BILINGUAL PUBLISHING GROUP

Journal of Environmental & Earth Sciences

https://journals.bilpubgroup.com/index.php/jees

ARTICLE

Eco-Friendly Amylase Production and Immobilization on Macadamia-Based Carbon Using *Aspergillus niger*

Siswoyo [1] **, *Farah Salma Elida*** [2] **, *Purwatiningsih*** [2] **, *Sutoyo*** [2] **, *Bambang Trianto*** [3]**, *Rudju Winarsa*** [2] **, *Andre Krestianto*** [3]**, *Kahar Muzakhar*** [2*]

[1] *Chemistry Department, Faculty of Mathematics and Natural Sciences, Jember University, Jember, 68121, Indonesia*

[2] *Biology Department, Faculty of Mathematics and Natural Sciences, Jember University, Jember, 68121, Indonesia*

[3] *PT Perkebunan Nusantara I Regional 5, Blawan Plantation Unit, Kali Anyar Village, Ijen Subdistrict, Bondowoso, Indonesia*

ABSTRACT

This study demonstrates the valorization of macadamia nutshells, a lignocellulosic agricultural waste, as both a carbon source for amylase production and a support matrix for enzyme immobilization. Under optimized solid-state fermentation conditions, *Aspergillus niger* ICP2 synthesized amylase with a peak activity of 0.312 U/mL after 72 hours. A four-step purification process of the crude enzyme extract resulted in a 188.54-fold increase in specific activity, albeit with a final recovery yield of 0.0031%. In parallel, nutshells were carbonized at 600 °C, 700 °C, and 800 °C, then chemically activated with $ZnCl_2$. The carbon derived at 700°C exhibited superior physicochemical characteristics, including enhanced porosity and increased availability of functional groups, which enabled effective enzyme adsorption, improved catalytic performance, and enhanced reusability. Immobilized amylase on this support retained approximately 30% of its initial activity after five hydrolysis cycles, demonstrating moderate operational reusability and potential for repeated use in bioprocesses. In contrast, carbon materials from 600 °C and 800 °C showed lower stability and enzyme performance. These findings highlight the critical role of carbonization conditions in designing effective immobilization matrices and underscore the potential of macadamia nutshells as a renewable and sustainable resource for biocatalyst development.

*CORRESPONDING AUTHOR:

Kahar Muzakhar, Biology Department, Faculty of Mathematics and Natural Sciences, Jember University, Jember, 68121, Indonesia; Email: kaharmzk@unej.ac.id

ARTICLE INFO

Received: 4 June 2025 | Revised: 12 June 2025 | Accepted: 17 June 2025 | Published Online:30 June 2025
DOI: https://doi.org/10.30564/jees.v7i7.10349

CITATION

Siswoyo, Elida, F.S., Purwatiningsih., et al., 2025. Eco-friendly amylase production and immobilization on macadamia-based carbon using *Aspergillus niger*. Journal of Environmental & Earth Sciences. 7(7): 73–85. DOI: https://doi.org/10.30564/jees.v7i7.10349

This "biowaste-to-biocatalyst" strategy exemplifies a circular bioeconomy model with implications for green chemistry, industrial biocatalysis, and environmental sustainability.

Keywords: Macadamia Nutshell Waste; Activated Carbon; Amylase; Immobilization; Reusability

1. Introduction

Macadamia nutshells, a widely abundant by-product of agricultural processing, are primarily composed of cellulose, hemicellulose, and lignin, with cellulose serving as the principal contributor to their remarkable mechanical strength[1–3], a characteristic that has traditionally confined them to low-value applications. However, recent advances in research have illuminated the untapped potential of macadamia nutshells for more advanced and commercially viable uses, including their role as carriers for pesticides[4, 5], abrasives[6, 7], and paint thickeners[8, 9], thereby expanding the scope of their industrial applications. Beyond these novel uses, the considerable abundance of structural polysaccharides in macadamia nutshells offers an enticing opportunity to repurpose this underutilized agricultural waste as a low-cost, renewable substrate for the production of industrial enzymes[10], aligning with the growing need for sustainable and environmentally responsible biotechnological processes.

