Research Article

Stability Improvement of Immobilized α-amylase using Nano Pore Zeolite

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Background: Enzyme engineering by immobilization techniques has proven to be well compatible with the other chemical or biological approaches aiming to improve enzyme's functions and stability. Zeolites are porous alumino-silicates with a wide range of porosity and particle size along with the other remarkable properties such as high surface area, high stability against a wide range temperatures, pHs, as well as organic solvents.
Objectives: Nano-zeolites are a class of advanced materials that have special properties that has made them ideal candidate for a wide range of applications.
Materials and Methods: In this study, a nano-zeolite which has been synthesized and characterized in our previous work, was used to immobilize α-amylase and activated with glutaraldehyde as a bi-functional agent to improve enzyme properties.
Results: Studies have shown an increased stability of the immobilized enzyme compared to the free enzyme against a range of temperature change and pHs as well. Also the stability of the immobilized enzyme was increased with respect to storage. The calculated binding efficiency shows that the immobilized α-amylase conserved 58.44% of its native activity.
Conclusions: Using nano pore zeolite for covalent attachment of the α-amylase resulted in an increased resistance of this enzyme against denaturation. The immobilized enzyme demonstrated higher stability compared to the free enzyme at higher temperatures and pH variations. Immobilization also caused an increase in the enzyme stability during storage.

Keywords: α-amylase; Enzyme Immobilization; Nano pore zeolite; Stability

1. Background

Enzyme stability is an important issue, as it has taken a wide range of applications in a diverse field of the industries, especially in terms of application and technology. Enzyme engineering, through immobilization techniques, i.e. adsorption, multipoint, and multi subunit covalent binding, as well as entrapment, is a preferred approach for improving enzyme properties, such as, stability, specificity, activity, and inhibition by reaction products (1). Immobilized enzymes have shown to display a much better operational properties than the free enzymes in the harsh condition. This process can maintain the activity of the enzyme for a long time and allows enzyme re-application in the industrial reactors (2).

There are different organic and inorganic nano-materials that can be used as a support for enzyme immobilization to ensure the highest retention of the enzyme activity and stability (3-6).

Zeolites are porous alumino-silicates with a wide range of porosity and particle size and remarkable properties such as high surface area, high stability against variation in the temperature and pH, in addition to organic solvents. These materials are resistant to micro-organisms and radiation respectively. They have specific properties such as ability to exchange ions, adsorption, molecular sieving, and catalysis, in addition to proper conductivity. As well, these materials are cost effective, and non-toxic with respect to the health issues. Therefore, they have found numerous applications in the recent years and can be considered as a suitable candidates for a variety of technological

applications such as water purification and softening, separation of gases, nuclear industry, and sensors (6-14). They can also act as suitable supports for immobilization of enzymes (15-18). α-amylase is among starch hydrolyzing enzymes with a wide range of applications in various industries such as textile, paper industries, detergent applications, and fermentation. Therefore, an improved operationally stable α-amylase through immobilization would gain importance, especially with respect to economical point of view. In this study, thermal, pH, and operational stability (multifold application) of the immobilized α-amylase were studied and compared with the corresponding free enzyme. α-amylase immobilized onto the nanopore zeolite according to the procedure described by Fernandes *et al*. (2004) (19). Glutaraldehyde was used for covalent immobilization of the enzyme which is quite simple, efficient method, and also among the most popular technologies for enzyme immobilization (20). Subsequently, the immobilized enzyme stability was studied against a range of temperature and pHs. This would be of practical importance for further applications.

2. Objectives

Nano-zeolites are a class of advanced materials that have special properties that has made them ideal candidate for a wide range of applications. In this study, a nano-zeolite which has been synthesized and characterized in our previous work, was used to immobilize α-amylase and activated with glutaraldehyde as a bifunctional agent to improve enzyme properties. Nano-zeolite immobilization could be considered as an effective method for improving α-amylase stability, and, as a suitable selection for its commercial applications on a large-scale industry.

