Iraqi Journal of Science, 2023, Vol. 64, No. 12, pp: 6093- 6103
DOI: 10.24996/ijs.2023.64.12.2

ISSN: 0067-2904

Enzymatic Assay of Immobilized β-D-Galactosidase Enzyme on Magnetite Nanoparticle

Ruaa Lateef Noor Al-Hajjar*, Ekhlass M. Taha, Ahlam M. Farhan, Naser Shaalan
Department of Chemistry, College of Science for Women, University of Baghdad, Baghdad-Iraq

Received: 18/8/2022 Accepted: 22/1/2023 Published: 30/12/2023

Abstract

According to the high operational costs, low stability, and reusability of enzymes, immobilization by nanoparticle gathering has increased in recent years. Iron oxide nanoparticles (magnetite nanoparticles, Fe_3O_4) have been prepared by mixing one volume of iron dioxide ions and two volumes of iron trioxide ions with HCl *via* the precipitation of iron salts by NH_4OH. The features of magnetic nanoparticles have been studied by Atomic Force Microscopy (AFM), X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy, and Scanning Electron Microscopy (SEM). The prepared Fe_3O_4 was used in the adsorption method to immobilize the galactosidase enzyme. The immobilized enzyme has been compared with the crude one by optimizing time, PH, temperature, substrate solution concentration, and enzyme solution concentration. As a result, the immobilized enzyme has exhibited higher activity over long storage times, and a wide PH and temperature range than crude enzyme. In addition, whatever the substrate and enzyme concentrations, the activity of the immobilized enzyme increased over the non-immobilized one. As a result, a lower Km value for immobilized D-galactosidase indicates a higher affinity for the substrate than crude D-galactosidase.

Keywords: Immobilization of enzymes, Magnetite nanoparticle, β-Galactosidase enzyme, Kinetic of enzyme.

المقايسة الأنزيمية لإنزيم β−D−galactosidase المُثبت على جسيمات الحديد النانوية

رؤى لطيف نور الحجار*، إخلاص محيي الدين طه، أحلام محمد فرحان، ناصر ضياء شعلان
قسم الكيمياء, كلية علوم البنات, جامعة بغداد, بغداد, العراق

الخلاصة

وفقًا لتكاليف العمل المرتفعه بالأنزيمات، وانخفاض استقراريتها وعدم أمكانية أعادة أستخدامها فإن تقييد الأنزيم (تثبيته) عن طريق الجسيمات النانوية نالت مزيدا من الأهتمام في السنوات السابقه. تم تحضير جزيئات أكسيد الحديد النانوية Fe_3O_4 بخلط حجم واحد من أيون الحديد ثنائي التأكسد وحجمين من أيون الحديد ثلاثي التأكسد مع حامض الهيدروكلوريك HCl عن طريق ترسيب أملاح الحديد بواسطة أضافة هيدروكسيد الأمونيوم NH_4OH الى المحلول. تمت دراسة مميزات الجسيمات النانوية المغناطيسية بواسطة الفحص المجهري للقوة الذرية (AFM)، وحيود الأشعة السينية (XRD)، والتحليل الطيفي بالأشعة تحت الحمراء (FTIR) ، والفحص المجهري الإلكتروني (SEM). تم استخدام Fe_3O_4 المحضر لتثبيت إنزيم β−D− galactosidase بطريقة

*Email: Ruaa.lateef1105a@csw.uobaghdad.edu.iq

الامتزاز . وقد تمت مقارنة الإنزيم المثبت مع الأنزيم الخام عن طريق تحسين الوقت، ودرجة الحموضة، ودرجة الحرارة، وتركيز المادة الأساس، وتركيز محلول الإنزيم. نتيجة لذلك، أظهر الإنزيم المثبت نشاطًا أعلى لوقت التخزين الطويل ومدى واسع لدرجة الحموضة ودرجة الحرارة مقارنة بالإنزيم الخام. وكذلك، مع زيادة تركيز المادة الأساس وتركيز الإنزيم في محلول القياس يزداد نشاط الإنزيم المثبت مقارنة بالخام. أيضا، يشير صغر قيمة Km للأنزيم المثبت الى الفته الكبيره للمادة الأساس مقارنة بالأنزيم الخام.

1. Introduction:

β-Galactosidase enzyme (EC3.2.1.23) is a tetramer of four identical polypeptide chains, each containing 1023 amino acids, in monoclinic crystal form with four tetramers in the asymmetric unit 13. Galactosidases can be derived from recombinant sources, plants, animals, and microbes such as fungi, bacteria, and yeasts. [1]. Due to the high operational costs associated with utilizing enzymes in large-scale industrial processes as well as their poor stability and reusability, immobilizing the enzyme on support materials has gained significant momentum in recent years. It is well known that such technology may protect and/or stabilize enzymes against chemical and environmental assaults. Additionally, it is vital to note that the immobilized enzymes can be retrieved and employed again in a large-scale, continuous operation [2]. Immobilization means reducing or eliminating motion in a body or a part by mechanical means. The immobilization technique emerges as a preferred method for preserving the enzyme. Due to recent developments in nanotechnology, a variety of nanoparticles, nanofibers, and mesoporous materials have been produced as carriers [3,4]. In addition to being less harmful than certain physicochemical manufacturing techniques, biosynthesis of nanoparticles is more distinctive and trustworthy since it can be utilized to generate vast numbers of nanoparticles with good form and size that are also free of combination. Due to their distinctive qualities, such as low toxicity and superparamagnetism, magnetite (Fe_3O_4) nanoparticles have received a lot of interest in recent years [5,6]. Most of the enzymes on magnetic nanoparticles showed surprisingly excellent stability against heat, pH, and other denaturants. Compared to free enzymes, enzymes immobilized on magnetic nanoparticles were shown to be more resistant to denaturation caused by organic solvents and less inhibitory to their particular inhibitors. The enzymes attached to magnetic nanoparticles have been employed independently in a variety of sectors, including biomedicine, biotechnology, environmental science, bioanalysis, medicine, and numerous other applications [7-11]. Many scientists have concluded that after making the necessary modifications to magnetic nanoparticles, magnetic nanoparticles have become an excellent supports for the immobilization and stabilization of enzymes. Enzymes immobilized on magnetic supports have obtained remarkably high operational stability and maintained good activity even after several repeated uses in batch processes [12-15].

