Effect of Immobilization on the Activity of Alpha-amylase from Naara Pearl Millet

Emmanuel Godwin Ankudey[1], Prince Addison-Gyan[2], Abdul Ganiwu Issah[1]
[1](*Department of Chemical Engineering, Kwame Nkrumah University of Science and Technology, Kumasi. Ghana)*
[2](*Food and Drugs Authority, Accra. Ghana)*

ABSTRACT: *The effect of immobilization on the activity of crude alpha amylase enzyme from a local source was investigated. Activated carbon was produced from coconut husk and the enzyme was successfully immobilized by adsorption onto the carbon achieving a high enzyme retention of more than 71 %. The optimum temperature for the free enzyme was found to be 50°C whiles that of the immobilized enzyme shifted to 60°C, suggesting the micro-environment confers some degree of thermal stability effect on the enzyme. Immobilization also shifted the optimal pH of the alpha amylase from 6 to 7. Though kinetic studies show that there may be diffusional limitations in the immobilized enzyme system, the immobilized enzyme could be re-used up to 4 times with only a 34% reduction in activity. It has also been shown that immobilization greatly improves the retention of activity in storage. When stored at room temperature, the immobilized enzyme only lost 7% of its activity compared to almost 30% for the free enzyme over a 20 day period. This research shows that activated carbon offers tremendous potential for immobilization of alpha amylase for re-use and lower the cost of bioprocessing.*

KEYWORDS -*Alpha-amylase, activated carbon, immobilization, re-use, storage*

I. INTRODUCTION

The industrial use of enzymes such as alpha-amylase has gained significant recognition due to their catalytic efficiency and specificity under low conditions. Alpha-amylase, a glycoside hydrolase, is an enzyme used extensively in food processing, pharmaceuticals, textiles, and biofuels. It catalyzes the hydrolysis of α-1,4 glycosidic bonds in starch to produce glucose, maltose, and dextrins [1,2,3]. Despite its broad application in these areas, there are still gaps in maintaining the stability and reusability of the enzyme, leading to wastage and an increase in production cost [4,5].

To address this limitation, immobilizing free alpha-amylase onto a solid surface such as activated carbon from coconut husk can stabilize the enzyme, improve its reusability, robust recovery, and longevity during hydrolysis. Immobilization involves the attachment of alpha-amylase onto solid supports such as activated carbon, chitosan, silica, and polymeric microspheres, enabling repeated enzyme utilization and simplified separation from reaction mixtures. These supports are usually carefully selected based on their solubility in aqueous medium, capacity to bind the enzyme, mechanical stability, and they should not have any detrimental effect on the enzyme activity [6].

Immobilization, however, imposes certain hindrances and limitations, such as steric hindrance, reduced substrate accessibility, and potential structural conformational changes in the enzyme. These factors may influence key kinetic parameters, altering enzyme-substrate affinity, catalytic efficiency, and activity [7, 8]. Hence, it is crucial to optimize and balance immobilization conditions such as pH, temperature, and the use of cross-linking agents to maintain enzyme activity and maximize process efficiency.

Different techniques of immobilization have been applied for the attachment of enzymes onto surfaces, including adsorption [6], covalent binding [9], entrapment [10], encapsulation [11], and cross-linking [12], each offering unique advantages and limitations. Among all these techniques, adsorption immobilization onto activated carbon has been reported by Rodrigues *et al*, 2013 [13] and Brena *et al,* 2016 [14] to be the most advantageous due to its high surface area, porosity, and cost-effectiveness. Also, the extensive surface area of the activated carbon presents numerous binding sites for enzyme molecules, facilitating effective adsorption while maintaining the enzyme's structure. Furthermore, adsorption maintains enzyme functionality, enables straightforward retrieval and reusability, and prevents the use of harsh chemical treatments that might denature the enzyme, rendering it an excellent option for large-scale biocatalytic applications [15].

This study aims to investigate the effect of immobilizing crude alpha-amylase on activated carbon obtained from coconut husk and compare the impact of the immobilization on the enzyme when compared to free alpha-amylase based on the effect of pH, temperature, thermal stability, and storage on the enzyme's activity. The source of the alpha amylase for this study is germinating grains of the *Naara* variety of Pearl millet found in Ghana and very popular with local brewers of several alcoholic and non-alcoholic drinks. Also, the study will further investigate how immobilization impacts the immobilized enzyme's reusability, recovery, and longevity during hydrolysis.