Macadamia nutshells, a non-edible lignocellulosic waste, offer a sustainable and cost-effective alternative to chitosan for enzyme immobilization. Unlike chitosan, which requires energy-intensive deacetylation[11, 12], carbonized macadamia nutshells are more eco-friendly and readily available. They exhibit superior mechanical strength and thermal stability, making them better suited for reuse in harsh industrial[2, 13] conditions where chitosan may degrade at elevated temperatures[14–16]. In this study, we demonstrate that macadamia nutshells derived from *Macadamia integrifolia* not only serve effectively as a substrate for amylase production by the fungus *A. niger* through solid-state fermentation but also as a precursor for the generation of activated carbon, which, in turn, functions as an efficient support material for the immobilization of the amylase enzyme, enhancing its stability and reusability for potential industrial applications.

2. Materials and Methods

2.1. Macadamia Nutshells as a Source for Substrate in Amylase Production and for Activated Carbon Preparation

Macadamia integrifolia nutshell waste, obtained from the Macadamia Plantation of PTPN I Blawan in Kalianyar Village, Bondowoso Regency, Indonesia, was utilized both as a substrate for amylase production and as a source material for the preparation of activated carbon used in enzyme immobilization. The nutshell waste was pulverized to a fine powder (50–200 mesh) to serve as the fermentation substrate, while activated carbon was produced through specific treatments as detailed in the following methodology.

2.2. Maintenance of Amylolytic Fungal Strain *A. niger* ICP2 and its Amylase Production

Amylase production was carried out using the amylolytic fungal strain *A. niger* ICP2, an indigenous filamentous fungus isolated from coffee pulp waste collected in East Java, Indonesia. This strain was selected based on its demonstrated potential to produce extracellular amylase in preliminary screening assays. For routine inoculum maintenance and preparation of working culture stocks, *A. niger* ICP2 was sub-cultured on Potato Dextrose Agar (PDA) medium and incubated at 30 °C for 3 days to promote optimal mycelial growth and sporulation. The resulting fungal biomass and spores were subsequently used as the inoculum source for the submerged fermentation process in amylase production. Amylase production was conducted using the solid-state fermentation (SSF) method. The SSF medium (100 g total substrate), prepared in a 2000 mL bottle flask, consisted of sterilized macadamia nutshell powder and distilled water in a 2:3 (w/v) ratio, without the addition of external nutrients or minerals. The initial pH of the medium was approximately 6.8. After sterilization, the medium was inoculated with 1 mL

of a spore suspension of *A. niger* ICP2 at a concentration of 1.33 x 10^8 spores/mL, using a substrate-to-inoculum volume ratio of 10:1 (v/v)[17]. Fermentation was carried out at 37 °C for 4 days to determine the optimal incubation period for maximum amylase production. Enzyme activity was monitored at 24-hour intervals throughout the incubation period. To recover the crude amylase produced under SSF conditions, Natrrium Chloride (NaCl) extraction was performed by adding a solution containing 0.01% sodium azide and 1% NaCl to the fermented substrate at a 1:1 volume ratio (v/v), followed by shaking for 6 hours. The solid residue was then separated from the liquid phase by centrifugation at 4000 *g* for 10 minutes. The resulting supernatant, containing the crude amylase, was filtered through a 40 μm membrane and stored at 4 °C for further analysis.

2.3. Amylase Purification

Amylase purification began with ammonium sulfate precipitation. One milliliter of crude enzyme extract was subjected to fractional precipitation using ammonium sulfate at saturation levels ranging from 20% to 100%. Each mixture was homogenized and incubated on ice for 30 minutes, followed by centrifugation at 13000 rpm for 20 minutes. The resulting pellets were resuspended in 20 mM acetate buffer (pH 5.0). The optimal saturation level for amylase recovery was determined based on enzyme activity assays and subsequently applied to a 500 mL volume of crude extract. The precipitate was then dissolved in 10 mL of the same acetate buffer and dialyzed using a 10–12 kDa molecular weight cut-off cellulose membrane to remove residual ammonium sulfate. Completion of dialysis was confirmed by the absence of ammonium ions (NH_4^+), as indicated by Nessler's reagent.

Further purification was performed using a Mono Q HR 5/5 anion exchange column (Pharmacia), pre-equilibrated with the same acetate buffer. Elution was carried out via fast protein liquid chromatography (FPLC) using a linear NaCl gradient from 0 to 0.5 M. Active fractions containing purified amylase were pooled, dialyzed again against acetate buffer, and subjected to a final polishing step using gel filtration chromatography with a Pharmacia Superdex 75 column. The purified amylase was stored at 4 °C for subsequent use in enzyme immobilization.