2. Materials and methods

2.1. Chemicals

Table 1: The chemicals used in laboratory work and their product companies and countries

Chemicals	Product company	Country
▪ β-Galactosidase enzyme ▪ O-Nitro phenyl-β-D-Galactoside substrate	Nanjing Duly Biotech Co. Ltd	China
▪ ammonium hydroxide (NH_4OH) ▪ hydrochloric acid (HCl) ▪ Iron(II) chloride tetrahydrate ($FeCl_2.4H_2O$)	Thomas Baker (Chemicals) Private Limited	India
▪ Iron(III) chloride hexa-hydrate ($FeCl_3.6H_2O$)	Qualikemis Lifesciences Private Limited	India
▪ Sodium phosphate dibasic hepta-hydrate ($Na_2HPO_4 \cdot 7H_2O$)	S D Fine Chem Limited	India
▪ Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)	PanReac AppliChem	Spain
▪ Citric acid ▪ Boric acid	HiMedia	India

2.2. Nanoparticle synthesis

For magnetite nanoparticle synthesis**,** $FeCl_2.4H_2O$ (400 mg) and $FeCl_3.6H_2O$ (1.08 g) were dissolved in HCL (5 mL, 2.0 M) in a three-neck round-bottom flask (the solution color is yellow). The solution of NH_4OH (50 mL, 0.7 M) was then added drop by drop until the color changed to black (about 30 minutes). The precipitate and supernatant were obtained by filtering the sample. The precipitate of Fe_3O_4 was then washed with methanol to remove all the impurities (Figure 1).

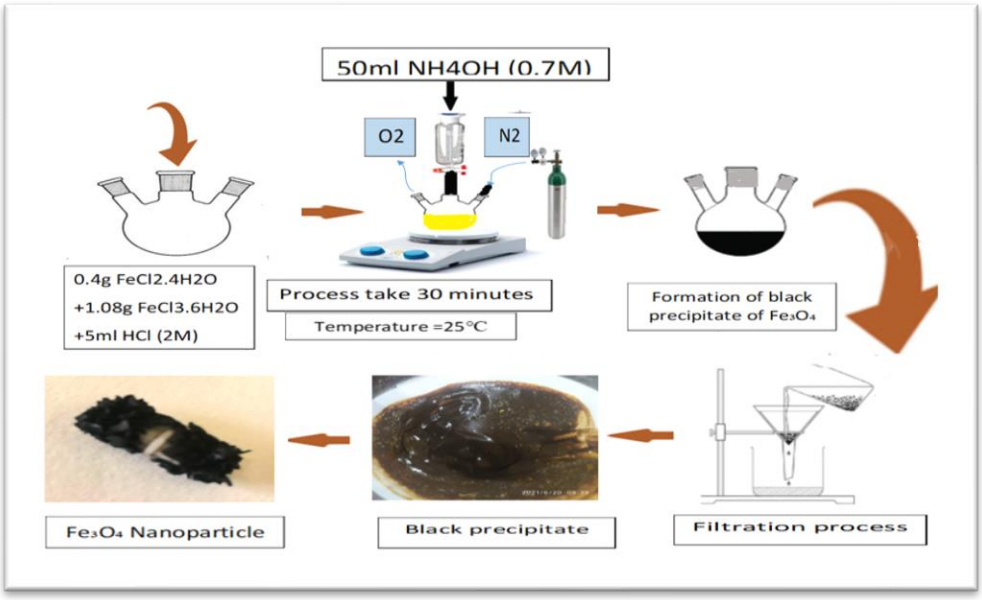


Figure 1: Schematic representation of magnetite Fe_3O_4 nanoparticle synthesis [4]

2.3. Immobilization procedure

For β-galactosidase enzyme immobilization onto magnetic nanoparticle by adsorption method, it was dissolved Fe_3O_4 (500 mg) with phosphate buffer (5 mL, 50 Mm, pH = 6.5). The mixture was sonicated by an ultrasonication device for 30 minutes to disperse the particles. Crude enzyme (β-galactosidase) (100 mg) was dissolved in the phosphate buffer (10 mL) before adding to the dispersed solution. The mixture was incubated at 15 ºC for 4 hours with shaking. Following that, a strong magnet was placed in the tube to collect the precipitant, filter the solution, and keep the supernatant with the immobilized enzyme. Finally, the precipitant was

washed three times with phosphate buffer to remove any impurities. The produced components are stored in a refrigerator at 15 ºC until use. The activities of particles that stuck to the magnet (immobilized), the supernatant, and crude enzyme (non-immobilized) have been determined.