II. MATERIALS AND METHODS

2.1 Materials and Chemicals

Millet grains, coconut husk were obtained from the local market in Kumasi, Ghana.
Sodium citrate, sodium phosphate, citric acid, potassium hydroxide, Lowry protein assay kit, 3,5-dinitrosalicylic acid (DNS), starch were obtained from Thermo Fisher Scientific, UK.

2.2 General Statistical Analysis

All experiments were conducted in at least triplicates and the reported data points are the means of these measurements

2.3 Preparation of Alpha Amylase

250 g of the cereal were washed, rinsed with distilled water and steeped in water for 24 hours. After draining the water, the grains were evenly spread in a compact layer on Petri dishes lined with moistened filter paper. The Petri dishes were covered and placed in the dark at room temperature to encourage germination. Moisture content was maintained by periodically adding drops of water to the filter paper. After 72 hours, seeds with shoots longer than 1 cm were considered viable. 10 g of the viable sprouting seeds were ground in 50 mL of pre-chilled 0.05 M citrate buffer at pH 6.0. The resulting homogenate was then centrifuged at 5,000 rpm for 20 minutes. The supernatant was collected as the crude enzyme, and it was stored at 4°C.

2.4 Preparation of Activated Carbon

The coconut fibers were cleaned by rinsing them in deionized water, and they were then dried in an oven at 105°C for five hours. After drying, the samples were cut into various irregular shapes, measuring between 5 to 10 cm, placed in a crucible before carbonization at 450°C for 30 minutes. The samples were immediately immersed in deionized water and then left to cool overnight. Subsequently, the carbonized coconut fibers were saturated with a 1.5 M KOH solution at a weight ratio of 4 (carbon) to 1 (KOH) for 24 hours, followed by heating at 700°C for 150 minutes to activate it. After activation, the samples were washed several times with deionized water, until a pH of 7 was obtained. The activated carbon was then dried at 120°C for 60 minutes and then crushed to the desired 2 mm particle size.

2.5 Immobilization of α-amylase on activated carbon

To immobilize the enzyme, 5 ml of crude alpha-amylase solution was mixed with 2 g of activated carbon and shaken continuously in an orbital shaker at 100 rpm for 1 hour at room temperature. The mixture was then filtered, and the activated carbon

residue was washed with distilled water to remove un-adsorbed soluble enzymes. Finally, the amount of protein/enzyme adsorbed onto the activated carbon was determined. The enzyme-bound activated carbon was stored in a wet state in a Falcon tube for further use.

2.6 Determination of the amount of immobilized enzyme

The amount of enzyme used for the immobilization was determined by quantifying the protein content in the free alpha amylase, the supernatant, and the washing solution using the Lowry method. 1600 µl of 2X Lowry concentrate (consisting of copper sulfate, sodium potassium tartrate, and sodium carbonate) was combined with 1600 µl of the protein solution. The mixture was then left to incubate at room temperature for at least 10 minutes to allow the formation of the copper-protein complex. Subsequently, 800 µl of Folin reagent was added and thoroughly mixed to prevent rapid decomposition or precipitation of the reagent. The sample was then allowed to incubate for 30 minutes, during which the blue color was formed and the absorbance was determined at 750 nm. The amount of immobilized enzyme was determined by subtracting the amount of protein determined in supernatants and washing solutions from the amount of initial protein used for immobilization using bovine serum albumin (BSA) [16].

2.7 Activity assay of α-amylase

To determine the activity of the α-amylase, the Bernfeld method was applied [17]. The activity of the α-amylase was analyzed by first preparing 1 ml of the reacting mixture consisting of an equal volume of 1% starch and α-amylase in phosphate buffer (0.02 M, pH 7) at 25°C for 3 min. The reaction was then terminated by adding 1 mL of DNS reagent, and the mixture was heated in a boiling water bath for 5 minutes. The blank contained all the assay reagents except the enzyme to account for any background absorbance. The amount of reducing sugar (glucose) was measured at 540 nm. The enzyme activity was expressed in terms of units, where one unit of activity is defined as the amount of enzyme that catalyzes the conversion of one milligram of substrate per minute under specified conditions (such as temperature, pH, and substrate concentration).

