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Research article

The covalent immobilization of β-galactosidase from *Aspergillus oryzae* and alkaline protease from *Bacillus licheniformis* on amino-functionalized multi-walled carbon nanotubes in milk

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ABSTRACT

This study aimed was to covalently immobilize *β-galactosidase* from *Aspergillus oryzae* and protease from *Bacillus licheniformis* on amino-functionalized multi-walled carbon nanotubes. In this study, a two-level factorial design was employed to investigate the impact of seven continuous variables (activation pH, glutaraldehyde molarity, activation time (0–8 h), buffer solution pH (8-0), buffer solution molarity, *MWCNT-NH_2-glutaraldehyde* quantity, and stabilization time (0–180 h)) on the immobilization efficiency and enzymatic activity of protease and *β-galactosidase*. Furthermore, the effect of time on the percentage of enzymatic activity was examined during specific intervals (24, 48, 72, 96, and 120 h) of the immobilization process. The analysis of variance results for protease enzymatic activity revealed a notable influence of the seven variables on immobilization efficiency and enzymatic activity. Additionally, the findings indicate that activation time, buffer pH, *MWCNT-NH_2-glutaraldehyde* quantity, and stabilization time significantly affect the activity of the protease enzyme. The interplay between buffer pH and stabilization time is also significant. Indeed, both activation time and the quantity of *MWCNT-NH_2-glutaraldehyde* exert a reducing effect on enzyme activity. Notably, the influence of *MWCNT-NH_2-glutaraldehyde* quantity is more significant ($p < 0.05$). In terms of beta-galactosidase enzymatic activity, the study results highlight that among the seven variables considered, only the glutaraldehyde molarity, activation time, and the interplay of activation time and the quantity of *MWCNT-NH_2-glutaraldehyde* can exert a statistically significant positive impact on the enzyme's activity ($p < 0.05$). The combination of activation time and buffer solution molarity, as well as the interactive effect of buffer pH and MWCNT–NH_2–glutaraldehyde, can lead to a significant improvement in the stabilization efficiency of the protease of carbon nanotubes. The analysis of variance results demonstrated that the efficiency of covalently immobilizing *β-galactosidase* from *Aspergillus oryzae* on amino-functionalized multi-walled carbon nanotubes is influenced by the molarity of glutaraldehyde, buffer pH, stabilization time, and the interplay of activation time + buffer pH, buffer pH + activation time, activation time + buffer molarity, and glutaraldehyde molarity + *MWCNT-NH_2-glutaraldehyde* ($p < 0.05$). Through the optimization and selection of optimal formulations, the obtained results indicate enzyme activities and stabilization efficiencies of 64.09 % ± 72.63 % and 65.96 % ± 71.77 % for protease and beta-galactosidase, respectively. Moreover, increasing the enzyme stabilization time resulted in a reduction of enzyme activity. Furthermore, an increase in pH, temperature, and the duration of milk storage passing through

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the enzyme-immobilized carbon nanotubes led to a decrease in enzyme stabilization efficiency, and lactose hydrolysis declined progressively over 8-h. Hence, the covalent immobilization of *β-galactosidase* from *Aspergillus oryzae* and protease from *Bacillus licheniformis* onto amino-functionalized multi-walled carbon nanotubes is anticipated to be achievable for milk applications.

1. Introduction

Enzymes are a group of proteins produced by living organisms. These proteins act as vital catalysts (biocatalysts), speeding up the rate of biochemical reactions without undergoing significant consumption. They possess the capability to initiate and direct these reactions [1]. Indeed, enzymes play a crucial role as living catalysts in biological processes. They make biochemical reactions active in living organisms by reducing the activation energy and speeding up the rate of these reactions [2]. Thus, vital life processes like digestion, respiration, cell synthesis and renewal, and other crucial activities are made possible through enzymes [3]. Each type of enzyme typically only reacts with one, or a couple, of substrates. In other words, each enzyme can catalyze a specific reaction. This feature is due to the three-dimensional structure and active sites of enzymes, which bind to specific molecules and activate biochemical reactions [4]. Therefore, the role of enzymes in the functioning of cells and the execution of diverse processes in living organisms is highly significant. Enzymes play a crucial role in the absorption and digestion of nutrients, the decomposition and synthesis of molecules, the regulation of cellular activity, the transfer of energy, and the functioning of other biological systems. Without enzymes, performing these biological activities would be challenging in terms of speed and efficiency. Therefore, enzymes are recognized as the most crucial factors in biological processes, and they have numerous applications in biological and biochemical studies. By improving our understanding of enzyme functions and their effects on cells, we can enhance biological processes and utilize their various applications in different fields,such as pharmaceutical industries, food industries, biotechnology, and biosensing [5]. In the food industry, enzymes can be used to produce fruit juices, bread fermentation, dairy production, and chocolate production. Enzymes also play a crucial role in the pharmaceutical industry, functioning as catalysts in the synthesis processes of drugs and the production of antibiotics. Milk is a rich source of enzymes, which play a significant role in the digestion and breakdown of nutrients in the milk. *B-galactosidase*, commonly known as lactase, is an enzyme responsible for hydrolyzing lactose in milk. In cow's milk, the average amount of lactose is approximately 4.4 %–6.8 %, while in human milk, the lactose content is higher, constituting about 7 % of the dry matter. The enzyme lactase facilitates the digestion of lactose by breaking it down into glucose and galactose within the body. Those who need lactase can take this enzyme as a supplement or in other available forms [6]. In this metabolism, the β-glycosidic bond in D-lactose is hydrolyzed to form D-galactose and D-glucose. Subsequently, these two monosaccharides can be efficiently absorbed through the intestinal wall, facilitating their entry into the bloodstream [7].

The enzyme protease of milk, plays a crucial role in hydrolyzing proteins into peptides and amino acids during digestion. This process enhances the digestion and better absorption of proteins in the body [8]. Besides protease, milk contains other enzymes that play a crucial role in digestion and the breakdown of nutrients. For instance, lipase or enzymes responsible for the hydrolysis of fats into glycerol and fatty acids, amylase utilized in the digestion of starch and carbohydrates into simple sugars, alkaline phosphatase, which plays a pivotal role in pH regulation of milk, and various other enzymes present in milk in trace amounts. These enzymes also exist as part of microbial compounds [9]. For example, beta-galactosidase enzyme from *Aspergillus oryzae* and protease enzyme from *Bacillus licheniformis* are notable instances. These two enzymes can be employed in biotechnological processes and utilized as microbial metabolites in food and dairy industries [10]. In contrast to chemical catalysts, utilizing enzymes derived from bacteria and fungi offers advantages such as cost-effectiveness and environmental friendliness. However, the application of these enzymes is not without its challenges. Challenges such as the isolation of enzymes from the reaction mixture, complex downstream processing, and the potential for product contamination contribute to the practical challenges associated with their application. Hence, enzyme stabilization is a crucial technology that holds significance in overcoming these challenges [11].

Homogeneous phase catalysis facilitates easy recovery and reuse of the biocatalyst. This method facilitates continuous processes, offering the capability for utilization in various reactor types and operational conditions while enhancing reaction control. Moreover, it enhances operational stability and can potentially improve enzyme properties and increase its resistance to inhibitors. In addition, enzyme stabilization leads to a stronger and less soluble structure [11,12].

Various methods are available for enzyme stabilization; however, in industry, simple and high-cost approaches are often employed. Common methods include physical stabilization (such as adsorption or physical entrapment) and chemical stabilization (such as covalent bonding and cross-linking). Covalent bonding is considered a more suitable method for stabilizing all enzymes in various applications, as the stabilized enzyme efficiently retains its activity. Moreover, selecting a suitable matrix for enzyme stabilization is aligned with the nature of the matrix, the simplicity of the method, and the targeted utilization of the enzyme [13,14].

Nanostructures such as nanoparticles, nanofibers, nanotubes, and nanocomposites are novel supports for enzyme stabilization. Due to their large surface area, these compounds can adsorb enzymes to a significant extent, thereby enhancing the volumetric activity of the enzyme. Among various nanomaterials, carbon nanotubes possess unique structural, mechanical, thermal, and biocompatibility features. By functionalizing nanomaterials, their efficiency can be improved [15,16]. This process involves attaching desirable functional groups to the surface of nanomaterials to achieve desired properties. This functionalization can impact the dispersion and interaction of nanomaterials with enzymes, consequently altering the catalytic activity of the stabilized enzyme. Therefore, this article aims to examine the properties of stabilized enzymes and the impact of various factors on the covalent immobilization of

β-galactosidase from *Aspergillus oryzae* and [10].

2. Materials and methods

2.1. Materials

Various materials and products have been employed in this research. The *β-galactosidase* enzyme from *Aspergillus oryzae* has been procured in powdered form from the company Troy in Virginia, USA. Additionally, the protease enzyme from *Bacillus licheniformis* is also supplied in liquid form by the same company. For the experiments, *MWCNT-NH_2 carbon nanotubes* with a purity of 99 %, a diameter ranging from 10 to 30 nm, and a length of 10 μm were purchased from Carbotec in India. Additionally, some chemical substances such as bovine serum albumin, casein, sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, lactose, sodium sulfite, phenol, and dextrose were purchased from Merck, Germany. For the experimental analysis, 3,5-dinitrosalicylic acid (DNS) from Alfa Aesar in Germany was utilized. Additionally, sodium potassium tartrate (Rochelle salt) was purchased from VWR Prolabo Chemicals in Belgium, and sodium azide was purchased from Merck Millipore in Germany. Additionally, L-Tyrosine was purchased from the German company Carl Roth GmbH + Co. KG, and nitrocellulose membrane filters with a pore size of 0.45 μm and a membrane diameter of 47 mm were utilized from the German company ISO-LAB.

