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# Immobilization of Alpha-Amylase on Chitosan-Coated Iron Oxide Nanomagnetic Nanoparticles

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ORIGINAL ARTICLE

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# Immobilization of Alpha-Amylase on Chitosan-Coated Iron Oxide Nanomagnetic Nanoparticles

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**Abstract**

**Amylases are widely used enzymes across various industries such as clinical chemistry, pharmaceuticals, food processing, detergents, textiles, and paper manufacturing. Immobilization can enhance their effectiveness. Nanostructured materials have become important in many fields, including magnetite nanoparticles ($Fe_3O_4$) for immobilizing enzymes. The study aimed to immobilize alpha-amylase extracted from Bacillus subtilis onto iron oxide nanoparticles coated with chitosan to compare its catalytic efficacy to its free form. Iron oxide nanoparticles were synthesized and coated. The substrate underwent amylase introduction with glutaraldehyde, stabilizing formation on chitosan-iron oxide nanoparticles. XRD, FT-IR, FE-SEM, and DLS were used to analyze immobilized amylase on chitosan + iron oxide nanoparticles. To conclude, we assessed the efficacy of immobilized enzyme versus free enzyme at different pH and temperature levels. Spectroscopy indicated a strong electrostatic bond between chitosan and $Fe_3O_4$ nanoparticles, causing chitosan to bind onto their surface during alkaline conditions. Alpha-amylase is bonded to the substrate through a Schiff base bond. Optimal temperature for free and immobilized enzymes is 40 °C, and optimal pH is 7.5. The study found that the immobilized enzyme was more active than the free enzyme at ideal pH and temperature, and examined their inactivation in the 40–60 °C range. Free enzymes showed a sharp activity decline, while immobilized enzymes had a slower decline. This study measured the Vmax and Km values of free and immobilized enzymes. The free enzyme had a Vmax of 61.52 μmol/min.mg and a Km of 0.0082 mg/ml. The immobilized enzyme had a Vmax of 48.43±0.6383 μmol/min.mg and a Km of 0.0084±0.0016 mg/ml. It also retained 47% activity after 7 cycles. This study shows a new approach to using enzyme-bound magnetic nanoparticles in various industries like paper and detergent.**

***Keywords:*** Immobilitation, Alpha amylase, Chitosan-iron oxide nanoparticles

## 1. Introduction

Enzymes, which serve as biological catalysts, are responsible for the regulation of diverse biochemical reactions. In the contemporary era, the feasibility of employing microorganisms as biotechnological agents for the production of crucial industrial enzymes has provoked enthusiasm among researchers examining the extracellular enzymatic activity of various microorganisms. Amylases hold great significance in various industrial applications, particularly in the sectors of brewing, detergent and food production, owing to their widespread utilization [1, 2].

Amylases refer to a class of enzymes that catalyze the process of hydrolyzing starch molecules into various products, which encompass maltose, dextrin, as well as smaller glucose polymers [3]. Alpha-amylase (EC 3.2.1.1) is currently deemed the most frequently used endoamylase within academic literature [4]. Exoamylases exhibit the ability to cleave α-1,4 glycosidic linkages similar to β-amylase (EC 3.2.1.2). The

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mode of action of Exoamylases involves the hydrolysis of the substrate from its non-reducing terminal, leading to the generation of relatively shorter chain end-products [5].

Alpha-amylase (E.C.3.2.1.1) refers to a specific type of hydrolase enzyme that effectively catalyzes the hydrolysis of internal alpha-1, 4 glycosidic bonds that are present within starch substrates. As a result of this catalytic activity, alpha-amylase is able to generate various products including glucose and maltose. This enzyme is classified as a calcium metalloenzyme, which means that its activity is dependent on the presence of a metal cofactor [6]. α-Amylase is widely recognized as an enzyme of significant significance owing to its prominent function in starch hydrolysis and the potential repertoire of activities it offers through enzymatic hydrolysis.

One of the aforementioned activities entails the manufacturing of glucose and fructose syrup through the hydrolysis of starch. Amidst the diverse array of enzymes utilized in the production of fermented foods, amylases occupy a prominent position as a vital component. The market for amylases has witnessed considerable expansion in recent times, galvanized by its versatile application spectrum. The first enzyme to be commercialized, among others, were the alpha-amylases. The enzyme sector has significant implications on various fields, thereby holding vital relevance in the global market. The enzymatic properties of amylases render them highly versatile in their potential applications in both the food industry, as well as in non-food starches, as supported by numerous empirical studies [6].

Various techniques have been employed hitherto in order to attain stable amylase. The isolation and subsequent screening of enzymes from extreme microorganisms, particularly thermophilic bacteria, and the implementation of genetic manipulation techniques in other microorganisms are crucial approaches in modern enzyme research. Additionally, enzyme stability can be enhanced via various means including protein engineering, enzyme stabilization and the addition of specific additives, as elucidated in reference [6]. The overall utilization of enzymes in the industrial sector, excluding pharmaceutical applications, is estimated to be approximately 1.5 billion euros. In the Food and Medical industries, the usage of enzymes presents challenges in terms of separation from the product and their limited single-use lifespan. Consequently, the suitability of enzymes in fluidic form is questionable, and the cost-effectiveness of their utilization is hindered owing to the high expenses involved. The application of stabilization techniques has been shown to augment the potential for reusability of enzymes across numerous instances, resulting in a noteworthy reduction in enzyme and product costs [7]. Thus, the stabilized enzyme form is considered to be a more advantageous alternative in comparison to its free variant owing to its ability to facilitate continuous flow processes as well as the facile recovery of stabilized enzymes, thereby permitting low-cost operations in industrial processing [8].

The implementation of chitin/chitosan for biocatalyst stabilization has extended the scope of biotechnological utilization. Chitosan, owing to its non-toxic properties, biocompatibility, possession of appropriate functional groups, and susceptibility to both chemical and enzymatic hydrolysis, is predominantly utilized as an adjuvant component in various formulations containing both minor and major molecular entities. Based on the findings as reported in various researches, it appears that the most optimal medium for stabilizing α-amylase is a composite material of chitosan nanoparticles which are modified by magnetite, along with glutaraldehyde and polyethyleneimine components. Based on the studies, this combination has demonstrated the greatest efficacy, with a reported efficiency of 100. Over the past few years, there has been a significant amount of research focused on investigating the feasibility of employing inorganic and organic nanoparticles not exceeding a 30 nm diameter as an optimal platform for enzyme stabilization. Nanoparticles confer a pronounced surface area in which enzyme binding can occur, thus leading to heightened enzyme loading at the matrix surface and an enhancement in stabilization performance. The preeminent advantage of nanoparticles relative to other minerals is their capacity to mitigate diffusion restrictions. Enzymatic molecules affix themselves to the surface of impermeable particles, thereby exposing their active sites to a significant amount of contact with substrates [9].

The aim of the present study is to examine the biochemical characteristics of alpha-amylase enzyme when immobilized in a nanomagnetic substrate equipped with chitosan and subsequently compare these properties with those of the enzyme in its free form.

## 2. Materials and methods

### *2.1. Stabilization of amylase on chitosan substrate + magnetic iron nanoparticles*

In order to enhance the stability of alpha-amylase through the use of magnetic iron and chitosan nanoparticles, a total of 5.4 grams of $FeCl_3$ $6H_2O$ and 1.99 grams of $FeCl_2$ $4H_2O$ were incorporated into

100 milliliters of distilled water contained within an Erlenmeyer flask. A 28% concentration of $NH_4OH$ was added to the mixture, resulting in an increase in pH to 10. The Erlenmeyer flask was subjected to $N_2$ gas for a duration of two hours at a temperature of 80 °C.

