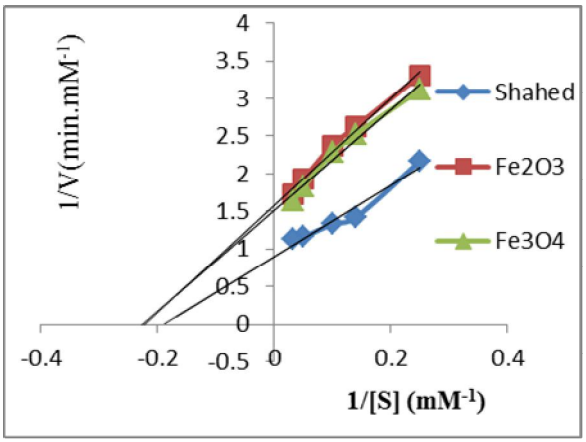


**Fig.5** Lineweaver-Burk plot of urease at presence of nano-$Fe_2O_3$ and nano-$Fe_3O_4$ (0.006mg/ml) at 40°C, pH =7.2.

As shown in Fig.3 and Fig.4, increasing the concentration of nanoparticles , have a negative effect on activity of urease and as shown in Fig.5 ,inhibitory effect of nano-$Fe_2O_3$ is more than nano $Fe_3O_4$.

**Table 1.** Kinetic parameter of Vmax and Km at various nano-$Fe_2O_3$ concentrations at 40°C, pH=7.2.

| Concentration of $Fe_2O_3$ | Km (mM) | Vmax ($mM.min^{-1}$) | Vmax/Km ($min^{-1}$) |
|---|---|---|---|
| 0 mg/ml | 4.52 | 1.03 | 0.22 |
| 0.002mg/ml | 4.50 | 0.787 | 0.17 |
| 0.004mg/ml | 4.52 | 0.713 | 0.15 |
| 0.006mg/ml | 4.53 | 0.634 | 0.13 |

As see on tables 1 and table 2, Kinetic parameters Km and Vmax enzyme obtained from Lineweaver–Burk plot in absence of nanoparticles is equal 4.52mM and 1/03mM.$min^{-1}$ respectively. In presence of na-

**Table 2.** Kinetic parameter of Vmax and Km at various nano-$Fe_3O_4$ concentrations at 40°C, pH=7.2.

| Concentration Of $Fe_3O_4$ | Km (mM) | Vmax ($mM.min^{-1}$) | Vmax/Km ($min^{-1}$) |
|---|---|---|---|
| 0 mg/ml | 4.52 | 1.03 | 0.22 |
| 0.002mg/ml | 4.53 | 0.813 | 0.17 |
| 0.004mg/ml | 4.56 | 0.739 | 0.16 |
| 0.006mg/ml | 4.57 | 0.668 | 0.14 |

noparticles, value Km is fix (4.52±0.05) but Vmax is decrease. That show inhibitory mechanism of nanoparticles was non competitive - inhibition. kinetics data are tabulated on table 1and2.

The activity of enzyme was measured against of different concentration of EDTA with of 0.006mg/ml of each of nanoparticles . For the protection with thiols, urease was preincubated With different concentrations of EDTA from the range 0.004 to 0.008 mg/ml and each of Nanoparticles (0.006mg/ml) that results shown in Figs 6 and 7.

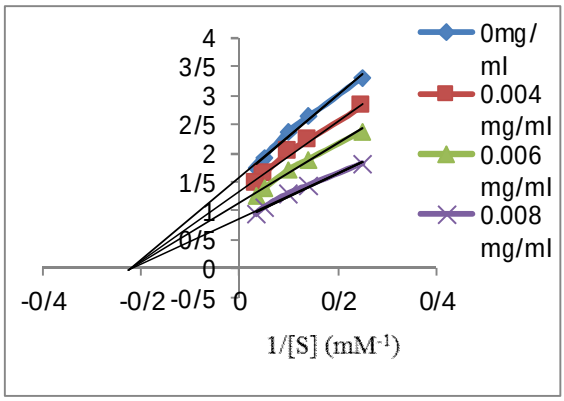


**Fig.6**. Lineweaver-Burk plot of urease at various EDTA concentrations with nano-$Fe_2O_3$ (0.006mg/ml) at 40°C, pH=7.2.

