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Covalent immobilization of Aspergillus oryzae β-galactosidase and Bacillus licheniformis protease with Amino-Multi Walled Carbon Nanotubes

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ABSTRACT

This study was carried out with the aim of covalent immobilization of Aspergillus oryzae beta-galactosidase and Bacillus licheniformis protease on multi-walled amino-carbon nanotubes. In this method, fractional 2k design was used to study the effect of seven continuous factors (activation pH, glutaraldehyde molarity, activation time, buffer solution pH, buffer solution molarity, MWCNT-NH3-glutaraldehyde amount and stabilization time) on the stabilization efficiency and enzyme activity. . Design-expert software was used to analyze data and draw graphs. The results showed that the aforementioned factors predict the level of enzyme activity of Bacillus licheniformis protease and Aspergillus oryzae beta-galactosidase with correlation coefficients of 0.80 and 0.92 at the rate of 77 and 88%, respectively. Also, the correlation coefficient of the covalent fixation efficiency model of Aspergillus oryzae beta-galactosidase and Bacillus licheniformis protease on multi-walled carbon nanotubes was 0.89 and 0.82, respectively, and the studied factors were able to determine the covalent fixation beta efficiency, respectively. Aspergillus oryzae galactosidase and Bacillus licheniformis protease on multi-walled amino-carbon nanotubes predict 83 and 77%, respectively.

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1- Introduction

Galactoligosaccharides are a mixture of lactose-derived saccharides consisting of two to eight saccharide units and one glucose residue. Galacto-oligosaccharides are present in the milk of almost all mammals and have a prebiotic effect that affects the composition of the digestive microflora, which is beneficial for the health and well-being of the host [1]. Currently, galactooligosaccharides are mainly produced from lactose through a trans-galactosylation reaction by the enzyme β-galactosidase, this mechanism involves the release of glucose and the formation of an enzymatic galactosyl complex (EGal) due to the cleavage of the β-1,4 glycosidic bond of lactose by a nucleophilic amino acid [2]. The EGal complex can subsequently convert galactose to a fructose molecule to form lactulose or to other lactose molecules to form galactoligosaccharides, both reactions being competitive with each other [3]. Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a biologically active disaccharide that is used as a medicine and as a prebiotic substance in functional foods. Lactulose is produced by chemical synthesis [4], but in recent years, the development of processes that are more compatible with the environment has received more attention; In the meantime, β-galactosidase from *Aspergillus oryzae* is an enzyme that is widely used in the dairy industry to produce lactose-free products as a medicinal supplement for people with lactose intolerance [5]; On the other hand, allergy to cow's milk protein is an immune reaction of the body against cow's milk proteins and is one of the most common allergies among babies. Allergy to cow's milk protein, which is seen as a common disease in the world, can be greatly reduced by consuming cow's milk without protein [6]. Proteases are enzymes that convert proteins into peptides and amino acids by hydrolysis. Therefore, they can be used in the production of cow's milk, which does not cause sensitivity to protein; *Bacillus licheniformis* protease is a serine peptidase that forms peptides by hydrolyzing proteins and is used in various applications such as food supplements, food and beverage processes, ingredient development and protein processing [7]. But despite the advantages such as cost-effectiveness and environmental friendliness of enzymes obtained from bacteria and fungi compared to chemical catalysts, its use is difficult due to the difficulty of separating the enzyme from the reaction mixture, complex downstream processing and the risk of product contamination [8]; Therefore, enzyme immobilization is a key technology to overcome these disadvantages. Heterogeneous phase catalysis allows for easy recovery and reuse of the biocatalyst, which in turn enables the development of continuous processes and application in different types of reactors and operating modes with better reaction control. In addition, it increases the operational stability, it may increase the specificity of the enzyme and resistance to inhibitors, and the stabilization of the enzyme makes its structure more rigid and insoluble [9]. Various methods can be used for enzyme immobilization, but the industry always uses simple and cost-effective methods. The most used methods are based on physical (adsorption or physical trapping) and chemical (covalent bonding and cross-linking) immobilization [10]; Covalent binding is a more suitable method for all enzymes and for all applications because the immobilized enzyme does not lose its activity easily; Also, choosing the right matrix for enzyme immobilization is related to the nature of the matrix, the simplicity of the method, and the targeted use of the enzyme [11]. Also, different forms of nanostructures such as nanoparticles, nanofibers, nanotubes and nanocomposites have been used as new

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supports for enzyme stabilization. These compounds have a large surface area, which leads to high enzyme loading and, as a result, high enzyme volumetric activity. Among the different forms of nanomaterials, carbon nanotubes have unique structural, mechanical, thermal and biocompatibility properties [12]. The performance of nanomaterials can be improved by the process of surface functionalization. The surface functionalization of nanomaterials includes linking desired functional groups on their surface to obtain nanomaterials with desired properties. This functionalization can affect their dispersion and interaction with enzymes, thus significantly changing the catalytic activity of the immobilized enzyme[13]. Therefore, the purpose of this article is to identify the properties of the immobilized enzymes and investigate the enzyme activity and immobilization of beta-galactosidase from *Aspergillus oryzae* and protease of *Bacillus licheniformis* on amino-multi-walled carbon nanotubes.