The production of $Fe_3O_4$ nanoparticles was carried out, wherein subsequent to their formation, a washing procedure was performed with water in order to neutralize the pH. A nanoparticle-laden colloidal solution is subjected to a two-hour freezing process, resulting in the production of nanoparticle powder. A chitosan compound weighing 0.25g was dissolved in a solvent comprising 50 mL of 1% acetic acid. Two grams of iron oxide nanoparticles were incorporated into the system, and subsequently stirred for a duration of two hours in order to achieve optimal dispersion of the nanoparticles. A quantity of fifty milliliters of NaOH was employed to facilitate the coating of chitosan onto iron oxide nanoparticles. The pH of the substance was neutralized through a process of washing with deionized water.

The stabilization of alpha-amylase is facilitated by chitosan and iron oxide particles, as reported in reference 117. In order to achieve stabilization of the alpha-amylase enzyme obtained from Bacillus subtilis on the substrate, the addition of 1 gram of chitosan and iron oxide nanoparticles was required, followed by immersion of the resultant mixture in a 30 mL solution of 2.5% W/V glutaraldehyde for a period of 2 hours at a temperature of 25 degrees Celsius. Through this process, the substrate is activated, thereby facilitating its ability to effectively bind with the enzyme. Subsequently, in order to eliminate residual glutaraldehydes that failed to react, as well as those that did not adhere to the substrate, the latter was subjected to multiple rinses utilizing deionized water. Fifty milliliters of alpha-amylase solution (6 mg/mL) was introduced into the chitosan and iron oxide nanoparticle substrate (2 g) and subjected to magnetic stirring at a temperature of 25 °C for a duration of two hours, thereby facilitating the binding of the enzyme with the substrate. Ultimately, enzymes immobilized onto a chitosan substrate supplemented with iron oxide nanoparticles were extracted by means of a substantial magnet and subsequently subjected to purification through rinsing with acetate buffer, resulting in the elimination of any unconsumed amylase [10]. In order to achieve stabilization of alpha-amylase, it is recommended to incorporate a combination of 1 gram chitosan and iron oxide nanoparticles into 30 milliliters of 2.5% weight/volume glutaraldehyde solution, which should be maintained at 25 °C for 2 hours. This phenomenon elicits the activation of the substrate to form a binding complex with the enzyme. The substrate must be thoroughly washed to eliminate any residual glutaraldehydes. Fifty milliliters of alpha-amylase should be added to form a substrate-complex, and the reaction must be allowed to continue for a duration of two hours. Enzymes were extracted from the chitosan + iron oxide nanoparticles through the utilization of a magnet and an acetate buffer, whilst unreacted amylase was subsequently eliminated [11].

### *2.2. Determination of physical and chemical properties of magnetic nanoparticles - chitosan - enzyme*

FE-SEM (TESCAN BRNO-Mira3 LMU model), XRD (Bruker Axs D8-Advance X-ray (Ettlingen, Germany))., FT-IR (Bruker Tensor 27 spectrophotometer), AFM model (DME DS 95-200), DLS (Malvern Zetasizer Nano-ZS system) and TGA methods were used to characterize the alpha-amylase enzyme stabilized on magnetic + chitosan nanoparticles.

### *2.3. Determination of specific enzyme activity*

The determination of enzyme activity is contingent on the prevailing conditions and ultimately reflects the quantity of functional enzyme present. At every phase of the purification process, the overall enzyme activity and specific activity were determined utilizing the approach explicated hereafter.

The following formula was used to determine the total enzyme activity:

**Total activity = $OD^{280}$ nm read from each step Sample volume (ml) × 1000 (absorption to activity conversion factor).**

In order to ascertain the specific activity, the quotient obtained by dividing the activity detected in each individual step by the protein concentration measured in the corresponding step was calculated.

### *2.4. Effect of pH on the activity and stability of the enzyme*

To assess the enzymatic activity across a range of different pH values, a total of 100 µl of both the stabilized and free enzymes were introduced individually into 500 µl of a 0.2% gelatin solution, which had been dissolved in buffers characterized by varying pH values separated by increments of 0.5 units, spanning a range of pH values from 3 to 10. Following a period of 30 minutes under incubation conditions at 37 °C, the enzymatic activity was subsequently assessed. In order to evaluate the pH stability

of the enzyme, a preliminary step was taken prior to substrate addition. Specifically, 100 µl of stabilized and unbound enzyme was introduced into a solution comprising 500 µl of buffer, with varying levels of pH, and subsequently left for a duration of one hour. Over the course of time, a fixed quantity of substrate (S) amounting to 500 microliters (µl) was added to individual samples, and subsequently, the absorption spectrum was measured at a wavelength of 570 nanometers (nm). The highest level of absorption was designated as 100% activity, and subsequent outcomes were evaluated in juxtaposition [10]

### *2.5. Effects of temperature on the activity and stability of the enzyme*

In order to assess the impact of temperature on the efficacy and constancy of both free and stabilized enzymes, the foremost step involved the identification of an optimal pH buffer displaying maximal enzyme activity. Subsequently, a comprehensive evaluation of the effect of diverse temperatures on the enzyme activity was conducted, followed by the measurement of the impact of varying temperatures (ranging from 20 to 70 °C in intervals of 10 °C) on the performance of free and stabilized enzymes in the presence of substrate. In order to achieve this objective, a reaction mixture comprising 0.1 mL of either stabilized or free enzyme solution and 0.5 mL of 0.2% gelatin, previously liquefied in 20 mM Tris-HCl buffer with a pH of 8.5, was subjected to incubation at various temperature ranges for a duration of 30 minutes. The enzymatic activity was subsequently estimated utilizing a standardized activity measurement method as prescribed in literature [10].

### *2.6. Determination of half-life of free and stabilized alpha-amylase enzyme*

To ascertain the half-life of both unbound and stabilized enzyme, a volume of 1.5 mL of enzyme was maintained within the temperature range of highest activity, specifically 40–60 °C in 5 °C increments. At hourly intervals, 0.1 mL of enzyme was extracted and subjected to buffer and substrate optimization. In this study, a mixture was employed to evaluate the residual activity of the enzyme with respect to its initial state (represented as time zero and defined as 100% activity). The residual activity was subsequently plotted on a logarithmic scale against time, enabling determination of the enzyme's half-life via the calculation of its slope.

### *2.7. Evaluation of kinetic parameters of free and stabilized enzyme*

The present study aimed to examine the kinetic parameters, namely Km and Vmax, through an investigation of the activity of both free and stabilized enzymes across varying substrate concentrations. Initially, five distinct concentrations of the enzyme substrate were solubilized within a Tris-HCl buffer possessing a 20 millimolar concentration at a pH value of 7. The concentrations employed in the present experimental study were designated as 0.05, 1, 2, 4 and 8 mg/mL. In order to measure enzyme activity, it was deemed necessary to expose substrate solutions of varying concentrations to a fixed quantity of enzyme for a period of sixty minutes. Following this incubation period, absorption was recorded utilizing established techniques. This measurement was taken against an appropriate control sample that contained identical amounts of substrate. Additionally, 100 µl of buffer was incorporated in lieu of enzyme solution during the reading process. The Michaelis-Menten plot was constructed through the graphical representation of the adsorbent values gathered at varying concentrations, employing the aid of GraphPad Prism software, version 6 [10].