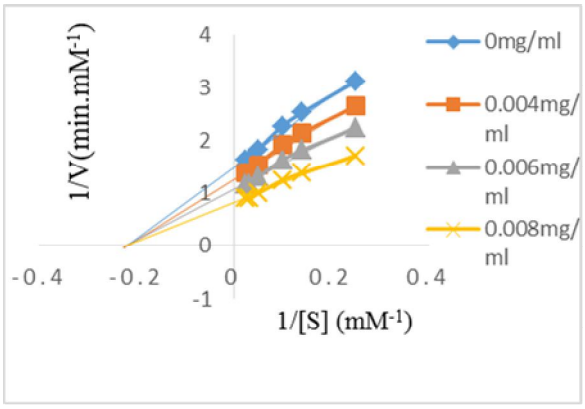


**Fig.7**. Lineweaver-Burk plot of urease at various EDTA concentrations with nano-$Fe_3O_4$(0.006mg/ml) at 40°C, pH=7.2

**Table 3.** Kinetic parameter of Vmax and Km at various EDTA concentrations with nano-$Fe_2O_3$(0.006mg/ml) at 40°C, pH=7.2.

| Concentration of EDTA+$Fe_2O_3$ (0.006mg/ml) | Km (mM) | Vmax ($mM.min^{-1}$) | Vmax/Km ($min^{-1}$) |
|---|---|---|---|
| 0 mg/ml | 4.5 | 0.63 | 0.14 |
| 0.004mg/ml | 4.5 | 0.746 | 0.16 |
| 0.006mg/ml | 4.5 | 0.886 | 0.19 |
| 0.008mg/ml | 4.5 | 1.15 | 0.25 |

**Table 4.** Kinetic parameter of Vmax and Km at various EDTA concentrations with nano-$Fe_3O_4$(0.006mg/ml) at 40°C, pH=7.2.

| Concentration of EDTA+$Fe_3O_4$ (0.006mg/ml) | Km (mM) | Vmax ($mM.min^{-1}$) | Vmax/Km ($min^{-1}$) |
|---|---|---|---|
| 0 mg/ml | 4.5 | 0.668 | 0.14 |
| 0.004mg/ml | 4.5 | 0.789 | 0.17 |
| 0.006mg/ml | 4.5 | 0.929 | 0.20 |
| 0.008mg/ml | 4.5 | 1.21 | 0.26 |

As see on tables 3and table 4, at the concentration of 0.004-0.008mg/ml of EDTA, amount of Vmax enzyme was increased and Km was fix that show protection effect EDTA on activity of Urease [1].

## 4. Discussion

In this study, the effects of nano-$Fe_2O_3$ and nano-$Fe_3O_4$ on the enzyme activity were studied specifically, the results of which show that the nanoparticles were inhibitory effect on the urease . The kinetic parameters for the enzyme obtained from Lineweaver–Burk plot show that Km is equal to 4.52mM and Vmax is equal to 1.03Mm.$min^{-1}$. The results showed that Nano-$Fe_2O_3$ and Nano-$Fe_3O_4$ act as non competitive inhibitor for urease. The inhibition of urease by nanoparticles is said to result from the reaction of these nanoparticlese with a sulfhydryl group of Cys in the mobile flap of the active site of the enzyme [31,33 ] in a reaction analogous to the formation of sulfides. The enzyme requiring the presence of free –SH groups are generally inhibited by nanoparticlese and the nanoparticles that form the most insoluble sulfides are the strongest inhibitors. The time-dependent inhibition of urease with nanoparticles in absence of urea appears to be due to the interaction of nanoparticles with the –SH groups of the enzyme protein. For the protection with thiols, urease was preincubated With different concentrations of EDTA from the range 0.004 to 0.008 mg/ml and each of Nanoparticles (0.006mg/ml). When that Urease pre-incubated with different concentrations EDTA and each of nanoparticles (at range 0.006mg/ml),syncholorary, EDTA with nanoparticles made a complex ,SO, EDTA was

capable of preventing the inactivation Urease with nanoparticles while Researchs showed that the immobilized Urease with $Fe_2O_3$ and $Fe_3O_4$ magnetic nanoparticles has activity not affected by wide change of pH, longer period of storage without losing catalytic activity and high thermal stability[51]. These results are expected to open up a new possibility for the enzyme immobilization as well as a new application of magnetic nanoparticles. Immobilization of enzyme onmagnetic nanoparticle offers several advantages compared to other conventional support because of easy product isolation by using a permanent magnet,low cost, facile preparation procedure and high chemical stability of enzyme onmagnetic nanoparticle. This low cost root paves the way for immobilization of other industrially important enzymes easily. The key step in the enzymatic process lies in successful immobilization of the enzyme that allows for its recovery and reuse[51]. The optimum pH and temperature profiles of the immobilized enzymes has compared to free form. The thermal stability of the urease was increased upon immobilization. This support is a promising material for storage and enzyme immobilization.[52,53] ,such, another research evidenced that this nanoparticles have positive effect on activity of lysozyme[54,55]. It has been reported that the presence of $Fe_2O_3$ magnetic nanoparticle can increase the β-sheet and α-helix contents. On the contrary, the contents of γ-random coil and T-turns will be reduced in the presence of nano-$Fe_2O_3$ [56, 57]. So can conclude that the presence of nano-$Fe_2O_3$ can increase the activity of lysozyme.

