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Fabrication of robust layered double-hydroxide nanoparticles for α-Amylase immobilization: Enhancing catalytic performance, stability, and prospective sustainable biocatalysis applications

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Abstract

A key component in the creation of sustainable processes is the design of novel biocatalysts that increase the stability and reusability of enzymes by immobilization. This study successfully designed an α-amylase biocatalyst using the co-precipitation approach, based on Zn-Al-layered double-hydroxide (LDH) nanoparticles (NPs). The enzymatic activity of the immobilized α-amylase (LDH/Amy) with a particle size of 255 +/- 36 nm was maintained with a high immobilization yield of 97% and an intermediate loading capacity of about 54.81 mg/g of enzyme/LDH. Zeta potential analysis showed that electrostatic interactions influenced enzyme immobilization on the LDH NPs. LDH/Amy exhibited a higher K_m and increased V_{max}, indicating enhanced catalytic activity. Studies on thermal stability revealed that LDH/Amy was more resistant than the free form. Reduction of the activation energy and enhancement of the half-life ($T_{1/2}$) revealed improved stability of LDH/Amy. In addition, LDH/Amy maintained 90% of its initial activity following 5 consecutive runs and 97.6% of its initial activity following a 25-day storage period. The findings presented in this study indicate the potential utility of LDH NPs as an ecologically benign, low-cost biocatalyst that can promote α-amylase in the hydrolysis of starch for use in pharmaceutical and industrial processes.

Keywords : α-Amylase immobilization; layered double hydroxides; half-life; nanoparticles; operational stability; starch hydrolysis

1. Introduction

Enzymes are significant in biotechnology given they are used in a wide range of fields, including biotechnology, pharmaceuticals, analytical chemistry, food technology, and textiles, and their versatility is driving the reduction in chemical use, which in turn is propelling the worldwide enzyme market. Their uses constantly expanding to meet the demands of the global industrial setting. The enzyme market is expected to grow to $14.7 billion by 2025[1]. However, the lack of low-cost technologies to overcome enzyme application constraints hinders their extensive industrial use. Enzymes frequently lack sufficient stability in the intended media, which is a significant disadvantage. Considering that even minimal conformal changes can result in a substantial decrease in activity. Enzymes can become inactive in various settings, including high or low pH levels, physical pressures, and contacts between gaseous liquids and organic materials[2]. In this context, producing enzymes that can be immobilized on suitable substrates has proven to be a promising strategy. The enzyme's functionality can be improved in several ways with this strategy, including improved catalytic stability and reusability, as well as better control over the catalytic process. [3,4]. The commercial applicability of immobilized enzymes is contingent on the immobilization approach efficiency, chemical and thermal stability, water solubility of the support, and residual enzyme activity. Typical immobilization techniques include adsorption, affinity immobilization, covalent binding, or entrapment with polymeric and inorganic materials with varying properties [5,6]. Despite the great efficiency of each approach, there are still several obstacles to the commercialization of immobilized enzymes due to the specificity of each enzyme or substrate[7]. When enzymes are immobilized, they demonstrate enhanced selectivity and specificity, improved control over operations, simpler product recovery without catalyst contamination, and, in the case of protease, a significant decrease in the autolysis process rate. Additionally, immobilization is one of the most favored methods of improving enzymes towards stabilization due to lower inhibition by reaction products, selectivity towards non-natural substrates, and superior functional qualities compared to soluble enzymes [8,9].

α-amylases are ubiquitous enzymes that are utilized in a variety of applications industries such as biotechnology, pharmacology, analytical chemistry, food, and textile. They account for approximately 25%-33% of the global market and are one of the most popular [10]. α-Amylase (EC.3.2.1.1) are endo-amylases that hydrolyze the internal α-1, 4 glycosidic bonds between neighboring glucose units in the straight amylose chain of starch and other polysaccharide polymers to glucose, maltose, and dextrin to generate a variety of products. α-amylase's industrial usage is limited due to its short half-lives, poor operational and storage stabilities, and high processing costs that make recovery and reuse challenging[11,12]. However, the

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efficiency of the immobilized enzyme, particularly at the nanoscale, significantly increases compared with the enzyme's soluble or free forms [5]. In addition, enzymes are reusable after immobilization, making them a good alternative to chemicals in several industries [6]. Previous studies have explored several types of supports and methods for efficiently immobilizing α-amylase, but the outcome was frequently a decline in enzyme activity. In the process of immobilization, enzymes may become denatured and lose activity. It is brought on by deformations, particularly if there are many contacts between the matrix and the enzyme [13].

Layered double hydroxides (LDHs) represent a class of synthetic hydrotalcite-type anionic clays that possess anion-exchange characteristics and resemble cationic clay minerals[14] . LDHs are typically considered inorganic hosts that help guest organic species maintain chemical and thermal stability. Such compounds can be described as "green materials" since they are non-toxic, readily available precursors, and easy and inexpensive to produce in an aqueous media with less hazardous waste products. Additionally have a large surface area, making them ideal for immobilizing enzymes [15]. LDHs are a class of layered materials that are composed of positively charged metal hydroxide films that became stable via interlamellar anions. LDHs are made up of interlayer anion-stacked brucite (Mg(OH2))-like layers. Anions occupy the interlayer space as seen in Figure 1 to offset the positive charge caused by the replacement of some of the divalent cations in the brucite-like sheet with trivalent ions, resulting in positively charged sheets[16].