### *2.8. The ability to reuse the stabilized enzyme*

To assess the recyclability of the stabilized enzyme, a solution consisting of 10 milligrams of iron-chitosan oxide nanoparticles containing the enzyme stabilized in 2 milliliters of phosphate-buffered saline at a pH of 7 was prepared. This solution was subsequently utilized to carry out the hydrolysis of one milliliter of a 1.2% starch solution in phosphate buffer. Following a 15-minute incubation period, magnetic separation was employed to isolate the enzyme-nanoparticle conjugates from the reaction mixture, and a one-milliliter volume of DNS reagent was added to the resulting supernatant for further analysis. Subsequently, the solution was subjected to a thermal treatment by means of immersion in a preheated water bath for a duration of 15 minutes, followed by a cooling phase. Upon completion, the solution underwent a volumetric augmentation via the addition of a specifically measured quantity of deionized water, and the extent of its adsorption was recorded at a wavelength of 540 nm. Buffer and starch were incorporated into the isolated precipitate utilizing a magnet, following which the enzymatic activity was evaluated subsequent to an elapsed period of 15 minutes, employing the aforementioned methodology. The aforementioned procedure was implemented over the course of seven consecutive days,

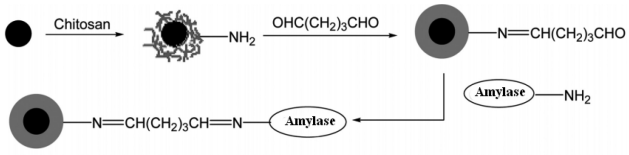


*Fig. 1. Preparation of alpha-amylase stabilized on iron-chitosan oxide nanoparticles.*

with a frequency of once per diem as denoted by the reference [10].

## 3. Results and discussion

### *3.1. Preparation of alpha-amylase stabilized on iron-chitosan oxide nanoparticles*

Iron oxide-chitosan nanoparticles stabilized alpha-amylase enzyme. Fig. 1 depicts synthesis mechanism of chitosan-coated $Fe_3O_4$ nanoparticles. Opposite charges in compounds caused strong interaction between chitosan nanoparticles and $Fe_3O_4$. Chitosan formed on $Fe_3O_4$ nanoparticles in an alkaline solution. Chitosan $Fe_3O_4$-nanoparticles were created and used as a substrate for covalent attachment of alpha-amylase via Schiff base bond. The reaction occurs between C=O in glutaraldehyde and $NH_2$ in the enzyme. Chitosan's mechanical properties improve due to a compression reaction during glutaraldehyde activation. Enzyme stabilization was done on nanoparticles with iron oxide, chitosan, glutaraldehyde, and amylase. The remaining amylase was removed and protein measured using the Bradford method.

### *3.2. Investigation of the properties of enzymes stabilized on nanoparticles FT-IR spectroscopy*

FT-IR spectroscopy was used to confirm amylase enzyme binding to iron oxide + chitosan nanoparticles through spectra comparison of free and stabilized enzymes with iron oxide nanoparticles. Fig. 2 displays spectra of free and stabilized enzyme on nanoparticle substrate. Stabilization reduces enzyme intensity. Absorption bands at 3430 $cm^{-1}$ and 1640 $cm^{-1}$ correspond to OH and $NH_2$ group vibrations in chitosan. The 2930 $cm^{-1}$ band shows CH vibrations in chitosan. Peaks at 1073 $cm^{-1}$ and 1413 $cm^{-1}$ correspond to C–O and C–N vibrations. The 1658 $cm^{-1}$ peak indicates C=O tensile vibrations in chitosan and may also be indicative of amide groups in amylase enzyme. Several bands at 1035, 1072, 1094, 1143, and 1168 $cm^{-1}$ indicate sulfide and disulfide vibrations, while peaks at 3600 $cm^{-1}$-3000 may be related to O–H amino group vibrations in chitosan.

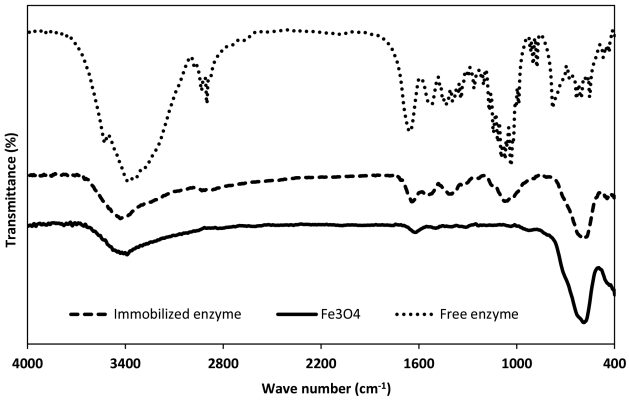


*Fig. 2. FT-IR spectrum of iron oxide nanoparticles, alpha-amylase stabilized on iron oxide nanoparticles + chitosan and alpha-amylase enzyme.*

### *3.3. XRD patterns*

The X-ray diffraction (XRD) pattern of iron oxide nanoparticles was ascertained within the angular interval of $2\Theta = 10–80°$. The aforementioned pattern is characterized by distinct angles, specifically, 2 theta values of 30.01, 35.81, 43.38, 54.01, 57.41 and 62.98 that have a direct correlation with the (2 2 0), (3 1 1) and (4 0 0), (4 2 2), (5 1 1), and (4 4 0) planes. Upon conducting a thorough analysis as shown in Fig. 3, it was discovered that the aforementioned peaks were congruent with the established trajectory of iron oxide magnetite nanoparticles. This comparison ultimately revealed a correlation between the reported peaks and iron oxide nanoparticles with a spinel spatial structure, as was evidenced by sources 95 and 109.

### *3.4. FE-SEM images*

As shown in Fig. 4, the morphology of the synthesized nanoparticles was investigated utilizing a field diffusion scanning electron microscope. It was observed that both samples manifested a uniform spherical configuration, yet magnetite-chitosan nanoparticles exhibited a significantly elevated surface-to-volume ratio in light of their rough exterior. In contrast, iron oxide nanoparticles exhibit a greater degree of dispersal as compared to $Fe_3O_4$-chitosan nanoparticles with a propensity towards accumulation.

### *3.5. Examination of transmission electron microscopy (TEM) images*

As shown in Fig. 5, the morphology and properties of nanoparticles were assessed through the use of a transient electron microscope (TEM). The images portray that the iron oxide magnetic nanoparticles that have undergone synthesis are endowed with a homogenous disposition and possess particle sizes