2- Materials and methods

1-2- Materials

β-galactosidase from *Aspergillus oryzae* (powdered enzyme) and *Bacillus licheniformis* protease (soluble enzyme) were purchased from the company Troy(VA, USA) with a density of 14.1 g/cm3 and a melting point of 100°C and a molecular weight of 340 g/mol). Amino-MWCNT (purity: 99%, diameter 10-30 nm, length 10 µm) from the company brand (Karnataka, India) and bovine serum albumin, casein, sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, lactose, sodium sulfite, phenol and D-glucose were purchased from the company (Merck, Germany). 3,5-dinitrosalicylic acid (DNS) was purchased from Alfa Aesar (Kandel, Germany). Sodium potassium tartrate (Rochelle salt) was purchased from VWR Prolabo Chemicals (Leuven Belgium). Sodium azide was purchased from Merck Millipore (Darmstadt, Germany). L-Tyrosin from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) was purchased. Nitrocellulose membrane filters (pore diameter 0.45 µm, membrane diameter 47 mm) were purchased from ISO-LAB (Wertheim, Germany).

2-2- Methods

2-2-1-immobilization of enzymes

Beta-galactosidase of *Aspergillus oryzae* and protease of *Bacillus licheniformis* were covalently immobilized on (MWCNT-NH3) separately. The effect of parameters such as enzyme to matrix ratio, pH and molarity of immobilization solutions, immobilization time, etc. on the activity and efficiency of immobilization was estimated and the immobilization conditions were optimized.

In enzyme immobilization, carbon nanotubes were first functionalized and then incubated with enzyme until covalent bond formed between enzyme and created functional groups. It was done according to the method of Cakmakci et al. (2016) [14].

To determine the beta-galactosidase activity of *Aspergillus oryzae*, equation 1 was used, and the basis of the measurement method is the increase in the amount of reducing sugar due to the progress of lactose hydrolysis [15].

$$\text{Activity Yield(\%)} = \frac{\text{Activity of immoboiized enzyme}}{\text{Activity of soluble enzyme}} \times 100$$

eq.1

For immobilization yield equation 2 was used:

$$\text{Immobilization Yield(\%)} = \frac{\text{Enzyme used for Immobilization} - \text{enzyme in filtrate}}{\text{Enzyme used for Immobilization}}$$

eq.2

2-2-2-Determination of lactase activity

At first, 1% (w/v) lactose solutions, in the amount of 5 ml, were prepared using 25 mM sodium dihydrogen phosphate solution (pH 4.5 for free enzyme and 5.5 for immobilized enzyme). Then, 200 µL of free AOG (Aspergillus oryzae β-galactosidase) or 0.317 g of immobilized AOG were mixed with it, respectively, and reacted in a chamber with gentle shaking at 55 °C for 60 minutes. 200 µl of the reaction mixture samples were added to 1800 µl of distilled water and boiled for 10 minutes to inactivate the enzymes. The amount of D-glucose formed was determined by measuring its absorbance at a wavelength of 575 nm using an ultraviolet (UV) spectrophotometer, according to the method of Miller (1959). One unit of AOG activity was defined as the amount of enzyme that formed 1 µmol of D-glucose from lactose every minute under the optimal conditions of the activity test.

2-2-3-Determination of alkaline protease activity

5 ml of casein solutions (1% w/v) prepared using 25 mM sodium phosphate solution (pH 7.5) with a volume of 200 µl of free BLP (*Bacillus licheniformis* protease) or 0.286 g of immobilized BLP at 70 °C was reacted for 60 minutes. Then, 400 microliters of reaction mixture samples were added to 3600 microliters of distilled water and boiled for 10 minutes to inactivate the enzyme. The amount of L-tyrosine formed was determined by measuring its absorbance using an ultraviolet spectrophotometer (UV-6300PC, Radnor, USA) at a wavelength of 274 nm (Lewis, 1980). One unit of activity was defined as the amount of enzyme that forms 1 micromol of L-tyrosine from casein every minute under the optimal conditions of the activity test.

2-2-4- Statistical analysis

In this method, fractional 2^k design is used to study the effect of seven continuous factors according to Table 1 (activation pH, glutaraldehyde molarity, activation time, buffer solution pH, buffer solution molarity, MWCNT-NH3-glutaraldehyde amount and stabilization time) on immobilization efficiency and enzyme activity. The first type error level in this study was considered equal to 0.05. Design-expert software version 13 was used to analyze data and draw graphs.