2.4. Method for kinetic parameters determination

The method includes the preparation of buffers, substrates, and enzyme solutions, as mentioned below:

Citrate buffer (400 mM, pH = 4.0 at 25 °C): The preparation of citrate buffer was made by weighting citric acid ($C_6H_8O_7$) (7.68 g) and dissolving it in deionized water (80 mL). At 25 °C, the pH of the solution was adjusted to 4.0 using NaOH (1.0 M), and the volume was increased until it reached 100 mL (M.Wt. of citric acid is 192.124 g/mol).

Borate buffer (200 mM, pH = 9.8 at 25 °C): The preparation of borate buffer was made by weighting boric acid $B(OH)_3$ (1.2 g) and dissolving it in deionized water (80 mL). At 25 °C, the pH of the solution was adjusted to 9.8 using NaOH (1.0 M), and the volume was increased until it reached 100 mL (M.Wt of boric acid = 61.833 g/mol).

o-Nitrophenyl-β-D-galactoside substrate solution (ONP-Gal) (10 Millimolar): The preparation of substrate solution was done by dissolving *o*-Nitrophenyl-β-D-galactopyranoside (15 mg) in deioinized water (5 mL) (M.Wt of ONP-Gal = 301.26 g/mol).

β-Galactosidase enzyme solution: A solution of 0.05-0.10 units/mL of the β-galactosidase enzyme in cold deionized water was prepared immediately before use.

3. Results and discussion

3.1. Synthesis of MNPs (Fe_3O_4)

3.1.1. AFM analysis for MNPs (Fe_3O_4)

The topography of the nanomaterials is investigated using atomic-force microscopy (AFM). AFM has been studied to analyze the diameter of the nanoparticles to verify the morphology and size of the synthesized MNPs samples, as shown in Figure 2. The average size distribution of particles of MNPs tested by AFM has an average diameter of less than 71 nm. The amorphous and granular shapes are shown in Figure 3.

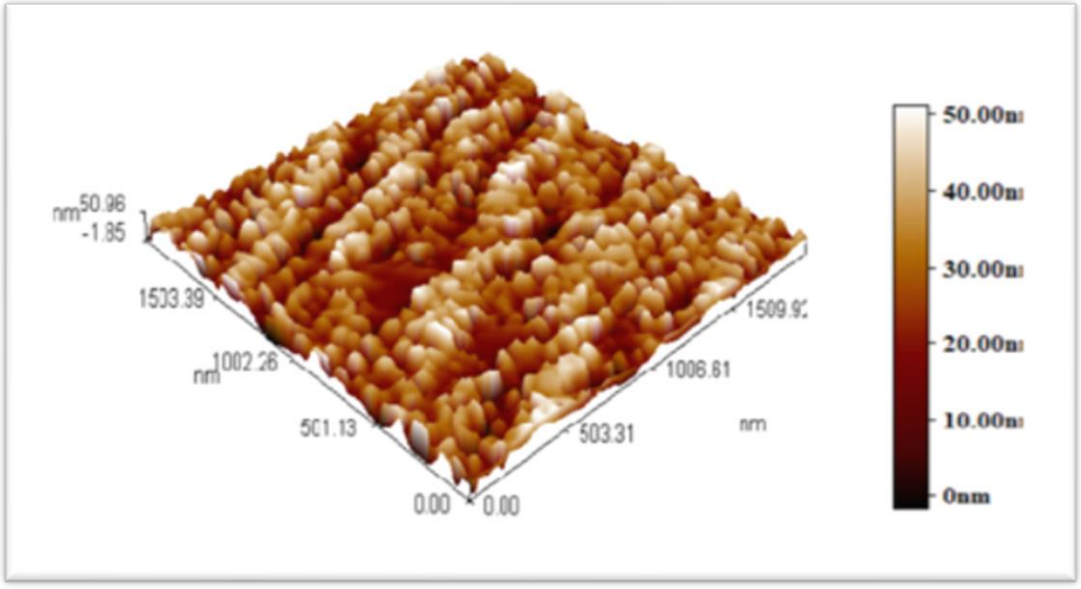


Figure 2 : Atomic Force Microscopy (AFM) images of MNPs' diameter and 3D topography

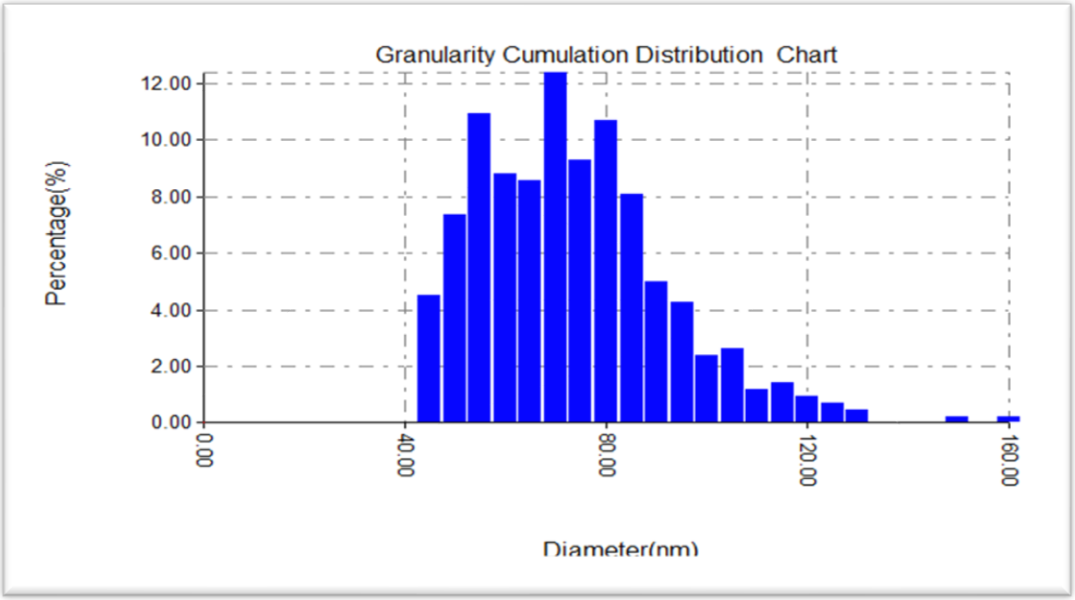


Figure 3 : Atomic Force Microscopy (AFM) images of MNPs' bars graph and granularity distribution