2.4. Amylase Activity Assay

Amylase activity was quantified using the blue value method, based on the starch-iodine color reaction[18, 19] with a few modifications. Iodine forms a deep blue complex with intact starch, and as amylase hydrolyzes starch, the intensity of this color decreases. The enzyme was incubated with a 1% starch solution, and the reaction was stopped with hydrochloric acid. An iodine reagent (1% KI and 0.1% I_2) was added in a 1:1 ratio, and absorbance was measured at 580 nm. A standard curve using known starch concentrations was used to determine residual starch. Amylase activity was expressed in units per milliliter (U/mL), where one unit is defined as the amount of enzyme that hydrolyzes 1 mg of starch per minute. Activity was calculated using **Equation (1)**:

$$\frac{U}{mL} = \frac{(C_{Control} - C_{Sample}) \times V_t}{t \times V_e} \quad (1)$$

where C is starch concentration (mg/mL), V_t is total volume, t is time (min), and V_e is enzyme volume (mL).

2.5. Synthesis of Activated Carbon from Macadamia Nutshells

Activated carbon derived from macadamia nutshells was synthesized through two steps: carbonization and chemical activation. Dried macadamia nutshells were carbonized in a furnace at 600 °C, 700 °C, and 800 °C for 1 hour. The resulting carbon was pulverized and sieved to a 60-mesh particle size. For activation, the carbon was soaked in a 1 M zinc chloride ($ZnCl_2$)[20] solution at a 1:10 (g/mL) ratio at 80 °C for 2 hours, then dried at 120 °C for 12 hours. The activated carbon was subsequently treated with 0.5 M HCl for 12 hours on a shaker to remove residual activating agents, followed by drying at 120 °C for another 12 hours. Finally, the carbon was washed with distilled water until the pH reached approximately 6.7, and then dried once more at 120 °C for 12 hours.

2.6. Immobilization of Amylase

Amylase immobilization was carried out using macadamia nutshell-based activated carbon, which was carbonized at different temperatures: 600 °C, 700 °C, and 800 °C

as described above. First step immobilization, the amylase was diluted in 20 mM acetate buffer (pH 5) at a 1:1 (v/v) ratio, and its concentration was measured by absorbance at 280 nm. Immobilization was performed by suspending the activated carbon in the enzyme solution at a ratio of 1:8 (g/mL), followed by shaking at 100 rpm at room temperature for 4 hours. Then, the suspension was allowed to stand for 30 minutes, and the remaining enzyme in the supernatant that was not adsorbed onto the activated carbon was measured again at 280 nm. After this step, the supernatant was removed, and the activated carbon containing amylase was washed with 20 mM acetate buffer (pH 5) to remove any non-adsorbed enzyme. The amount of enzyme bound or adsorbed onto the activated carbon, reflecting the immobilized amylase, was determined by subtracting the residual enzyme in the supernatant from the initial amount of enzyme.

2.7. Reusability Evaluation of Immobilized Amylase

The reusability analysis of immobilized amylase was performed by evaluating its relative hydrolytic activity, expressed as a percentage, against a 1% starch substrate in 20 mM acetate buffer (pH 5). The assay involved adding a defined amount of immobilized enzyme to the starch solution at a ratio of 1:8 (g/mL). Hydrolytic activity was assessed using the blue value method as previously described, and the procedure was repeated over five consecutive cycles to evaluate the stability and reusability of the enzyme. After each cycle, the immobilized enzyme was washed and re-equilibrated with the same acetate buffer before reuse.

3. Results and Discussion

The utilization of macadamia nutshell waste as both a substrate for microbial amylase production and a precursor for activated carbon-based adsorbent used in enzyme immobilization presents a multifaceted and innovative approach to sustainable biotechnology. This dual-purpose strategy not only contributes to waste minimization by valorizing an abundant agricultural byproduct, but also enhances the efficiency and sustainability of bioprocesses through the integration of low-cost, renewable materials. In the present study, this concept has been successfully demonstrated, showing that macadamia nutshell waste can serve as an effective carbon source for the production of amylase under SSF conditions, while the same biomass can be thermochemically converted into activated carbon for enzyme immobilization. These findings support the broader application of agro-industrial residues in circular bioeconomy frameworks, where waste streams are transformed into high-value biotechnological inputs, thereby promoting environmentally friendly and economically viable alternatives for industrial biotechnology.