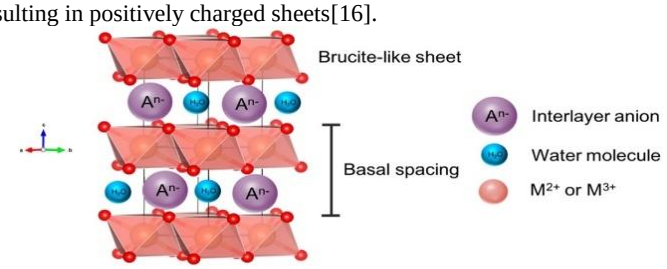

Fig 1. A schematic structure of layered double hydroxides. Reproduced under a Creative Commons Attribution Non-Commercial No Derivatives License (CC BY-NC-ND) from Ref. [16].

This mineral's chemical formula is expressed as $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}(A^{m-})_{x/m}.yH_2O$, where A^{m-} is an exchangeable anion like CO_3^{2-}, Cl^-, or OH^- and M^{2+} and M^{3+} are metal cations such Mg^{2+}, Zn^{2+}, Al^{3+}, Cr^{3+}, or Fe^{3+} [17,18]. Hence, LDHs are one of the most promising options among the several supports proposed for α-Amylase immobilization owing to their biocompatibility, lamellar structure, and composition along with their insolubility in most solvents, tolerance to high temperatures and pH, etc. In terms of biocompatibility and environmental sustainability, LDHs containing carbonate-intercalated Zn^{2+} and Al^{3+} are often preferred. Indeed, they are exceptional nanomaterials because they are inexpensive and simpler to develop. The co-precipitation procedure is usually employed in their production being an advantageous immobilization approach that functions easily and robustly, negating the need for the use of unstable or inexpensive chemicals, given that an immobilized enzyme eventually finds use as an industrial biocatalyst[15]. Enzyme immobilization on LDH occurs via enzyme adsorption on the nanoparticle support [18]. It is among the least expensive and easiest methods of immobilizing an enzyme since it relies on mild interactions, which often cause minimal disruption to the protein structure. Moreover, it usually requires few immobilization stages and no reagents[19]. Further, immobilization usually occurs on the solids' outer surface, which diminishes the bulky substrates' diffusion limits for the immobilized enzymes. Even so, it is regrettable that there aren't many studies in the literature about the immobilization of α-amylase on Mg/Al-LDHs [20]. Moreover, no studies have been conducted on the comparative study of free and LDH-immobilized α-amylase enzymes. Motivated by these distinct attributes, the purpose of this study is to shed a spotlight on the potential of LDH as a nanomaterial for immobilizing the α-amylase enzyme from Bacillus Sp. and creating biohybrid materials that can accelerate the hydrolysis of starch, resulting in the formation of oligosaccharides. We aimed to enhance the catalytic efficiency and specific activity of the α-amylase immobilized form; hence, we explored the immobilization of α-amylase on unmodified Zn/Al-LDH. The immobilized α-amylase was tested to evaluate its kinetic and stability parameters. Finally, the reusability and storage stability of the immobilized α-amylase was assessed.

2. Materials and Methods

2.1 Materials

Enzyme α-Amylase (EC 3.2.1.1) from Bacillus sp. (A6814), and other materials were bought from Sigma-Aldrich and utilized without further purification. Chemicals were all analytical grade. Deionized water was utilized throughout the study.

2.2 Methods

2.2.1 Layered double hydroxide nanoparticles (LDH NPs) synthesis

LDH NPs were prepared by co-precipitation with [Zn]/[Al] = 1/2 at a constant pH with modifications to the approach outlined by Starukh et al. [21]. In brief, 0.1 mol $ZnCl_2$ and 0.05 mol $AlCl_3.6H_2O$ in 200 ml distilled water were added dropwise under vigorous mixing to 200 ml of an aqueous solution of 0.2 M NaOH and 0.1 $CaCO_3$. After the addition, the temperature was increased to 85 °C, and the precipitate was further aged for 16 h. The LDH NPs produced were collected by centrifugation (10000 rpm for 15 min) and washed thoroughly with deionized water until they reached pH 7. The produced NPs were then dried at 40 °C. The sample is labeled Zn-Al/LDH.

2.2.2 α-amylase immobilization on Zn-Al/ LDH

For the immobilization of α-amylase on Zn-Al/LDH suspensions containing 20 mg of LDH samples and 10 mL of 0.1 M of phosphate buffer (pH 6.5) and α-amylase with different concentrations (50,100, 200 mg/mL) were gently shaken at 40 rpm for 4 h. Following the immobilization process, LDH with immobilized enzyme (LDH/Amy) was collected by centrifugation (10000 rpm for 15 min), washed several times with buffer, and air dried at 25 ºC. Both the precipitant and the filtrates were

collected for further investigation. The protein content in the supernatant was compared to the protein value of the initial concentration to compute the loaded protein, which was done using the Bradford technique [22].

Immobilization yield %:

The immobilization yield was calculated as the enzyme yield that was immobilized by adsorption onto the LDH NPs support and calculated by the following equation.

$$\text{Immobilization yield (\%)} = \frac{\textbf{Activity of immobilized enzyme}}{\textbf{A}-\textbf{B}} \boldsymbol{x100} \quad \text{(Eq. 1)}$$

where A is the initial activity of free α-amylase added, and B is the activity of α-amylase in supernatant. Amylolytic activities were assayed under standard assay conditions.