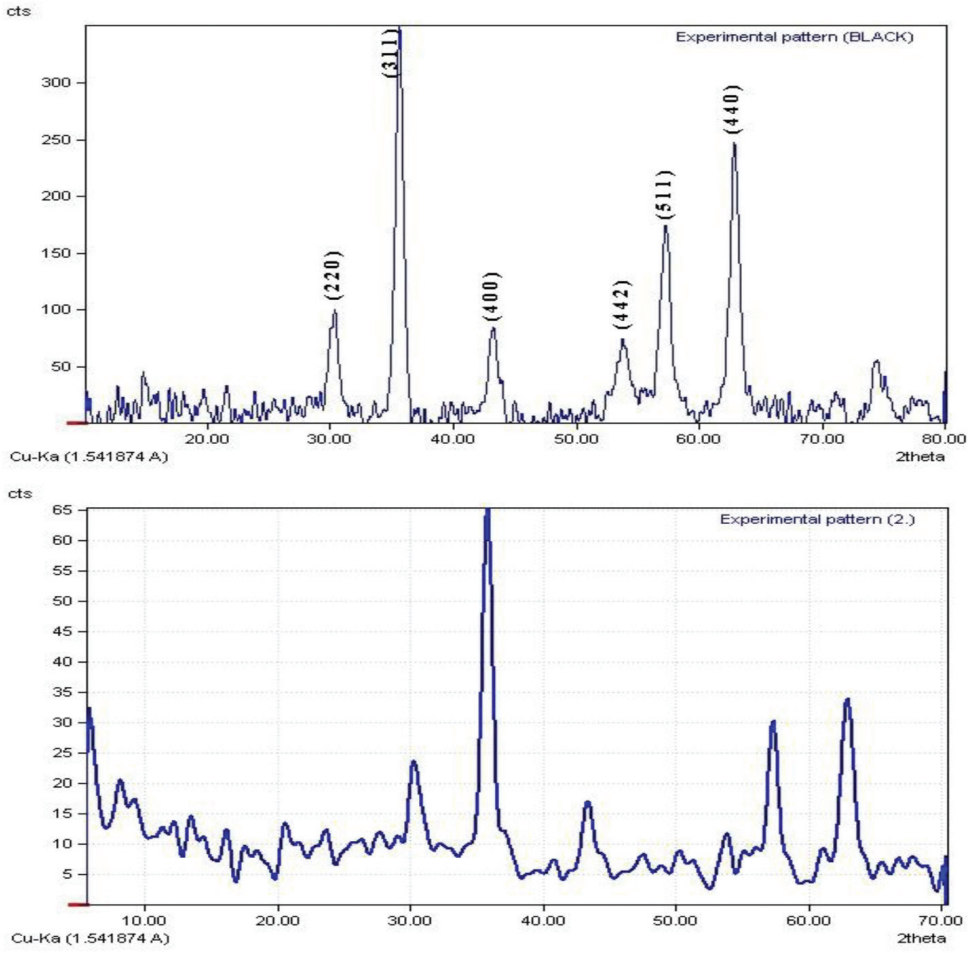


*Fig. 3. X-ray diffraction pattern of iron oxide + chitosan (a) nanoparticles and amylase stabilized on iron oxide + chitosan (b) nanoparticles.*

characterized by a typical diameter of 25 nm (determined by the utilization of the image J tool). The study revealed that nanoparticles coated with chitosan and chitosan-enzyme exhibit a propensity for aggregation, resulting in their conglomeration in a spheroidal and clustered pattern.

### *3.6. AFM atomic force microscope*

As shown in Fig. 6, the findings from AFM microscopy employing two-dimensional and three-dimensional imaging methods demonstrate that the application of a coating effectively inhibits the self-aggregation properties of iron oxide nanoparticles. The present study revealed that the surface roughness of iron oxide-chitosan nanoparticles in their uncoated state was 38.7 nm, which was observed to significantly increase to 60.37 nm with the application of a coating composed of iron oxide nanoparticles and chitosan-amylase. Enhancing the degree of surface roughness in the coated specimen establishes the efficaciousness of its coating. The AFM imagery of magnetic nanoparticles comprised of iron oxide evinces the presence of numerous minute spherical nanostructures observable on the surface of the silicon sheet.

### *3.7. Determination of zeta potential and size of synthesized nanoparticles*

As shown in Fig. 7, the zeta potential of magnetic nanoparticles The zeta potential of magnetic iron oxide nanoparticles, magnetic particles of iron oxide + chitosan and magnetic particles of iron oxide + chitosan + enzyme are about −26.28, −27.2 and −3, respectively. 27-mV was determined. Zeta potential is an indicator of colloidal dispersion. The large size of the zeta potential indicates an increase in electrostatic repulsion between adjacent particles and scattering. In this study, the zeta potential of magnetic iron oxide nanoparticles showed a negative value, which means that the net charge of the dispersed body is negative. The zeta potential, which is in the range of −30 mV to + 30 mV, is more suitable due to having sufficient

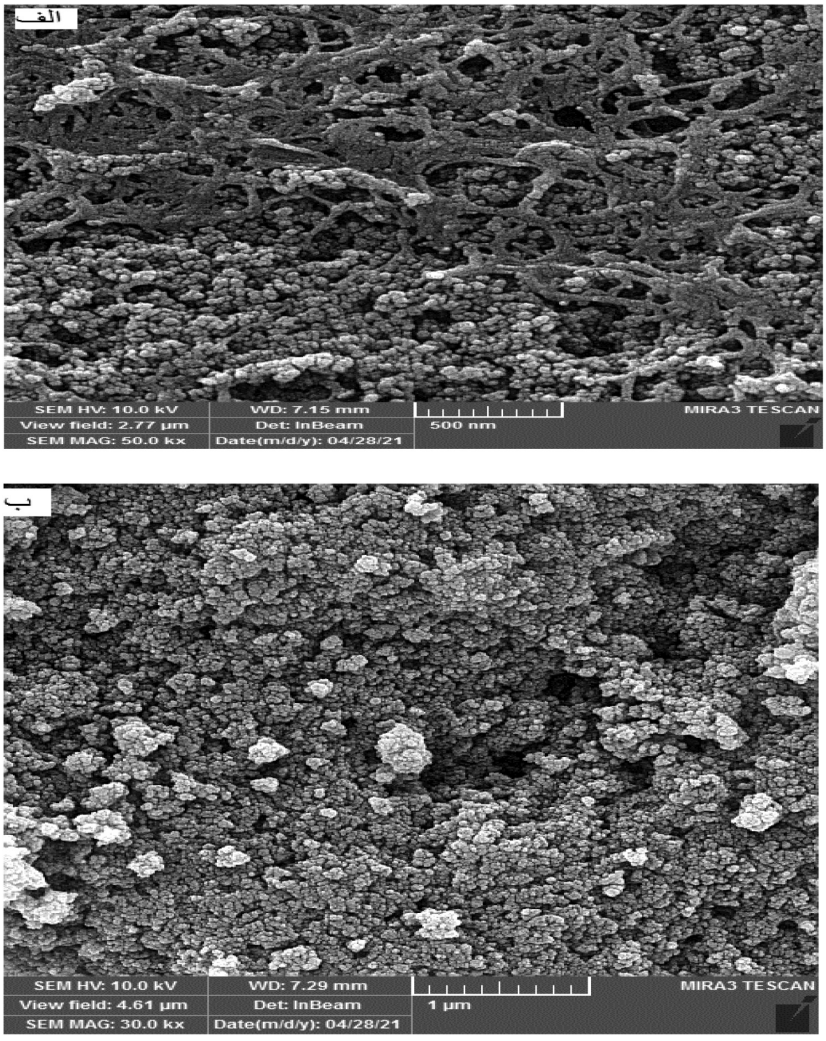


*Fig. 4. FE-SEM images of iron oxide + chitosan nanoparticles (a) iron oxide nanoparticles + chitosan + alpha amylase (b).*

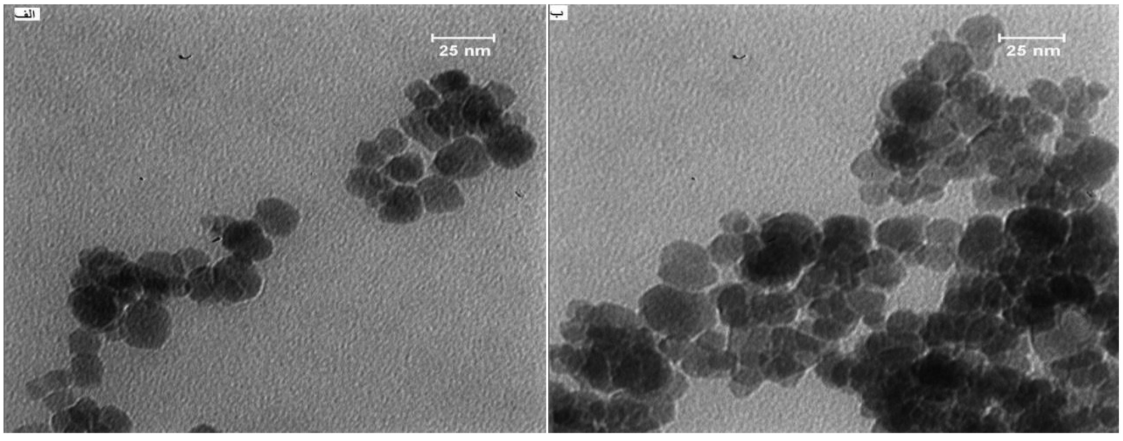


*Fig. 5. Transmission electron microscopy (TEM) images at 25 nm scale, iron oxide-chitosan nanoparticles (a), iron oxide nanoparticles + chitosan + enzyme (b).*