3.1. Amylase Production

Macadamia nutshell waste showed considerable potential as a substrate for amylase production under SSF. The successful use of macadamia nutshell waste for amylase production under SSF addresses a notable gap in the literature. No prior studies have reported this application, making the findings both novel and significant. As shown in **Figure 1**, *A. niger* ICP2 produced amylase effectively when cultivated on this lignocellulosic substrate. Enzyme production began 24 hours after inoculation and reached its peak on the third day of incubation, with an activity of 0.312 U/mL. This peak suggests that the macadamia nutshells provided sufficient nutrients and a favorable environment to support fungal growth and enzyme synthesis during the early stages of fermentation, confirming their suitability as a carbon source in SSF systems. However, a sharp decline in amylase activity was observed on the fourth day, ultimately resulting in no detectable enzyme activity in subsequent days. This decrease is likely attributed to nutrient depletion [21–25] and the accumulation of metabolic by-products as previously reported, which may exert inhibitory effects on fungal metabolism and interfere with amylase biosynthesis [26, 27]. Such patterns are commonly observed in SSF processes, where prolonged incubation often leads to environmental stress within the fermentation matrix, thereby reducing microbial efficiency and overall enzyme productivity [23–25, 28].

The success of amylase production in this study aligns with previous reports demonstrating the ability of *Aspergillus* species to synthesize hydrolytic enzymes from various agro-industrial residues under SSF conditions [29–31]. In particular, substrates such as coffee pulp, coffee husk, and other lignocellulosic wastes have been effectively utilized to support the growth and enzymatic activity of these fungi [32–36]. The comparable performance of macadamia nutshell waste ob-

served in this study reinforces its potential as a viable and value-added substrate for microbial fermentation. Typically, discarded or underutilized, macadamia nutshells represent an abundant agricultural by-product that can be repurposed for enzyme production, contributing to both waste minimization and resource circularity.

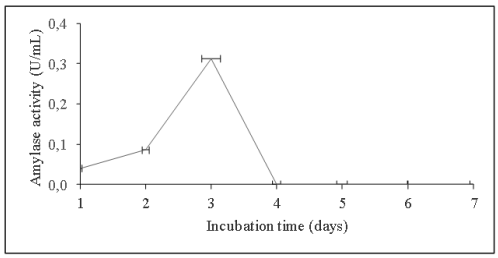

Figure 1. Time-course of amylase production by *A. niger* ICP2 cultivated on macadamia nutshell waste under SSF at 37 °C. Enzyme activity (U/mL) was measured at 24-hour intervals over a 7-day incubation period to determine the optimal production time. The highest amylase activity, 0.312 U/mL, was observed on day 3.

This approach not only supports environmental sustainability by reducing organic waste but also enables the development of cost-effective bioprocesses suitable for regions rich in agricultural biomass. Given the promising results with macadamia nutshells and the proven success of other substrates like coffee pulp [33, 36], future research should focus on optimizing fermentation parameters such as moisture content, pH, substrate particle size, and inoculum concentration to further enhance enzyme yields and improve process efficiency. Such efforts would solidify the role of agriculture-waste-based SSF in sustainable enzyme biotechnology.

3.2. Amylase Purification

The purification of amylase from *A. niger* ICP2 was achieved through a sequential process involving ammonium sulfate precipitation, dialysis, anion exchange chromatography (Mono Q), and gel filtration (Superdex 75) (**Table 1**). The crude extract contained 345,670 mg of total protein with 34,523 U of enzymatic activity, yielding a specific activity of 0.10 U/mg. Ammonium sulfate precipitation at optimized saturation significantly reduced the total protein to 7,745 mg with minimal activity loss (31.405 U), enhancing the specific activity to 4.05 U/mg and achieving a purification fold of 40.60. Dialysis further removed residual salts, raising the specific activity to 5.27 U/mg. This was followed by anion exchange chromatography, which enriched the enzyme fraction to a specific activity of 15.56 U/mg. Final purification using gel filtration chromatography increased the specific activity to 18.83 U/mg, culminating in a 188.54-fold purification.

Table 1. Stepwise Purification Profile of the Amylase Enzyme.