Immobilization efficiency (EE)

The immobilization efficiency of the enzyme was evaluated in terms of specific activity using starch as the substrate under standard assay conditions using equation (2):

$$\text{Immobilization efficiency} = \frac{\boldsymbol{Specific\ activity\ of\ immobilized\ enzyme}}{\boldsymbol{specific\ activity\ of\ free\ enzyme}} \quad (\boldsymbol{Eq.2})$$

Loading capacity (LC)

The initial examination of the enzyme loading capacity was conducted using a modified version of the method reported by Verma and Raghav [4]. The appropriate amounts of α-amylase were added to 1 mL phosphate buffer (pH 6.5, 50 mM), 2 mL distilled water, and 100 mg LDH NPs to create the reaction mixtures. The liquids were agitated for 30 minutes to guarantee even mixing, and then they were allowed to incubate. Following that, centrifugation (1000 rpm for 10 minutes) was employed to collect LDH/Amy samples. The supernatant was 10x diluted. Using colorimetric analysis, as published in the Bradford Assay [22], with minor modifications, we measured the loading capacity, which quantifies the enzyme loaded in the LDH NPs. The immobilized enzyme (LDH/Amy) was incubated for 12 hours at 5°C in 200µL of phosphate buffer (pH 6.5). The solution was gently agitated for 3 minutes with a 1.8 mL Bradford reagent aliquot to facilitate protein binding. The absorbance was measured at 595 nm. The standard was bovine serum albumin (BSA). Using Equation (3), the enzyme loading capacity was calculated; Loading capacity $mg \cdot g^{-1}$:

$$\frac{\textbf{Initial protein concentrtion} - \textbf{Amount o protein in LDH}_{\textbf{Amy}}}{\textbf{Total mass of LDH NPS}}$$

2.2.3 α-Amylase activity assay

The assay for free and immobilized α-amylase activity was conducted utilizing Bernfeld's method [23], with minor adjustments made as needed. 100 µL of α-amylase solution or 20 mg LDH/Amy was incubated for 5 min in 1.5 mL of 50 mM phosphate buffer (pH 6.5) with 1% NaCl. Subsequently, 400 µL of a 2% starch solution was added, and the mixture was incubated at 40 °C for 15 min at 10 rpm. Next, 3 mL of DNS reagent (3, 5-dinitro salicylic acid reagent, Sigma-Aldrich Spruce Street, St. Louis, USA) was added and the mixture was then maintained at 90 °C for 15 min, and finally cooled to room temperature (23 ± 2 °C). The resulting orange color was detected spectrophotometrically at 540 nm. Similarly, a blank was produced without free α-amylase. One α-amylase activity unit (U) is defined as the amount of enzyme that liberates 1.0 µmol of reducing sugar/min with maltose as a standard, under standard assay conditions. The residual enzyme activity was calculated using the following equation (4):

Residual Activity ($Umg.g^{-1}$) =

2..2.4 Particle size and zeta-potential measurements

The mean particle size, size distribution (polydispersity index, PDI), and surface charge (zeta potential), of the LDH NPs and LDH/Amy were measured using a Malvern Zetasizer Nano (Malvern Instrument Ltd., Malvern, UK).

2.2.5 Comparative studies of free and immobilized α-Amylase

2.2.5.1 Effect of pH and temperature on enzyme activity

By assessing the activity of free and immobilized α-amylase at various temperatures (20–80 °C), the effects of temperature were investigated. The effects of varying pH levels on the activity of free and immobilized α-Amylase were assessed. Before testing, Free α-amylase e and LDH/Amy were pre-incubated using different buffers at pH 4.5-8. To obtain this pH range, 50 mM citrate buffer (pH 5), 50 mM potassium phosphate buffer (pH 6-7), and 50 mM Tris-Hcl buffer (pH 8–9) were used. Residual enzyme activity was measured under standard assay conditions.

2.2.5.2 Evaluation of kinetic parameters

Enzyme kinetic parameters are considered a quantitative measure of the amount of active enzyme and its efficiency level is defined as the amount of substrate converted per time unit considering the initial enzyme concentration [19,24]. Using varying initial substrate concentrations (starch at 0.01–30.0 mg/ml). In the current study, the Michaelis-Menten constant (Km) and apparent maximum velocity (Vmax) of the free and immobilized enzyme were calculated using the Lineweaver-Burk plot with non-linear regression analysis using the Graph-Pad Prism program version 9 (GraphPad Software, San Diego, CA, USA) following Eq. (5)[19].

$$\mathbf{V} = \frac{\mathbf{V_{max}}\ [\boldsymbol{S}]}{\mathbf{K_m} + \mathbf{S}} \quad (\mathbf{Eq5})$$

where V and V_{max} are the starting and maximum rates of reaction ($\mu mol \cdot min^{-1}$), respectively, and K_m is the Michaelis–Menten constant (mM). [S] denotes the concentration of the substrate (mM). In addition, K_{cat} and the catalytic efficiencies (k_{cat}/K_m) of free and immobilized enzymes were calculated.

2.4.5.3 Thermal stability of free and immobilized enzyme

The free and immobilized α-amylase were incubated for 2 h at varying temperatures between 50 and 80 °C before the addition of the substrate to investigate the temperature stability. After 2 h intervals, the residual enzyme activity was determined under standard assay conditions. The residual enzyme activity was determined by considering the initial enzyme activity at 0 min as 100%. The assayed residual activity was used to calculate the half-life of the free and immobilized α-amylase

2.4.5.4 Determination of thermodynamic parameters

The deactivation rate constant (*kd*) was calculated from the first-order rate expression when log of residual activity (%) was plotted as a function of time (min) at the temperatures used for inactivation as follows [24];

$$ln\frac{Et}{E0} = -Kdt \qquad \text{(Eq. 6)}$$

K_d is the deactivation rate constant, which was calculated from the plots of Ln [E_t/E_0] versus t (min).

