Immobilization of Lactase Enzyme on Aleppo Bentonite

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Abstract - **This research was conducted at Idlib University, located in northwestern Syria, during the years 2023 and 2024. Lactase was immobilized on Aleppo Bentoniteby adsorption. A surfactant (SDS) was added, and the immobilization yield, immobilization efficiency, enzyme recovery rate, temperature, and optimum pH were studied. The results demonstrated the success of the lactase enzyme immobilization process on Aleppo Bentonite. They also demonstrated that adding a surfactant (SDS) increased the efficiency of the enzyme immobilization process. The Aleppo Bentonite treated with 0.5 N hydrochloric acid using a magnetic stirrer was the best, with an immobilization yield of 90%. The optimum pH for enzyme immobilization was 6.6, and the immobilized enzyme demonstrated greater resistance to pH drops in acidic media. The optimum temperature for enzyme immobilization was 70°C. The results also showed that the immobilized enzyme demonstrated greater stability at high temperatures and was capable of hydrolyzing 80% of lactose after three hours. Furthermore, the immobilized enzyme retained 60% of its original activity after six consecutive cycles. The study concluded that activated Aleppo Bentoniteis a good support for β-galactoside enzyme immobilization.**

Keywords: Aleppo Bentonite, lactose, lactase, beta-galactosidase, enzyme fixation.

I. INTRODUCTION

Lactose is the main sugar found in dairy products. For the body to absorb it, it must be broken down in the intestine by the enzyme lactase. If there is a deficiency in the enzyme, lactose cannot be broken down, which can cause symptoms of lactose intolerance (abdominal pain, bloating, gas, cramps, diarrhea). Infants are usually born with sufficient lactase to digest the lactose in breast milk (Ugidos-Rodríguez *et al*., 2018). Factors that can influence the severity of symptoms include metabolic processes and the gut microbiota (the community of healthy bacteria that helps digest food). Sometimes, new or worsening lactose intolerance can occur as a result of other conditions such as celiac disease, small intestinal bacterial overgrowth, or viral or parasitic infections (Micic *et al*., 2019), so it has been suggested that this condition could be treated by adding the lactase enzyme exogenously to milk (Ibba *et al*., 2014). Enzymes have received much attention from researchers due to their useful applications in the technological, pharmaceutical, and food industries, in addition to their unique properties, such as catalytic efficiency, specificity, and their operation under moderate conditions (moderate temperatures and moderate pH) (Karakus *et al*., 2008). New research is based on immobilizing enzymes on solid supports due to their advantages over free enzymes. Because free enzymes have limited industrial use, reusability, and low recovery yields, immobilizing enzymes on solid supports has received significant attention recently. Important advantages of immobilized enzymes compared to free enzymes include the ability to run enzyme reactions continuously, rapid completion of reactions, control of product quantity, and the absence of enzyme loss in the reaction solution (thus saving time in the purification step to remove the enzyme from the product system) (Karakus *et al*., 2008). Immobilized enzymes can also be used to remove bitterness from citrus juice and in food packaging to extend shelf life and improve food quality. Packed (Yushkova *et al.,* 2019). The most important requirements for supports used to immobilize enzymes are: mechanical and thermal strength, microbial resistance, non-toxicity, renewable potential, low cost, and a large surface area for enzyme reactions (Li *et al.,* 2004). Several studies indicate that clay minerals can be used as solid supports to immobilize enzymes due to their great catalytic potential (An *et al.,* 2015). Given that Aleppo Bentonite is a clay material abundant in our country and contains highly porous and non-toxic silicate compounds, research has focused on its use as a carrier in chromatography. This is achieved after purification and thermal and chemical treatment to make it suitable for use in various chromatographic applications. This leads to enriching chromatographic carriers and increasing the possibility of its use in various fields (Samani, 2013). Aleppo sap has also been used as a natural material to remove radioactive elements from wastewater from nuclear reactors (Aboujamous, 1992), heavy metals from laboratory water, and industrial dyes (Kassem, 2015).