3. Materials and Methods

3.1. Synthesis of Nano Pore Zeolite

Nano pore zeolite was synthesized according to our previous work. Briefly, Zeolite A was synthesized in three steps. In step one, clinoptilolite was dissolved in alkaline solution as the Si source. In step two, the Al solution was prepared from aluminum sulfate or sodiumaluminate and in the step three, the zeolite was synthesized in the hydrothermal conditions (21).

The solutions of Si and Al with specific calculated mass ratios were mixed together. Synthesis of the nano-zeolite requires a high speed mixing system. Hence, the stainless steel reactor was equipped with a homemade high shear mixer and mixing was performed at 1950 rpm. The mixture was heated at 90°C and stirred for 2 h in a stainless steel reactor equipped with a heating jacket. The synthesized nano-zeolite was filtered applying centrifuging at 4000 rpm for 10 minutes. The product was washed, dried, and characterized by X-Ray Diffraction (XRD), X-Ray Fluorescence (XRF), Scanning Electron Microscopy (SEM), and Tunneling Electron Microscopy (TEM) techniques.

3.2. Activation of Nano Pore Zeolite Supports

The activation process of the nano-zeolite was performed according to the method described by Fernandes *et al*. (2004). A 2.5% (V/V) glutaraldehyde solution was prepared in 0.1 M sodium acetate buffer, pH 6.0 as a bifunctional agent. The mixture was left to react under reflux for 2 h at 30°C.

3.3. Enzyme Immobilization

α-amylase immobilization was carried out by adding 3.0 mL of an enzyme solution containing 66 µg α-amylase in 0.1 M sodium acetate buffer, pH 6.0, to 0.2 g activated zeolite particles. The mixture was slowly stirred for 4 h at 4°C; the solids were separated and washed with the same buffer to extract unreacted enzyme. The obtained solids were checked for the enzyme activity.

3.4. Enzyme Activity Assay

Activity assay of α-amylase was performed following to the procedure of Apar and Ozbek (2005) (22). The assay was performed at room temperature, using soluble starch as substrate and iodine solution as a stopper. 200 µL of the prepared enzyme was added to 1000 µL of 0.2% soluble starch in acetate buffer 50 mM, pH 5.9. The mixture was incubated for 10 min at 30°C. Subsequently, 200 µL of the reaction mixture was added to 5 ml iodine solution to stop the reaction. The absorbance was measured at 620 nm as a function of time for 2 min. The assay of the immobilized enzyme was carried out in the same condition, except, for preventing interruption in the reaction before doing spectrophotometric measurements, the nano-zeolite particles were separated from the reaction mixture.

3.5. Enzyme Binding Efficiency

Subsequent to performing immobilization under an appropriate condition, the solids were separated. The supernatant was collected, dialyzed against water, and was lyophilized. Using Bradford method, the protein

content was determined (23) by dissolving the lyophilized sample in the sodium acetate buffer. The binding efficiency (amount of retained protein that remains active) was calculated as a ratio between the percentage of the immobilized active enzyme and the percentage of the retained protein (24). For statistically validating the obtained results, the experiments were performed three times.

3.6. Thermal Stability of Immobilized and Free α-Amylase

Thermal stability studies were carried out by suspending both of the free and immobilized enzymes in 0.1 M acetate buffer, pH 6.0 and incubating the mixture in water bath in varying tempratures, ranging from 25°C to 85°C. Following to the incubation time, the mixture was immediately transferred into an ice bath and then tested for remaining activity.

3.7. Storage Stability

To determine the stability of the immobilized the enzyme during storage, the immobilized and the native enzymes were stored at 4°C. Subsequently, aliquots were assayed for the enzyme activity according to the method described previously at regular intervals of the time.