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude Extract	345,670	34,523	0.10	100	1
Ammonium Sulfate Precip.	7,745	31,405	4.05	0.02	40.60
Dialysis	5,402	28,452	5.27	0.02	52.74
Mono-Q Anion Exchange	1,436	22,341	15.56	0.0042	155.78
Superdex 75 Gel Filtration	1,075	20,242	18.83	0.0031	188.54

Purifying amylase prior to immobilization is essential for achieving high immobilization efficiency, catalytic performance, and long-term reusability of the biocatalyst. Crude enzyme extracts often contain a mixture of interfering proteins, nucleic acids, lipids, phenolic compounds, and other secondary metabolites, which can nonspecifically adsorb to the immobilization matrix [37–39]. These impurities may alter the binding efficiency or occupy functional groups on the support surface, reducing the number of accessible sites for the target enzyme [40–42]. Moreover, proteolytic enzymes in the crude extract can degrade the immobilized enzyme over time, negatively impacting reusability and storage stability [43–45]. From a structural biology perspective, enzyme immobilization depends on the availability and reactivity of surface residues such as lysines, carboxyl groups, and hydroxyl groups. The presence of contaminating proteins or polymers can sterically hinder or mask these reactive groups, leading to random orientation or weak binding, which ultimately decreases catalytic efficiency [46–49]. A purified enzyme allows for a controlled and predictable interaction with the support matrix, ensuring reproducible activity profiles and higher recovery of functional enzyme post-immobilization [37, 38, 50–53]. In addition, impurities may cause diffusional limitations in porous support materials, block active sites, or contribute

to non-productive binding, all of which lower the apparent activity of the immobilized enzyme. Purified enzymes show better mass transfer properties and substrate accessibility due to uniform binding orientation and minimal steric interference[54, 55]. Furthermore, immobilization onto activated carbons or charged surfaces relies on electrostatic and hydrophobic interactions; these interactions are highly sensitive to protein composition and purity[56].

Therefore, purification not only maximizes the performance of the immobilized enzyme system but also aligns with industrial and regulatory standards for enzyme-based bioprocesses, particularly in the food, pharmaceutical, and biofuel sectors where product purity and enzyme reuse are critical.

3.3. Effect of Carbonization Temperature and $ZnCl_2$ Activation on Amylase Immobilization Efficiency

Carbonization temperature is a critical factor influencing the development of pore structure and the adsorption performance of carbon materials[57].

Table 2. Residual amylase concentration (%) measured at 280 nm in the supernatant after immobilization using non-activated and $ZnCl_2$-activated macadamia nutshell-derived carbon at various carbonization temperatures.

Carbonization Temperature (°C)	Residual Amylase Activated (%) in Supernatant	
	Non-Activated	Activated
600	67	36
700	19	0
800	76	18

As shown in **Table 2**, carbonization at 700 °C resulted in the highest amylase adsorption capacity, attributed to the formation of an optimal porous architecture. The influence of chemical activation using $ZnCl_2$ was also evaluated. It has been reported that, for enzyme immobilization to be effective, the carbon-based support material must first undergo activation to enhance essential surface properties particularly surface area, porosity, and the density of functional groups capable of interacting with enzymes. This activation process, which can be carried out through chemical or physical methods, introduces reactive groups, such as carboxyl, hydroxyl, or amino functionalities, onto the carbon surface. These groups facilitate the formation of covalent bonds or strong non-covalent interactions between the enzyme and the support, thereby improving both immobilization efficiency and enzyme stability[56, 58–61].

Activated and functionalized carbon has demonstrated the ability to increase enzyme loading capacity to over 99%, while preserving enzymatic activity close to its native state. Moreover, it significantly extends the operational stability of the enzyme during repeated use cycles[56, 58, 61]. When further functionalized with crosslinking agents such as glutaraldehyde or with metal ions, the carbon support can provide more specific and stable attachment sites for enzymes, thereby reducing the likelihood of enzyme leaching throughout the process[56, 59, 61].

The results demonstrated that carbon activation markedly enhances enzyme immobilization. Across all tested carbonization temperatures (600 °C, 700 °C, and 800 °C), $ZnCl_2$-activated carbon consistently exhibited superior adsorption efficiency compared to its non-activated counterpart. At 600 °C, the residual amylase concentration was 67% for non-activated carbon and decreased to 36% for $ZnCl_2$-activated carbon. The extent of carbonization in carbon-based support materials, such as activated carbon or carbon nanotubes, plays a crucial role in determining their suitability for enzyme immobilization. Low carbonization levels typically result in a limited number of adsorption sites, reduced surface area, and a lower density of functional groups. These factors diminish the material's capacity to effectively bind enzymes during immobilization, leading to poor enzyme loading and weak interaction between the enzyme and support[56, 62]. Consequently, insufficiently carbonized materials often yield biocatalyst systems with lower efficiency and stability. Optimizing the carbonization process is therefore essential to enhance the structural and chemical properties of the support and to improve enzyme immobilization performance[56, 62]. Notably, at 700 °C, non-activated carbon retained 19% of the amylase, while no residual amylase was detected in the solution with activated carbon, indicating complete adsorption and highly efficient immobilization under these conditions. This finding highlights 700 °C carbonization combined with $ZnCl_2$ activation as the optimal condition for enzyme immobilization.