## 5. Conclusion

To our knowledge this is the first study on the effects of nano-$Fe_2O_3$ and nano-$Fe_3O_4$ on the activity of Urease.

As see in result section nano-$Fe_2O_3$ and nano-$Fe_3O_4$ had noncompetitive inhibitory effect on activity of Urease ,such,different concentrations EDTA decrease inhibitory effect of nanoparticles, Thus nano-$Fe_2O_3$ and nano-$Fe_3O_4$ might have some toxic effect on biomolecules. Hence we suggest that prior of use of nanoparticle on various industry, different aspect of interaction of nano particles with various proteins must be considered.

## References

[1] K. Hund-Rinke, M. Simon, Environmental Science and Pollution Research 13 (2006) 225.

[2] V. Stone, H. Johnston, M.J.D. Clift, Geoscience and Remote Sensing, IEEE Transactions 6 (2007) 331.

[3] Y. Wang, B. Wang, M.T. Zhu, M. Li, H.J. Wang, M. Wang, H. Ouyang, Z.F. Chai, W.Y. Feng, Y.L.

Zhao, Toxicology letters 205 (2011) 26.

[4] J.J. Wang, B.J.S. Sanderson, H. Wang, Mutation Research - Genetic Toxicology and Environmental 628

(2007) 99.

[5] S.B. Lovern, J.R. Strickler, R. Klaper, Environmental Science and Technology 41 (2007) 4465.

[6] N. Lu, Z. Zhu, X. Zhao, R. Tao, X. Yang, Z. Gao, Biochemical and Biophysical Research Co 370 (2008)
675.
[7] X.H. Chen, L. Zhang, Y.X. Weng, L.C. Du, M.P. Ye, G.Z. Yang, R. Fujii, F.S. Rondonuwu, Y. Koyama,
Y.S. Wu, Biophysical Journal 88 (2005) 4262.
[8] G. Oberd, E. Oberd, J. Oberd, Environmental Health Perspectives 113 (2005) 823.
[9] S.T. Stern, S.E. McNeil, Toxicological Sciences 101 (2008) 4.
[10] C. Buzea, I. Pacheco, K. Robbie, Comparative Biochemistry and Physiology 143 (2006) 123.
[11] M. Heinlaan, A. Ivask, I. Blinova, H.C. Dubourguier, A. Kahru, Chemosphere 71 (2008) 1308.
[12] W.G. Kreyling, M. Semmler, F. Erbe, P. Mayer, S. Takenaka, H. Schulz, G. Oberd, A. Ziesenis,
Journal of Toxicology and Environmental Health Part A 65 (2002) 1513.
[13]. R. L. Edelstein, C. R. Tamanaha, et al. Biosensors and Bioelectronics 14 (2000) 805-813.
[14]. JM. Nam, CC. Thaxton, CA. Mirkin, Science301 (2003) 1884-1886.
[15]. R. S Molday and D. Mackenzie, Journal of immunological methods 52 (1982) 353-367.
[16]. J. Yoshida, T. Kobayashi, J Magn Magn Mater 194 (1999) 176-184.

[17]. K. Donaldson, X. Y. Li, et al. Journal of Aerosol Science 29 (1998) 553-560.
[18]. B. Fahmy and S. A. Cormier, Toxicology in Vitro 23 (2009) 1365-1371.
[19] M. Bystrzejewski, S. Cudzilo, A. Huczko, H. Lange, G. Soucy, G.C. Sanchez, W.Kaszuwara, Biomol. Eng. 24 (2007) 555–558.
[20] J.M. Perez, F.J. Simeone, Y. Saeki, L. Josephson, R. Weissleder, J. Am. Chem. Soc. 125 (2003) 10192–10193.
[21] J. Gao, H. Gu, A.B. Xu, Acc. Chem. Res. 42 (2009) 1097–1107.
[22] A. Fornara, P. Johansson, K. Petersson, S. Gustafsson, J. Qin, E. Olsson, D. Ilver,
A. Krozer, M. Muhammed, C. Johansson, Nano Lett. 8 (2008) 3423–3428.
[23] P. Pouponneau, J.C. Leroux, S. Martel, Biomaterials 30 (2009) 6327–6332.
[24] Y. Yong, Y. Bai, Y. Li, L. Lin, Y. Cui, C. Xia, J. Magn. Magn. Mater. 320 (2008)
2350–2355.
[25] L. Wang, X. Gan, Microchim. Acta 164 (2009) 231–237.
[26] Z. Shan, Q. Wua, X. Wang, Z. Zhou, K.D. Oakes, X. Zhang, Q. Huang, W. Yang,
Anal. Biochem. 398 (2010) 120–122.
[27] T.T. Baby, S. Ramaprabhu, Talanta 80 (2010) 2016–2022.
[28] M.C. Urbina, S. Zinoveva, T. Miller, C.M. Sabliov, W.T. Monroe, C.S.S.R. Kumar, J. Phys. Chem. C 112 (2008) 11102–11108.
[29] Y. Li, X. Li, J. Chu, C. Dong, J. Qi, Y. Yuan, Environ. Pollut. 158 (2010) 2317–2323.
[30]. K,Barbara . potency.Enzymatic 68(2011)262-
269.DOI:10.1016/j.molcat.2010.11.015.