2.8 Effect of temperature on the activity of the free and the immobilized α-amylase

The effect of temperature on enzyme activities was studied at various temperatures using a thermostatically controlled water bath under standard assay conditions for both free and immobilized α-amylase. The reaction mixtures were incubated at different temperatures of between 30°C and 80°C for 5 hours, with continuous agitation at 150 rpm to allow ample time for enzyme-substrate interaction, and activities at the various temperatures were assayed using the Bernfeld method [17].

2.9 Effect of pH on the activity of the free and the immobilized α-amylase

The pH dependence of the immobilized α-amylase activity was determined at pH values of 3.0, 6.0, and 9.0, under the same assay conditions as previously described, using the Bernfeld method at room temperature**.**

2.10 Repeated batch operational stability of the immobilized α-amylase

To evaluate the reusability of the immobilized α-amylase, a successive enzyme assay was performed under the same experimental conditions. After each reaction cycle, the immobilized enzyme was recovered by simple filtration using Whatman filter paper, which separated the activated carbon-enzyme complex from the reaction mixture. The recovered immobilized α-amylase was then washed gently with 5–10 mL of cold distilled water to remove any residual substrate and reaction by-products. The immobilized enzyme was washed gently using a buffer solution to minimize mechanical disturbance that could dislodge the enzyme from the support material. After washing, the immobilized enzyme was reintroduced into a fresh reaction mixture to measure its retained enzymatic activity for the next cycle.

2.11 The effect of storage on the free and the immobilized α-amylase

The effect of storage stability on both free and immobilized α-amylase was assessed by measuring their residual activity after 20 days of storage, and this was compared with their initial activity. The enzyme samples were stored at 4°C and at room temperature. The activity of the enzymes under these conditions was expressed as a percentage of the residual activity after storage and compared to the initial activity.

III. RESULTS AND DISCUSSION

3.1 Carbonization and activation of coconut husk

A summary of the results from carbonization and activation of coconut husk is presented in Table 1 and the FTIR of the activated carbon is shown in Fig. 1. Activated carbon was produced from coconut husk by first carbonizing it at 450°C, followed by chemical activation with KOH at 700°C. A carbonization temperature of 450°C was selected based on previous reports indicating its effectiveness in breaking down lignocellulosic biomass while preserving sufficient structural integrity for subsequent activation processes [18, 19]. This temperature is often cited as optimal for balancing the elimination of volatile matter with the carbon yield of biomass-derived carbon materials. In this study, an initial weight of 4500 g of coconut husk was used for carbonization, resulting in 1350 g of carbonized material, indicating a weight loss of 70% and a carbonization yield of 30%. From the 1350 g of carbonized material, 1010 g was used for chemical activation with potassium hydroxide (KOH) as the activating agent. The mass ratio of the activating agent to the carbon material typically ranges from 1:1 to 3:1, depending on the desired textural properties. Previous studies by (Li *et al*., 2018) [20] and Deng *et al*., 2010 [21] has shown that a ratio of 2:1 or 3:1 can significantly enhance surface area and microporosity. Therefore, in this study, a 3:1 mass ratio of KOH to carbon material was used, which involved mixing 3030 g of KOH with 1010 g of carbonized carbon material and thermally activating it at 700°C in a controlled furnace environment. This activation temperature was chosen based on the prior works in [20], [21] and [22], which demonstrated great success in producing high surface area activated carbon with well-developed porosity.

A summary of the results is shown in Table 1.The yield based on the carbonized sample was 96.04%, and the overall yield based on the initial coconut husk weight was 21.56%. This result aligns with findings by Tan *et al*., [23] and Ohimor *et al*., 2021 [24] on coconut husk-based activated carbon, in which they reported an overall yield of 20.16% and 21%, respectively.