However, further increasing the carbonization temperature to 800 °C led to a noticeable decline in adsorption efficiency, as indicated by the elevated residual amylase concentrations, 76% for non-activated carbon and 18% for $ZnCl_2$-

activated carbon. This reduction in performance is likely due to thermal degradation of the carbon structure, which may involve pore collapse or a loss of surface functional groups essential for enzyme attachment. Consistent with these findings, several studies have reported that while increasing the carbonization temperature can enhance the adsorption efficiency of carbon materials, this improvement occurs only up to an optimal temperature. Beyond this threshold, excessively high temperatures can induce structural deterioration, compromising the material's adsorption capacity. Studies on materials such as graphene oxide and biochar have demonstrated that extreme temperatures can cause surface deformation of the support and enzyme denaturation, resulting in a significant decline in adsorption capacity [63, 64]. In addition, high temperatures may accelerate enzyme desorption from the support due to weakened physical or chemical interactions between the enzyme and the material [65]. Research has shown that carbonization within the range of 500 °C to 1000 °C, depending on the precursor biomass, typically yields carbon with high surface area and well-developed porosity, both of which are critical for effective adsorption [57, 66–68]. In contrast, carbonization at temperatures exceeding this optimal range, such as 1200 °C, has been shown to cause pore shrinkage or collapse and a significant reduction in surface functional groups, ultimately leading to diminished adsorption efficiency [57, 66, 68]. These observations emphasize the importance of optimizing carbonization temperature to balance structural integrity and functional group availability for effective biomolecule immobilization.

Overall, these results confirm that $ZnCl_2$ activation, particularly when applied to macadamia nutshell carbonized at 700 °C significantly enhances the material's capacity for amylase immobilization. This indicates that, with proper thermal and chemical treatment, macadamia nutshell-derived carbon serves as a promising, sustainable support matrix for enzyme immobilization applications.

3.4. Reusability of Immobilized Amylase

Current literature shows no studies on using macadamia nutshell biomass as a matrix for amylase immobilization or its reusability. While supports like lignin, chitosan, banana peel cellulose, and gellan hydrogels show high reuse potential (up to 11–12 cycles), nutshells remain unexplored [69–72]. Synthetic materials such as nanoparticles and polymer composites have been studied [69, 71, 73–75], but lignocellulosic hard-shell biomass like macadamia, despite its strength, stability, and abundance, has not been utilized. This gap presents a promising direction for developing low-cost, sustainable biocatalyst supports.

The reusability of immobilized enzymes plays a pivotal role in determining their suitability for industrial applications, especially in processes involving multiple cycles of catalysis. In this work, the catalytic stability of amylase immobilized on $ZnCl_2$-activated carbon derived from macadamia nutshells was investigated over five successive hydrolysis cycles. The carbon supports were prepared by carbonizing the biomass at three different temperatures: 600 °C, 700 °C, and 800 °C, to assess how thermal treatment affects support durability and enzyme retention. Enzyme activity was measured after each cycle to evaluate performance changes over time. The data, summarized in **Table 3**, show enzyme activity (U/mL) for each reuse cycle, while **Figure 2** illustrates the residual enzyme activity expressed as a percentage of the initial activity, facilitating a clear comparison of the immobilization efficiency across treatments.

Table 3. Activity (U/mL) of immobilized amylase on activated carbon prepared at different carbonization temperatures (°C).