3.1.2. Scanning electron microscope (SEM) of Fe_3O_4 MNPs

The scanning electron microscope (SEM) is a direct technique for determining nanoparticle size and surface morphology. It is used to investigate the morphology, topography, and average particle size. SEM generates a range of signals at the surface of solid specimens using a concentrated beam of high-energy electrons. The external appearance, texture, chemical composition, and crystalline structure and orientation of components that make up the sample are all revealed by the signals derived from electron sample interactions. The morphology and size of the synthesized MNPs samples were verified using SEM, which was utilized to assess the diameter of the nanoparticles as illustrated in Figure 4. An SEM image of the magnetite nanoparticle (Fe_3O_4) has been attached below at 100 nm.

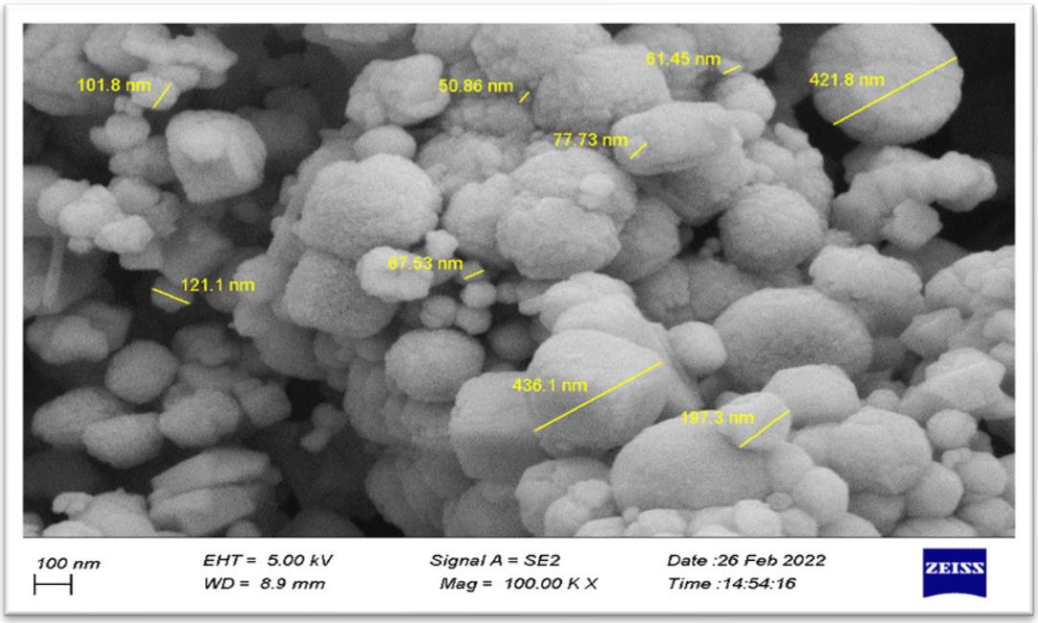


Figure 4 : Scanning electron microscope (SEM) images of MNPs' Fe_3O_4

3.1.3. X-ray diffraction analysis (XRD)

XRD is used for phase identification and characterization of the crystal structure of the nanoparticles. The prepared nanoparticles had the following XRD crystal sizes and 2-theta values: crystal size (20 nm) and theta value (20.52), crystal size (17 nm) and theta value (23.068), crystal size (13 nm) and theta value (46.485), crystal size (14 nm) and theta value (62.784), crystal size (13 nm) and theta value (66.796), as shown in Table 2 and Figure 5.