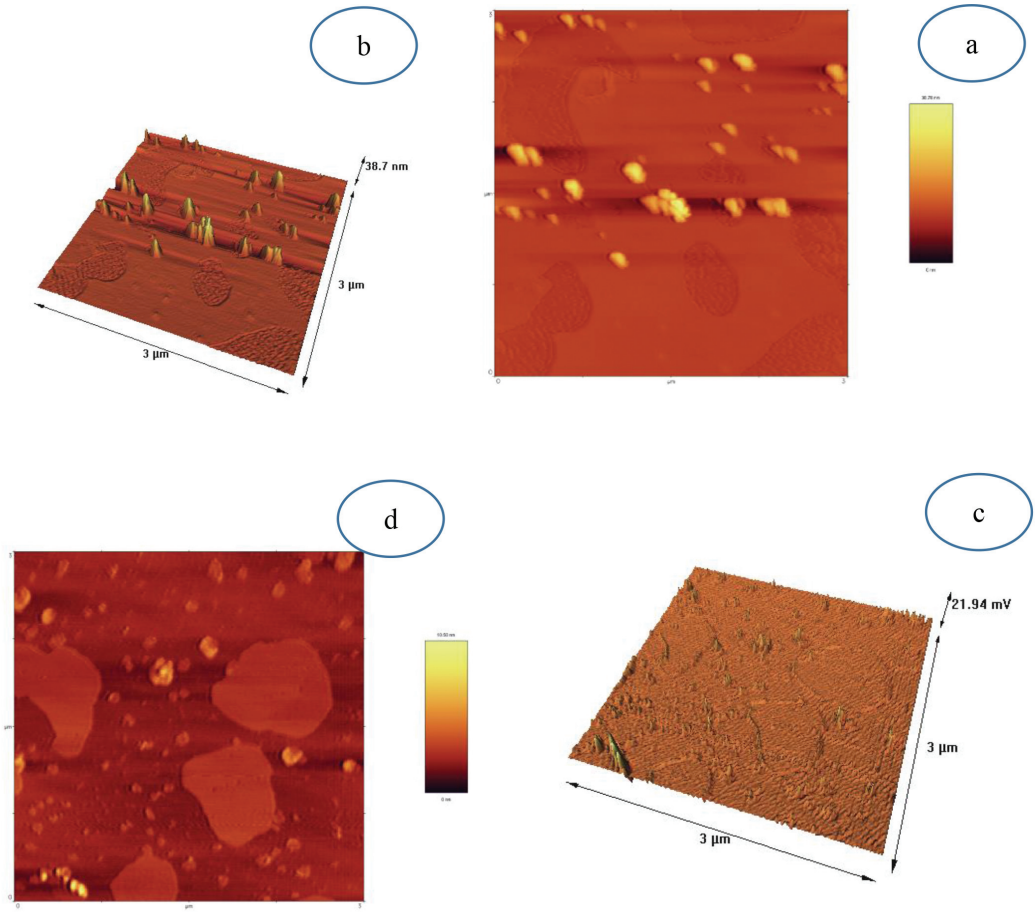


*Fig. 6. Atomic force microscope (AFM) images of magnetic nanoparticles. Two-dimensional (a) and three-dimensional (b) images of magnetic iron oxide-chitosan nanoparticles. Two-dimensional (c) and three-dimensional (d) image of magnetic iron oxide-chitosan-amylase nanoparticles.*

repulsive force to achieve physical colloidal stability [12].

The present study reveals that the average dimensions of iron oxide nanoparticles, iron oxide + chitosan nanoparticles, and enzyme immobilized on iron oxide + chitosan nanoparticles were determined to be 23.5 nm, 34.6 nm, and 22.08 nm, correspondingly. As shown in Fig. 8, the PDI values of 0.3320, 0.3770, and 0.3940 were computed, indicating the nanoparticles' repulsion, stability, and dispersion. This observation corroborates the notion that the nanoparticles are in a state of repulsion, stable, and well-dispersed.

### *3.8. TGA results*

As shown in Fig. 9, the findings from the thermal analysis conducted on magnetite nanoparticles revealed a reduction in particle weight by approximately 21% when subjected to a temperature of 800 °C. The weight loss observed at temperatures up to 237 °C can be attributed to the adsorption of water on the surface of the particle. It can be inferred that a proportion of approximately 5% of this weight loss can be attributed to the presence of moisture within the particle. It is apparent that the particles exhibit a considerable degree of thermal stability up to 200 °C. Within this range, no discernible mass reduction is observed. After being subjected to temperatures up to 384 degrees Celsius, a weight loss of 21% was demonstrated. According to available reports, coated particles have been found to exhibit greater thermal stability in comparison with their uncoated counterparts [10].

### *3.9. The effect of temperature on the activity of free and stabilized enzymes*

As shown in Fig. 10, the present study aimed to determine the optimal temperature range for both free and stabilized enzymes in terms of their maximum activity. It was found that the optimal temperature for free enzymes was 40 °C, whereas for stabilized enzymes, it was 50 °C. The enzymatic activity of the stabilized enzyme has demonstrated superiority

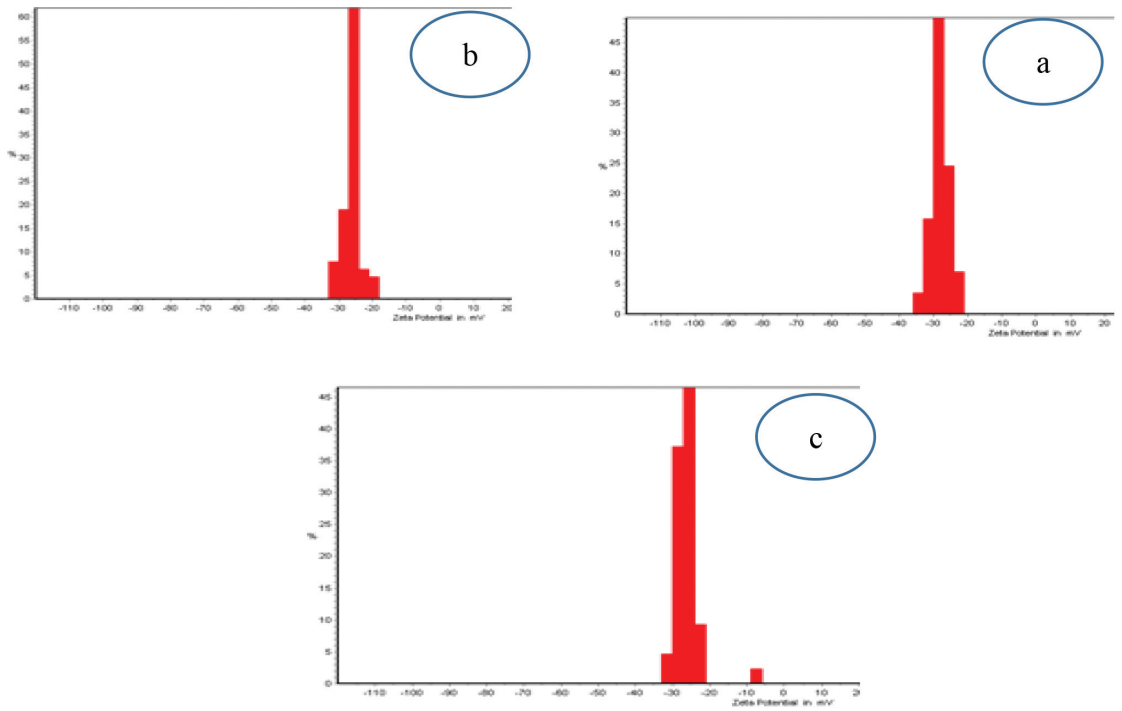


*Fig. 7. Zeta potential of iron oxide magnetic nanoparticles (a), iron oxide magnetic particles + chitosan (b) and iron oxide magnetic particles + chitosan + enzyme + (c).*

vis-à-vis the free enzyme as temperature increases. Nevertheless, an upward trend in temperature ranging from 40 to 70 °C has resulted in a decrease in the observed enzymatic activity. The enzymatic activities at a temperature of 50 °C, with and without stabilization, were determined to be 70% and 77%, respectively.