2.2. Methods

2.2.1. Enzyme immobilization

In this study, β-galactosidase enzyme from Aspergillus oryzae and protease enzyme from Bacillus licheniformis were immobilized separately onto carbon nanotubes (MWCNT-NH2) through a covalent bonding method. Initially, the carbon nanotubes were activated to introduce suitable functional groups for enzyme binding, likely involving treatment with acid chlorides or sulfonyl chlorides. The enzymes were then prepared in buffer solutions at optimal pH and temperature to maintain their activity. Subsequently, the activated carbon nanotubes were mixed with the enzyme solutions under controlled conditions to facilitate covalent bonding between the enzymes and the functional groups on the nanotubes. This process included incubation and gentle mixing to ensure uniform enzyme attachment. Post-immobilization, thorough washing and purification steps were performed to remove any unbound enzymes or impurities from the enzyme-bound carbon nanotubes, ensuring the purity of the immobilized enzymes. Finally, the immobilized enzymes underwent characterization using techniques enzyme activity assays to confirm successful immobilization and evaluate enzyme stability and activity. This detailed description of the enzyme immobilization process is crucial for understanding the experimental methodology employed in this study, elucidating the mechanisms by which enzymes interacted and bonded with carbon nanotubes via covalent attachment. Optimal immobilization conditions were optimized through this study. In enzyme stabilization, carbon nanotubes are first functionalized using glutaraldehyde and then treated with the enzyme by the incubation method to form covalent bonds with the created functional groups.

To determine the activity of *β-galactosidase Aspergillus oryzae*, equation (1) was employed, which measures the increase in reducing sugar content due to the progression of lactose hydrolysis. Additionally, to assess the activity of *Bacillus licheniformis* protease, equation (2) was utilized based on the increase in -L-tyrosine concentration resulting from protein hydrolysis. These activities were measured using a UV–Vis spectrophotometer with the Bradford [17] method.

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$$IU \Big/ mg\ Enzyme = \frac{Released\ Reducing\ sugar\ (\mu mol)}{Enzyme\ used\ (mg)\ x\ Duration\ of\ reaction\ (min)} \tag{1}$$

$$IU \Big/ mg\ Enzyme = \frac{Released\ L-tyrosin\ (\mu mol)}{Enzyme\ used\ (mg)\ x\ Duration\ of\ reaction\ (min)} \tag{2}$$

Additionally, equation (3) is employed for the efficiency of immobilization.

$$\text{Immobilization Yield } (\%) = \frac{\text{Enzyme used for immobilization} - \text{enzyme in filtrate}}{\text{Enzyme used for immobilization}} \times 100 \tag{3}$$

Equation (4) is also used to calculate the efficiency of activity.

$$Activity\ Yield\ (\%) = \frac{Activity\ of\ immobilized\ enzyme}{Activity\ of\ soluble\ enzyme} x100 \tag{4}$$

The characteristics of the immobilized enzymes were evaluated over time by determining parameters such as optimal pH, optimal temperature, and stability.

2.3. Tests

2.3.1. Lactase enzymatic activity

In this study, the first step involved preparing a 1 % lactose solution for the assessment of lactase activity. Subsequently, 5 mL of this solution was mixed with a sodium dihydrogen phosphate solution at a concentration of 25 mM (with a pH of 4.5 for free enzyme and pH 5.5 for immobilized enzyme). Then, 200 μL of free enzyme AOG and 0.317 g of immobilized enzyme AOG were added to the solution. The reaction proceeded at a temperature of 55 °C for 60 min. Then, 200 μL of free enzyme AOG and 0.317 g of immobilized enzyme AOG were added to the solution. The reaction proceeded at a temperature of 55 °C for 60 min. After adding 200 μL of the reaction samples to 1800 μL of distilled water, the mixture was boiled for 10 min to deactivate the enzymes. After this step, the amount of D-glucose was determined by measuring its absorption at a wavelength of 575 nm using a UV spectrophotometer. This method involved determining the concentration of D-glucose, serving as an indicator of lactase enzyme activity, by measuring its absorbance. In other words, one unit of AOG activity represents the amount of enzyme that, under optimal conditions, forms 1 μM of D-glucose from lactose per minute.

2.3.2. Determining the activity of alkaline protease

To measure the activity of the protease, a 5-mL solution containing 1 % casein, prepared in a sodium phosphate buffer with a concentration of 25 mM and a pH of 7.5, is mixed with either 200 μL of the free enzyme or 0.286 g of the immobilized enzyme for the reaction. This reaction was carried out in a gently shaken container at 70 °C for 60 min. Following this, 400 μL of the collected reaction mixture samples were combined with 3600 μL of distilled water and heated for 10 min to deactivate the enzyme. The tyrosine concentration was assessed through absorbance measurements employing a UV spectrophotometer (UV–6300PC, Radnor, USA) at a wavelength of 274 nm (Lewis, 1998). One unit of enzymatic activity (IU) is defined as the amount of the enzyme that, under optimal assay conditions, produces 1 μmol of L-tyrosine from casein per minute [18].

2.3.3. Standard curve of bovine serum albumin

This curve, based on the Bradford protein assay method Moghassemi, Hadjizadeh and Omidfar [19], was constructed using absorbance values measured in a UV spectrophotometer at a wavelength of 595 nm. The standard curve was generated by preparing aqueous standard solutions of bovine serum albumin at various concentrations. The calibration equation was determined as $Y = 0.29958x$ with an R^2 value of 0.99.

2.3.4. Standard curve of L-tyrosine

The standard curve of L-tyrosine was plotted based on the method introduced by Basheir and Elbashir [20]. To achieve this, the standard curve of glucose was plotted based on the absorbance values measured in a UV spectrophotometer at a wavelength of 274 nm. A calibration equation was established by preparing standard glucose solutions at different concentrations, resulting in $Y = 0.00134x$ with an R^2 value of 0.99.

2.3.5. The standard curve of D-glucose

The standard curve of D-glucose was plotted using absorbance values measured in a UV spectrophotometer at a wavelength of 575 nm. A calibration equation was established by preparing aqueous standard solutions of D-glucose at different concentrations, resulting in $Y = 0.00104x$ with an R^2 value of 0.99 based on the DNS method Slein [21].

Following this, we examined the impact of temperature and pH on the Enzyme Stabilization Efficiency, as well as the effect of enzyme stabilization time on enzymatic activity. In addition, the impact of wash frequency and storage time on the enzyme activity and stabilization process in milk was assessed.

2.3.6. Thermal stability of immobilized lactase enzyme

In the initial step, 100 μL of free enzyme and 0.317 g of immobilized enzyme β-galactosidase from *Aspergillus oryzae* were reacted with 2.5 mL of 25-mM sodium phosphate buffer at various pH and temperatures (30–95 °C) for 1 h in a reaction vessel. Then, all free and immobilized enzymes were cooled in an ice bath for 10 min to halt the temperature effect. Finally, the remaining activities were determined using the standard activity assay method by adding 2.5 mL of 2 % lactose buffered solution (w/v) (at optimal pH).

2.3.7. Operational stability and storage of stabilized lactase enzyme

Operational stability and storage of the stabilized enzyme were determined by implementing the standard activity measurement method in 15 repeated batch experiments, performed every two days during storage. The stabilized β-galactosidase from *Aspergillus oryzae* was used to determine the storage stability in 0.1 M sodium phosphate buffers (pH opt) containing 0.02 % (w/v) sodium azide. The enzyme was stored in a refrigerator at +4 °C until further use. Before each use, the immobilized enzymes are filtered and washed on a membrane filter using vacuum suction with 15 mL of sodium phosphate buffer (0.1 M, optimal pH) and 15 mL of distilled water.

2.3.8. Thermal stability of free and immobilized alkaline protease

Initially, 100 μL of free alkaline protease from *Bacillus licheniformis* or 0.319 g of immobilized alkaline protease from *Bacillus licheniformis* were incubated in an incubator at various temperatures (30–95 °C) for 1 h with 2.5 mL of 25-mM sodium phosphate buffer at different pH levels. Subsequently, all free and immobilized enzymes were cooled in an ice bath for 10 min to halt the temperature effect. Finally, the residual activities were determined based on the standard activity measurement method by adding 2.5 mL of 2 %

(w/v) buffered casein solution (pH opt).

2.3.9. Operational and storage stability of stabilized protease enzyme

The operational and storage stability of immobilized alkaline protease from *Bacillus licheniformis* was determined by performing the standard activity measurement method in 15 repeated batch experiments every two days over a four-week storage period. The immobilized BLP used for assessing storage stability was stored in 0.1 M sodium phosphate buffers (pH opt) containing 0.02 % (w/v) sodium azide in a refrigerator at +4 °C until further use. Before each use, the stabilized enzymes were filtered and washed on a membrane filter under vacuum suction, using 15 mL of 0.1 M sodium phosphate buffer (pH opt) and 15 mL of distilled water.

2.4. Statistical analysis

In this method, 2k fractional factorial designs were employed to study the effects of seven continuous variables (activation pH, glutaraldehyde molarity, activation time, buffer solution pH, buffer solution molarity, amount of MWCNT–NH_2–glutaraldehyde, and stabilization time) on the stabilization efficiency and enzymatic activity of protease and beta-galactosidase. The sensitivity of the type I error in this study was set at 0.05 (Table 1). Design-Expert software version 13 was utilized for data analysis and graph plotting.