Decimal reduction time (*D*-value) at a specific temperature was defined as the time needed to lose 90 % of the initial enzyme activity. The *D*-value and the half-lives ($t_{1/2}$) of the enzyme were determined as shown in Eqs. (7) and (8) [25]:

$$D\text{-value} = \frac{\ln 10}{kd} \quad Eq.\ 7 \qquad t_{1/2} = \frac{\ln 2}{kd} \quad Eq.\ 8$$

The activation energy for denaturation (*Ea*) was determined using Arrhenius equation (9):

$$\mathbf{lnKd = lnA - \frac{E_a}{R} \times \frac{1}{T}} \qquad \text{(Eq. 9)}$$

where T is the absolute temperature (K), A is the Arrhenius frequency of the collision factor, E_a is the activation energy for thermal denaturation (kJ/mol), and R for the universal gas constant (8.314 × 10−3 kJ/mol K).

2.2.5.5 Leaching Study

To evaluate the leaching capability of immobilized α-amylase, suitable aliquots of LDH/Amy were incubated in phosphate buffer solution (50 mM, pH 6.5) at 30 ℃ for 3 h at 150 rpm.. Following centrifugation, leaching% was calculated according to Eq. (10)

$$\text{Leaching\%} = \frac{\text{amount of protein in the supernatant}}{\text{amount of immobilized protein}} \text{X100} \qquad \text{(Eq. 10)}$$

2.2.5.6 Reusability of the immobilized enzyme

The immobilized α-amylase's operational stability was tested using repeated hydrolysis reaction assays[24]. Ten mL of starch solution (0.100 g/l) was used for each test, and the immobilized enzyme was at 40 ℃ and pH 6 (50 mg LDH/Amy). The enzyme was filtered out of the reaction media following each measurement of activity. Following the addition of a new complete medium to the reaction flask containing the previously employed enzymes, activity measurements were performed under standard assay conditions. Ten successive reaction cycles (each lasting 900 s) were used to test the reusability of the immobilized enzyme. The initial activity was assumed to be 100%, and the percentage residual activity RA (%) was calculated accordingly after each cycle.

2.2.5.7 Storage stability of immobilized enzyme

Storage stability of the free α-Amylase and LDH/Amy biocatalysts was also examined at 4 ℃ for 25 days in sodium phosphate buffer (50 mM, pH 6.5). The initial activity was assumed to be 100% and residual activities were determined under standard reaction conditions.

2.6 Statistical analysis

All treatments were performed in triplicate. Analysis of variance (one-way ANOVA) followed by student's t-test where ($p < 0.05$) was designated as the significance cutoff for differences in mean values. This was done using SPSS 24 (SPSS Inc., Chicago, IL, USA) software. The findings are expressed as the mean ± SD and all experimental measurements were performed in triplicate.

3. Results

Co-precipitation is the most common method used to prepare LDHs and is often described as a simple, robust, and economic "one pot" method (Waheed et al., 2023). Coprecipitation from aqueous solutions containing mixed metal ions (Zn^{2+}/Al^{3+}=2:1) with a base (0.1 N NaOH, pH≈10) was used to produce LDHs. The white nanoparticle precipitate produced was washed up several times, dried, and then reacted with the enzyme for immobilization. The immobilized α-amylase (LDH/Amy) was examined for its enzymatic activity and physicochemical properties.

3.1 Enzyme loading capacity

The correlation between α-amylase loading capacity, its enzymatic activity, and its specific activity which is defined as units of activity per milligram of α-amylase; are described in Table 1. The greater the α-amylase concentration in LDHs, the higher its enzymatic activity and loading capacity at the enzyme concentration used in this investigation (Fig. 2). With NPs enzyme ratio of 1:2, LDH NPs have the greatest loading capacity, as revealed in Fig. 2. Nonetheless, the relationship between the specific activity and the enzymatic activity and loading capacity was inversely correlated. Several studies have reported similar findings on enzyme.

Additionally, they noticed that while the enzyme's loading capacity increased, the specific activity decreased[26]. Moreover, they pointed out that as the concentration of the enzyme rose, the loading efficiency did too. Nevertheless, the enzyme activity decreased considerably. They attributed these findings to enzyme-substrate restriction at high concentrations due to its inclusion in the immobilization matrix.

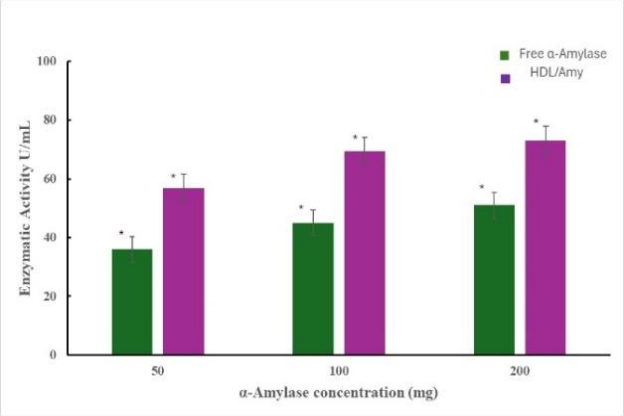

Fig. 2. Enzymatic activity of free and immobilized α-amylase (LDH/Amy) at different concentrations 2 with starch as substrate The results are expressed as the mean (n = 3) and the bars in the columns represent the standard deviation. (*) Significant 2 differences when comparing the free and enzymes at the same concentration (p > 0.05).