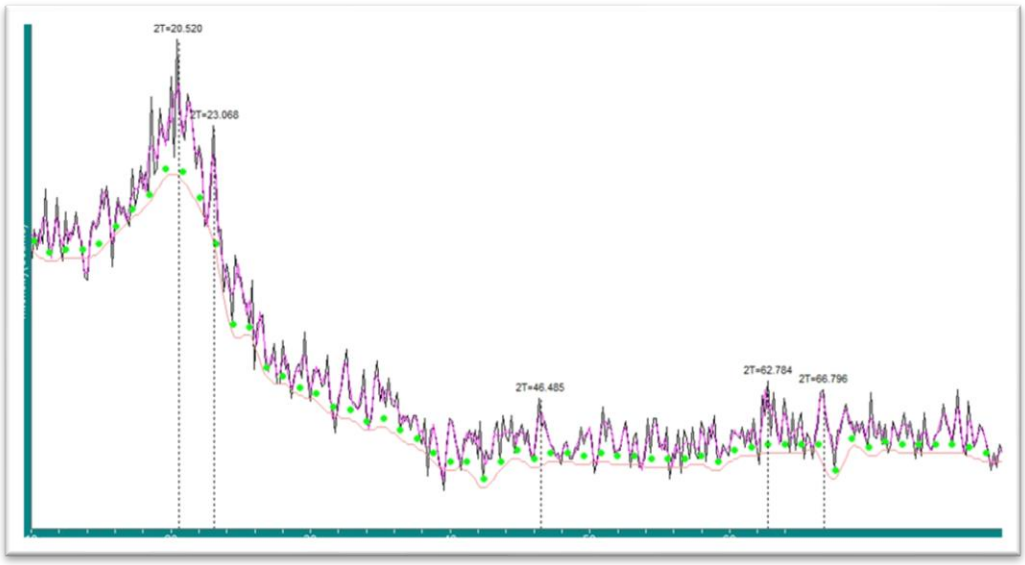


Figure 5: X-ray diffraction (XRD) of Fe_3O_4 peaks clarifies 2-theta values

Table 2: Summary of the XRD information of MNPs Fe_3O_4 peaks

2-Theta	d(nm)	BG	Height	I%	Area	I%	FWHM	Crystal size (nm)
20.52	0.43247	123	47	100	112	100	0.405	20
23.068	0.38524	100	40	85.1	109	97.3	0.463	17
46.485	0.19519	21	24	51.1	90	80.4	0.638	13
62.784	0.14788	26	25	53.2	99	88.4	0.673	14
66.796	0.13994	22	26	55.3	111	99.1	0.726	13

3.1.4. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy can be used to assess the presence of chemical functional groups in functionalized nanomaterials as well as the structure of a material [16]. The values of the vibration and absorption spectra have been shown in Table 2. The result of FT-IR confirmed the formation of MNPs as the strong peak for Fe_3O_4 was presented at 559 nm, as shown in Figure 6.

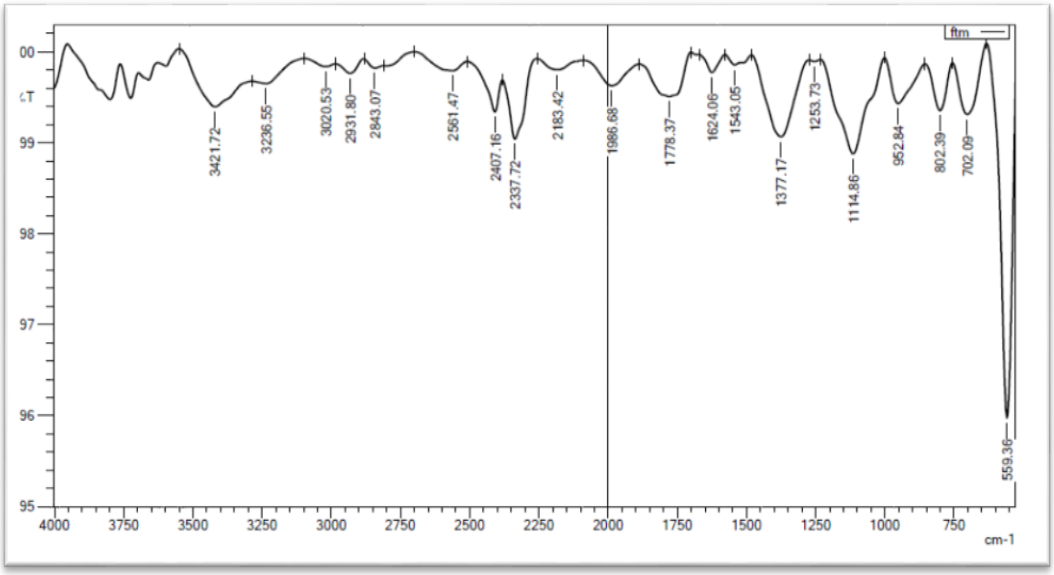


Figure 6 : FT-IR analysis of MNPs' Fe_3O_4

3.2. Factors affecting enzyme activity:

Studying the factors of kinetics, that effect immobilization is a very important part and is considered an integral study for the first part of our research (the immobilization of enzymes). All data have been performed in triplicate and reported as mean values.

3.2.1. Effect of time

The higher activity of immobilized enzyme over crude enzyme was investigated by measuring activity every 24 hours for five days. Immobilized enzyme exhibits a slight increase in activity after the first 24 hours and reaches its peak after 72 hours. Then, it started decreasing slightly until the last day (120 hours). As contrasted with crude enzymes, which record their highest activity on the first day and begin to decline until they reach the third day of storage, then begin their straight phase until the last day (Figure 7 A).

3.2.2. Effect of pH

The comparison of free and immobilized enzyme activity has been studied for a pH range between 3 and 12, while keeping other factors constant. Free and immobilized enzymes show higher activity at pH 9 (the optimum pH) over other pH values. The immobilized enzyme demonstrated high stability against a wide range of pH and maintained a higher activity level over time than the free enzyme (Figure 7 B). The pH values in the acidic range (less than 7) are considered unfavorable for enzyme reactions, according to our findings, because the enzyme's NH_2 group tends to be protonated in an acidic environment. Therefore, the kinetics of an immobilized enzyme in an acidic condition is much smaller than that in a under neutral or basic environment. A high pH value has given higher activity and stability for native and immobilized enzymes and higher reaction efficiency than a low pH value.