3. Results and discussion

3.1. Results of protease enzyme activity

The study examined the influence of seven variables, including activation pH, glutaraldehyde molarity, activation time (Fig. 1: a), buffer solution pH, buffer solution molarity, amount of MWCNT–NH_2–glutaraldehyde (Fig. 1: b), and stabilization time, on the stabilization efficiency and enzymatic activity. Results of the variance analysis indicate that the seven mentioned variables significantly impact the stabilization efficiency and enzymatic activity (Table, 2). The analysis demonstrates that these variables are effective in controlling the variations in stabilization efficiency and enzymatic activity, and the model significantly fits the data ($p < 0.05$). Moreover, the results indicate that activation time(Fig. 1: a), buffer pH, the amount of *MWCNT-NH_2-glutaraldehyde* (Fig. 1: b), and

Table 1
Statistical design.

Run	A:Act. pH	B:Molarity of glutaraldehyde (mM)	C:Activation time(h)	D:Buffer pH	E:Molarity of the buffer solution(mM)	F:MWCNT-NH2-glutaraldehyde amount(mg/mg Enzyme)	G:Immobilization time(h)
1	3	100	8	8	25	100	8
2	8	100	1	8	100	25	1
3	3	500	1	3	100	25	1
4	3	100	1	8	25	25	8
5	8	100	8	8	25	25	1
6	5.5	300	4.5	5.5	62.5	62.5	4.5
7	8	100	8	3	100	25	1
8	5.5	300	4.5	5.5	62.5	62.5	4.5
9	3	100	8	8	100	25	1
10	3	500	1	3	25	100	8
11	3	100	1	8	100	100	8
12	8	500	1	3	100	100	8
13	8	500	1	8	25	100	8
14	8	500	8	3	25	100	8
15	3	500	1	8	100	100	1
16	5.5	300	4.5	5.5	62.5	62.5	4.5
17	3	500	8	3	25	25	1
18	3	100	1	3	100	100	1
19	3	100	8	3	25	100	1
20	8	100	1	3	25	100	8
21	8	100	8	8	100	25	8
22	8	500	1	3	25	100	1
23	3	500	8	8	25	25	8
24	3	100	1	8	25	100	1
25	8	500	1	8	25	25	1
26	3	500	8	3	100	100	1
27	3	500	8	3	100	25	8
28	8	500	8	8	100	25	1
29	8	100	8	8	100	100	1
30	8	500	8	8	100	100	8
31	3	100	8	3	25	25	8
32	5.5	300	4.5	5.5	62.5	62.5	4.5
33	8	100	1	3	100	25	8
34	5.5	300	4.5	5.5	62.5	62.5	4.5
35	8	500	1	3	25	25	8

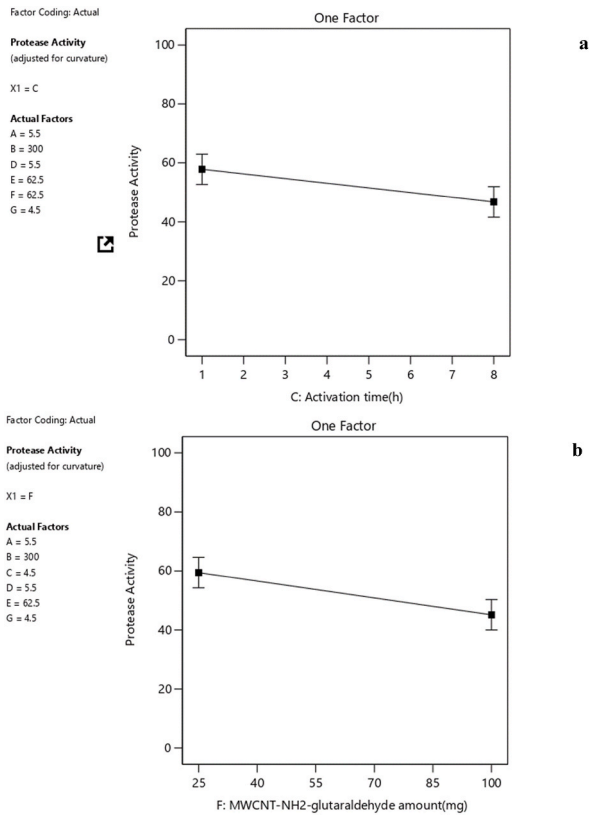

Fig. 1. illustrates the contour plot of the effect Activation time (h) (a) and MWCNT-NH2-glutaraldehyde amount (mg) (b) on enzyme activity.

stabilization time have a significant impact on the protease enzyme activity. The interactive effect between buffer pH and stabilization time is also significant. Indeed, activation time and the amount of *MWCNT-NH_2-glutaraldehyde* have a reducing effect on enzyme activity, and among them, the impact of the *MWCNT-NH_2-glutaraldehyde* amount is more significant ($p < 0.05$).

When the pH deviates from the neutral range, the amino acid residues of the enzyme become ionized. These ionic alterations can potentially modify the enzyme's three-dimensional structure, and reduce its activity [22].

Proteases play an essential role in the protein breakdown and degradation. Various factors, including pH, substrate, temperature, stability, active site location, and catalytic mechanisms, play a crucial role in optimizing the activity of proteases. Proteases are categorized into three groups—acidic, neutral, and alkaline—based on the optimal pH for their activity. Furthermore, neutral proteases have limited industrial applications and are mainly produced by species of *Bacillus* and *Aspergillus*. Proteases play a crucial role in organisms' nutrition by hydrolyzing proteins [23]. They break down large polypeptide molecules into smaller ones, which can be absorbed by cells. Intracellular proteases also play a crucial role in protein remodeling processes within the cell, as well as contributing to the metabolic balance between protein degradation and synthesis [24]. Regarding the negative impact of *MWCNT-NH_2-glutaraldehyde* on protease activity may involve interference with the enzyme's natural structure and function or alteration of the optimal conditions for enzyme activity and ultimately leads to a reduction in protease activity. Proteases play a crucial role in breaking down large polypeptide molecules into smaller ones that can be absorbed by cells. Moreover, intracellular proteases contribute significantly to protein remodeling processes within the cell and help maintain the metabolic balance between protein degradation and synthesis [10]. The negative impact of MWCNT-NH2-glutaraldehyde on protease activity may involve interference with the enzyme's natural structure and function or alteration of the optimal conditions for enzyme activity, ultimately leading to a reduction in protease activity [10,25].

The multi-walled carbon nanotube (*MWCNT*) as a nanomaterial can effects enzyme activities. The interaction of carbon nanotubes with enzymes can alter the structure and activity of the enzymes. The *MWCNT-NH_2-glutaraldehyde* can bind to the protease enzyme, altering its physical structure or catalytic mechanism. Moreover, protease enzyme's reaction may be influenced due to changes in the optimal conditions for enzyme activity [26]. The presence of *MWCNT-NH_2-glutaraldehyde* may lead to changes in the pH, temperature, or other required parameters for protease activity. As a result, the enzyme's activity may decrease. Consequently, the presence of *MWCNT-NH_2-glutaraldehyde* may lead to a reduction in the activity of the protease enzyme, exerting a negative impact on its activity. Nevertheless, to delve deeper and gain a better understanding of these effects, more research is needed to acquire more precise information regarding the extent and mechanisms of these influences. Over time, enzymes may experience structural changes and reduced stability due to external factors such as heat, humidity, and pH. These alterations may result in a decline in enzyme activity

and its incapacity to fulfill its functions over time. Furthermore, enzymes may react with oxidizing agents over the long term, such as oxygen in the air and other oxidants, leading to their oxidation. This oxidation can lead to structural changes in the enzyme and decreased activity [27].

In some cases, enzymes may experience degradation and digestion, and various factors in this process can contribute to deactivating the enzyme. This degradation and digestion can lead to a reduction in enzyme activity, occurring over time through the activation of the enzyme. If the activation time of *Bacillus licheniformis* protease on amino-functionalized multi-walled carbon nanotubes decreases, it is likely due to structural changes and reduced stability of the enzyme during activation. The covalent immobilization of β-galactosidase from *Aspergillus oryzae* and protease from *Bacillus licheniformis* on amino-functionalized multi-walled carbon nanotubes can be positively influenced by the immobilization time and the pH of the buffer. The term "immobilization time" refers to the period during which enzymes and carbon nanotubes come into contact and establish covalent beta interactions between them. A more extended immobilization time can provide the best opportunity to form of these connections, leading to a significant improvement in the performance of enzymes on the surface of nanotubes [28].

Additionally, the pH of the buffer solution used in the immobilization process can have a significant impact on the performance of enzymes and nanotubes [29]. The buffer pH must be within a suitable range for enzyme activity and facilitate covalent beta interactions between enzymes and nanotubes. Selecting the suitable pH can increase the adsorption of enzymes on the surface of nanotubes and, consequently, lead to a significant improvement in enzyme activity [30].