Studies show that enzyme stabilization on nanoparticles improves their resilience to high temperature and other forces. Research is ongoing in this area on various substrates [13] studied the effect of temperatures on amylase activity, using magnetic iron oxide nanoparticles. Stabilized enzyme is more active than free enzyme and has higher stability. Akhund et al. [14] found optimal activity of stabilized amylase at 45 °C. In this study, enzyme activity was optimal at 40 °C, depending on enzyme type, stabilization, and testing. This matches the findings of Daval et al. [15] where both free and stabilized amylase also had optimal activity at 40 °C.

Ahmad et al. [16] found that the optimal temperature for amylase enzyme on magnetite-chitosan nanoparticles was 45 °C for free amylase and 55 °C for stabilized amylase. At 70 °C, free and stabilized enzymes activity decreased by 42% and 11% respectively. Carrier binding may strengthen and make enzyme molecular structures more mobile at high temperatures. Stabilized enzymes can maintain their active structure at higher temperatures. Temperature increase led to rapid decline in α-amylase activity. Free α-amylase activity was about 44% at 70 °C, while stabilized enzyme activity was estimated at 67% at the same temp. The enzyme's thermal stability improved due to stabilization, which can be attributed to protective covalent bonds and substrate stability [16].

### *3.10. The effect of pH on the activity and stability of amylase stabilized and free*

The investigation depicted in the Figs. 11 and 12 indicated that the peak performance of both unbound and secured enzymes was ascertained at pH values of 7.5 and 8.5, respectively. At these pH levels, the enzymatic activity was assessed as 100% relative. The activity spectrum of enzymes, whether in their unbound or stabilized state, is within a pH range spanning from 3 to 10. Nevertheless, stabilized enzymes demonstrate a greater degree of enzymatic activity compared to their free, soluble counterparts. The present study observed a positive correlation between pH and enzyme activity, suggesting an optimum enzyme activity at alkaline pH conditions. The present study aimed to determine the free enzyme activity of the tested enzyme under varying pH conditions. Based on the experimental outcomes, it was found that the enzyme's activity was reduced to 11% and enhanced up to 79% at the lowest and highest pH values, respectively. In contrast, the enzyme

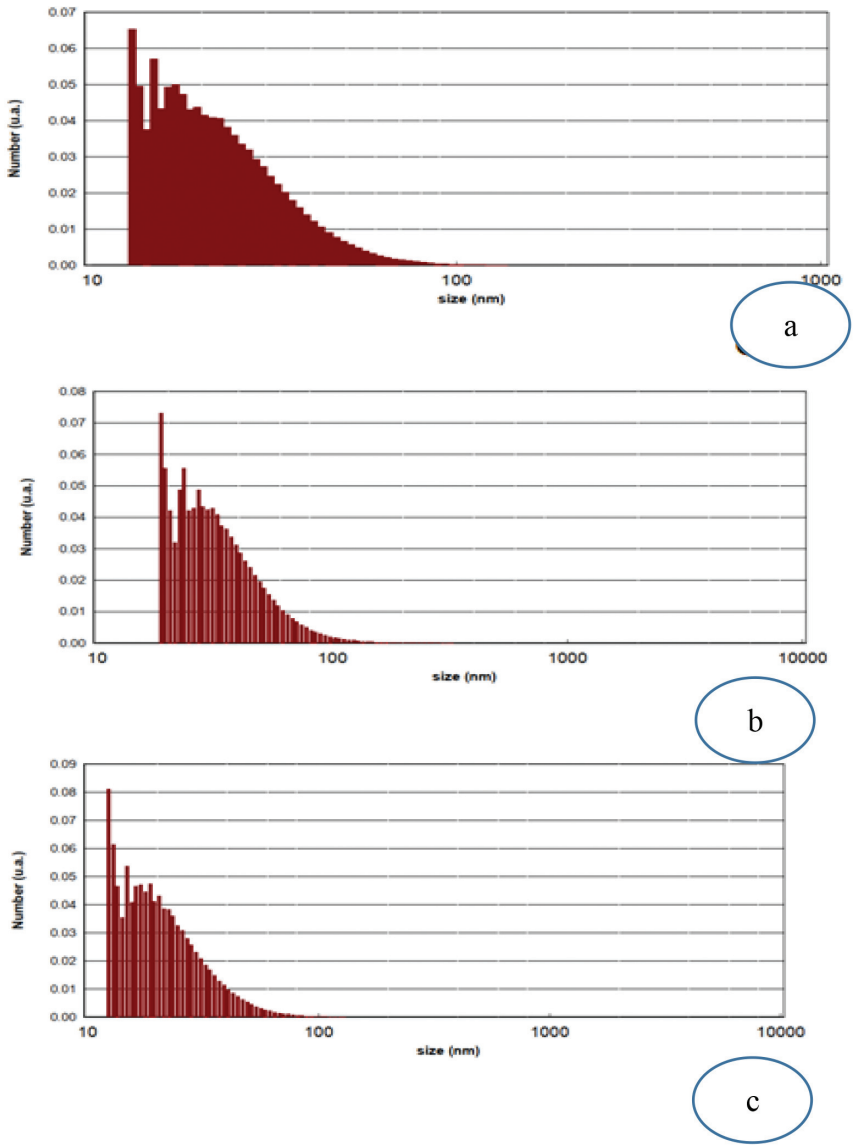


*Fig. 8. Size dispersion of iron oxide nanoparticles (a), iron oxide nanoparticles (chitosan (b)) and iron oxide nanoparticles (chitosan + amylase (c)).*

stabilization at 23% and 82.5% exhibited its maximum activity at pHs 3 and 10, respectively.

According to a study by Ahmad et al., in the year 2020, the present study utilized stabilization methods for amylase enzyme through incorporation onto magnetite-chitosan nanoparticles with an aim to ascertain their catalytic properties. The findings reveal that the α-amylase activity is augmented and stabilized when the pH is increased to 8.0. Nevertheless, the stabilized enzyme exhibited superior stability in alkaline pH conditions compared to the unbound enzyme, as reported by a previous study [16]. The stabilized enzyme exhibited notable stability by retaining its initial activity at 93.2% and 78.3% after 60 minutes when subjected to pH 9 and pH 10, respectively. In comparison, the free enzyme demonstrated a significantly lower maintenance rate of activity, at 61% and 12%, respectively. It has been conserved. Furthermore, the enzymatic activity of the stabilized enzyme exhibited a 65.6% retention rate at a pH of 11 over the course of 60 minutes, while the unbound enzyme experienced a decline in activity of 95%. The findings of the report suggest that stabilized α-amylase exhibits lower sensitivity to free enzyme and a greater ability to adapt to changes in pH. According to recent findings, an enzyme employed in the detergent sector is required to exhibit robust endurance against rigorous environmental conditions, including elevated pH levels within the range of 8 to 10.5. The enhanced enzymatic activity observed in chitosan nanoparticles may be attributed to the multipoint binding of the enzyme to $Fe_3O_4$, thereby