3.2.3. Effect of temperature

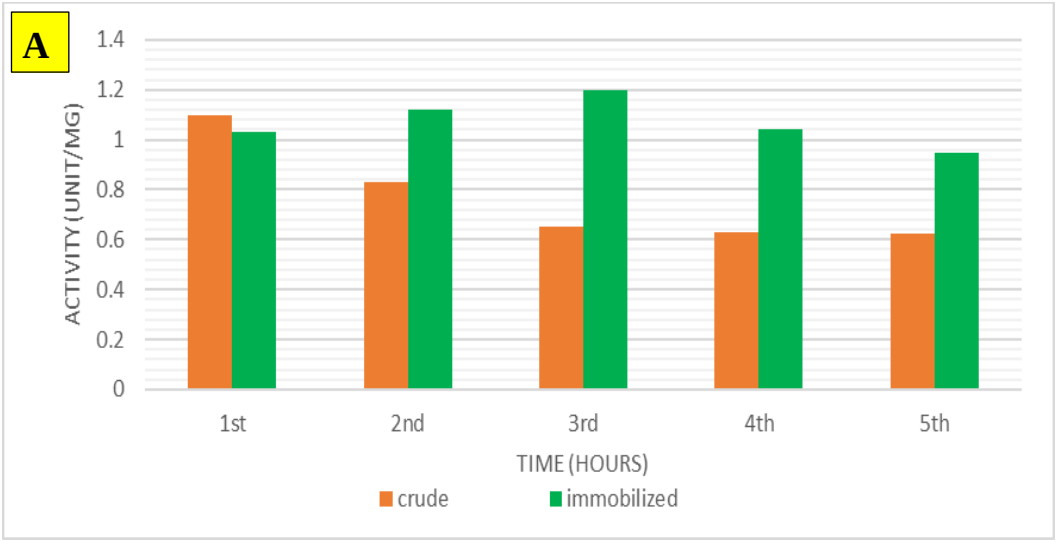
The effect of temperature on the activity of native and immobilized enzymes has been studied at a range between 5 and 45 °C while keeping the other factors constant. It has been noticed that as well as being centigrade smaller, crude and immobilized enzymes show higher activity. The immobilized enzyme records higher activity for all temperature ranges than the crude enzyme. As a result, our finding confirms that immobilized enzyme is less sensitive to temperature change and has more heat resistance than free enzyme (Figure 7C).

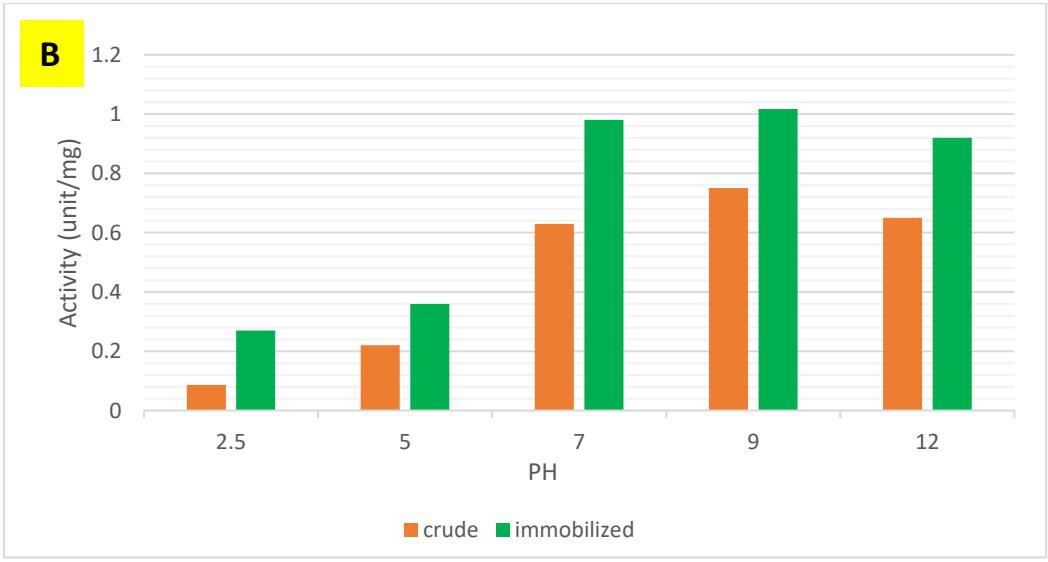
3.2.4. Effect of enzyme-substrate concentration

Studying the activity of native and immobilized enzymes was done by changing substrate concentrations while the rest of the other factors remained fixed. The study included a range of substrate concentrations ranging from 50 to 250 mg/mL. As the substrate concentration in the solution increased, so did the activity of all forms of the β-D-galactosidase enzyme. The immobilized enzyme showed higher activity along the range of substrate concentrations over the free one and recorded its highest activity at 250 mg/mL (Figure 7 D).

3.2.5. Effect of enzyme concentration

Different concentrations of enzyme solution (free and immobilized) ranging between 0.1 and 0.9 mg/mL were studied while keeping the other factors constant. It was found that there is a directly proportional relationship between enzyme concentration and their activity. As a result, the efficiency of reactions increases as the concentration of enzymes increases. The activity of the enzyme (immobilized or not) increased as the enzyme concentration in the solution increased. Immobilized enzyme has recorded higher activity for all the range of concentration over the native one (Figure 7 E).