Table 1. Statistical plan for Immobilization and activity of enzymes

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7
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

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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

3-Results and discussions

In this study, the results of variance analysis of the effect of seven continuous factors, activation pH, glutaraldehyde molarity, activation time, buffer solution pH, buffer solution molarity, amount of MWCNT-NH3-glutaraldehyde and immobilization time on immobilization efficiency and enzyme activity are shown in Table 2. The effect of these seven factors on both immobilization efficiency and enzyme activity was significant ($P<0.05$) and the lack of fit of the model also was non-significant and this shows the ability of the model fit to respond to changes.

The effect of factors on the enzymatic activity of *Bacillus licheniformis* protease on multi-walled amino carbon nanotubes

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with R2=0.80 and Adjusted R^2=0.77 was obtained as equation no 3.

Protease Activity =52.30-5.59C+1.57D-7.13F+23.23G+11.15DG
eq.3

The R2 index is high and indicates the appropriateness of the obtained model, and this model can predict 77% of the changes.

in conditions where the pH significantly deviates from the neutral range, the amino and carboxylic groups of the amino acids of the enzyme convert to anionic form. These ionic changes can change the three-dimensional structure of the enzyme and weaken the absorption of the enzyme to the substrate and decrease its activity. In addition, at high temperatures, thermal energy enters the enzyme and this causes changes in its three-dimensional structure. This change in structure may reduce or eliminate enzyme activity. Also, the presence of heavy metal ions can bind with enzymes and change the structure of the enzyme and reduce its activity or deactivate it. These ions can bind to the active groups of the enzyme and cause changes in its three-dimensional structure [16, 17]. As a result, in the conditions of extreme pH, high temperature, the presence of heavy metal ions and other undesirable substances, the strong structure of neutral proteases is destroyed, and this causes a decrease or even inactivation of the enzyme activity [18].

Table 2. Analysis of variance the effect of factors on the covalent immobilization of *Aspergillus oryzae* 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	3.02NS	143.07NS
B-Molarity of glutaraldehyde (mM)	0	0	576.19**	902.05**
C-Activation time(h)	851.95 **	741.74NS	3220.84**	78NS
D-Buffer pH	73.46 **	264.93NS	50.23NS	825.81**
E-Molarity of the buffer solution(mM)	0	308.17NS	957.32**	0.15NS
F-MWCNT-NH2-glutaraldehyde amount(mg)	1418.17 **	47.86NS	5.07NS	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	283.31NS
BE	0	0	0	942.54**
BG	0	0	126.16NS	0
CE	0	1952.21**	0	0
CF	0	0	1324.25**	0
DE	0	0	122.03NS	0
DF	0	1712.58**	0	402.13**

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DG	3552.03 **	0	0	0
FG	0	0	140.89NS	0
Curvature	6561.92	2604.43	1171.91	7856.77
Residual	176.86	208.42	35.66	68.61
Lack of Fit	176.86NS	208.42NS	35.66NS	68.61NS
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

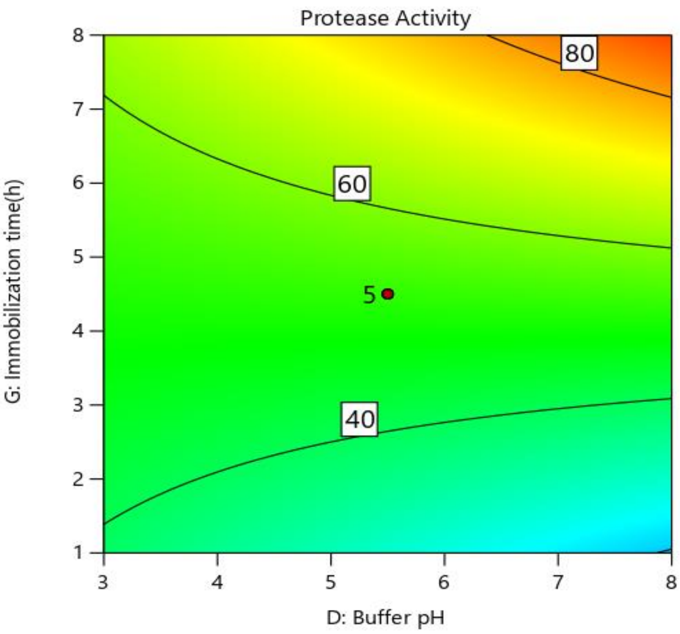

Figure 1. Contour plot of the effect of buffer pH and incubation time on enzyme activity**.**