Aleppo bentonite has a good ability to remove phosphate from aqueous solutions, removing more than 50-80% of the dissolved phosphate at concentrations ranging from 5-100 mg/L. Therefore, Aleppo bentonite ore can be used as a carrier when treated with phosphorus solutions. It can be used as a slow-release phosphate fertilizer, just as it is important as an

adsorption support, useful in treating phosphate-enriched nutrient pollution in the natural environment (Ziad *et al.,* 2019). Furthermore, it was shown that bentonite treated with hydrochloric acid at boiling temperature produced an adsorbent with a high capacity to absorb carbon dioxide and sulfur dioxide, which led to increased gas retention due to the structural properties of bentonite (Venaruzzo *et al.,* 2002).

II. MATERIALS AND METHODS

2.1 Preparation of the Phosphate Buffer Solution:

The phosphate buffer solution was prepared according to the method described in the British Pharmacopoeia. Pharmacopoeia Commission, 2015), by following the following steps:

- 750 ml of distilled water was added to a 1000 ml volumetric flask.
- 27.22 g of KH_2PO_4 was then weighed and added to the flask. The mixture was then placed in an ultrasonic device until completely dissolved.
- 0.305 g of $MgCl_2$ was then weighed and added to the solution. The pH of the solution was then adjusted to 6.6 by adding NaOH dropwise with continuous stirring. The volume of the solution was then brought to 1000 ml by adding distilled water up to the mark on the flask.

2.2 Preparation and Activation of the Aleppo Bentonite:

The Aleppo Bentonite was prepared and activated according to the method (Al-Essa, 2018) by following the following steps:

2.2.1 Step One: Preparation of the Aleppo Bentonite before Activation:

- The Aleppo Bentonitewas mechanically ground using a mortar and pestle, then sifted using a sieve with 0.5 mm holes.
- The ground Bentonite was placed in an oven for four hours at 100°C to remove impurities and moisture.

2.2.2 Step 2: Activating the Aleppo Bentonite with hydrochloric acid at different concentrations (N) (0.1-0.5-1) in two ways:

2.2.2.1 Method 1: Activating the Aleppo Bentonite using the reverse refrigeration method:

- 15 g of the Aleppo Bentonite prepared in the first step was dissolved in a reverse refrigeration flask in 200 ml of hydrochloric acid at different concentrations (N) (0.1-0.5-1).

- The mixture was then distilled for 7 hours at 70°C, and the mixture was placed in an oven at 100°C for 4 hours.

2-2-2-2 Method 2: Activating the Aleppo Bentonite using the magnetic stirrer method:

- 15 g of the Aleppo Bentonite prepared in the first step was dissolved in 200 ml of hydrochloric acid at different concentrations (N) (0.1-0.5-1).
- The mixture was placed in a magnetic stirrer for 4 hours at 70°C for each concentration separately.

2.2.3 Step 3: Drying the Aleppo Bentonite:

- The Aleppo Bentonite prepared in the second step using both methods was dried in an oven at 100°C for 4 hours. After drying, the Aleppo Bentonite was ground and sieved using a 0.5 mm mesh sieve.

2.3 Immobilization of the Lactase Enzyme (Beta-galactosidase) on the Aleppo Bentonite:

- The lactase enzyme produced by Kluyveromyces lactis (Lactase 5200-HA) was selected, as the average enzyme activity was 5200 U/g (where U1 of enzyme is defined as the amount of enzyme capable of forming 1 μmol of glucose from lactose under specified conditions) (Pham *et al.,* 2019).

Lactase was immobilized on Aleppo Bentonite using various concentrations of hydrochloric acid (0.1-0.5-1) using magnetic stirrers and reverse refrigeration (Husain *et al.,* 2011) methods, following the following steps:

Step 1:

1) 3 g of Aleppo Bentonite activated with various concentrations of hydrochloric acid (0.1-0.5-1) using magnetic stirrers and reverse refrigeration (RF) methods were weighed.
2) 30 ml of phosphate buffer solution (pH 6.6) was added, along with 0.2 g of surfactant (SDS) to increase the efficiency of the immobilization process.
3) 900 μL of lactase enzyme was added in drops and placed in a magnetic stirrer. Stirring continued at room temperature for 2 hours, and the enzyme immobilization yield was measured.