3.2 Enzymatic activity of free and immobilized α-amylase

Fig. 3 shows the enzymatic activity of free and immobilized α-amylase at various substrate concentrations. Using starch as the substrate, α-amylase activity in LDH NPs incubated with the lowest substrate concentration (1%) exhibited a significant difference ($p > 0.05$) between the free and immobilized enzyme (24.74±1.2 U.mL−1 and 29.97±1.5 U.mL−1, respectively). Additionally, the immobilized enzyme in LDH NPs showed higher activity at all tested concentrations than the free form at similar concentrations (Fig 3). Remarkably, the enzymatic activity of the free and immobilized forms varied significantly ($p > 0.05$) at comparable concentrations examined. The immobilized enzyme's increased catalytic activity may be the result of decreased diffusion resistance, which allows the substrate and product to flow freely and increases the amount of enzyme-substrate contact in the LDH NPs. The higher enzymatic activity of immobilized α-amylase may also be attributable to LDHs molecule binding sites, allowing hydroxyl functional group interactions with the enzyme. It can be inferred that the immobilization process used in this study did not impair enzyme activity because α-amylase activity was sustained after the immobilization method. Similarly, improved enzymatic activity of α-amylase upon immobilization has been reported previously [12,25,27].

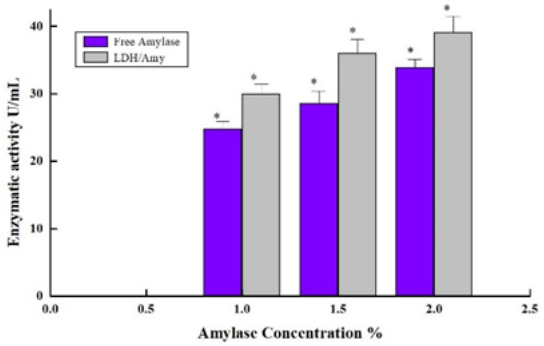

Fig. 3. Enzymatic activity of free and immobilized α-amylase (LDH/Amy) at different substrate concentrations. The results are expressed as the mean (n = 3) and the bars in the columns represent the standard deviation. (*) Significant differences when comparing the free and LDH/Amy at the same concentration (p > 0.05).

Table 1. Comparison of different concentrations of immobilized α-amylase loading capacity, and their specific activity.

Substrate	α-Amylase (mg)	Loading capacity ($mg \cdot mg^{-1}$)	Enzymatic activity ($U \cdot mg^{-1}$)	Specific activity ($U \cdot mL \cdot mg^{-1}$)	Immobilization efficiency %	Immobilization yield%
Starch	50	41.42	56.85	1.37	1.52	80
	100	84.81	139.26	1.64	2.27	97
	200	162.12	172.92	1.06	1.413	95

3.3 Zeta potential and particle size

Positive charges on the lamellae of free LDH NPs responsible for holding interlaminar anions are linked to polarity; the values, which range from 10 to 35 mv, are comparable to those described in the literature [28,29] . A sharp drop in the Z-potential from +26 mV (LDH NPs) to -18 mV (LDH/Amy) was achieved when amylase was immobilized to the support with values that are comparable to pure amylase. The immobilization of amylase through electrostatic interactions between the enzyme and the support is confirmed by the shielding effect of LDH NPs charges. In addition, a greater particle size of the suspended LDH/Amy NPs (255 +/- 36 nm) was noted in comparison to the pure LDH (185 +/- 9 nm).

3.4 Characterization of free and immobilized α-amylase

3.4.1 Effect of temperature

The immobilized α-amylase enzyme must exhibit strong temperature stability for industrial applications. The findings displayed in Figure 4A demonstrate that the immobilized α-amylase exhibits superior thermal stability in contrast to the free enzyme. These findings indicate that at higher temperatures, LDHs have a protective effect on α-amylase activity. In addition to inhibiting aggregation and unfolding of the enzyme protein, immobilization lowers conformational flexibility, thermal vibrations, and enzyme mobility [3,27,30]. Hence, the free enzyme was more responsive to temperature changes between 50 and 80°C than the immobilized enzyme. At 55 °C, the activity of the immobilized enzyme declined gradually. However, the activity of the free enzyme fell significantly as the temperature increased, primarily because of thermal denaturation. This is because of the restriction of enzyme mobility upon immobilization, which protects the protein's three-dimensional structure against heat denaturation. In addition, stabilization to the enzyme's stereochemical structure might be responsible for the rise in thermal stability and ideal operating temperature for the immobilized enzyme [2,10]. Similar findings were reported by previous studies for free and immobilized α-amylase [26,31,32].