On the other hand, according to the optimal conditions for the specific activity of proteases, they can be classified based on their differences in parameters such as pH, substrate, temperature, stability, active site, and catalytic mechanisms. The properties of proteolytic enzymes depend on the nature of amino acids near the hydrolysable band [19]. Proteases are divided into three categories based on the pH suitable for activity: acidic proteases, neutral proteases and alkaline proteases. The maximum activity of acidic proteases is in the range of pH 2 to 6, neutral proteases in the range of pH 6.5 to 7.5 and alkaline proteases in the range of pH 8 to 11. Extracellular microbial proteases play a vital role in the nutrition of organisms by hydrolyzing large polypeptide molecules into smaller molecules that can be absorbed by the cell. In all cellular systems, there is a balance between metabolic processes that include protein synthesis and degradation, and

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intracellular proteases play a vital role in these protein renewal processes. Neutral proteases have few industrial applications compared to other types of proteases and are usually produced by Bacillus and Aspergillus species [20, 21]. Regarding the negative effect on protease activity, it can be said that the negative effect of MWCNT-NH3-glutaraldehyde amount on protease enzyme activity can be due to interference with the normal structure and function of the enzyme or a change in the optimal conditions for enzyme activity.[22]

MWCNT (multi-walled carbon nanotube) as a nanomaterial, usually exerts effects on the activity of enzymes. Carbon nanotubes can bind to enzymes and change the structure and activity of the enzyme. Here, MWCNT-NH3-glutaraldehyde binds to the protease enzyme, possibly causing a change in the physical structure or catalytic mechanism of the protease. In addition, the protease enzyme reaction may be affected due to changes in the optimal conditions for enzyme activity. For example, the amount of MWCNT-NH3-glutaraldehyde may cause a change in pH, temperature or other parameters required for protease enzyme activity, which reduces its activity [23, 24].

Therefore, the amount of MWCNT-NH3-glutaraldehyde can reduce the activity of the protease enzyme and have a negative effect on its activity. On the other hand, the delay or negative effect of activation time on enzyme activity can occur due to various factors. Over time and exposed to external factors such as heat, humidity and pH, enzymes may experience structural changes and decrease enzyme stability. These changes can lead to the inability of the enzyme to perform its tasks and reduce its activity. In the long term, enzymes may interact with air oxygen and other oxidizing agents and become oxidized. This phenomenon is known as oxidation and can lead to structural changes of the enzyme and decrease its activity [25]. In some cases, enzymes may be broken down and digested. In this process, agents such as proteases and peptidases enter the enzyme and deactivate it. This decomposition and digestion can occur by reducing enzyme activity and being affected by enzyme activation time. Therefore, if the activation time of *Bacillus licheniformis* protease on multi-walled amino-carbon nanotubes leads to a decrease in the activity of this protease, it is probably due to structural changes and a decrease in enzyme stability during activation [26].

Covalent immobilization of *Bacillus licheniformis* protease on multi-walled amino-carbon nanotubes can be positively influenced by immobilization time and buffer pH. Immobilization time refers to the time that enzymes and carbon nanotubes come into contact with each other and form covalent beta bonds between them. A longer immobilization time can provide the best opportunity for the formation of these connections and significantly improve the performance of enzymes on the surface of nanotubes [27].

Also, the buffer pH of the solution used in the immobilization process can have an important effect on the performance of enzymes and nanotubes. The pH of the buffer should be in a range that is suitable for the activity of enzymes and can also facilitate beta covalent bonds between enzymes and nanotubes. Choosing the right pH can increase the adsorption of enzymes on the surface of nanotubes and, as a result, significantly improve the activity of enzymes. Finally, the covalent immobilization of *Bacillus licheniformis* protease on amino-multiwalled carbon nanotubes is positively affected by the immobilization time and buffer pH. Also longer immobilization time and suitable buffer pH can significantly improve the performance of enzymes on the surface of nanotubes and facilitate beta covalent bonds between enzyme and nanotubes [28].

2-3-The efficiency of covalent immobilization of *Bacillus licheniformis* protease on multi-walled amino carbon nanotube

As mentioned, protease is one of the biologically active enzymes and Bacillus species are one of the most important producers (strains) of this enzyme. It can catalyze the hydrolysis reaction of protein molecules to peptides and amino acids under alkaline conditions. The results of protease enzyme immobilization efficiency in Table 2 show that this efficiency is mostly influenced by the enzyme immobilization time, the interaction effect of activation time + buffer solution molarity, as well as the interaction effect of buffer pH and MWCNT-NH3-glutaraldehyde (Figure 2). Meanwhile, the interaction effect of activation time and buffer solution molarity is higher than all factors ($p<0.05$) and these effects are positive, so that the combination of activation time and buffer solution molarity increases, as well as the interaction effect of buffer pH and MWCNT-NH3 - Glutaraldehyde increases the stabilization efficiency of *Bacillus licheniformis* protease enzyme on multi-walled amino-carbon nanotubes. The equation of the effect of factors on the efficiency of covalent stabilization of *Bacillus licheniformis* protease on multi-walled amino carbon nanotubes with R2 = 0.82 and Adjusted R^2 = 0.77 was obtained as equation number 4.