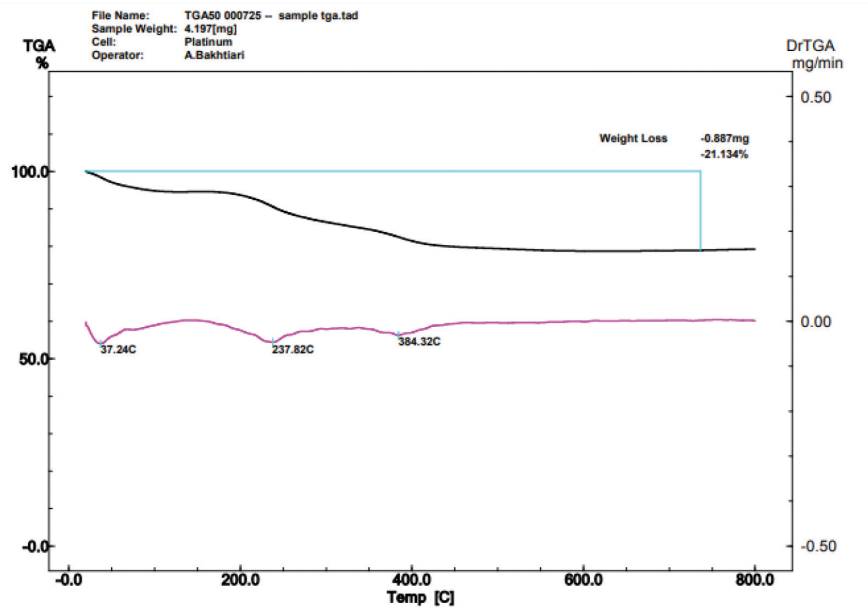


*Fig. 9. Scheme of thermal investigation of alpha-amylase enzyme stabilized on iron oxide-chitosan magnetite nanoparticles.*

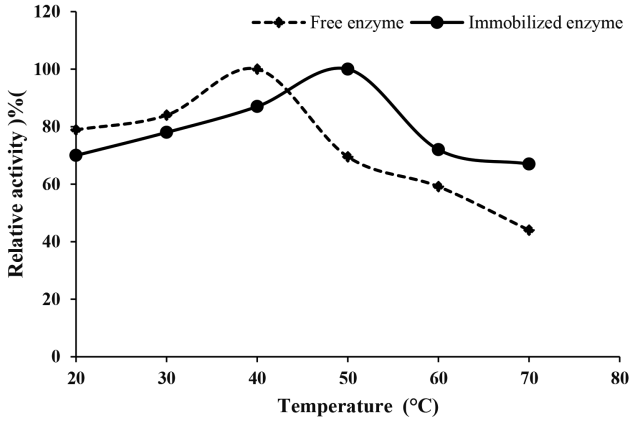


*Fig. 10. Diagram of the effect of temperature on the activity of free and stabilized amylase enzyme on iron oxide + chitosan nanoparticles.*

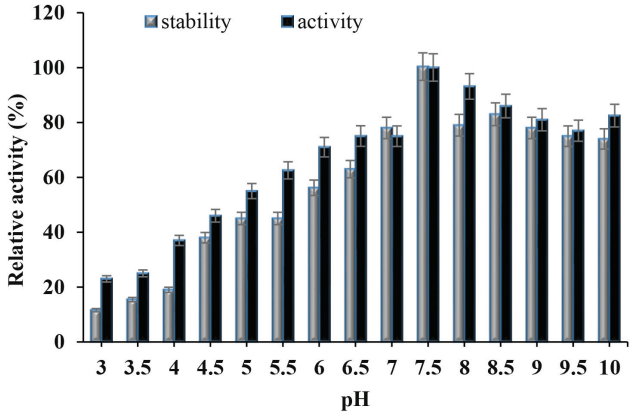


*Fig. 11. Investigation of the effect of pH on the activity and stability of free alpha-amylase enzyme.*

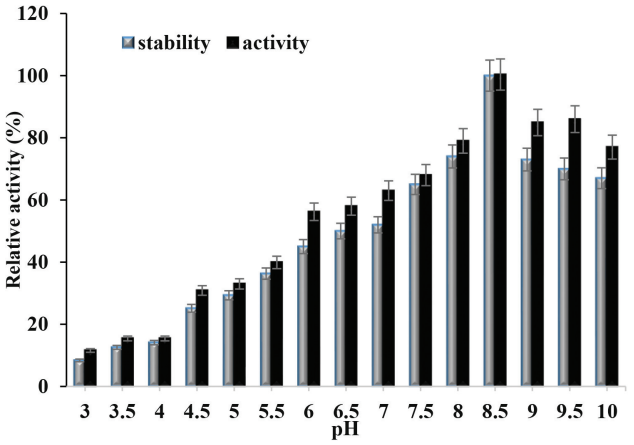


*Fig. 12. Investigation of the effect of pH on the activity and stability of free alpha-amylase enzyme.*

increasing the heterogeneity of enzyme molecules through alterations in pH [16].

### *3.11. Determining the half-life of free and stabilized enzyme*

To assess the half-life or temperature inactivation of both the unfettered and stabilized enzyme, an investigation was conducted to measure enzyme activity within the temperature range of 40 to 60 degrees Celsius, with increments of 5 degrees Celsius. In the Fig. 13, the half-lives of the free enzyme were determined at three different temperatures, namely 30 °C, 40 °C, and 50 °C, resulting in values of 200, 150, and 157 minutes, respectively. In contrast, the enzymatic