Step 2:

The contents of the beaker were poured into glass tubes and then centrifuged at 3500 rpm for 10 min to separate the solid phase from the liquid phase.

Step 3:

1) 2 ml of the supernatant from the inverted cooler and magnetic stirrer were taken separately to assay enzyme activity.
2) 0.1 g of lactose was added and incubated for half an hour at 37°C.
3) Glucose was measured using a glucose assay kit.

Step Four:

- Free Enzyme: 1 ml of phosphate buffer solution (pH 6.6) was added with 0.1 g of lactose and 50 μl of enzyme, then incubated for half an hour at 37°C to assess the activity of the free enzyme.

The enzyme immobilization yield (IY) was determined by measuring the enzyme activity in the control solution and the activity of the unbound enzyme in the washing solution according to the following equation (Fabra *et al.,* 2020):

Immobilization yield (IY%) = [(C - UB)/C] × 100 -------- (1)

Where C indicates the enzyme activity of the control sample, and UB indicates the activity of the unbound enzyme in the washing solution, respectively.

- The enzyme immobilization efficiency (IE) was determined according to the following equation developed by Sheldon and van Pelt, 2013:

Immobilization Efficiency (IE%) = (observed activity)/(immobilized activity) × 100 ------ (2)

- The immobilized enzyme activity recovery (AR) was determined according to the equation developed by Sheldon and van Pelt, 2013:

Activity Recovery (AR%) = (observed activity)/(starting activity) × 100 ------ (3)

Where:

Observed activity: The activity of the immobilized enzyme measured upon first use after washing.
Immobilized activity: The enzyme activity in the control solution minus the enzyme activity in the washing solution.
Starting activity: The enzyme activity in the control solution.

2.4 Experimental Design and Statistical Analysis:

The statistical analysis of the current research results was conducted using IBM SPSS 21, applying the Paired Samples tTest at a significance level of 95%. Data were considered significant if the probability value (P) was less than 5%. Graphs were drawn using Graphpad Prism 7.

III. RESULTS AND DISCUSSION

3.1 Enzyme Loading Efficiency:

Lactase was immobilized on Aleppo Bentonite treated with hydrochloric acid at different concentrations (1-0.5-0.1) using magnetic stirrers and reverse osmosis.

3.2 Yield of the Lactase Enzyme Immobilization Process Using the Magnetic Stirrer Method:

The study showed that the immobilization process using the magnetic stirrer method yielded the best immobilization yield when treating Aleppo Bentonite with hydrochloric acid at a concentration of 0.5 N, reaching 90%. However, at concentrations of N (0.1-1), the yield was lower, reaching 18.10-24.70%, respectively. This is attributed to the fact that the 0.5 N concentration increased the effective specific surface area of the Aleppo Bentonite, thus increasing the number of sites on which the enzyme adsorption occurs, thus increasing immobilization. However, no effective specific surface area was achieved at low and high N concentrations (0.1-1). Optimal conditions for enzyme immobilization were not provided due to their effect on the surface structure of the Aleppo Bentonite, as shown in Figure (1).
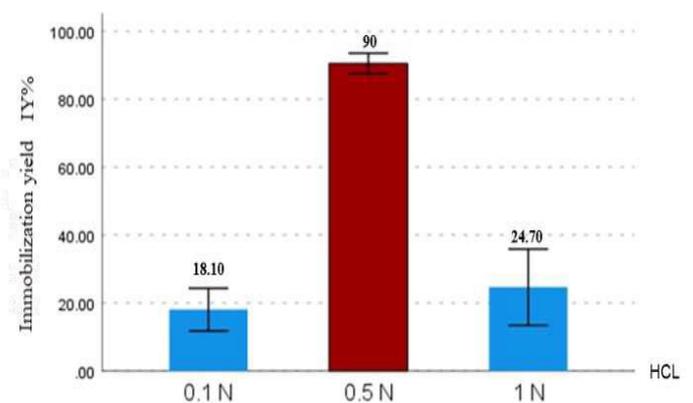
Figure (1): The efficiency of the lactase enzyme immobilization process on the Aleppo Bentonite treated with the magnetic stirrer method at different concentrations