Protease Immobilization =57.416+5.09C-3.01D-3.32E-1.29F-27.81G+8.17CE+7.76
eq.4

The value of the R2 index is high and indicates the appropriateness of the obtained model, and this model can predict 77% of the changes in the efficiency of covalent immobilization of *Bacillus licheniformis* protease on multi-walled carbon nanotubes.

Increasing of activation time and molarity of the buffer solution as well as the interaction effect of buffer pH and MWCNT-NH3-glutaraldehyde can significantly increase the efficiency of *Bacillus licheniformis* protease enzyme immobilization on multi-walled amino-carbon nanotubes. Activation time refers to the time in which the enzyme interacts with the buffer solution. By increasing the activation time, more opportunity is provided for the interaction of the enzyme with the carbon nanotubes, which can lead to better binding and stabilization of *Bacillus licheniformis* protease on the surface of the nanotubes. This binding and immobilization improves the immobilization efficiency of the enzyme [29]. In addition, the molarity of the buffer solution also plays a role in increasing the efficiency of enzyme stabilization. By increasing the molarity of the buffer solution, more ions and buffer molecules will be present in the environment. These ions and molecules can act as adsorbents for Bacillus licheniformis protease and carbon nanotubes and cause better stabilization of the enzyme on the nanotubes [30].

Also, the interaction between buffer pH and MWCNT-NH3-glutaraldehyde can also affect the enzyme immobilization efficiency. Buffer pH can change the electric charge level of carbon nanotubes and protease. By adjusting the pH to a suitable value, the electric charge of the surface of nanotubes and protease converges and a better connection is established between the enzyme and the nanotubes, which again improves the immobilization efficiency of the enzyme [26].

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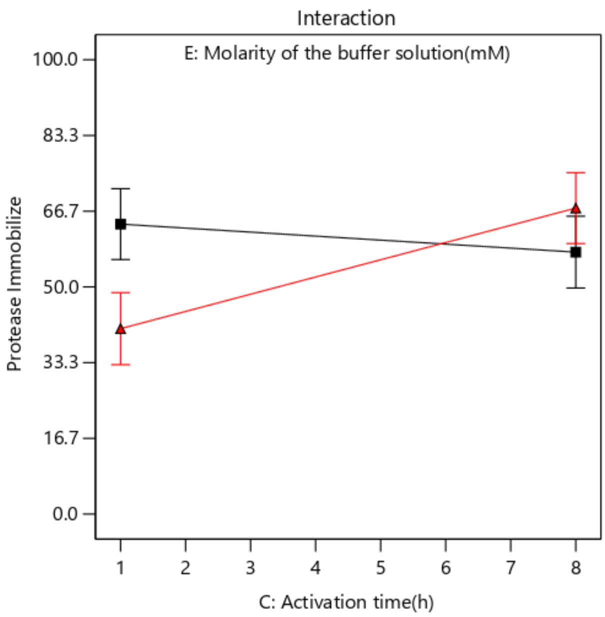

Figure 2- Interaction of buffer solution molarity and activation time on the efficiency of protease enzyme immobilization

Therefore, by increasing of activation time and molarity of the buffer solution and by properly adjusting the pH of the buffer and MWCNT-NH3-glutaraldehyde, the immobilization efficiency of the protease enzyme can be delayed or the negative effect of the activation time on the enzyme activity can occur due to various factors. Over time and exposed to external factors such as heat, humidity and pH, enzymes may experience structural changes and decrease enzyme stability. These changes can lead to the inability of the enzyme to perform its tasks and reduce its activity. In the long term, enzymes may interact with air oxygen and other oxidizing agents and become oxidized. This phenomenon is known as oxidation and can lead to structural changes of the enzyme and decrease its activity. In some cases, enzymes may be broken down and digested. In this process, agents such as proteases and peptidases enter the enzyme and deactivate it. This decomposition and digestion can occur by reducing enzyme activity and being affected by enzyme activation time [25, 26]. As a result, if the activation time of *Bacillus licheniformis* protease on multi-walled amino-carbon nanotubes leads to a decrease in the activity of this protease, the reason is probably related to the structural changes and decrease in the stability of the enzyme during activation.

3-3-Aspergillus oryzae beta-galactosidase enzyme activity

Beta-galactosidase is an enzyme capable of hydrolyzing glucosides and lactose. A group of these enzymes are produced by bacteria and can play a significant role in processes such as dairy production, sugar production, and also in the breakdown of sugars and saccharides in the human digestive system. One of the beta-galactosidase enzymes is the beta-galactosidase enzyme of *Aspergillus oryzae*. *Aspergillus oryzae* is a fungus that can be found in natural environments and has the ability to produce various enzymes [31].