3.4.2 Effect of pH

Fig. 4B shows the pH stability of the free and immobilized α-amylase in an aqueous buffer solution at various pH levels (5.0–9.0). α-Amylase activity retained enzymatic activity in its immobilized form at all pH values examined, compared to the free form. Enzymes and NPs are pH-dependent owing to their unique structures. This demonstrated that the LDH NPs were predominantly microporous hence, they adapted well to the pH range studied [19]. The free and immobilized enzymes showed their highest activities at pH 6.5 and 6, respectively (Fig.4B). When compared to the free enzyme, the optimum pH for the LDH/Amy shifted toward a more acidic range, which aligns with different studies[31,33]. Possible explanations for this change include ionic interactions or hydrogen bonding between the enzyme and the NPs [18,34]. When anion concentrations close to the support surface are greater than in the solution, the charged supports shift the optimal pH of α-amylase toward lower pHs [35]. Carbonate ions attached to LDHs alter the surrounding environment of the immobilized α-amylase by attracting protons in the solution. As a result of this occurrence, the ideal pH for immobilized α-amylase drops below that of free α-amylase. Immobilized α-amylase had a relative activity of 80% on LDHs at pH 5.0, compared to the optimal pH activity. At pH 7.0, LDHs remain substantially stable, but their stability rapidly decreases at pH levels that are too alkaline. The evaluation of α-amylase e activity at pH 8.0 revealed that while it was only 19% in its free form, it remained at approximately 60% in the immobilized form. This decrease in stability is most likely the result of hydroxide ions replacing carbonate ions. Consequently, it is expected that the immobilized α-amylase structure will be disturbed, leading to a substantial decrease in its activity. Previous studies have demonstrated that in the immobilized form, the enzyme activity was stable across a wide range of pH values (4.0 and 9.0). Conversely. the activity of the free enzyme was lower at the same values [10,36]. The stability examination at various pH values in this work suggests that immobilized α-amylase has promise for use in starch hydrolysis processes.

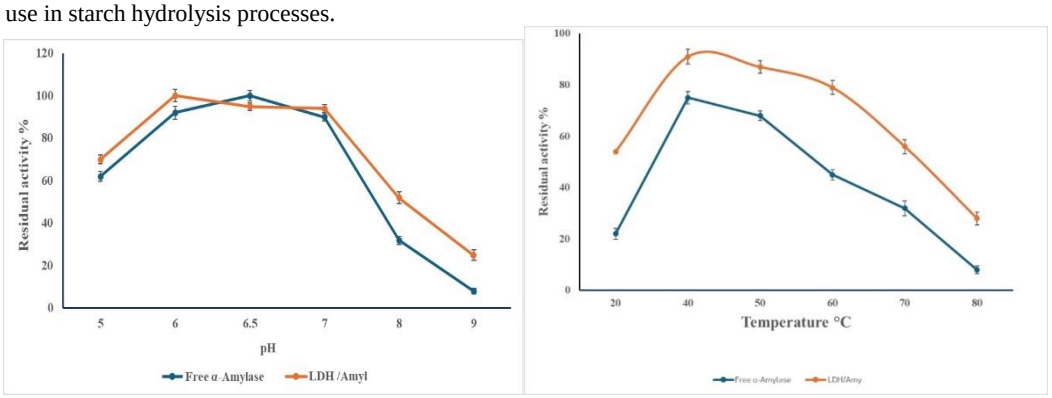

Fig 4 Biochemical characterization of free and immobilized enzyme catalysis: A) temperature profile (assay temperature range: 20–80 °C) and B) pH profile (pH range: 4.5–9; B of free and immobilized α-amylase (LDH/Amy). Each point reflects the average of three experimental results ± SD. Enzyme assay was carried out under standard assay conditions.

3.4.3 Evaluation of Kinetic parameters

To study the effects of immobilization on α-amylase e activity, it was essential to compare the two kinetic parameters of α-amylase, K_m, and V_{max}. Michaelis-Menten plot demonstrated K_m and V_{max} values for substrate hydrolysis rates at different substrate concentrations (Fig.5, Table 2).). The immobilized enzyme had a higher Vmax value than the free enzyme (59.88 U/mg protein-1 min-1). In addition, the immobilized enzyme exhibited a higher Km value (3.089 mg/mL) than the free enzyme. Enzyme immobilization increases enzyme activity, as evidenced by a higher Vmax value for the immobilized enzyme. In addition, the catalytic efficiency, enzymatic efficiency, and KCat were increased by 0.74,1.3 and 0.65 fold, respectively. Enhanced orientation of the α-amylase molecule on the supporting surface can increase active sites and substrate affinity[2,3,27,37].The low mass transfer limitation of the diffusion layer surrounding the enzyme particles corresponds to the observed rise in Vmax after immobilization, which did not limit the diffusion of starch toward the LDH-immobilized α-amylase. It is also possible that the observed changes in catalytic characteristics are due to the stability of the three-dimensional protein structure that occurs when the enzyme binds to the NPs [32]. Free and immobilized α-amylase showed similar outcomes in the studies conducted by Bruna et al. and Abdel-Mageed et al. [3,20,27]

Table 2. Kinetic parameters of free and immobilized α-amylase on Zn-Al-CO_3-LDH nanoparticles.

Kinetic parameter measured	Free α-amylase	Immobilized α-amylase LDH/Amy
K_m (mg/mL)	2.798	3.089
V_{max} (U/mg protein)	40.98	59.88
K_{Cat}	0.1366	0.1996
Enzymatic efficiency (V_{max}/ K_m)	14.64	19.38
Catalytic efficiency K_{Cat}/K_m	0.048	0.0646

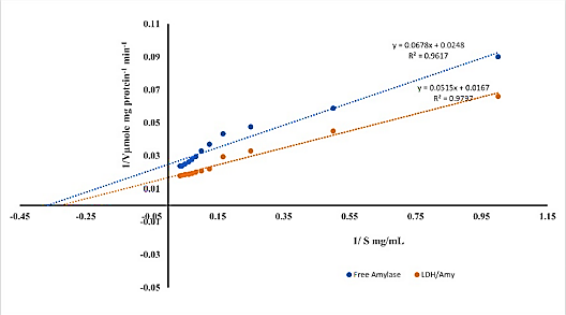

Fig 5 Lineweaver-Burk plot for the determination of kinetic constants free and immobilized amylase using starch as enzyme substrate. All data points are means of triplicate.