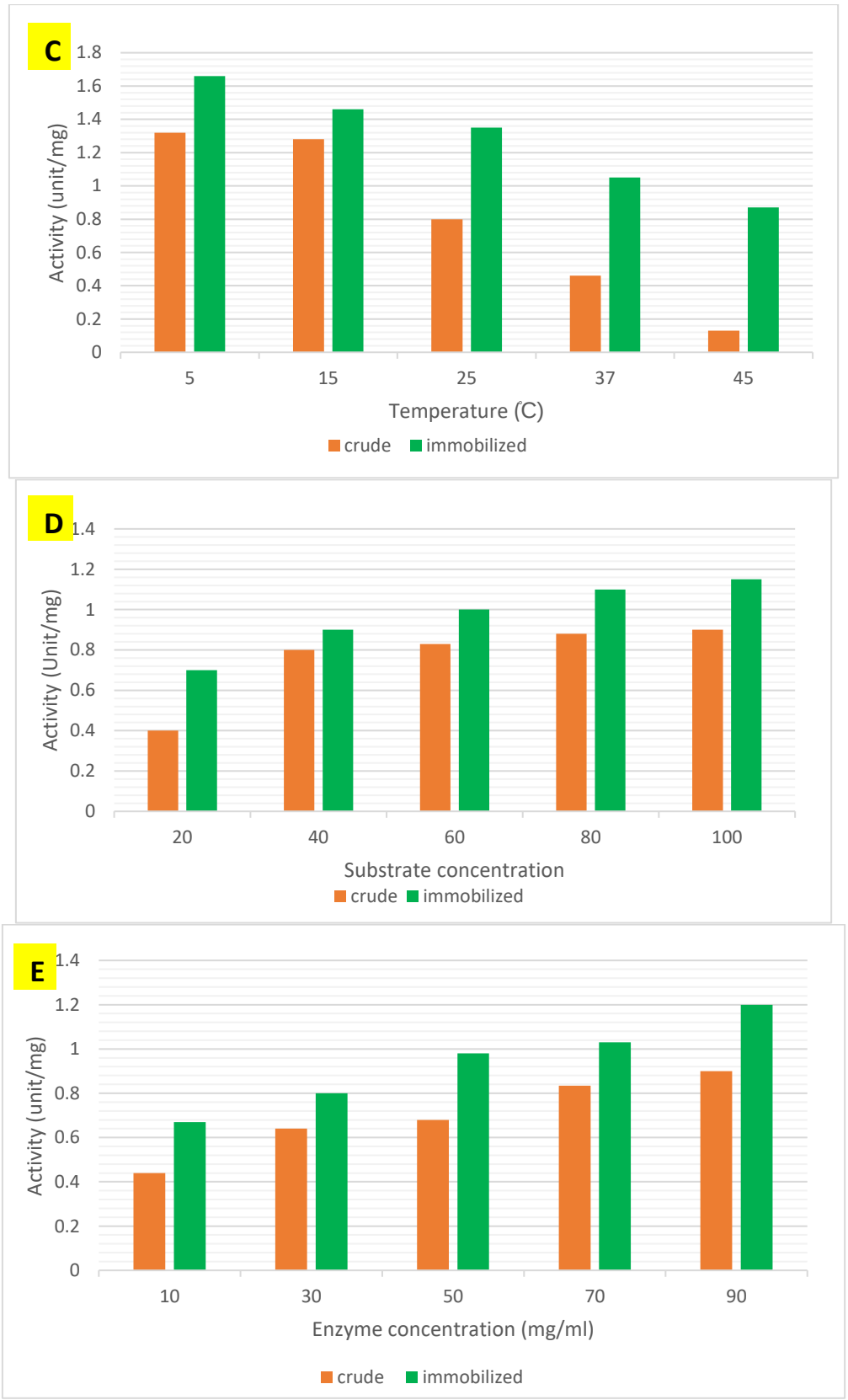


Figure 7 : The Effect of **A**. Time, **B**. pH value, **C**. Temperature, **D**. Substrate concentration, and **E**. Enzyme concentration on crude and immobilized β-galactosidase enzyme

Table 3: The Optimum time, pH, temperature, substrate concentration and enzyme concentration for native and immobilized β-D-galactosidase enzyme working.

Enzyme	Time (h)	pH	Temperature (°C)	Substrate concentration (mg/mL)	Enzyme concentration (mg/mL)
Native β-D-galactosidase	24	9	5	100	90
Immobilized β-D-galactosidase	72	9	5	100	90

3.3. Determination of Km and Vmax

Lineweaver-Burk plots were used to estimate kinetic factors (Km and Vmax) for enzymes using different concentrations of o-nitrophenyl-β-D-galactopyranoside (ONPG) as enzyme substrate at pH 7.

$$\frac{1}{V_o} = \frac{Km}{Vmax\,[S]} + \frac{1}{Vmax}$$

Where:
V_o= Initial velocity
$Vmax$ = Maximum velocity
Km = Michaelis constant
$[S]$= Substrate concentration

As the Km value decreases, the enzyme's affinity for its substrate increases, as demonstrated by immobilized β-galactosidase, which has a lower Km and a higher affinity for substrate than native β-galactosidase. In addition, the immobilized form of an enzyme has a higher Vmax value than the native form, and this indicates that a higher amount of substrate converts to products per unit time. By dividing Vmax by km, the catalytic efficiency of enzymes has been determined. The term "catalytic efficiency" means that as the rate of chemical reaction increases, the reaction will be more efficient. Immobilized enzyme is more efficient than the crude β-galactosidase enzyme (Table 4)

Table 4 : The initial and maximum velocity, Mechalis constant, and catalytic efficiency of β-D-galactosidase enzymes in native and immobilized forms.