The minor loss observed after activation (3.96% weight loss) indicates that the activation conditions were well-optimized to enhance pore structure formation without compromising material stability. The combination of high temperatures and chemical activation promotes the development of appropriate pores which enhance the adsorptive capacity of the carbon. The presence of a large surface area and functional groups such as hydroxyl, carbonyl, and carboxyl groups plays a vital role in enzyme attachment to activated carbon [25, 26]. These functional groups facilitate both physical adsorption and covalent bonding, thereby enhancing enzyme stability and maintaining catalytic activity under various conditions.

The FTIR spectrum of the activated carbon reveals the presence of key functional groups responsible for enzyme interaction on the surface. Broad absorption bands at 3734 cm^{-1} correspond to O–H stretching vibrations, indicating the presence of hydroxyl groups, such as carboxylic acids and alcohols, which are capable of forming hydrogen bonds with the enzyme [27]. Peaks observed in the 2000 cm^{-1} range is characteristic of alkyne (–C≡C–) and C=C=O functional groups, while bands at 1578 cm^{-1} and 1431 cm^{-1} are indicative of aromatic C=C stretching and possible metal–ligand complexation, respectively [28].

Table 1: Summary of Carbonization and activation of Coconut husk.

Precursor	**Coconut husk**
Initial weight (g)	4500g
Weight after carbonization (g)	1350g
% weight loss after carbonization	70%

Weight of sample activated (g)	1010g
Weight after activation (g)	970g
Percentage yield (%) after carbonization	30%
Percentage yield (%) after activation based on the initial weight of the coconut husk	21.56%
Carbonization temperature (°C)	450°C
Activating agent	KOH
Activating temperature (°C)	700°C
Percentage yield of activation based on the weight of the carbonized sample (%)	96.04%

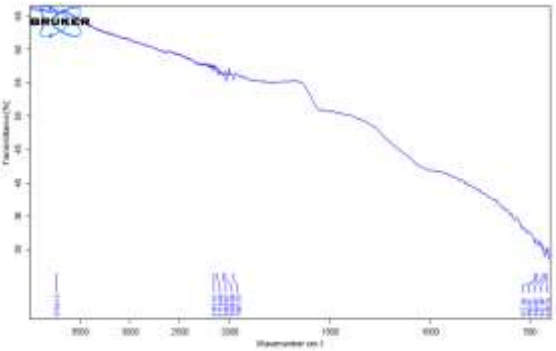

Figure 1: FT-IR Spectrum of activated carbon from coconut fibers.

Multiple strong absorption bands were observed in the 400-500 cm^{-1} (fingerprint) region which reflects the complex surface chemistry of the activated carbon. These functional groups collectively contribute to enzyme adsorption through hydrogen bonding, electrostatic forces, and hydrophobic interactions, thereby enhancing the enzyme's binding affinity and functional stability to the activated carbon surface [27, 28].

3.2 Immobilization and Enzyme Activity

2.25 mL of the crude α-amylase was obtained after extraction, and upon immobilization, 1.78 mL of the enzyme was successfully bound to the activated carbon. This indicates that 79.1% of the free α-amylase was bound to the activated carbon surface, and this high percentage shows the suitability of activated carbon from organic sources as a good binding surface for enzyme immobilization. (Table 2).

Table 2: Parameters of the free and immobilized α-amylase

Parameter	**Crude Free Enzyme**	**Immobilized Enzyme**
Volume of Enzyme Extract (mL)	2.25	–
Unbound Enzyme Volume (mL)	0.47	–
Bound Enzyme Volume (mL)	-	1.78
Assay Volume (mL)	1.0	1.0
Reaction Time (min)	10	10
Absorbance	0.08562	0.06123
Product Concentration (µmol/mL)	6416	4587
Total Product Formed (µmol)	1,068,443	763,797
Enzyme Activity (µmol/min)	642	459
Protein Concentration (mg/mL)	2.1	2.1
Relative Activity (%)	100	71.4

The enzyme also retains 71.4% of its catalytic efficiency which suggests that the immobilization protocol was highly effective, allowing robust enzyme attachment while largely preserving functionality. The slight decrease in activity following immobilization may be attributed to factors such as diffusional limitations, conformational changes, or minor enzyme deactivation during the process. Nonetheless, these findings underscore the suitability of activated carbon as a viable support material for α-amylase immobilization, maintaining substantial enzymatic performance post-immobilization. Furthermore, the reduction in the activity of the immobilized enzyme

is a result of a potential reduction in substrate availability at the enzyme's active sites, which can result from the chemical bonding and diffusion constraints imposed by the polymeric support, as reported by Tufan *et al*., 2022 [29]. The immobilization process can also cause structural modifications to the enzyme, potentially altering its substrate affinity and leading to decreased accessibility of substrates to the active sites, as highlighted by Datta *et al.*, 2022 [30].