Cycle	Activity (U/ml) of Immobilized Amylase at Carbonized Different Temperature (°C)		
	600	700	800
1	0.53	0.71	0.52
2	0.38	0.49	0.40
3	0.24	0.31	0.00
4	0.07	0.23	0.00
5	0.08	0.21	0.00

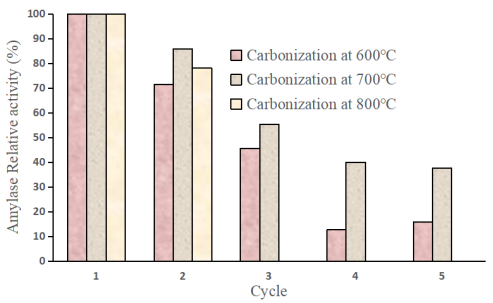

Figure 2. Residual amylase activity (%) over reuse cycles. The optimum carbonized support (700 °C) retained 85.9% activity in Cycle 2, followed by 55.3%, 40.0%, and 37.6% in Cycles 3–5.

In the first cycle, all immobilized enzyme systems demonstrated full activity (100%), indicating successful immobilization and initial compatibility of the enzyme with all

three support types. However, distinct performance trends emerged over the subsequent cycles. The carbon support carbonized at 700 °C showed superior reusability, retaining 85.9% of its initial activity in Cycle 2, followed by 55.3%, 40.0%, and 37.6% in Cycles 3 to 5, respectively. This progressive decline suggests that while some enzyme leaching or conformational instability may occur, the carbon matrix carbonized at this temperature maintains adequate structural integrity and functional surface chemistry to stabilize the enzyme over multiple uses. The relatively high retention even after five cycles highlights the favorable porous architecture and appropriate balance of hydrophilic and hydrophobic interactions afforded by the 700 °C treatment.

By contrast, the 600 °C carbon support, although performing well in Cycle 1, showed a sharper decline with only 71.5% activity remaining in Cycle 2, dropping further to 45.6% in Cycle 3, and eventually reaching 12.7% and 15.8% in Cycles 4 and 5, respectively. Carbonization at insufficient or low temperatures tends to produce carbon with poorly developed porosity and limited surface functionality, which negatively impacts its ability to effectively immobilize enzymes [56, 58, 76]. Such materials offer weaker interactions between the enzyme and the support and provide minimal structural protection against denaturing environmental factors. Consequently, enzymes immobilized on low-temperature carbonized supports often exhibit poor stability, with a high tendency to leach or lose activity during repeated usage [58, 76]. The significant reduction in enzymatic activity across multiple cycles suggests rapid deterioration of the immobilization matrix, likely due to inadequate mechanical and chemical stability resulting from insufficient carbonization [56, 58, 76]. In contrast, carbon supports with well-formed pore architecture and effective surface activation have been shown to improve enzyme retention, maintain catalytic function, and support long-term operational use [56, 58]. Therefore, suboptimal carbonization conditions not only limit the adsorption and binding capacity of the material but also compromise the functional longevity of immobilized enzyme systems [56, 58, 76].

The immobilized enzyme on carbon prepared at 800 °C initially retained 78.1% of its activity in Cycle 2 but exhibited a complete loss of detectable activity in Cycles 3 through 5. This steep decline suggests that over-carbonization may have caused excessive pore collapse or volatilization of functional groups necessary for enzyme anchoring. The highly graphitized and possibly hydrophobic nature of the support at this temperature may have led to poor enzyme adhesion, structural instability, and increased enzyme leaching or inactivation. The lack of reusability beyond two cycles renders this treatment unsuitable for industrial applications requiring consistent enzyme performance over multiple batches.

These results collectively demonstrate that the physicochemical properties of activated carbon, strongly influenced by the carbonization temperature, play a critical role in determining the immobilized enzyme's operational stability. Carbonization at 700 °C appears to offer an optimal compromise between adequate porosity and surface chemistry for durable enzyme immobilization. This thermal treatment enables a sufficient surface area and pore volume to accommodate enzyme molecules while preserving enough polar functional groups to facilitate covalent or non-covalent interactions that enhance enzyme retention. Furthermore, $ZnCl_2$ activation enhances microporosity and provides additional binding sites, further supporting the enzyme structure during repeated catalysis cycles.