Enzyme	Vi	Vmax	Km	Vmax/Km
Native β-galactosidase	0.45	0.9	24	0.0375
Immobilized β-galactosidase	0.575	1.15	16	0.0718

4. Conclusion

Fe_3O_4 magnetite nanoparticle preparation and β-D-galactosidase enzyme immobilization have been conducted using simple and effective procedures. Fe_3O_4 MNPs' characteristics, such as size, diameter shape, surface morphology, crystal structure, and chemical functional groups, have been studied by AFM, SEM, XRD, and FT-IR techniques. Our research includes comparisons between free and immobilized enzymes for each of the kinetic factors (time, pH, temperature, substrate concentration, and enzyme concentration). The immobilized β-D-galactosidase was more stable and active than the native one over a wide range of hours, pH, and temperature. Furthermore, as the concentration of substrate and enzyme (free or immobilized) in a solution increases, so does free and immobilized enzyme activity. An immobilized enzyme has a lower KM value than a crude enzyme, indicating a higher affinity for substrate.

References

[1] D. M. Sedzro, S. F. Bellah, H. Akbar, and S. S. Billah, "Structure, Function, Application and Modification Strategy of β-Galactosidase," *Journal of Multidisciplinary Research and Reviews*, vol. 1, no. 1, pp. 10-16, 2018.

[2] J. Antonio, M. Rodríguez, G. Álvarez-Hernán, Y. Gañán, and A. Santos-Almeida, "Endogenous pH 6.0 β-Galactosidase Activity is Linked to Neuronal Differentiation in the Olfactory Epithelium," *Cells*, vol. 11, no. 2, p. 298, 2022.

[3] G. M. Saleh, "Green synthesis concept of nanoparticles from environmental bacteria and their effects on pathogenic bacteria," *Iraqi Journal of Science*, vol. 61, no. 6, pp. 1289-1297, 2020.

[4] H. Askaripour, M. Vossoughi, K. Khajeh, and I. Alemzadeh, "Magnetite nanoparticle as a support for stabilization of chondroitinase ABCI," *Artificial Cells, Nanomedicine, and Biotechnology*, vol. 47, no. 1, pp. 2721-2728, 2019.

[5] H. R. Barros, M. C. Santos, L. R. S. Barbosa, L. Piovana, and I. C. Riegel-Vidotti, "Physicochemical Study of the Interaction between Gold Nanoparticles and Lipase from *Candida* sp. (CALB): Insights into the Nano-Bio Interface," *Journal of the Brazilian Chemical Society*, vol. 30, no. 10, pp. 2231-2242, 2019.

[6] D. F. Hassan and M. B. Mahmood, "Biosynthesis of iron oxide nanoparticles using Escherichia coli," *Iraqi Journal of Science*, vol. 60, no. 3, pp. 453-459, 2019.

[7] Q. Husain, "Magnetic nanoparticles as a tool for the immobilization/stabilization of hydrolases and their applications," *Biointerface research in Applied Chemistry*, vol. 6, no. 6, pp. 1585-1606, 2016.

[8] J. X. Jingjing, Y. Wang, J. Sheng, F. Wang, and M. Sun, "Application of Iron Magnetic Nanoparticles in Protein Immobilization," *Molecules*, vol. 19, no. 8, pp. 11465-11486, 2014.

[9] I. L. Ardelean, L. B. Stoencea, D. Ficai, and A. Ficai, "Development of Stabilized Magnetite Nanoparticles for Medical Applications," *Journal of Nanomaterials*, 2017.

[10] R. Kozlowski, A. Ragupathi, and R. B. Dyer, "Characterizing the Surface Coverage of Protein-Gold Nanoparticle Bioconjugates," *Bioconjugate Chemistry*. vol. 29, no. 8, pp. 2691-2700, 2018.

[11] H. Rashid, M. A. Mansoor, B. Haider, R. Nasir, S. B. Abd Hamid, and A. Abdulrahman, "Synthesis and characterization of magnetite nano particles with high selectivity using in-situ precipitation method," *Separation Science and Technology*, vol. 55, no. 6, pp. 1207-1217, 2019.

[12] J. Wallyn, N. Anton, and T. F. Vandamme, "Synthesis, Principles, and Properties of Magnetite Nanoparticles for in Vivo Imaging Applications," *Pharmaceutics*, vol. 11, no. 11, p. 601, 2019.

[13] R. Ahmad and M. Sardar, "Enzyme Immobilization: An Overview on Nanoparticles as Immobilization Matrix," *Biochemistry and Analytical Biochemistry*, vol. 4, no. 2. ID,1000178 , 2015.

[14] A. M. Alshanberi, R. Satar, and S. A. Ansari, "Stabilization of β-Galactosidase on Modified Gold Nanoparticles: A Preliminary Biochemical Study to Obtain Lactose-Free Dairy Products for Lactose-Intolerant Individuals," *Molecules*, vol. 26, no. 5, p. 1226, 2021.

[15] N. Bashir, M. Sood, and J. D Bandral, "Enzyme immobilization and its applications in food processing," *International Journal of Chemical Studies*, vol. 8, no. 2, pp. 254-261, 2020.

[16] S. Ding, A. A. Cargill, I. L. Medintz, and J. C. Claussen, "Increasing the activity of immobilized enzymes with nanoparticle conjugation," *Current Opinion in Biotechnology*, vol. 34, pp. 242-250. 2015.

[17] L. Zhou, L. Ye, and Y. Lu, "Flexible and Effective Preparation of Magnetic Nanoclusters via One-Step Flow Synthesis," *Nanomaterials*, vol. 12, no. 3, p. 350, 2022.

[18] G. X. Liu, J. Kong, W. W. Lu, and W. T. Kong, "β-galactosidase with transgalactosylation activity from *Lactobacillus fermentum* K4," *American Dairy Science Association*, vol. 94, no. 12, pp. 5811-5820, 2011.