3.4.4 Determination of thermodynamic parameters

Both soluble and immobilized enzymes were incubated at various temperatures (50 mM phosphate buffer at pH 7). Data obtained revealed that immobilization using LDH NPs improved the stability of the enzyme-substrate reaction at high temperatures and extreme pH, in contrast to the soluble form (Table 3). The immobilized form of the enzyme exhibited greater stability compared to its free form. The substance's rate of heat inactivation was assessed at temperatures between 50 and 80 °C for both its free and immobilized forms (Fig. 6). The results demonstrated that the free enzyme lost 65% of its initial activity after 2 hours at 80 °C, while the immobilized form maintained more than half of its activity. When compared to free enzymes, immobilized amylase generally demonstrated increased stability[38]. The findings indicated that its unfolding at a high temperature may have been limited by the immobilization process. Hence, compared to soluble α-amylase, the immobilized form provided higher orderly and structurally stable confirmation. Since the thermodynamic data provides an additional means of evaluating enzyme stability and interpreting its catalytic and physiological features, the relationship between thermodynamic and catalytic results is evaluated. Data presented in Table 3 shows that the immobilized α-amylase required less activation energy than its soluble form, as demonstrated by the lower values of E. These outcomes validated the interactions between Amylase and LDH NPs. Compared to the free enzyme (70.84 kJ mol−1), the immobilized form's Ed value (64.01 kJ mol−1) was lower. Consequently, the fact that more energy was needed for the immobilized amylase to denature suggests that thermal stability could be improved upon immobilization. The immobilized form's half-lives and D-values are significantly higher than the free form, according to Table 3's thermodynamic data. Similar findings were also reported by Karam et al. [17], Ahmed et al. [39] and Desai et al. [25] wherein immobilization of the amylase enzyme resulted in a considerable extension of both half-lives and D-values. The formation of many covalent connections between the enzyme and its carrier, which results in a reduction in the enzyme's capacity to change shape and vibrate thermally, can be used to explain this phenomenon. Consequently, denaturation does not occur and the protein stays folded.

Table 3. Determination of thermodynamic and kinetic parameters of free and immobilized amylase on Zn-Al-CO_3 LDH nanoparticles.

Temperature °C	50 °C		60 °C		80 °C	
Enzyme	Free	LDH/Amy	Free	LDH/Amy	Free	LDH/Amy
k_d (min^{-1})	0.000965	0.0007225	0.001805	0.001312	0.006635	0.004675
$t_{1/2}$ (min)	760	960	384	528	105	144
D-value (min)	2383	3183	1274	1753	346.64	491.97

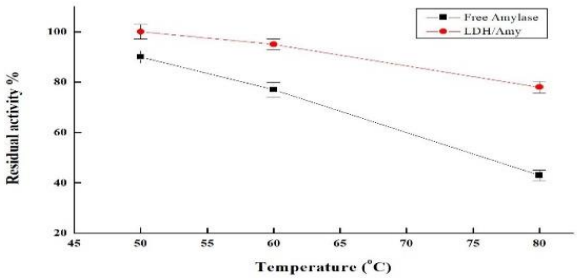

Fig. 6. Thermal stability study on free and immobilized α-amylase (LDH/Amy) at different temperatures from 50 and 80 ºC for 2 h intervals. The initial activity was taken as 100 %. Each point reflects the average of three experimental results ± SD

3.4.5 Enzyme leakage

Enzyme leakage can occur when proteins are not covalently linked to the matrix, particularly during washing with buffered solutions. Therefore, we have taken into account the potential reduction in activity resulting from α-amylase desorption during buffered solution washings. Nevertheless, upon analysis of the washing supernatants, no appreciable quantity or activity of protein was observed (Fig. 7). However, the amount of protein in the biomaterials that were directly examined matched the amount calculated using the indirect method. These findings verified that there was no discernible α-amylase e desorption following immobilization. They also indicated that there are no appreciable losses in amylase enzyme due to any other mechanism. Similar results by Bruna et al. [20] were reported for α-amylase immobilized.

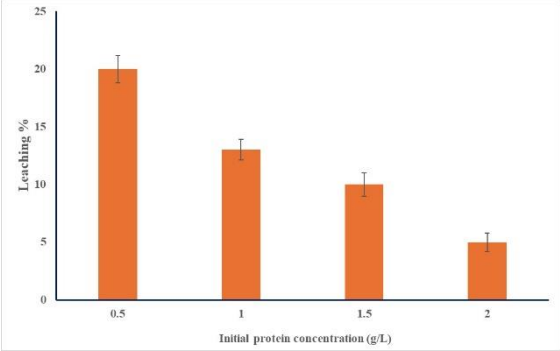

Fig. 7. Represents immobilized enzyme (LDH/Amy) leakage %. LDH/Amy was incubated in phosphate buffer solution (50 mM, pH 6.5) at 30 °C for 3 h at 150 rpm. Each point represents the average of three experiments ±SD.