4. Results

4.1. Characterization of Nano Pore Zeolite

Several methods such as XRD, XRF, SEM, TEM, were used to analyze the synthesized nano-zeolite. The XRD pattern of the synthesized nano-zeolite is shown in Figure 1. XRD technique has been used for the identification and quantification of zeolite A phase. Several reports are available on using XRD method for estimating crystallinity (25). Crystallinity has been estimated for the synthesized samples according to following formula:

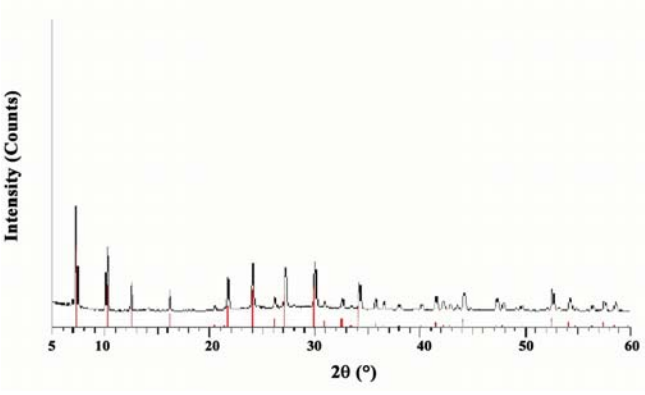

Figure 1. XRD pattern of the synthesized nano-zeolite as support of enzyme, the pattern indicates a crystalline structure of nano-zeolite

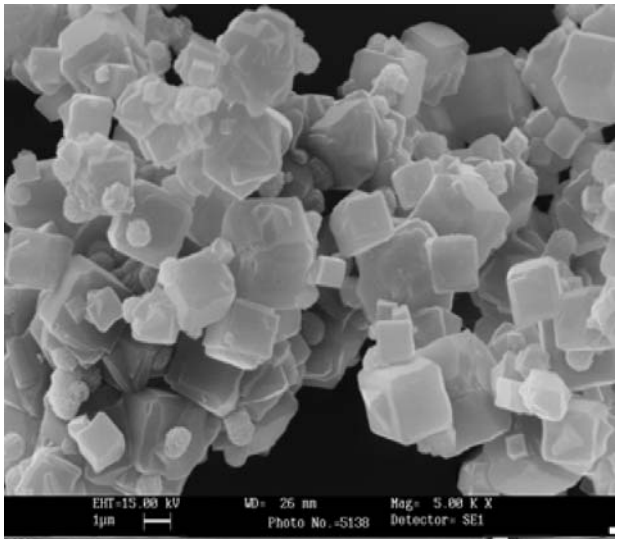

Figure 2. SEM image and morphologic structure of the nano-zeolite used as support for enzyme immobilization, the image indicates a cubic crystalline structure of the nano-zeolite particle

$$\Sigma \text{ Relative intensities of sample} = \Sigma \text{ Relative intensities of standard} \times 100\% \quad (1)$$

The crystallinity of the synthesized nan-zeolite was 96.4%, according to the Eq. (1).

SEM image of the synthesized nano-zeolite is shown in Figure 2. Cubic crystalline structure of the zeolite particle is being well seen in the SEM image. The particle size of the product can be estimated by these images, 100-200 nm for nano-zeolite.

TEM image of the nano-zeolite is shown in Figure 3. According to the TEM image, particle size of the nano-zeolite synthesized by a high shear mixer. The high speed mixing system has resulted in obtaining nano-zeolite particles in a size range of 100-200 nm.

4.2. Efficiency of Enzyme Immobilization

The amounts of the enzyme and protein linked to the nano-zeolite under the best condition for immobilization was calculated. The results show that 23.2% of the available enzyme was immobilized. This is because the surface of the support was untreated; leading to low yield. As well, 25.31% of the protein was retained.

There are several different methods of assessment for calculating the binding efficiency of α-amylase. Colorimetric is among such methods. In this method,

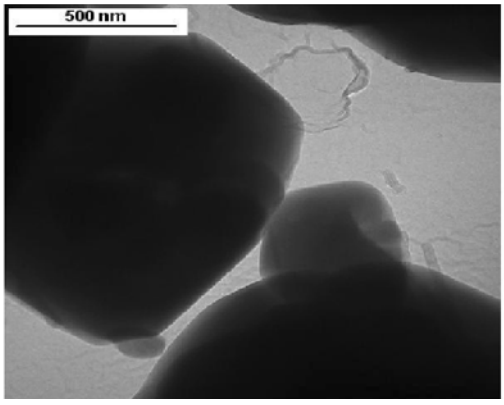

Figure 3. TEM image of the nano-zeolite as particle size determination method, the image indicates a cubical structure of the particle with high percentage of homogeneity