3.3 Yield of the Lactase Enzyme Immobilization Process Using the Reversed Cooling Method:

The results of the study showed that the enzyme immobilization process using the reverse cooling method reached its highest value when treating Aleppo Bentonite with 0.5 N hydrochloric acid, reaching 87%, while its value reached 18.33% and 9.45% at N concentrations (1–0.1), respectively. This is explained by the same reasons mentioned for the magnetic stirrer method, as shown in Figure (2).
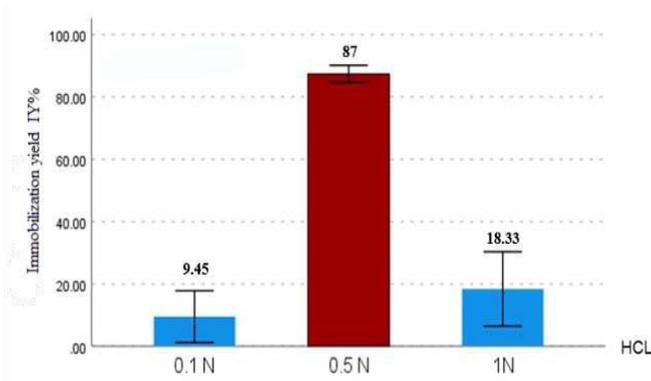
Figure (2): The yield of the lactase enzyme fixation process on the Aleppo Bentonite treated by the reverse refrigeration method at different concentrations

Based on the results presented in Figures (1 and 2), it was observed that:

The best immobilization yield of lactase on Aleppo Bentonite treated with hydrochloric acid was achieved using a magnetic stirrer at a concentration of 0.5 N, which was therefore adopted in subsequent tests.

Table (1) shows the results of the statistical analysis comparing the immobilization yield between the 0.5 N magnetic stirrer and the reversed coolant at concentrations of (0.1-0.5-1) N, in addition to comparing the immobilization yield between the 0.5 N magnetic stirrer and the magnetic stirrer at (0.1-1) N.

Table (1): Statistical study comparing the immobilization yield between the 0.5 N magnetic stirrer and the reversed coolant at different HCl concentrations

ANOVA	P value < 0.0001		
0.5 S	0.1R	0.5R	1R
	< 0.0001	0.265	< 0.0001
0.5 S	0.1 S		1S
	< 0.0001		< 0.0001

S: Magnetic stirrer, R: Reverse coolant.

From Table (1), we find that the best yield of the enzyme immobilization process on Aleppo Bentonite treated with hydrochloric acid was at a concentration of 0.5 N, as no significant differences were observed at a concentration of 0.5 N, and the value reached (P=0.265), while there were significant differences with the rest of the concentrations of hydrochloric acid used in treating Aleppo Bentonite, where (P<0.0001) separately, which supports the adoption of the 0.5 N concentration using the magnetic stirrer method in subsequent experiments in order to save the time needed to obtain Aleppo Bentonite. Comparing the results of our study with the result reached by (Ansari and Husain, 2012), in which the lactase enzyme extracted from Aspergillus oryzae was

immobilized on the celite layer as a support for bioaffinity, and the yield of enzyme immobilization was 71%. While the study (Tizchang *et al.,* 2020) immobilized the lactase enzyme on silicon dioxide nanoparticles using glutaraldehyde as an activating agent, the compound was then trapped within bacterial cellulose nanocrystals under optimal conditions. The enzyme immobilization efficiency was 87%. Overall, the immobilization yield results appear high when compared to previous studies of lactase enzyme loading onto other supports, which is consistent with our study.