3.5 Enzyme reusability

The industrial use of enzymes is highly required due to their economic and sustainable values. Industrial continuous bioprocesses require stable and economically reusable enzymes. From a commercial perspective, enzyme reusability is another crucial factor. Enzyme recovery following reaction completion may be a laborious procedure. However, this is minimized by immobilization. Enzymatic reusability was examined in the current study throughout 10 consecutive cycles. The immobilized α-amylase on LDH NPs showed a slight reduction in activity following every cycle. After five cycles, the residual activities for LDH/Amy were 90%, of their initial activities. After 10 consecutive cycles, LDH/Amy maintained 84% of the initial enzymatic activity (Fig. 8). This decline in activity is attributed to the discharge of α-amylase molecules that are lightly bound to the support. Hence, contrary to previous reports where a higher residual activity percentage was achieved in this study [32,39,40]. These findings suggest that there is adequate stability and reusability of the immobilized α-amylase using LDH NPs. Two main causes were found by the researchers to be responsible for the immobilized enzyme losing its catalytic activity: the enzyme's altered stereochemical structure and its diffusion during the recycling process. Over time, the immobilized enzyme's active site becomes distorted due to repeated substrate contact, which results in a loss of activity. Furthermore, loss of activity results from the active region of the immobilized enzyme being distorted by repeated interactions of the substrate with it [39,40] or, enzyme denaturation in sequence to consecutive procedures used. This includes repeated washing, shaking, and recycling within an entirely new hydrolytic cycle [18] .

3.6 Evaluation of Storage Stability

To assess storage stability, the activities of the immobilized and free enzymes were measured after being stored at 4 °C for 25 days. Residual activity was determined at regular intervals of time. The outcomes demonstrated that the enzyme's storage stability was improved by immobilization (Fig. 9) where LDH/Amy showed superior retention of residual activity for the free enzyme. Residual enzyme activity for immobilized and free α-amylase e was found to be 97.6%, and 58%, respectively, after storage for 25 days at 4 °C. Enhancement of enzyme storage stability upon immobilization has been reported previously [41]. Hence, it can be concluded that LDHs present a better option for α-amylase e stability, where immobilization prevents enzyme

deactivation and enhances the enzyme's storage stability. Stability is an inherent property of an enzyme and the presence of a high enzyme concentration in the matrix inhibits its denaturation.

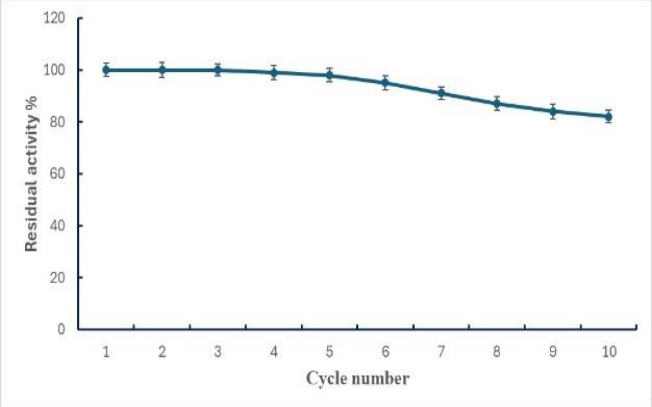

Fig. 8. Reusability of immobilized (enzyme (LDH/Amy) after 10 repeated hydrolytic cycles (initial activity was considered as 100%). Activity assay was performed under normal assay conditions. Each point presents the average of three experimental results ± SD

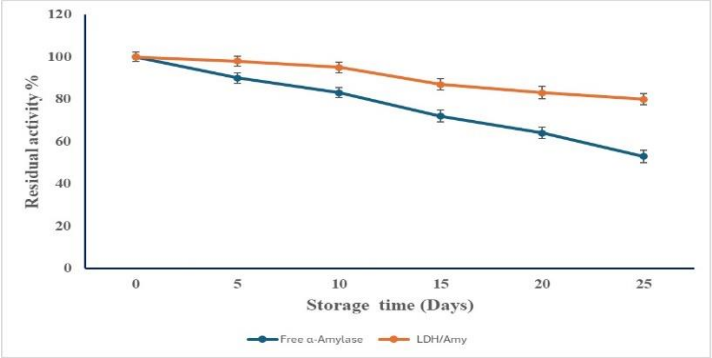

Fig. 9. Storage stability of free and immobilized α-amylase upon storage at 5 °C for 25 days. Initial activity at day zero was considered 100%. Each point presents the average of three experimental results ± SD.

Conclusion

The present investigation demonstrates the immobilization of α-amylase on LDH. The bio-nanocatalyst was synthesized to attain excellent reusability, appropriate activity, and high stability under various processing circumstances. we chose a nanoparticle delivery system for the synthesis of LDH with Al^{3+} and Zn^{2+} metals. LDHs were produced by the co-precipitation method. Quite interesting, α-amylase exhibited a high loading efficiency onto the surface of the LDH nanoparticles. The immobilization boosted the thermal stability in addition to the general stability of the enzyme across a broad pH range. Immobilization reduced the mobility of the enzyme protein structures and improved the catalytic performance, at different temperatures and pH levels. Immobilized enzymes on LDH-supports were efficiently restored through centrifugation for reusability. The formulated LDH/Amy, as a straightforward, environmentally benign, and economically viable biocatalyst, firmly demonstrates promise in medicinal and industrial processes. The LDH/Amy s' superior characteristics showed that LDH NPs are a promising matrix for immobilizing enzymes, not just α-amylase. The results of this study can potentially improve biosensing and bio-nanocatalyst immobilization.

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