3.2. The efficiency of protease enzyme immobilization on multi-walled carbon amino nanotubes

In light of the information provided, protease is one of the active biological enzymes produced by species of *Bacillus*. In alkaline conditions, this enzyme can catalyze the hydrolysis of proteins into peptides and amino acids [31]. This analysis indicates that the immobilization time (Fig. 2a), buffer solution molarity (, as well as the interactive effect of buffer pH and *MWCNT-NH$_2$-glutaraldehyde* (Fig. 2b) can lead to a significant improvement in the efficiency of protease immobilization on carbon nanotubes. In other words, adjusting the activation time, buffer concentration, and considering the pH interaction with the MWCNT–NH$_2$–glutaraldehyde can substantially enhance the protease immobilization efficiency on carbon nanotubes. In conclusion, increasing the combination of activation time, buffer solution molarity, and the interactive effect of buffer pH with MWCNT–NH$_2$–glutaraldehyde can significantly enhance the efficiency of protease immobilization on multi-walled carbon nanotubes. The activation time refers to the duration the enzyme interacts with the buffer solution. With an increase in activation time, there is more opportunity for the enzyme to interact

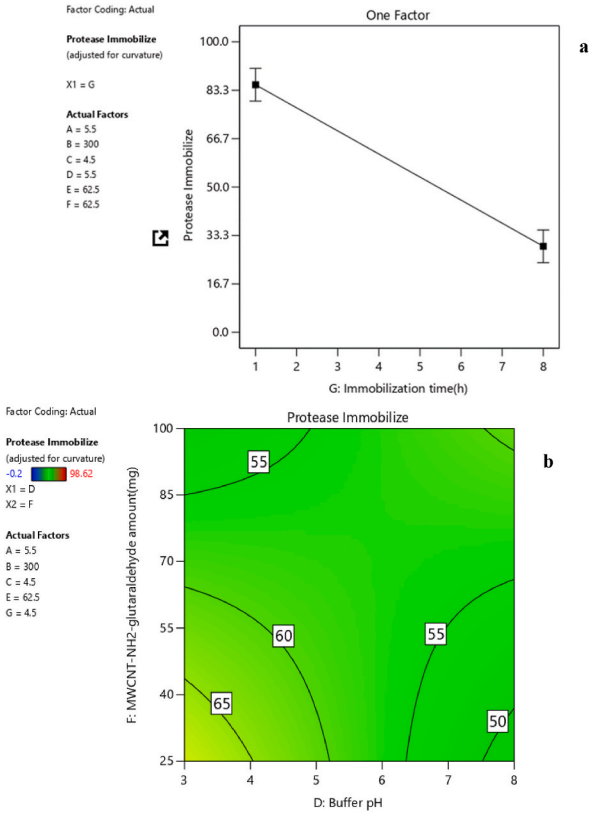

Fig. 2. The effect Immobilization time (h) (a) the mutual of Buffer pH and MWCNT-NH2-glutaraldehyde amount(mg) (b) on the efficiency of protease enzyme immobilization.

with carbon nanotubes [32], resulting in improved attachment and immobilization of *Bacillus licheniformis* protease onto the nanotube surface. The molarity of the buffer solution also contributes to the efficiency of enzyme immobilization. As the molarity of the buffer solution increases, a higher concentration of ions and buffer molecules becomes available in the environment. These can serve as adsorbents for both the protease and carbon nanotubes, leading to an enhanced immobilization of the enzyme onto the nanotubes [33]. Moreover, the interplay of buffer pH and *MWCNT-NH_2-glutaraldehyde* can also impact the efficiency of enzyme immobilization. When the pH is appropriately adjusted, the electrical charge on the surface of the nanotubes aligns with that of the protease, creating a stronger bond between the enzyme and the nanotubes. This, in turn, enhances the efficiency of enzyme immobilization [6]. By increasing the combination of activation time and the molarity of the buffer solution, alongside appropriate adjustment of the buffer pH and *MWCNT-NH_2-glutaraldehyde*, it is possible to enhance the efficiency of protease enzyme immobilization. During the activation time of *Bacillus licheniformis* protease on multi-walled carbon nanotubes, structural changes and enzyme stability reduction can be influenced by external factors such as temperature, humidity, pH, oxidation (under the influence of oxygen and oxidizing agents), as well as degradation (under the influence of proteases and peptidases). These factors play a significant role in enabling structural alterations and decreasing the stability of the enzyme. As a result, the activation time of *Bacillus licheniformis* protease on amino-functionalized multi-walled carbon nanotubes can be influenced by various factors. In general, a longer activation time typically leads to increased binding and stabilization of the protease onto carbon nanotubes [34].

Protease, derived from various species of Bacillus, stands out as a key biological enzyme. Under alkaline conditions, this enzyme effectively catalyzes the hydrolysis of proteins into peptides and amino acids. The optimization of parameters such as activation time, buffer solution molarity, and the interplay between buffer pH and MWCNT-NH2-glutaraldehyde can significantly enhance the efficiency of protease immobilization on carbon nanotubes. Activation time refers to the duration during which the enzyme interacts with the buffer solution. Longer activation times provide increased opportunities for the enzyme to interact with carbon nanotubes, thereby facilitating improved attachment and immobilization of Bacillus licheniformis protease onto the nanotube surface [10,26].

The molarity of the buffer solution is also crucial. Higher molarities result in a greater concentration of ions and buffer molecules in the environment, serving as effective adsorbents for both the protease and carbon nanotubes. Consequently, this leads to enhanced enzyme immobilization onto the nanotubes. Furthermore, the synergy between buffer pH and MWCNT-NH2-glutaraldehyde significantly impacts enzyme immobilization efficiency. Proper adjustment of pH aligns the electrical charge on the nanotube surface with that of the protease [35], fostering a stronger bond between them and thereby enhancing immobilization efficiency [36].

By optimizing activation time, buffer solution molarity, pH adjustment, and the presence of MWCNT-NH2-glutaraldehyde, it becomes feasible to boost the efficiency of protease enzyme immobilization. During the activation time of Bacillus licheniformis protease on multi-walled carbon nanotubes, external factors such as temperature, humidity, pH, oxidation, and degradation can influence structural changes and enzyme stability. Despite potential destabilizing factors, a longer activation time generally results in increased binding and stabilization of the protease onto carbon nanotubes [35,37].

Table 2
Variance analysis table of the effect of factors on the covalent immobilization of Aspergillus aureus beta-galactosidase and *Bacillus licheniformis* protease, as well as enzyme activity on multi-walled carbon nanotubes.

Factor	Protease Activity	Protease immob	Lactase Activity	Lactase Immob
Model	4158.82**	3614.26**	698.24**	12319.74**
A-Act.pH	0	0	3.02^{NS}	143.07^{NS}
B-Molarity of glutaraldehyde (mM)	0	0	576.19**	902.05**
C-Activation time(h)	851.95 **	741.74^{NS}	3220.84**	78^{NS}
D-Buffer pH	73.46 **	264.93^{NS}	50.23^{NS}	825.81**
E-Molarity of the buffer solution(mM)	0	308.17^{NS}	957.32**	0.15^{NS}
F-MWCNT-NH2-glutaraldehyde amount(mg)	1418.17 **	47.86^{NS}	5.07^{NS}	582.17**
G-Immobilization time(h)	16013**	21509.54**	1679.09**	1376.27**
AC	0	0	0	1799.70**
AD	0	0	0	2029.24**
AE	0	0	0	449.57**
AF	0	0	1196.60**	0
AG	0	0	0	283.31^{NS}
BE	0	0	0	942.54**
BG	0	0	126.16^{NS}	0
CE	0	1952.21**	0	0
CF	0	0	1324.25**	0
DE	0	0	122.03^{NS}	0
DF	0	1712.58**	0	402.13**
DG	3552.03 **	0	0	0
FG	0	0	140.89^{NS}	0
Curvature	6561.92	2604.43	1171.91	7856.77
Residual	176.86	208.42	35.66	68.61
Lack of Fit	176.86^{NS}	208.42^{NS}	35.66^{NS}	68.61^{NS}
R^2	0.8077	0/82	0/92	0.89
Adj-R^2	0.7733	0/77	0/87	0.83
CV%	23.22	26/78	9.05	17.93

NS: non-significant and **significant at 99.99 % probability level.

3.3. Activity of beta-galactosidase enzyme from Aspergillus oryzae

Similar to the study on the protease enzyme,Table 2 displays the impact of seven continuous variables—activation pH, glutaraldehyde molarity, activation time, buffer solution pH, buffer solution molarity, *MWCNT-NH$_2$-glutaraldehyde* quantity, and immobilization time—on both the efficiency of immobilization and the enzymatic activity of the *Aspergillus oryzae* beta-galactosidase enzyme.

According to the obtained results, glutaraldehyde molarity (Fig. 3b), activation time, and the interactive effect between activation time and the quantity of *MWCNT-NH$_2$-glutaraldehyde* (Fig. 3a) are the only factors among the seven considered in this study (mutual effect of activation tim (h) and the amount of MWCNT-NH3-glutaraldehyde (Fig. 3a), mutual effect of Molarity of glutaraldehyde (mM) and Immobilization time(h) (Fig. 3b), mutual effect of MWCNT-NH3-glutaraldehyde and Immobilization time(h) (Fig. 3c) and mutual effect of Buffer pH and Molarity of glutaraldehyde (mM) (Fig. 3d) that can significantly and positively affect the enzymatic activity of the *Aspergillus oryzae* beta-galactosidase enzyme ($p < 0.05$) (Fig. 3).

The β-galactosidase enzyme serves as a vital catalyst in various biological and industrial processes. It plays a vital role in the breakdown and metabolism of lactose, the sugar present in milk and other dairy products. Moreover, beta-galactosidase has wide-ranging applications in industries such as food and beverages, pharmaceuticals, and biotechnology [38].