Also, the porous structure and surface functional groups in the activated carbon facilitate physical adsorption and covalent bonding between the enzyme and the carbon surface, enhancing the stability and activity of the enzyme [25, 26]. However, the spatial confinement within the pores of activated carbon can also limit the movement of substrate molecules, thereby reducing the overall activity of the immobilized enzyme. Additionally, the microenvironment created by the activated carbon can affect the enzyme's conformation and dynamics, further impacting its activity [25].

3.3 Enzyme Kinetic Parameters

Assuming a simple Michaelis-Menten model for enzyme kinetics, the Lineweaver-Burk plot was used to determine the maximum velocity (V_{max}) and the Michaelis constant, K_m. A linearized form of the model equation is presented in equation 1as:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}} \qquad (1)$$

where V is the initial reaction rate, S, the substrate concentration, K_m, the Michaelis constant and V_{max} is the maximum reaction rate. [31, 32].
The plot is shown in Fig. 2 and the results summarized in Table 3.

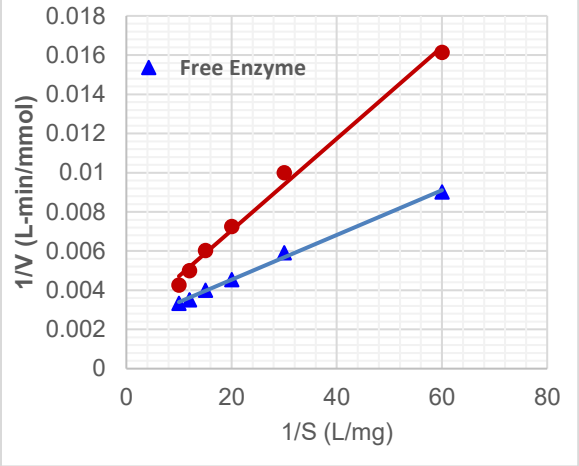

Figure 2: Lineweaver-Burk plot for kinetic parameters

In the model, the maximum reaction rate for the enzyme corresponds to when all active sites are occupied. Table 3 shows that immobilization of the enzyme has resulted in a decrease in the maximum velocity in starch conversion. This may be due to the inaccessibility of few active sites to the substrate as also observed in other studies. The moderate reduction in the V_{max} is also an indication that most of the points of attachment of the enzyme to the activated carbon support is probably not through the active site. It must also be noted that the present study was on the crude enzyme which presumably is slightly protected and not readily accessible even in the free state hence immobilization will tend to have little effect on the availability of the active sites.

Table 3: Summary of kinetic parameters

Enzyme State	**Kinetic parameters**	
	V_{max} (mmol/L.min)	**K_m (mg/L)**
Free enzyme	435	4.34
Immobilized enzyme	417	8.32

K_m is a dissociation constant and indicates the level of affinity of the enzyme towards a particular substrate, in this case starch. The high value of 8.32 mg/L obtained for the immobilized enzyme compared with 4.32 mg/L for the free enzyme is an indication of diffusional limitations in the activated carbon matrix. Other studies have reported such

increased K_m values on immobilization [33, 34, 35]. Conformational changes during immobilization and confinement in a new micro-environment may also contribute to such changes.

3.4 Effect of Temperature on Activity

The activity of both free and immobilized α-amylase was assessed between 30°C and 80°C under standard assay conditions using a thermostatically controlled water bath. As shown in Fig. 3, temperature significantly affected enzyme activity, but distinct trends were observed for the free and immobilized forms. Both forms of the enzyme showed a gradual increase in activity as the temperature increases up to 50°C in the case of the free enzyme and 60°C for the immobilized enzyme.