Aspergillus oryzae β-galactosidase enzyme activity is very important in the hydrolysis of glucosides and lactose. This enzyme releases glucose by cutting glucosides at the beta-1,4 position through the hydrolysis of the glucose-glucose bond. Also, by hydrolyzing the beta-1,4 bond of lactose, it directly leads to the production of glucose and galactose. Beta-galactosidase activity of *Aspergillus oryzae* is especially used in the dairy industry. This enzyme is able to break down lactose in milk into glucose and galactose. This process helps in the production of products such as sweets, lactose-free commercial dairy products and other low-lactose products. Also, the use of beta-galactosidase enzyme of *Aspergillus oryzae* in the decomposition of lactose in the human digestive system has also been considered. Other uses of beta-galactosidase enzyme in different industries include sugar production, food disintegrators, production of sweeteners and production of low-lactose foods [32].

In this research, the effect of seven continuous factors, pH of activation, molarity of glutaraldehyde, activation time, pH of buffer solution, molarity of buffer solution, amount of

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MWCNT-NH3-glutaraldehyde and immobilization time on the immobilization efficiency and enzymatic activity of the mentioned enzyme are shown in Table 2.

According to the obtained results, glutaraldehyde molarity, activation time and the interaction effect of activation time and the amount of MWCNT-NH3-glutaraldehyde alone are among the seven factors proposed in this research that can have a positive effect on the enzymatic activity of beta-galactosidase enzyme of *Aspergillus oryzae*. have significant (p>0.05) (Figure 3):

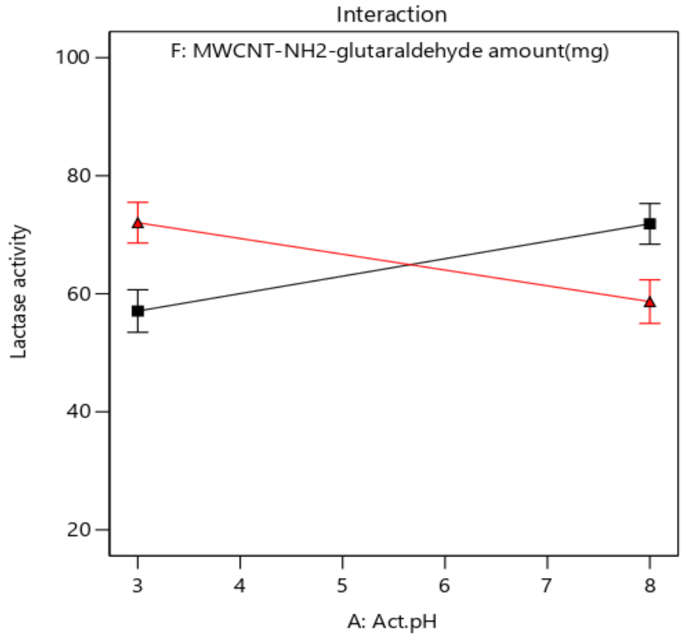

Figure 3- Interaction of pH activation and the amount of MWCNT-NH3-glutaraldehyde on the activity of *Aspergillus oryzae* beta-galactosidase enzyme.

The equation of the effect of factors on the activity of beta-galactosidase enzyme of Aspergillus oryzae on multi-walled amino carbon nanotubes with R2=0.92 and Adjusted R^2=0.87 was obtained as equation number 5.

LactaseActivity=64.91+0.35A+4.73B+11.57C-1.37D+5.92E+0.44F-7.03AF+2.18BG+7.21CF-2.15DE+2.34FG eq.5

The value of R2 index is very high and shows the appropriateness of the obtained model, and this model can predict 87% of the changes in the activation of *Aspergillus oryzae* beta-galactosidase enzyme on multi-walled carbon nanotubes. In the meantime, the contribution of activation time prediction is higher than all factors in a positive direction.

In general, this finding shows that the use of multi-walled amino-carbon nanotubes as a base for *Aspergillus oryzae* beta-galactosidase enzyme can be improved by increasing the molarity of glutaraldehyde and the amount of MWCNT-NH3-glutaraldehyde and the activation time. and increase the enzyme activity of beta-galactosidase [33]. Enzyme activation time can affect the enzymatic activity of beta-galactosidase activity of Aspergillus oryzae. For example, enzyme activation time can increase enzyme activity. A study showed that beta-galactosidase activity of *Aspergillus oryzae* at 50 degrees Celsius and pH 6.5 after 24 hours of activation was 10 times higher than the previous activity [26]. Also, other studies have shown that enzyme activation time can significantly improve *Aspergillus oryzae* β-galactosidase activity [34]. Therefore, the enzyme activation time can be used to improve the beta-galactosidase activity of Aspergillus oryzae. MWCNT-NH3-glutaraldehyde is a carbon nanotube modified by amino group (NH3) and glutaraldehyde. The amino group is attached to the carbon nanotube and glutaraldehyde is attached to the amino group. These structural changes can significantly improve the properties of carbon nanotubes, including increasing stability, increasing resistance to oxidation, and increasing the ability to bind to other chemicals. MWCNT-NH3-glutaraldehyde can play an important role in increasing the enzyme activity of beta-galactosidase of *Aspergillus oryzae*. In particular, this carbon nanotube can be used as a base to attach the enzyme to its surface. This connection can