Additionally, beta-galactosidase is extensively employed in the food industry for lactose hydrolysis, which is crucial for producing lactose-free dairy products suitable for lactose-intolerant individuals. Furthermore, this enzyme finds utility in the pharmaceutical sector for the formulation of medications, particularly those targeting lactose intolerance [39]. In biotechnology, beta-galactosidase is utilized as a reporter gene in genetic engineering techniques, aiding in the detection and quantification of gene expression. Its versatility and efficiency make beta-galactosidase a fundamental tool in various scientific and industrial applications [40].

These findings indicate that utilizing amino-functionalized multi-walled carbon nanotubes as a base for the *Aspergillus oryzae* beta-galactosidase enzyme, along with increased glutaraldehyde molarity and the quantity of *MWCNT-NH$_2$-glutaraldehyde*, along with

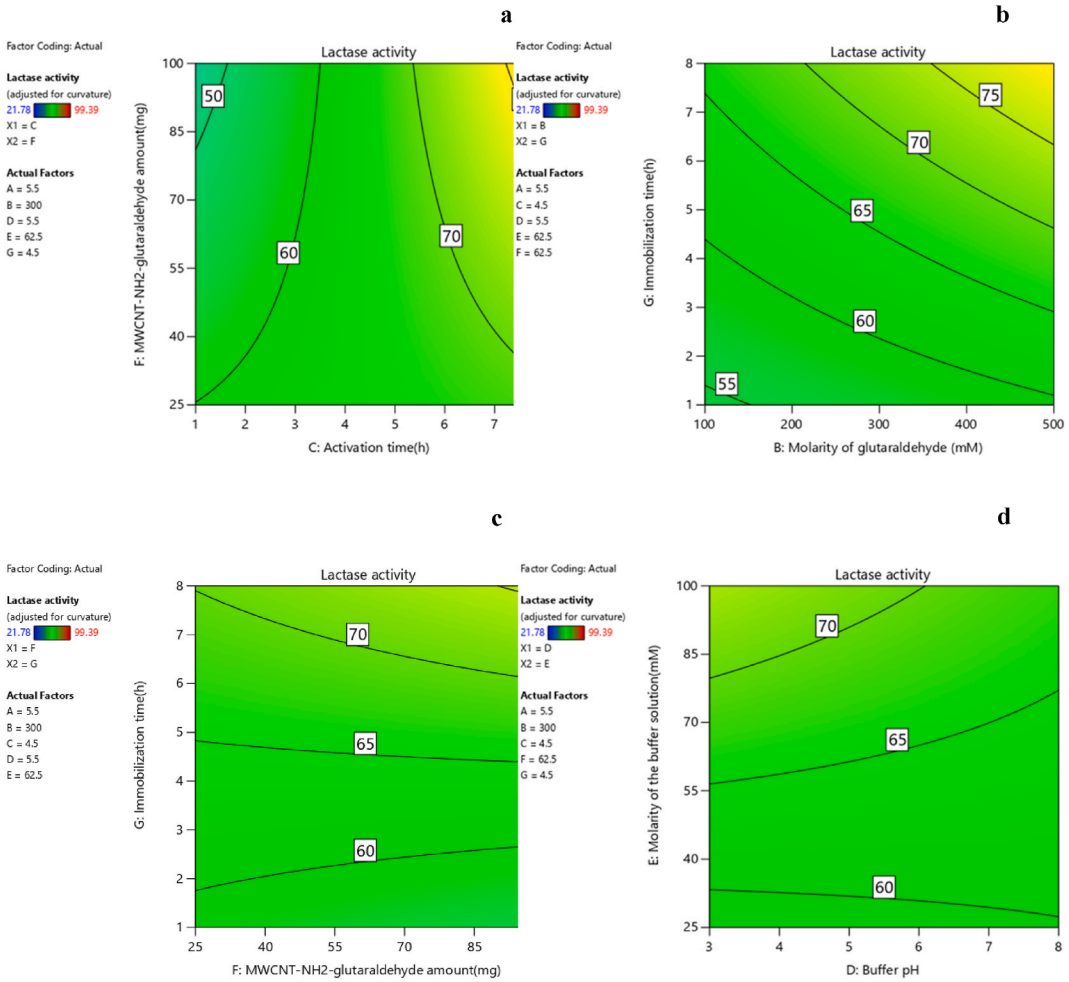

Fig. 3. The mutual effect of activation tim (h) and the amount of MWCNT-NH3-glutaraldehyde (a), mutual effect of Molarity of glutaraldehyde (mM) and Immobilization time(h) (b), mutual effect of MWCNT-NH3-glutaraldehyde and Immobilization time(h) (c) and mutual effect of Buffer pH and Molarity of glutaraldehyde (mM) (d) on the activity of Aspergillus oryzae beta-galactosidase enzyme.

extended activation time, can enhance and augment the enzymatic activity of β. Activation time can influence the enzymatic activity of *Aspergillus oryzae beta-galactosidase*. So that the enzyme activation time can lead to an increase in enzymatic activity. The findings revealed that the activity of *Aspergillus oryzae beta-galactosidase* increased by over tenfold at a temperature of 50 °C and pH 6.5 following a 24-h activation period, compared to its initial activity [41]. Furthermore, other studies have demonstrated that enzyme activation time can significantly enhance the activity of Aspergillus oryzae beta-galactosidase. Hence, enzyme activation time can be employed to improve the activity of *Aspergillus oryzae* beta-galactosidase [42].

MWCNT-NH2-glutaraldehyde is a carbon nanotube modified with an amino group (NH3) and glutaraldehyde. The amino group is attached to the carbon nanotube, and glutaraldehyde is linked to the amino group. These structural modifications can lead to significant enhancements in the properties of carbon nanotubes, including improved stability, increased resistance to oxidation, and enhanced capability for bonding with other chemical substances. The *MWCNT-NH$_2$-glutaraldehyde* composite can play a significant role in enhancing the enzymatic activity of *beta-galactosidase* from *Aspergillus oryzae*. This carbon nanotube can act as a scaffold for immobilizing enzymes onto its surface. This bonding can significantly enhance both the stability and enzymatic activity [6]. Moreover, *MWCNT-NH$_2$-glutaraldehyde* can serve as an activator for activating beta-galactosidase enzymes from Aspergillus oryzae. This activator can enhance enzymatic activity, thereby leading to a significant improvement in the enzyme's performance [43]. In a related study,

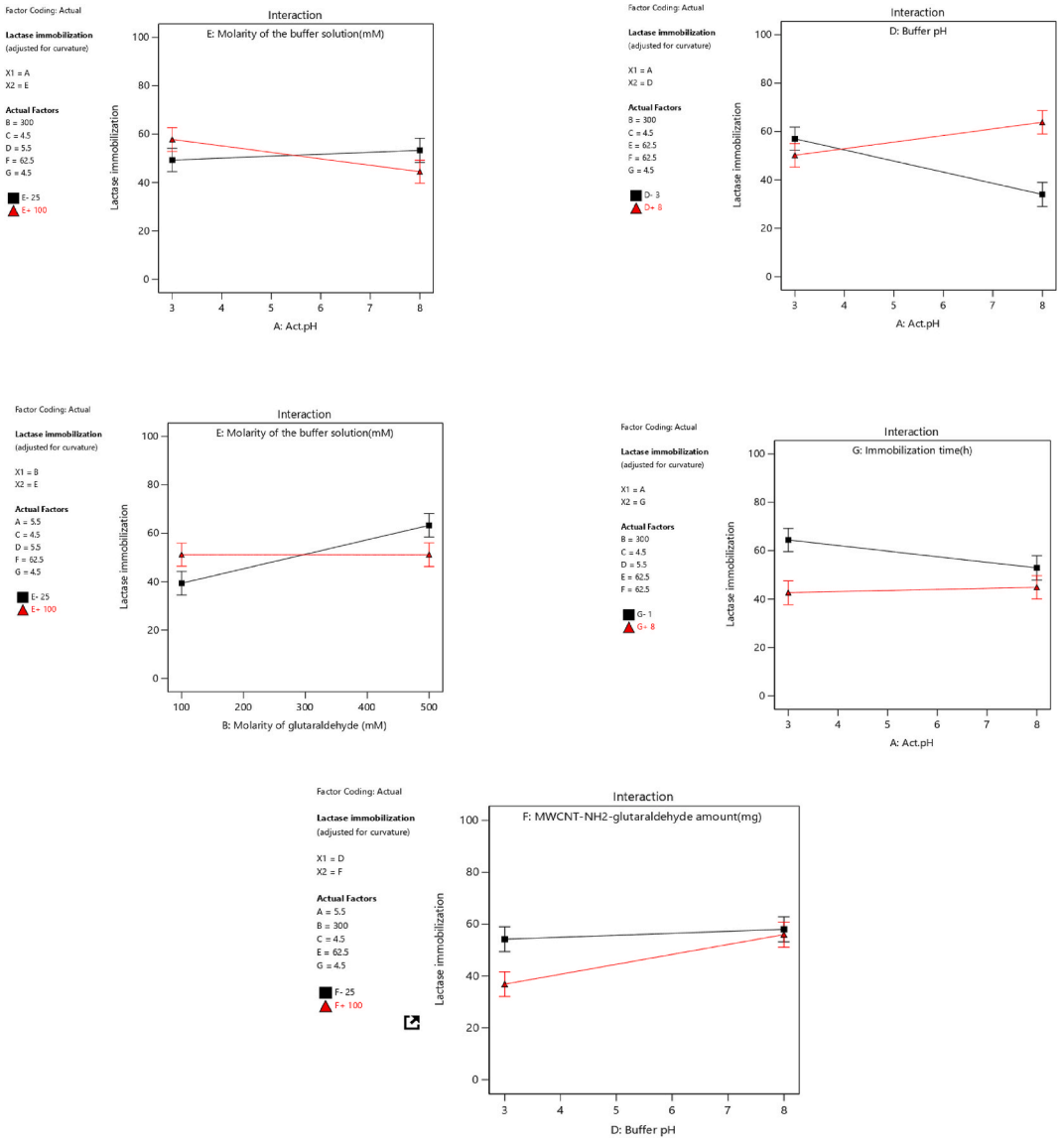

Fig. 4. The mutual effect of pH activation and Molarity of the buffer solution(mM) (a), mutual effect of pH activation and pH Buffer (b), mutual effect of Molarity of the buffer solution(mM) Molarity of glutaraldehyde (mM) (c), mutual effect of pH activation and Immobilization time (h) (d) and), mutual effect of pH Buffer and MWCNT-NH2-glutaraldehyde amount(mg/mg Enzyme) (e) on the efficiency of covalent immobilization of Aspergillus oryzae beta-galactosidase onto multi-walled amino-functionalized carbon nanotubes.