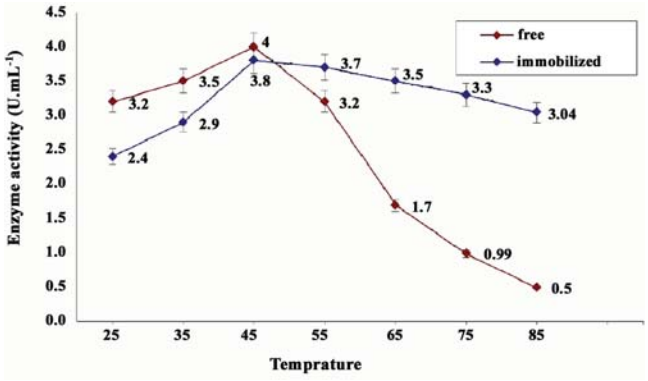

Figure 5. Thermal stability of the free (◆) and the immobilized enzymes (▲) (n=3). Both enzymes show a trend toward an increased enzymatic activity up to 45 °C. With further increase in the temperature, the activity of the immobilized enzyme decreases slowly, while the activity of the free enzyme decreases sharply

the absorption of enzyme solution was measured at λ=280 nm before and after immobilization process. Binding efficiency was calculated by applying following formula:

$$\text{Binding efficiency \%} = \frac{\text{Absorption before immobilization} - \text{Absorption after immobilization}}{\text{Absorption before immobilization}} \times 100 \quad (2)$$

Determining the binding efficiency, it was found that 58.44% of the immobilized protein has maintained its native activity.

4.3. Storage and Immobilized Enzyme Stability

Assessing immobilized and free enzyme activities during storage at 4°C has indicated that the immobilization processes has enhanced the stability of the enzyme. The result of storage stability at 4°C is shown in Figure 4. It should be added that when free and immobilized enzymes are stored at 4°C, the immobilized enzyme maintained 80% of its activity during 15 days of the storage, whereas, the native enzyme loses 100% of its initial activity within this period of time.

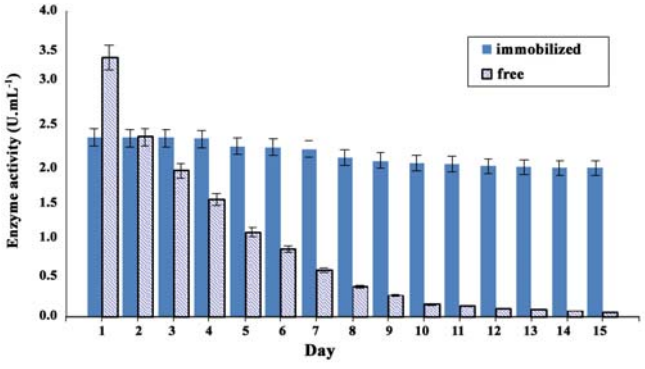

Figure 4. Storage stability of the free and the immobilized enzyme at 4°C (n=3), the graph shows the high storage stability of the immobilized enzyme compared to free enzyme during the time

4.4. Effect of Temperature on Free and Immobilized Enzyme Stability

The thermal stability curves of the immobilized and free enzymes at different temperatures ranging from 25°C to 85°C are shown in (Figure 5) As could be seen the free and immobilized enzymes behave differently when exposed to the heat. The immobilized enzyme has a higher resistance against thermal denaturation compared to the free enzyme. The immobilized enzyme maintained 75% and 82% of its initial activity after 45 min incubation at 85°C and 120 min incubation at 75°C, respectively. The native enzyme was absolutely inactive initially at 85°C and kept 10% of its activity following to 120 min incubation at 75°C. It seems that multipoint attachment which are acquired in the immobilization process leading to an improved denaturation resistance of the immobilized α-amylase.