[31]. A,Sinan., Y,yagmur., B,GÜla., D,Adil.,M.Yakup.process Biochemistry 38(2002)675-683.PII:S0032-9592(02)00198-X.

[32]. K, Barbara. Inactivation by heavy metal ions. J. Chem. Technol. Biotechnol. 52, 157–162.

[33]. K,Barbara., W,Zaborska., M,Chudy.. J. Inorg. Biochem. 98, 1160–1168.

[34]. R.L,Mulvaney.,J.M. Bremner Soil Biochem. 5 (1981) 153.

[35]. J, Laska., J,Wlodarczyk., W,Zaborska. Polyaniline as asupport for urease immobilization. J Mol Catal B: Enzymatic 1999;6:549.

[36]. J-P,Chen.,S-H, Chiu . Enzyme Microb Technol 2000;26:359.

[37]. E,Hearn., RJ,Neufeld. Process Biochem 2000;35:1253.

[38]. K,Barbara. Journal of Molecular Catalysis B: Enzymatic 59 (1–3): 9–21.

[39]. K,Barbara., E,Rudi.,B, Małgorzata. JBIC Journal of Biological Inorganic Chemistry 17 (7): 1123–1134.

[40]. K,Barbara.,C,Stefano. plant physiology and Biochemistry 43(2005)651-658.DOI:10.1016/J.Plaphy-2005.05.009.

[41]. M.A,Pearson., I.S,Park.,R.A, Schaller., L.O,Michel., P.A,Karplus., R.P,Hausinger. Biochemistry 39(2000)8575-8584.

[42].S, Ciurli., S,Manganic Handbook on metalloproteins ,mercel Dekker,Newyork,2001,pp.669-707.

[43]. N.E,Dixon., C,Gazzola., R.L,Blakeley., B,Zerner.j.Am.chem.soc.97(1975)4131-4133.

[44]. P.W,Riddles., R.K,Andrews., R.L,Blakeley. ,B, Zerner. Biochim. Biophys. Acta 743 (1983) 115.

[45]. M.J, Todd.,R.P, Hausinger. Bio l, J.1991. Chem. 266 (1991) 10260.

[46]. M.W,Winterbourn., ANAL.Analytical chemistry 39(1967). 971-974 .

[47]. D, Sonila and .C,Alqi. Podgorica,9(3):743-751.

[48]. P,Ga-eun.,O, Ha-na.,and A,samyoung.Bull.korean chem..soc.2009,Vol.30,No.9.

[49]. SJ. Tseng, JP. Hsu, J. Theor. Biol.145 (1990) 457–464.

[50]. J.P. Jaroslow, M. Mazzarello, et al, Mol Divers 15 (2011) 215-226.

[51]. B. Sahoo, S. Kumar Sahu , P.Pramanik. Journal of Molecular Catalysis B: Enzymatic 69 (2011) 95–102.

[52]. M. Tortajada, D. Ramon, D. Beltrand, P. Amoros, J. Mater. Chem. 15 (2005) 3859–3868.

[53]. G. Bayramoglu, H. Altınok, A. Bulut, A. Denizli, M.Y. Arıca, React. Funct. Polym. 56 (2003) 111–121.

[54]. B. Shareghi, S. Farhadian, M. Salavati-Niasarib, Journal of Nanostructures 1 (2012) 205.

[55]. S. Farhadian, B. Shareghi, M. Salavati-Niasari, R. Amooaghaeia, Journal of Nanostructures 1 (2011) 95.

[56]. J. Sun, R. Xu, Y. Yang, Journal of Chromatography B 879 (2011) 3053.

[57]. A. Bonincontro, G. Onori, Chemical Physics Letters 398 (2004) 260.