Settar and Jafari (2018) highlighted the significance of glutaraldehyde's role as an activator/stabilizer matrix in enhancing the operational and thermal stability of *β-galactosidase* (βG) for biotechnological applications. *Glutaraldehyde* has been extensively employed as a cross-linking agent and for matrix functionalization purposes, aiming to immobilize *β-galactosidase.* Under diverse operational conditions, Glutaraldehyde-treated immobilized *β-galactosidase* systems (IβGS) have found application in hydrolyzing whey and lactose in milk across batch reactors, packed-bed continuous reactors, and fluidized-bed reactors.

Moreover, molarity refers to the amount of glutaraldehyde present in 1 mol of the substance. Regarding the positive impact of glutaraldehyde on enhancing the enzymatic activity of beta-galactosidase from *Aspergillus oryzae*, it should be noted that glutaraldehyde is employed as an activator of the *beta-galactosidase* enzyme from *Aspergillus oryzae* [44]. Studies have indicated that increasing the molarity of glutaraldehyde can lead to an enhancement in the enzymatic activity of beta-galactosidase from *Aspergillus oryzae*. Another study demonstrated that increasing the molarity of glutaraldehyde to the molar level resulted in heightened enzymatic activity of beta-galactosidase from *Aspergillus oryzae*. Hence, the molarity of glutaraldehyde can be regarded as a significant factor in enhancing the enzymatic activity of *beta-galactosidase* from *Aspergillus oryzae* [6].

3.4. The covalent immobilization efficiency of Aspergillus oryzae beta-galactosidase onto multi-walled carbon nanotubes functionalized with amino groups

The variance analysis results revealed that the efficiency of Aspergillus oryzae beta-galactosidase immobilization on amino-functionalized multi-walled carbon nanotubes was impacted by several factors (pH activation and Molarity of the buffer solution (mM) (Fig. 4a), mutual effect of pH activation and pH Buffer (Fig. 4b), mutual effect of Molarity of the buffer solution(mM) Molarity of glutaraldehyde (mM) (Fig. 4c), mutual effect of pH activation and Immobilization time (h) (Fig. 4d), mutual effect of pH Buffer and MWCNT-NH2-glutaraldehyde amount(mg/mg Enzyme) (Fig. 4e)). These included the molarity of glutaraldehyde, buffer pH, *MWCNT-NH2-glutaraldehyde* incubation time, and interactive effects such as activation pH + activation time, buffer pH + activation pH, activation pH + buffer molarity, and glutaraldehyde molarity + *MWCNT-NH2-glutaraldehyde* ($p < 0.05$) (Fig. 4). The results indicate that pH plays a pivotal role in determining the level of immobilization efficiency. The impact of activation pH and buffer pH on the efficiency of covalent immobilization of beta-galactosidase from *Aspergillus oryzae* onto amino-functionalized multi-walled carbon nanotubes can be significant. Studies have indicated that buffer pH can significantly influence the efficiency of beta-galactosidase from *Aspergillus oryzae* immobilization on surfaces [45]. Specifically, the buffer pH can directly impact the surface electrostatic charge of amino-functionalized multi-walled carbon nanotubes, potentially leading to a significant enhancement in the efficiency of covalent immobilization of *beta-galacto*sidase from *Aspergillus oryzae*. Moreover, the activation pH can also have a direct impact on the efficiency of covalent immobilization of beta-galactosidase from *Aspergillus oryzae* onto amino-functionalized multi-walled carbon nanotubes. In line with the obtained results, a study has shown that the activation pH for covalent immobilization of beta-galactosidase from *Aspergillus oryzae* onto amino-functionalized surfaces should ideally range between 6 and 8 to achieve a significant enhancement in the efficiency of immobilization [46]. According to a study [38], beta-galactosidase from *Aspergillus oryzae* was immobilized onto an amino-functionalized support using a 5 mM buffer at pH levels of 5, 7, and 9. The immobilization process was completed within 30 min, retaining 75 to 80 percent of the enzymatic activity. The stability of the enzyme immobilized at pH 9 was significantly lower compared to the enzyme immobilized at pH 5 under all studied conditions; however, the differences reduced with an increase in the ionic strength of the inactive solution. The most likely explanation for these different pH-dependent stabilities could be attributed to variations in the enzyme's orientation upon the support surface. The immobilized enzyme at pH 5 exhibited higher stability compared to the free enzyme at both pH 5 and 9 (approximately 2- to 6-fold, respectively), whereas at pH 7, the free enzyme was notably more stable than the immobilized enzyme. The *MWCNT-NH2-glutaraldehyde* plays a crucial role in enhancing the efficiency of covalent immobilization of beta-galactosidase from *Aspergillus oryzae* onto multi-walled amino-functionalized carbon nanotubes. Specifically, *MWCNT-NH2-glutaraldehyde* can be utilized as a foundation for attaching enzymes to its surface. This attachment can significantly enhance the stability and enzymatic activity. This attachment can significantly enhance the stability and enzymatic activity. Moreover, *MWCNT-NH2-glutaraldehyde* can act as an activator of beta-galactosidase enzymes from Aspergillus oryzae. This activator can enhance enzymatic activity, consequently leading to a considerable improvement in the efficiency of covalent immobilization of beta-galactosidase from *Aspergillus oryzae* onto multi-walled amino-functionalized carbon nanotubes [38].

This activator can enhance enzymatic activity, consequently leading to a considerable improvement in the efficiency of covalent immobilization of beta-galactosidase from Aspergillus oryzae onto multi-walled amino-functionalized carbon nanotubes. The presence of the activator is likely to facilitate the binding of the enzyme to the carbon nanotubes, promoting a more stable and effective immobilization process [47]. This enhanced immobilization efficiency can be attributed to the activator's ability to modify the surface properties of the carbon nanotubes, creating favorable conditions for the attachment and retention of the enzyme [34]. As a result, the activated carbon nanotubes provide a conducive environment for the immobilized enzyme, potentially leading to improved catalytic performance and reusability. This synergistic effect between the activator and the carbon nanotubes demonstrates the potential for enhancing the practical applications of immobilized enzymes, particularly in biocatalysis and biotechnological processes [6].

In a similar study, Garrido, Ruiz-Moyano [48] reported the utilization of this activator for producing galactooligosaccharides in the food, dairy, and fermentation industries. Glutaraldehyde has shown notable stability in immobilizing βG against various physical and chemical denaturants, consequently enhancing its thermal and operational stability. This characteristic makes it more suitable for repetitive use in industrial-scale operations [43].

The molarity of glutaraldehyde and the activation time, as mentioned in the enzyme activation section, can significantly impact the enhancement of covalent immobilization efficiency of beta-galactosidase from *Aspergillus oryzae* on multi-walled amino-functionalized carbon nanotubes. Studies have indicated that increasing the glutaraldehyde molarity can lead to an improvement in the efficiency of

Table 3
The best treatment selected from among the available formulas.

	Number	Act. pH	Molarity of glutaraldehyde (mM)	Activation time(h)	Buffer pH	Molarity of the buffer solution (mM)	MWCNT-NH2-glutaraldehyde amount(mg)	Immobilization time(h)	Protease Activity	Protease Immobilize	Lactase activity	Lactase immobilization	Desirability
Optimum	1	**8.0**	**500.00**	**1.04**	**3.5**	**25.00**	**25.03**	**4.60**	**64.0**	**72.62**	**65.9**	**71.77**	**0.48**

enzyme immobilization on amino-functionalized surfaces [49]. Furthermore, the activation time directly affects the efficiency of covalent immobilization of beta-galactosidase from *Aspergillus oryzae* on multi-walled amino-functionalized carbon nanotubes. For instance, a study has demonstrated that the activation time significantly improves the efficiency of immobilization when prolonged [50]. In discussing the outcomes related to each significant factor's individual effects, it's apparent that this factor has the potential to enhance the influence of other crucial factors, such as the combined effect of pH activation + activation time, buffer pH + activation pH, activation pH + buffer molarity, and *glutaraldehyde molarity* + *MWCNT-NH2-glutaraldehyde*.