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significantly improve the stability and enzyme activity. Also, MWCNT-NH3-glutaraldehyde can be used as an activator to activate *Aspergillus oryzae* beta-galactosidase enzyme. This activator can increase the enzyme activity and thus significantly improve the function of beta-galactosidase enzyme of *Aspergillus oryzae* [35].

Also, the molarity of glutaraldehyde refers to the amount of glutaraldehyde present in one mole of the substance. Regarding the positive effect of glutaraldehyde on increasing Aspergillus aureus beta-galactosidase enzyme activity, it should be said that glutaraldehyde is used as an activator to activate the *Aspergillus oryzae* beta-galactosidase enzyme [36]. Studies have shown that increasing the molarity of glutaraldehyde can lead to an increase in the enzyme activity of beta-galactosidase of *Aspergillus oryzae*. For example, one study showed that increasing the molarity of glutaraldehyde from 0.01 to 0.05 M increased the enzymatic activity of *Aspergillus oryzae* beta-galactosidase [37]. Therefore, the molarity of glutaraldehyde can be used as an important factor in increasing the enzyme activity of beta-galactosidase of Aspergillus oryzae.

4-2- The efficiency of covalent immobilization of Aspergillus aureus beta-galactosidase on multi-walled amino carbon nanotube

The results of analysis of variance showed that the efficiency of the covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled amino carbon nanotubes was affected by the molarity of glutaraldehyde, buffer pH, MWCNT-NH3-glutaraldehyde fixation time and also the interaction effect of activation pH + activation time, buffer pH + activation pH, activation pH + buffer molarity, glutaraldehyde molarity + MWCNT-NH3-glutaraldehyde ($p < 0.05$) (Figure 4).

The equation of the effect of factors on the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotubes with R2 = 0.89 and Adjusted R^2 = 0.83 was obtained as equation number 6.

Lactase Immobilization =51.24-2.32A+5.97B-1.77C+5.73D-0.08E-4.83F-7.45G-8.61AC+9.15AD-4.33AE+3.44AG-5.98BE+3.82DF
eq.6

The value of the R2 index is very high and indicates the appropriateness of the obtained model, and this model can predict 83% of the changes in the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino. Meanwhile, the prediction contribution of the interaction of activation time and activation pH is higher than all factors in the opposite direction.

The results indicate that pH has played a decisive role in the immobilization efficiency. The effect of activation pH and buffer pH on the covalent immobilization efficiency of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino can be significant. Studies have shown that buffer pH can have a significant effect on the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase [38]. In particular, the pH of the buffer can have a direct effect on the electrical charge of the surface of amino multi-walled carbon nanotubes, which can significantly improve the efficiency of covalent immobilization of *Aspergillus aureus* beta-galactosidase. Also, activation pH can have a direct effect on the covalent immobilization efficiency of Aspergillus aureus beta-galactosidase on multi-walled carbon nanotube amino. In confirmation of this result, a study has shown that the activation pH for covalent immobilization of *Aspergillus aureus* beta-galactosidase on multi-walled carbon nanotube amino should be in the range of 6 to 8 in order to create a significant improvement in the efficiency of the immobilization process. MWCNT-NH3-glutaraldehyde can play an important role in increasing the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino. In particular, MWCNT-NH3-glutaraldehyde can be used as a base to attach the enzyme to its surface [39]. This connection can significantly improve the stability and enzyme activity. Also, MWCNT-NH3-glutaraldehyde can be used as an activator to activate *Aspergillus oryzae* beta-galactosidase enzyme. This activator can increase the enzyme activity and, as a result, significantly improve the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino [40, 41].