4.5. Effect of pH on Free and Immobilized Enzyme Activity

The effect of the pH on the activity of the free and the immobilized enzymes is shown in Figure 6. As could be inferred the pH activity curves of the native and the immobilized enzymes are similar, particularity in the range of acidic pHs, in addition to the optimum pHs for either of the studied enzymes. Both of the native and the immobilized enzymes have the maximum activ-

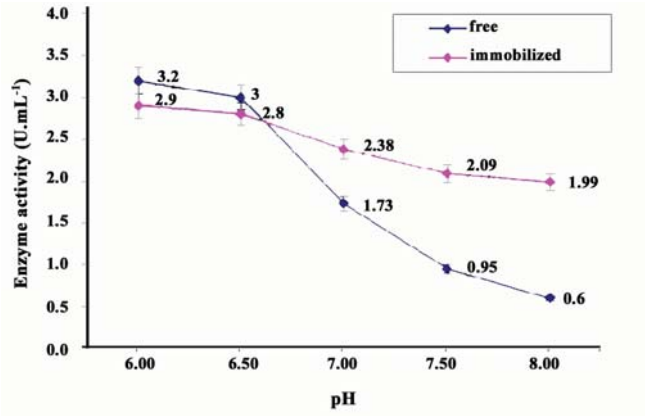

Figure 6. The pH stability of the free (◆) and the immobilized enzymes (▲). The stability of the immobilized enzyme decreases slowly up to pH 6.5. In contrast, the stability of the free enzyme decreases very sharply with the increased pH

ity at pH 6, however the immobilized enzyme presents a higher activity than that of the native enzyme in the alkaline range (pH 7.5 and pH 8.0). At pH 8 the immobilized enzyme kept 68% of its initial activity, whereas the native enzyme kept only 18% of its original activity. The observed change in the pH profile could be attributed to the pH change in the domain of the immobilized enzyme particles, resulting from nano particles characteristics.

5. Discussion

Due to many uses that α-amylase has received in a wide range of the industries, the enzyme has recently been the focus of an intense researches with an aime centered on it's the stabilization process.

Swarnalatha *et al*. have immobilized α-amylase using glutaraldehyde, onto magnetite nanoparticles prepared using gum acacia as the steric stabilizer. The use of this support has enabled higher immobilization of the α-amylase (60%), in contrast to the unmodified magnetite nanoparticles (~20%). The immobilization has also facilitated the reuse of the amylase for six cycles with 30% of loss in the initial enzyme activity (26).

Kumar *et al*. have used alginate as the affinity matrix for entrapment of the α-amylase and subsequent precipitation of the beads with calcium chloride. The entrapped enzyme had higher thermal stability compared to the free enzyme. The midpoint of the thermal inactivation for the enzyme increased by 6±1°C upon entrapment. The reusability of the beads was dependent on the bead size and could be reused for six cycles with ~30% loss in activity (27).

El-Batal *et al*. have studied the entrapment of the α-amylase onto butylacrylate-acrylic acid copolymer using gama-irradiation. Covering α-amylase with the surfactant has made the enzyme more stable than the uncovered form of the enzyme. The results showed an increase in the relative enzyme activity with an increased degree of hydration (28).

In this research, we have studied the storage stability of the α-amylase immobilized onto nano-zeolite as support. Results showed that the calculated binding efficiency of the immobilized α-amylase was 58.44% of its native enzyme activity. The results suggest that the immobilization of the α -amylase is a potentially useful approach on nano-zeolite, commercially.

6. Conclusions

The special properties, plus effectiveness of the nano-zeolite has made this compound to be considered as a suitable support for the enzyme immobilization. Using nano pore zeolite for covalent attachment of the α-amylase resulted in an increased resistance of this enzyme against denaturation. The immobilized enzyme demonstrated higher stability compared to the free enzyme at higher temperatures and pH variations. Immobilization also caused an increase in the enzyme stability during storage.

Therefore nano-zeolite immobilization could be considered as an effective method for improving α-amylase stability, and, as a suitable selection for its commercial applications on a large-scale industry application.

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