3.4.1. Optimization assessment and selection of optimal formulas

In order to optimize products, the response surface methodology (RSM) was utilized as it helps reduce the number of required experiments, resulting in time and cost savings. In this method, each variable is encoded within a range of −1 to +1. Essentially, the aim is to simplify the regression analysis. Following the model determination, optimization was conducted to achieve the optimal formulation in terms of the enzymatic activity of beta-galactosidase from *Aspergillus oryzae*, the protease from *Bacillus*, and the efficiency of the immobilization process for both enzymes (Table 3). Firstly, the low, high, and desired levels for each response were established. Subsequently, using the described models and the specified levels, the software introduced an appropriate combination of variables (denoted as X1, X2, and so forth). In the software, several desired points (formulations) were aimed for, with the maximum achievable Brix. There was a slight difference in these characteristics compared to the control sample. From these points, 35 formulations were selected for the product, and these formulations were subsequently prepared and tested for attributes such as enzymatic activity and the efficiency of the enzyme immobilization process. Based on the obtained and predicted responses, it's evident that the established models can be used for the indices.

3.4.2. Effect of enzyme immobilization time on enzyme activity

Fig. 5 (The effect of enzyme stabilization time on the amount of enzyme activity (Fig. 5a), effect of pH (Fig. 5b) and temperature on the efficiency of enzymatic stabilization (Fig. 5c) Lactose hydrolysis during enzyme immobilization) illustrates the changes in enzyme

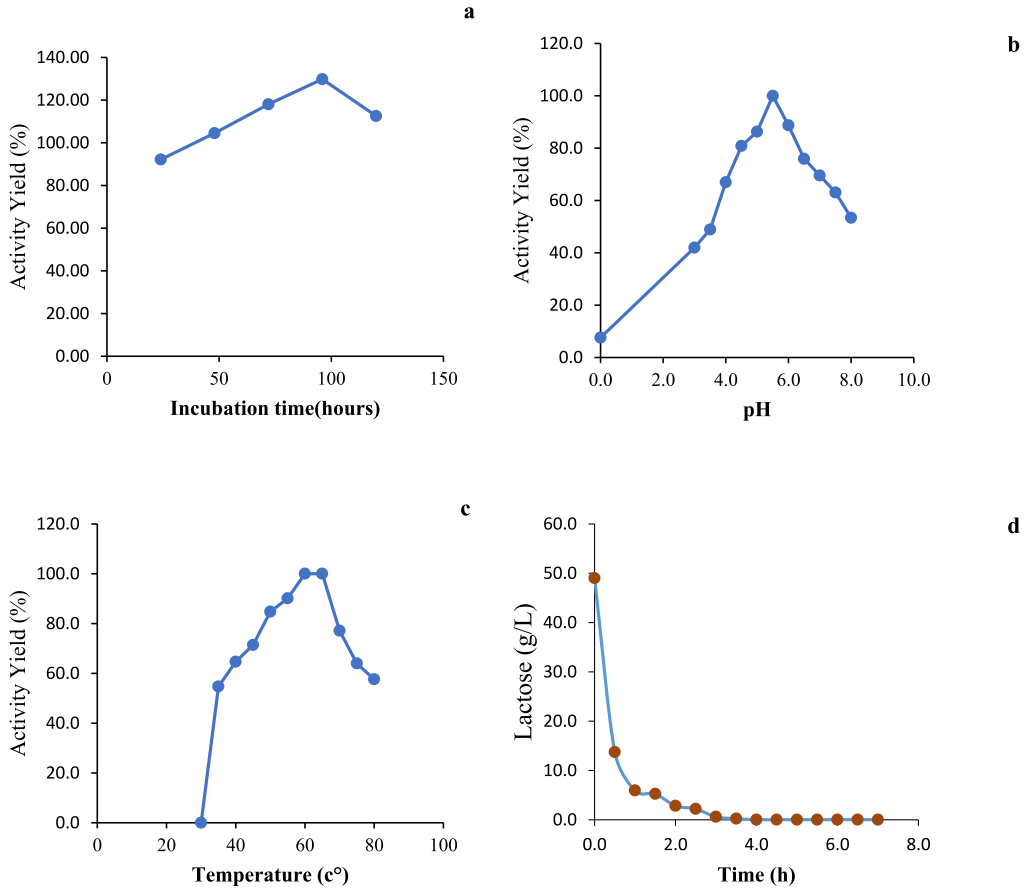

Fig. 5. The effect of enzyme stabilization time on the amount of enzyme activity (a), effect of pH (b) and temperature on the efficiency of enzymatic stabilization (c) Lactose hydrolysis during enzyme immobilization.

activity over the time of enzyme immobilization in percentage. The enzyme activity percentage has been measured during specific time intervals of the process (24, 48, 72, 96, and 120 h). The enzyme activity was 92.16 % during the initial hours, and it increased after 96 h. However, after 96 h, this trend shifts to a decreasing pattern. During the 96-h period, the enzyme activity was at 129.89 %, dropping to 112.56 % after 120 h. In interpreting these results, reference can be made to the findings of Klin and colleagues (2013), who investigated the immobilization of β-*d*-galactosidase from Kluyveromyces lactis on glutaraldehyde-activated chitosan fixed in a packed-bed reactor. They reported a significant enhancement in the enzyme's thermal stability in the presence of lactose. The full lactose hydrolysis for whey milk and lactose solution was achieved at a temperature of 37 °C, with a flow rate of up to 2.6 mL per minute. The maximum GOS concentration of 26 g per liter was obtained at a flow rate of 3.1 mL per minute, with an efficiency of 186 g per liter per hour at 1. For 15 days, the steady-state operation showcased the stability of the reactor in terms of lactose hydrolysis. This suggests that the decrease in lactose over time during storage has reduced the enzyme activity.

The maximum GOS concentration of 26 g per liter was obtained at a flow rate of 3.1 mL per minute, with an efficiency of 186 g per liter per hour at pH 1. Over the course of 15 days, the steady-state operation demonstrated the reactor's stability in terms of lactose hydrolysis. This indicates that the gradual decrease in lactose levels over time, possibly due to storage conditions, has led to a reduction in enzyme activity.The optimized conditions of flow rate and pH value resulted in the highest GOS concentration achieved, highlighting the importance of fine-tuning operational parameters for maximizing product yield in enzymatic processes. The efficiency of 186 g per liter per hour underscores the effectiveness of the enzyme in catalyzing the conversion of lactose to GOS at the specified conditions. The sustained stability of the reactor over the 15-day period signifies its reliability in maintaining consistent performance in lactose hydrolysis. However, the observed decline in enzyme activity as lactose levels decrease emphasizes the necessity of monitoring substrate concentrations and implementing proper storage practices to preserve enzyme functionality over extended periods [51]. Overall, these findings underscore the significance of operational optimization and substrate management in ensuring the long-term efficacy and stability of enzymatic reactions in industrial applications.

3.4.3. The effect of pH on the enzyme immobilization efficiency

The enzyme immobilization efficiency across different pH levels is illustrated in Fig. 5 as percentages. The enzyme immobilization efficiency increases with the pH rising from 0 to 5.5, resulting in an additional 6.7 % immobilization (100 %–6.7 %). However, beyond a pH of 5.5, as the environment becomes more alkaline, this percentage reduces to 4.53 %. In situations where conditions such as extreme pH, the presence of heavy metal ions, and other undesirable substances exist, the sturdy structure of neutral proteases is compromised, resulting in a decrease or even inactivation of enzyme activity [52]. Khalid and Arif [38] reported in a study that the stability of immobilized beta-galactosidase from *Aspergillus oryzae* at pH 9 was notably lower compared to the enzyme immobilized at pH 5 across all examined conditions. However, differences diminished as the ionic strength of the inactive solution increased.

3.4.4. The effect of temperature on the enzyme immobilization efficiency

The enzyme's immobilization efficiency has shown variations at different temperatures (Fig. 5). The enzyme's immobilization efficiency varied with temperature changes. Between 30 and 60 °C, it showed an upward trend (with a range of changes from 7.54 % to 100 %), whereas from 60 to 80 °C, it displayed a declining trend (with a range of changes from 100 % to 52 %). At higher temperatures, thermal energy is absorbed by the enzyme, altering its three-dimensional structure, potentially resulting in a reduction or loss of enzymatic activity. Additionally, the presence of heavy metal ions can interact with the enzyme, altering its structure and potentially reducing or deactivating its activity. These ions can bind to the active groups of the enzyme, causing alterations in its three-dimensional structure. In conditions such as high temperatures, the robust structure of neutral proteases is compromised, leading to a decrease or even inactivation of enzyme activity [4].

At higher temperatures, thermal energy is absorbed by the enzyme, altering its three-dimensional structure, potentially resulting in a reduction or loss of enzymatic activity. Additionally, the presence of heavy metal ions can interact with the enzyme, altering its structure and potentially reducing or deactivating its activity. These ions can bind to the active groups of the enzyme, causing alterations in its three-dimensional structure [53]. In conditions such as high temperatures, the robust structure of neutral proteases is compromised, leading to a decrease or even inactivation of enzyme activity.

Moreover, the denaturation of enzymes at high temperatures can be explained by the disruption of non-covalent bonds within the enzyme molecule. The increased thermal energy causes the enzyme to vibrate more vigorously, leading to the breaking of hydrogen bonds, ionic bonds, and hydrophobic interactions that help maintain the enzyme's specific shape [54]. As a result, the enzyme loses its native conformation and can no longer effectively bind to its substrate, thereby reducing its catalytic activity [55]. In summary, both high temperatures and the presence of heavy metal ions can disrupt the three-dimensional structure of enzymes, leading to a loss of enzymatic activity. This underscores the importance of maintaining optimal conditions for enzyme function in various biological and industrial processes.