3.4 Optimum Temperature and Thermal Stability:

The optimum temperature for the best immobilization yield (IY) was studied at 37, 50, 60, and 70°C. Temperature had a clear effect on the immobilization process. The higher the temperature, the higher the percentage of immobilized enzyme. This is attributed to the fact that increasing temperature leads to the separation of the cellulose layers, which increases the specific surface area of the cellulose and exposes more active sites on which the enzyme can adsorb. The results shown in Figure (3) showed that the highest value of the immobilization process yield was at a temperature of 70°C with a yield value of 96.3%, and the lowest value was 80.5% at 37°C, while at (50-60)°C it was (90.1-94.9%), respectively. It was found that the best temperature that gave the highest immobilization yield was at 70°C, where all P values were < 0.0001, meaning that there were significant differences between the samples. According to the previous results, it can be said that the higher the temperature, the higher the enzyme immobilization yield, as enzymes are active at high desired temperatures, because high operating temperatures prevent bacterial contamination and support catalysis (Katrolia *et al.,* 2019).
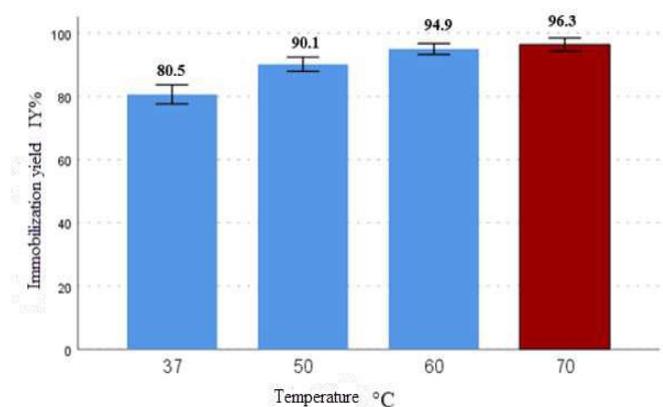

Figure (3): Efficiency of lactase immobilization on the Aleppo Bentonite at different temperatures

Comparing these results with the results obtained by (Zhou and Chen, 2001), in which the maximum activity of the immobilized lactase enzyme was obtained at approximately

50°C, it was found that the immobilized enzyme had an increased ability to withstand temperature.

The results in Figure (4) also showed that the best immobilization efficiency of lactase on the Aleppo Bentonite (IE) at the studied temperatures (37-50-60-70°C) was at 70°C, with a value of 96.23%. According to (Zhou and Chen, 2001), the increased immobilization rate at the optimum temperature of 70°C may be due to the ability of the immobilized enzyme to bond more easily to the support as the temperature increases until it reaches its optimum temperature.
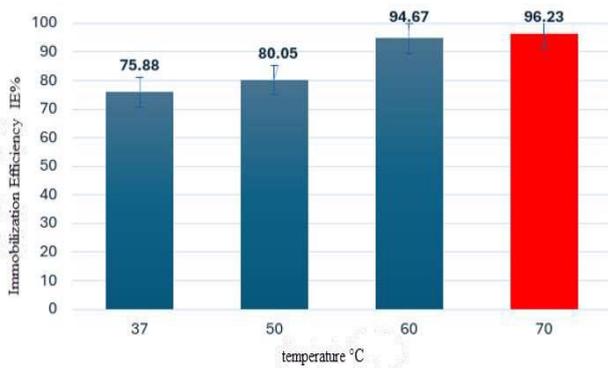

Figure (4): Efficiency of lactase immobilization on the Aleppo Bentonite at the studied temperatures