Other studies have reported similar optimal temperature especially for the free enzyme [36, 37, 38, 39]. The maximum activity, however, was lower for the immobilized enzyme. The decline in activity likely reflects conformational changes and unavailability of the entire active sites on the enzyme surface. The shift in the temperature optimum of 60°C indicates an improved thermal stability of the immobilized enzyme likely due to the stabilizing effect of the activated carbon matrix that reduces structural denaturation. By attaching enzymes to solid supports such as activated carbon, their conformational flexibility is restricted, and they are provided with a protective microenvironment that lowers the chances of thermal denaturation. Moreover, the support can help prevent aggregation or unfolding of enzyme structures at elevated temperatures, thereby improving their operational stability. Similar temperature optima shifts have been reported on immobilization of alpha amylase on various supports. [36, 40, 41].

At 80°C, both the free and the immobilized enzymes lost most of their activities as expected. At elevated temperatures, the three dimensional structure of the protein unfolds and the enzyme loses most activity. Such loss of activity by α-amylase derived from cereals is also reported in several studies [42, 43, 44].

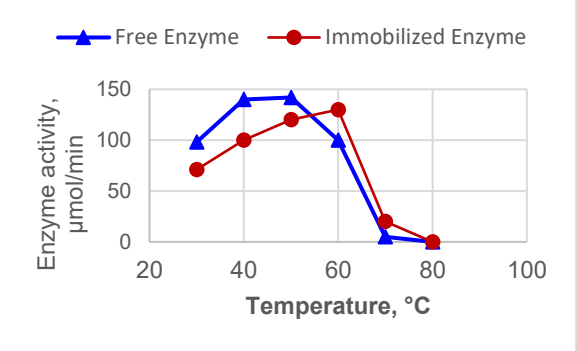

Figure 3: Effect of temperature on the activity

3.5 Effect of pH on Activity

Environmental conditions, notably pH, have a significant impact on enzyme activity, and exploring enzyme immobilization offers valuable insights into the intricate connections between the structure and function of enzyme proteins. The activity of the free and immobilized α-amylase was determined at various pH values (3.0, 6.0, and 9.0), and the activity was measured under the standard assay conditions, and the results are shown in Fig. 4. This study showed the optimum pH of the free α-amylase and the immobilized enzyme on activated carbon to be pH 6 and 7.0. The optimum pH of 7.0 observed for immobilized α-amylase suggests that the matrix provides a stable microenvironment, buffering pH fluctuations and maintaining enzyme activity by protecting the active site from denaturation [45]. This likely reflects the stabilizing effect of the matrix, which provides a stable microenvironment that buffers against pH fluctuations, helping to maintain the enzyme's structure and activity by preventing denaturation of the active site. In contrast, the optimum pH of 6 for free α-amylase is typically observed in its natural environment, where the enzyme works best in slightly acidic conditions [46]. These findings suggest that immobilization can enhance the overall performance of α-amylase, especially under industrial conditions where temperature and pH can vary. A study conducted by Mobasher, 2003 [45] showed a pH of 7.0 as the optimum activity for alpha amylase on chitosan-alginate. This further confirms that neutral pH provides the best conditions in a restricted environment for enzyme stability and efficiency. The chitosan-alginate substrate's properties and the

enzyme's structural integrity likely contribute to this optimal activity, similar to those observed in the activated carbon.