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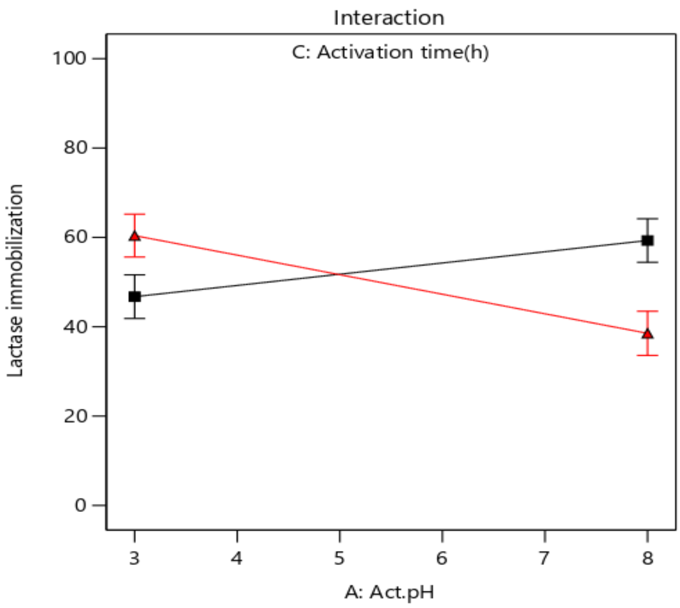

Figure 4. Interaction effect of pH activation and activation time on the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase onto multi-walled amino-functionalized carbon nanotubes

As mentioned in the enzyme activation effect section, glutaraldehyde molarity and activation time can play an important role in increasing the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino. Studies have shown that increasing the molarity of glutaraldehyde can lead to an increase in the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotubes. For example, one study has shown that increasing the molarity of glutaraldehyde from 0.01 to 0.05 M increased the efficiency of the covalent immobilization of *Aspergillus oryzae* beta-galactosidase [37]. Also, the activation time can have a direct effect on the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino. For example, one study has shown that the activation time for the covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino should be between 2 and 4 hours to achieve a significant improvement in the efficiency of the immobilization [2]. In explaining the results of the separate effect of each of the effective factors, it can be said that this factor can intensify the effect of other effective factors in its place, including the mutual effect of activation pH+activation time, buffer pH+activation pH , activation pH + buffer molarity, glutaraldehyde + MWCNT-NH3-glutaraldehyde molarity.

4- General conclusion

In this research, the effect of seven continuous factors on the immobilization efficiency and enzyme activity of *Aspergillus oryzae* beta-galactosidase on multi-walled carbon nanotube amino was investigated. The results showed that glutaraldehyde molarity, activation time and the interaction effect of activation time and the amount of MWCNT-NH3-glutaraldehyde are the only factors that can affect the positive enzyme activity. Also, the results showed that the efficiency of covalent immobilization of *Aspergillus oryzae* beta-galactosidase on multi-walled amino carbon nanotubes was affected by the molarity of glutaraldehyde, buffer pH, MWCNT-NH3-glutaraldehyde, immobilization time and the interaction effect of activation pH + activation time, activation pH + buffer molarity. , are the molarity of glutaraldehyde + MWCNT-NH3-glutaraldehyde.

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تثبیت کووالانت بتا گالاکتوزیداز آسپرژیلوس اوریزه و پروتئاز باسیلوس لیکنی فورمیس بر آمینو- نانولوله‌های کربنی چند دیواره‌ای

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چکیده

این مطالعه با هدف تثبیت کووالانت بتا گالاکتوزیداز آسپرژیلوس اوریزه و پروتئاز باسیلوس لیکنی فورمیس برروی آمینو- نانولوله‌های کربنی چند دیواره‌ای انجام شد. در این روش ازطرح 2k کسری برای مطالعه اثر هفت فاکتور پیوسته (pH فعال سازی، مولاریته گلوتارآلدئید، زمان فعال سازی، pHمحلول بافر، مولاریته محلول بافر، مقدارMWCNT-NH3 -گلوتارآلدئید و زمان تثبیت) بر روی راندمان تثبیت و فعالیت آنزیمی استفاده شد. از نرم افزارDesign-expert برای آنالیز داده ها و رسم نمودار ها استفاده شد. نتایج نشان داد که فاکتور های مذکور میزان فعالیت آنزیمی پروتئاز باسیلوس لیکنی فورمیس و بتا گالاکتوزیداز آسپرژیلوس اوریزه به ترتیب با ضریب همبستگی ۰/۸۰ و ۰/۹۲ به میزان ۷۷ و ۸۸ درصد پیش بینی می کنند. همچنین ضریب همبستگی مدل راندمان تثبیت کووالانت بتا گالاکتوزیداز آسپرژیلوس اوریزه و پروتئاز باسیلوس لیکنی فورمیس برروی آمینو- نانولوله‌های کربنی چند دیواره‌ای به ترتیب ۰/۸۹ و ۰/۸۲ بدست آمد و فاکتورهای مورد مطالعه توانستند به ترتیب میزان راندمان تثبیت کووالانت بتا گالاکتوزیداز آسپرژیلوس اوریزه و پروتئاز باسیلوس لیکنی فورمیس برروی آمینو- نانولوله‌های کربنی چند دیواره‌ای به ترتیب ۸۳ و ۷۷ درصد پیش بینی کنند.