The results also showed that the best enzyme activity recovery rate (AR) at temperatures (37-50-60-70°C) was at 70°C, with a value of 96.37%, for the same reason mentioned for the immobilization efficiency, as shown in Figure (5).
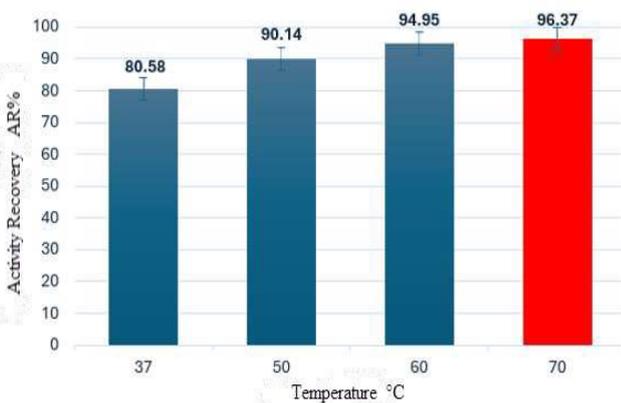

Figure (5): The percentage of lactase enzyme recovery at the studied temperatures

In a study (Popescu *et al.,* 2021) conducted at temperatures from 4-70°C and pH 6.55, it was found that the stability of the lactase enzyme was proportional to the increase in temperature, especially at 45°C, which is consistent with the enzymes studied, as the subsequent increase in temperature led to a decrease in enzyme activity. Therefore, at 60°C, the degree of hydrolysis was 30% for the enzyme. By comparing

the results of our study with the result reached by (Tizchang *et al.,* 2020), where the lactase enzyme was immobilized on silicon dioxide nanoparticles using glutaraldehyde as an activating agent, then the former compound was trapped within bacterial cellulose nanocrystals. Under ideal conditions, the ideal temperature for the free enzyme was 40°C and for the immobilized enzyme 50°C. After 45 days, the immobilized enzyme showed higher stability than the free enzyme, and the immobilized enzyme retained 80%. From its initial activity after 12 cycles of use, previous studies have generally shown that enzyme stability varies with increasing temperature. As the temperature increases, the percentage of immobilized enzyme increases. However, there were differences in the optimum temperature, with the highest temperature for enzyme immobilization without denaturation being 70°C.

3.5 Determining the optimal pH:

The optimal pH, which gives the highest yield for the immobilization process, was studied at different acidity levels (2, 4, 6.6, 8, 10), and a temperature of 37°C. The results are summarized in Figure (6). The immobilization yield reached its maximum value of 87% at pH 6.6, and the lowest value was 10.72% at pH 10. At pH levels (2, 4, 8), the value was (41.95-75.36-14.1%). According to the previous results, electrostatic forces were observed between the enzyme and the support (which are forces of attraction between different charges). When the pH was moderate or low, the immobilization yield was high, and the concentration of hydrogen ions was high, i.e., the charge was positive. It is known that the lactase enzyme has an apparent negative charge, meaning that it is adsorbed on sites that give an apparent positive charge. However, when the pH increased, the immobilization yield decreased significantly. This is due to the coverage of positive hydrogen ions on the active adsorption sites on the apparently negatively charged enzyme surface.

Figure (6): The efficiency of lactase enzyme immobilization on the Aleppo Bentonite at the studied pH values

This study is consistent with the results obtained by Ricardi *et al.* (2018) on the immobilization of lactase on a silica/chitosan composite support. The enzyme immobilized on silica composites showed high efficiency, reaching 62%. The optimum pH for both the free and immobilized enzyme was evaluated at pH 5.5, 6.0, 6.5, 7.5, and 8.0 at a constant temperature of 37°C. The relative activity of the free and immobilized enzyme was clearly pH dependent. The highest relative activity of the free and immobilized enzyme was observed at pH 7, indicating that the free and immobilized enzymes maintained their active conformation at this pH. The significant decrease in relative activity in more acidic and alkaline media can be attributed to enzyme inactivation.

IV. CONCLUSIONS

The results showed that immobilizing lactase on the Aleppo Bentonite 0.5 N hydrochloric acid-treated galbanum using a magnetic stirrer yielded better immobilization yields. The immobilized enzyme demonstrated greater resistance to the effects of pH in acidic media. The immobilized enzyme also demonstrated maximum activity at 70°C, opening the way for its use in the hydrolysis of lactose at high temperatures to address the problem of bacterial contamination.

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