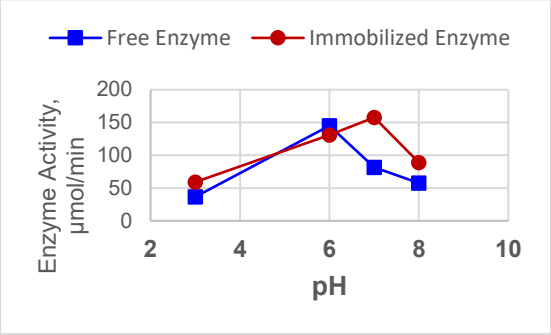

Figure 4: Effect of pH on the enzyme activity

3.6 Repeated batch operational stability of the immobilized α-amylase

The reusability of the immobilized α-amylase was assessed through repeated batch reactions for starch hydrolysis over three consecutive cycles. As shown in Fig. 5, the relative activity of the enzyme declined with each reuse, decreasing to about 66% of the initial rate after the third cycle. The gradual decline in activity is attributed to factors such as enzyme leaching, conformational destabilization, and partial denaturation due to repeated use. Despite the structural support provided by the activated carbon matrix, α-amylase may still undergo progressive deactivation caused by mechanical agitation, substrate exposure, and mild desorption. Nevertheless, the relatively moderate 34% loss over four cycles demonstrates satisfactory operational stability, which is essential for industrial applications where enzyme reuse lowers production costs. Immobilized enzymes provide both economic and environmental advantages, as they can be reused without full replacement in each cycle enhancing sustainability and reducing waste [47,48].

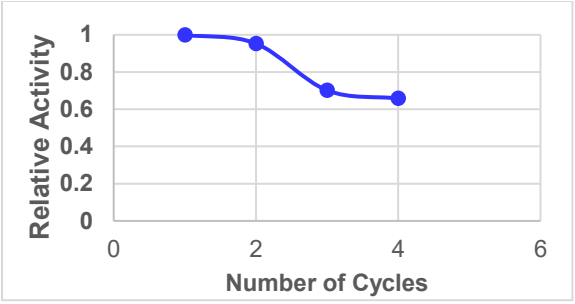

Figure 5: Effect of reusability on the activity

3.7 Storage stability of the free and the immobilized α-amylase

Table 4 presents the enzyme activities of both free and immobilized enzymes stored at 4°C and room temperature over time. The data indicate that after 20 days, the free enzyme showed a 10% decrease in activity, whereas the immobilized enzyme only experienced a 2% decline when stored at 4°C. When both enzyme types were stored at room temperature, the free enzyme exhibited a more significant loss of activity, with a 29.5% decrease, compared to just 7% for the immobilized enzyme. These results underscore the protective effects of enzyme immobilization, particularly at higher temperatures, where enzyme denaturation and activity loss are more pronounced.

Table 4: Storage stability of free and immobilized α-amylase

(A)

Days	**Enzyme activity at 4°C, umol/min**	
	Free	Immobilized
0	1.42,581	101,965
25	127,793	99,666

(B)

Days	**Enzyme activity at 25°C, umol/min**	
	Free	Immobilized
0	142,581	101,965
25	100,516	94,771

The porous structure of the activated carbon support material likely plays a role in improving the

enzyme-substrate interaction, offering a more favorable environment for efficient starch breakdown. This is consistent with previous studies, such as those reported by Zaidi *et al*., 2022 [49], which demonstrated that immobilized enzymes, including lipases, often exhibit higher catalytic activity compared to their free counterparts. The protective and stabilizing effects of immobilization are not limited to starch hydrolysis. A study by (Mateo et al., 2007 [50] on the immobilization of alpha-amylase on various polymeric supports found significant improvements in both enzyme stability and activity under diverse operational conditions. This reinforces the idea that immobilization, particularly on stable and porous supports like activated carbon, not only protects enzymes from environmental stresses but also enhances their overall catalytic efficiency.

These findings suggest that enzyme immobilization, particularly on materials like activated carbon, offers a dual advantage: it not only protects the enzyme from degradation during storage at various temperatures but also boosts its catalytic efficiency, especially in processes like starch hydrolysis. By maintaining enzyme activity over extended periods and under varying conditions, immobilization provides a cost-effective and sustainable solution for industrial applications, reducing the need for frequent enzyme replenishment while maximizing the overall reaction rate.

IV. CONCLUSION

Alpha-amylase, derived from local cereal was successfully immobilized on activated carbon produced from coconut husk, an agricultural waste. The optimal conditions of temperature and pH changed slightly upon immobilization which may be advantageous for the processing of different substrates.

By immobilizing the enzyme, it could be re-used up to 4 times without very significant loss in activity and its storage life at room temperature was significantly extended. We believe that further optimization of the immobilization process could further improve enzyme longevity and performance, offering even greater benefits for industrial biocatalysis.

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