In a similar study, cross-linked papain enzyme grains were formulated on commercial porous silica gel (CLEAs-MSG) to improve the performance and mechanical stability of CLEAs. The CLEAs-MSG demonstrated a stable structure due to the covalent bond between CLEAs and MSG, preventing enzyme leakage from larger pores. The optimal temperature for Papain CLEAs in MSG was 40–90 °C, with an optimal pH of 7.0, showing improvement compared to free Papain and CLEAs [56].

3.4.5. Lactose hydrolysis

The results indicate that an increase in enzyme immobilization time leads to a decrease in lactose levels (g/L) in the medium (Fig. 5). During the enzyme immobilization period and under the given conditions, lactose gradually hydrolyzes, decreasing from an initial concentration of 49 g per liter to 0 within 4 h post-enzyme immobilization. This decrease indicates the heightened activity of the

enzyme beta-galactosidase, which catalyzes lactose hydrolysis [26]. In a study conducted Klein, Fallavena [57], *β-D-galactosidase* from *Kluyveromyces* lactis was immobilized on glutaraldehyde-activated chitosan and utilized in a packed-bed reactor for continuous lactose hydrolysis and galactooligosaccharides (GOS) synthesis. It has also been reported that the enzyme's thermal stability significantly increases in the presence of lactose. Degrees. The maximum GOS concentration of 26 g per liter was achieved at a flow rate of 3.1 mL per minute with an efficiency of 186 g per liter per hour at pH 1. steady-state operation for 15 days evidenced the reactor's stability in lactose hydrolysis. This indicates the effect of storage over time, where the decrease in lactose in the environment has reduced the level of enzymatic activity.

It has also been reported that the enzyme's thermal stability significantly increases in the presence of lactose. The maximum GOS concentration of 26 g per liter was achieved at a flow rate of 3.1 mL per minute with an efficiency of 186 g per liter per hour at pH 1. Steady-state operation for 15 days evidenced the reactor's stability in lactose hydrolysis. This indicates the effect of storage over time, where the decrease in lactose in the environment has reduced the level of enzymatic activity.

The presence of lactose can have a stabilizing effect on the enzyme's thermal stability, potentially protecting the enzyme from denaturation at high temperatures. This could be attributed to specific interactions between lactose molecules and the enzyme, which help maintain the enzyme's structure and function under thermal stress. The achievement of a high GOS concentration at a specific flow rate and pH value demonstrates the importance of optimizing operating conditions for maximizing enzymatic efficiency in industrial processes. The stability of the reactor over a 15-day period further supports the effectiveness of the enzyme in catalyzing lactose hydrolysis under controlled conditions [58].

The observed decrease in enzymatic activity over time due to the reduction in lactose levels highlights the importance of monitoring and maintaining substrate concentrations to sustain enzyme activity in long-term operations. This emphasizes the need for proper storage and handling practices to preserve the efficacy of enzymes in various applications [59].

3.4.6. Comparison of enzyme activity and stability between the optimum and 30-day samples

What is observable in Fig. 6 is the enzymatic activity level (Fig. 6a) and the enzyme stabilization efficiency (Fig. 6b). In the case of the optimum sample, these were 82.92 % and 40.56 %, respectively, whereas for the selected sample, they were obtained as 54.97 % and 49.48 %, respectively.

The impact of the number of milk passes through the tube (a) and the duration of storage (days) (b) on the level of stabilized enzyme activity in milk.

Throughout each reuse of the tube for milk passage in both the optimized sample and the sample under specific conditions (Sample 30), there was a decline in the preserved enzyme activity. The range of variation for Sample 30 reduced from 100 to 34.81, while for the optimized sample, it reached 43.91 over 15 iterations (Fig. 7a). Furthermore, the enzyme activity levels observed in both the optimized sample and Sample 30 exhibited a decline over the storage periods, as depicted in Fig. 7b, reducing from 100 to 72.94 and 66.94, respectively. It is anticipated that with each use of the immobilized enzyme bed, a gradual release of the enzyme into the solution will occur, mirroring the stepwise introduction of the enzyme into the milk as it traverses through the tubes. Consequently, there is a gradual decrease in enzymatic activity over time [60]. Enzymes, due to their proteinaceous composition, undergo structural modifications over time in different conditions [61], resulting in changes to the structural properties of these biocatalysts [62]. The alterations observed in both samples indicate a reduced retained enzyme activity during the 28-day storage period. In a similar study, Wang et al. (2010) reported that the cross-linking of papain in commercial porous silica gel increased both storage stability and thermal stability. Moreover, due to its optimal size and active properties, *CLEAs-MSG* demonstrated excellent reusability [56].

4. Conclusion

The results of the analysis of variance for the protease enzyme activity demonstrate a notable influence of the seven specified variables on both the efficacy of enzyme stabilization and its activity. Furthermore, the findings demonstrate that activation time, buffer pH, the quantity of *MWCNT-NH2-glutaraldehyde*, and the immobilization duration have a substantial impact on the protease enzyme activity. Also, the interaction effect between buffer pH and immobilization time is significant. In particular, the activation time and the quantity of *MWCNT-NH2-glutaraldehyde* have a decreasing effect on enzyme activity, with the latter showing a more significant impact ($p < 0.05$). In the context of beta-galactosidase enzyme activity, the findings demonstrate that among the seven variables considered in this study, only the molarity of glutaraldehyde, activation time, and the interactive effect between activation time and the quantity of *MWCNT-NH2-glutaraldehyde* exhibit a significant and positive impact on the enzyme activity of *Aspergillus oryzae* beta-galactosidase ($p < 0.05$). The combination of activation time and buffer solution molarity, along with the interactive influence of buffer pH *and MWCNT-NH2-glutaraldehyde*, may lead to a substantial improvement in the protease stabilization efficiency on carbon nanotubes. Additionally, the interactive effects of activation pH + activation time, buffer pH + activation pH, activation pH + buffer molarity, and glutaraldehyde molarity were found to be significant. The optimization experiments and selection of optimal formulas revealed that the activity and stabilization efficiency of the protease and beta-galactosidase enzymes, respectively, can be achieved at levels of 63.72 ± 0.64 % and 77.71 ± 0.65 %. With an increase in enzyme immobilization time, there was a reduction in enzymatic activity. Additionally, an increase in pH, temperature, and the storage duration of milk in recovered carbon nanotubes with the enzyme led to sdecreased enzyme activity and lactose hydrolysis. Therefore, it is expected that the covalent immobilization of *Aspergillus oryzae* beta-galactosidase and *Bacillus licheniformis* protease onto multi-walled carbon nanotubes is feasible and can be applied to milk.

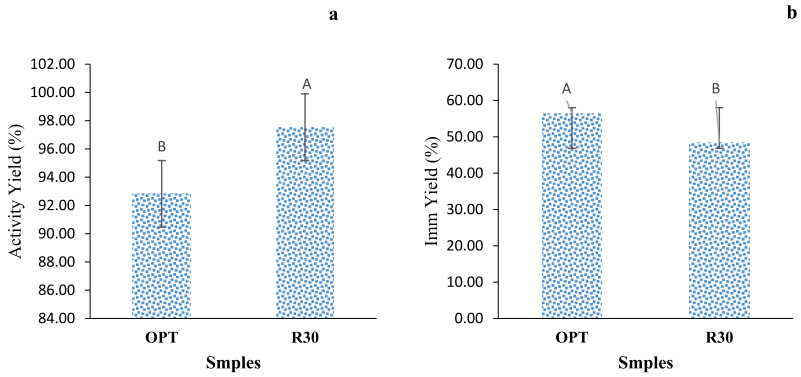

Fig. 6. Examining the amount of enzyme activity (a) and stabilization (b) between the selected samples and 30 thighs.

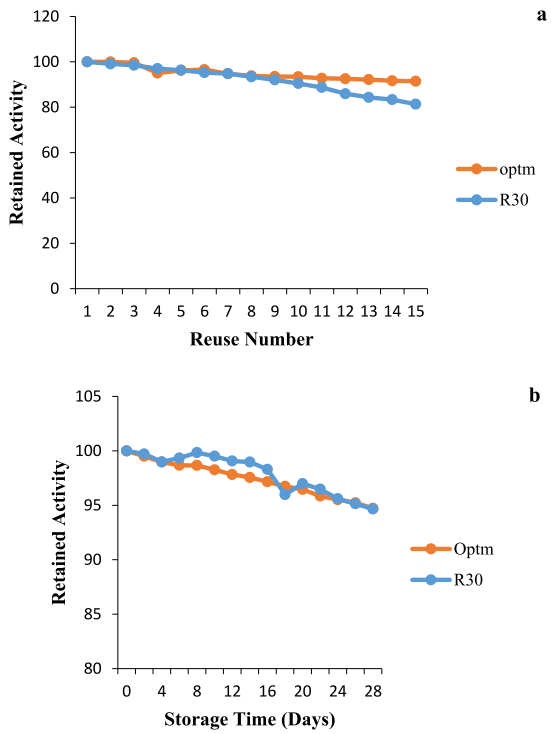

Fig. 7. The effect of Reuse Number (a) and Storage Time (Days) (b) Retained Activity Anzym in milk.

CRediT authorship contribution statement

Alan Yaseen Taher: Writing – original draft, Investigation, Conceptualization. **Mohammad Alizadeh:** Writing – review & editing, Validation, Software, Project administration, Data curation. **Yakup Aslan:** Writing – review & editing, Validation, Project administration, Data curation.

Declaration of competing interest

The authors declare that they do not have any special conflict of interests.

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