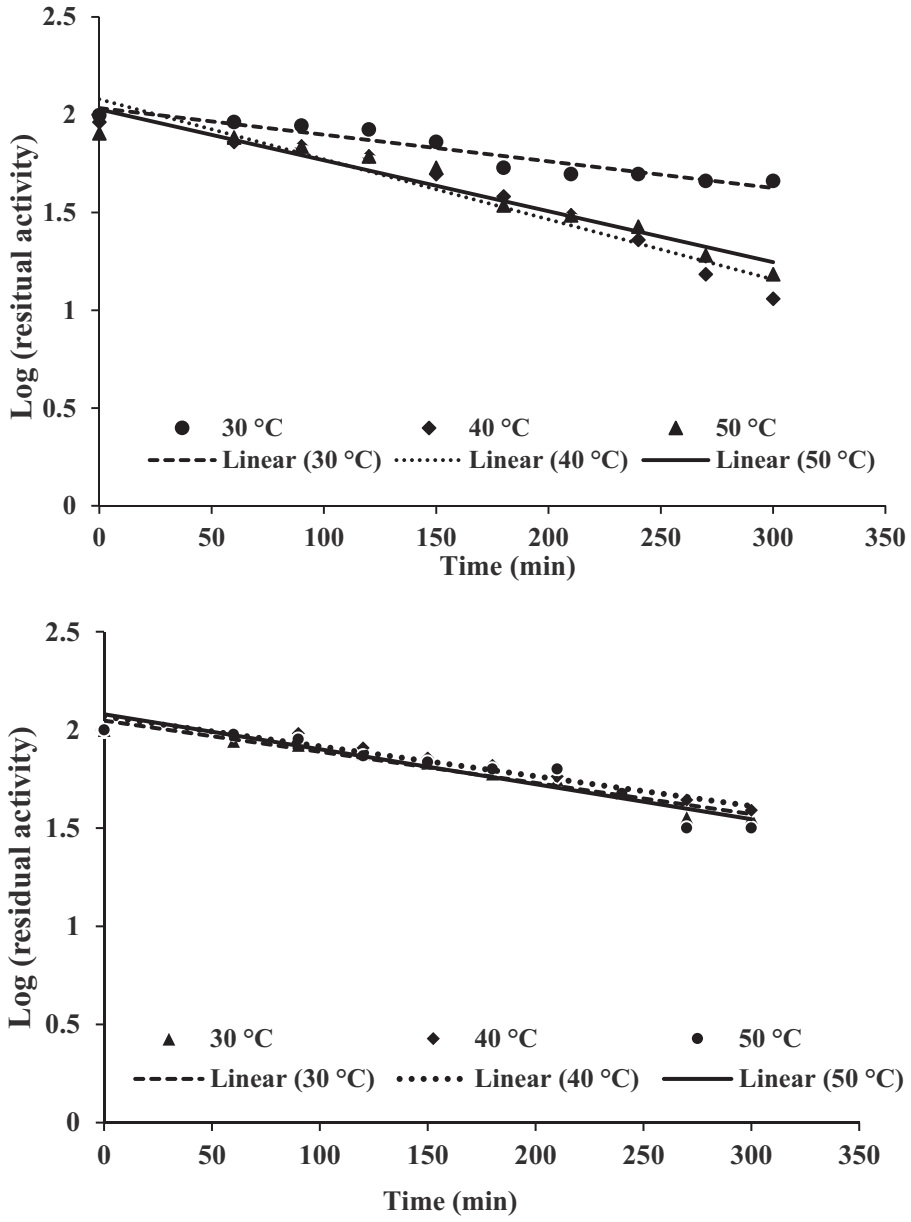


*Fig. 13. Logarithm of the residual activity of the enzyme stabilized at temperatures of 30 °C, 40 °C and 50 °C.*

half-life stabilized on iron oxide + chitosan nanoparticles was assessed to be 235, 233, and 240 minutes, respectively. According to the findings of Daval et al. [15], the utilization of magnetic nanoparticles in the stabilization of alpha-amylase enzyme resulted in the retention of 66% of its activity even after a span of 20 days. Comparatively, the free enzyme displayed a mere retention of 18% of its activity.

### *3.12. Determination of kinetic parameters of the enzyme*

As shown in Figs. 14 and 15, the aim of this study was to explore the kinetic parameters of alpha-amylase, specifically Km and Vmax, through exposure to various substrate concentrations. An approach involving the use of Prism GraphPad software was adopted to construct Michaelis-Menten curves for the free and stabilized forms of the enzyme. The findings elucidated that the maximum velocity of unbound enzyme was comparatively lesser than that of the steady-state. The findings derived from the GraphPad software indicate that the utmost velocity (Vmax) exhibited by the unbound enzyme was 1.371 mol/min.mg ± 61.52. Additionally, the Michaelis-Menten constant (Km) was observed to be 0.003 mg/ml ± 0.0082. The present study aimed

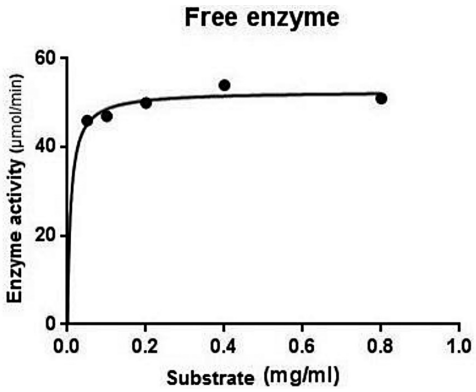


*Fig. 14. Mikaelis-Menten diagram of free amylase enzyme.*

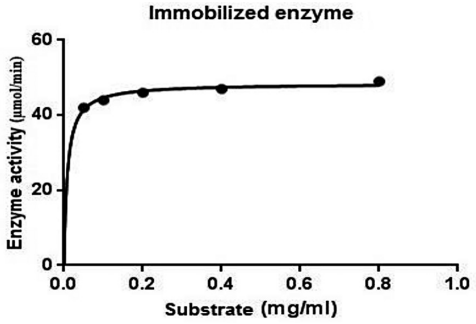


*Fig. 15. Mikaelis-Menten diagram of amylase stabilized on magnetic iron oxide-chitosan nanoparticles.*

to evaluate the kinetic parameters of an enzyme stabilized on chitosan. Our findings indicate that the maximum velocity of the enzyme-catalyzed reaction was 48.43 ± 0.383 6 mol / min.mg, while the Michaelis-Menten constant was determined to be 0.15003006 0.15 mg/ml/km/ml. These findings demonstrate the potential significance of utilizing chitosan as a stabilizing agent for enzymatic reactions.

In the study conducted by Sohrabi et al., In the year 2014, the determination of Km values for amylase enzyme immobilized on magnetic iron oxide nanoparticles, both in its free and stabilized states, resulted in values of 6.27 and 4.77 mmol, respectively. The present study yielded consistent results with regard to the Vmax values obtained for both free and stabilized enzymes, which were determined to be 2.44 and 11.58 µmol / mg / min, respectively, as reported in a previous work [13].

### *3.13. Results of re-use of stabilized alpha-amylase enzyme over time*

As depicted in Fig. 16, the enzymatic activity exhibits a decrease as the time elapsed increases from zero up to seven times. On the fourth iteration, the enzyme exhibited a retention of 72% of its initial activity, whereas following seven successive iterations, the protein's activity was observed to decrease to 47%. Throughout the enzymatic storage duration within the phosphate buffer, the enzyme has been sheltered from full inactivation within the storage medium via interactions with iron-chitosan oxide nanoparticles, thereby providing a platform for sustained enzymatic activity for a period of seven days. Montazeri and colleagues conducted a comparable investigation in their scholarly inquiry. In a study conducted in 2018, it was discovered that by immobilizing the alpha-amylase enzyme on magnetic iron oxide nanoparticles, the stabilized enzyme exhibited notable retention of activity, with 36.8% of its function consistent over 4 consecutive cycles. These results are consistent with previous findings [17, 18].

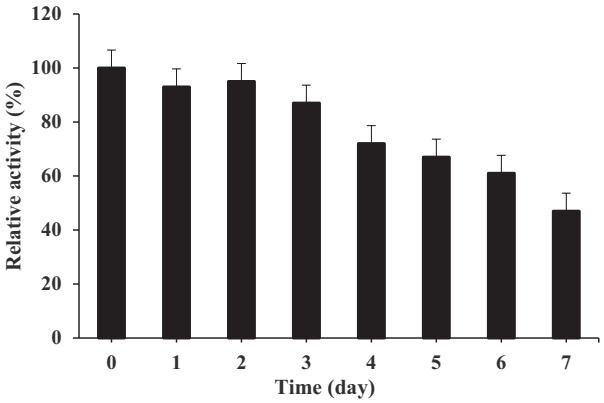


*Fig. 16. Residual activity of amylase enzyme stabilized in iron-chitosan substrate during 7 days.*

## 4. Conclusion

The present investigation entails the synthesis of iron oxide-chitosan nanoparticles followed by the stabilization of alpha-amylase through the supplementation of glutaraldehyde to the aforementioned substrate. The binding of amylase enzyme to the substrate was validated by means of X-ray diffraction (XRD), Fourier-transform infrared spectrometry (FT-IR), Field Emission Scanning Electron Microscopy (FE-SEM), and zeta potential measurements. There have been numerous instances. The findings of the study indicate that the stabilized form of the enzyme exhibited comparatively greater activity when subjected to a temperature of 40 °C and an optimal pH level of 7.5 in comparison to the free form of the enzyme. The half-life of the stabilized enzyme was observed to be extended compared to that of the free enzyme. As such, during the process of thermal inactivation, it was established that the stabilized enzyme exhibited a comparatively slower loss of initial

activity, requiring more time to attain 50% of its original activity than the free enzyme at the relevant temperature range under investigation. The investigation also revealed that the stabilized enzyme exhibited elevated values of maximum velocity (Vmax) and Michaelis-Menten constant (Km) compared to those of the free enzyme.

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