4. Conclusion

This study highlights the comprehensive valorization of macadamia nutshells (*Macadamia integrifolia*), a lignocellulosic agricultural waste, as a sustainable resource for biocatalyst development. Through solid-state fermentation using *Aspergillus niger*, the nutshells served as an effective substrate for amylase production, achieving optimal enzyme activity within 72 hours. Simultaneously, the same biomass was transformed into activated carbon supports through controlled carbonization (600 °C, 700 °C, and 800 °C), followed by $ZnCl_2$ chemical activation. Among the tested conditions, activated carbon derived at 700 °C exhibited the most favorable characteristics enhanced porosity, abundant functional groups, and structural integrity, resulting in the most effective enzyme immobilization. Amylase immobilized on this support retained over 30% of its initial activity after five catalytic cycles, indicating notable operational stability and reusability. Importantly, this dual-purpose application of macadamia nutshells not only demonstrates their effectiveness in supporting enzyme biosynthesis but also establishes their utility as precursors for high-performance immobilization matrices.

This integrated approach reflects a sustainable "biowaste-to-biocatalyst" strategy, aligning with circular bioeconomy principles by converting agricultural residues into value-added products for industrial biotechnology. Moreover, the success of this system with amylase suggests potential applicability for other industrial enzymes, paving the way for broader bioprocessing applications. Future research should focus on scaling this approach, optimizing immobilization conditions for various enzymes, and evaluating long-term performance in continuous systems to fully realize its industrial potential.

Author Contributions

Conceptualization, K.M.; methodology, K.M, S., F.S.E., S., R.W, B.T. and A.K.; software, K.M. and F.S.E.; validation, S., S., F.S.E. and K.M.; formal analysis, F.S.E. and K.M.; investigation, F.S.E, R.W., S. and K.M.; resources, K.M., B.T. and A.K.; data curation, F.S.E. and K.M.; writing—original draft preparation, F.S.E. and K.M.; writing—review and editing, F.S.E. and K.M.; supervision, K.M.; project administration, F.S.E.; funding acquisition, K.M. All authors have read and agreed to the published version of the manuscript.

Funding

This research is part of a funded project under the 2024 Hibah Penelitian Produktivitas Guru Besar (Professor Research Productivity Grant) scheme at Jember University.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data supporting this study will be provided by the corresponding author upon reasonable request.

Acknowledgments

We gratefully acknowledge Djoko Andi P. Prasetija of PT. SADYA BALAWAN (SABA), a company specializing in chemicals and enzyme/bioproducts for industrial applications, located in Kawasan Industri Tengsaw, Jl. Alternatif Tarikolot-Sukahati, Citeureup Bogor, West Indonesia, 16810, for his valuable technical support and the generous provision of in-kind materials essential to this research.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

[1] Ali, S.A., Sonego, M., Salavati, M., et al., 2024. Influence of structure and geometry on the compressive deformation behavior of Macadamia integrifolia and Bertholletia excelsa shells: A validated finite element simulation study. Advanced Engineering Materials. 26(4), 1-15. DOI: https://doi.org/10.1002/adem.202300723

[2] Cortat, L.O., Zanini, N.C., Barbosa, R.F.S., et al., 2021. A sustainable perspective for macadamia nutshell residues revalorization by green composites development. Journal of Polymers and the Environment. 29(10), 3210-3226. DOI: https://doi.org/10.1007/s10924-021-02080-y

[3] Khan, M.S., Islam, M.M., Epaarachchi, J., et al., 2023. Exploring the prospects of macadamia nutshells for bio-synthetic polymer composites: a review. Polymers. 15(19), 4007. DOI: https://doi.org/10.3390/polym15194007

[4] Pakade, V.E., Maremeni, L.C., Ntuli, T.D., et al., 2016. Application of quaternized activated carbon derived from macadamia nutshells for the removal of hexavalent chromium from aqueous solutions. South African Journal of Chemistry. 69, 88-97. DOI: https://doi.org/10.17159/0379-4350/2016/v69a22

[5] Wu, C., Zhang, G., Liu, J., et al., 2022. Influence of the interaction between activation conditions on the pore structure and CO_2 uptake of the prepared macadamia nutshell-based activated carbon. International Journal of Energy Research. 46(12), 17204-17219. DOI: https://doi.org/10.1002/er.8385

[6] Dao, T.M., Luu, T.L., 2020. Synthesis of activated carbon from macadamia nutshells activated by H_2SO_4 and K_2CO_3 for methylene blue removal in water. Bioresource Technology Reports. 12, 100583. DOI: https://doi.org/10.1016/j.biteb.2020.100583

[7] Gong, Y., Li, S., Hu, Y., et al., 2025. Acidic and alkaline deep eutectic solvents pretreatment of macadamia

nutshells for production of cellulose nanofibrils and lignin nanoparticles. International Journal of Biological Macromolecules. 300, 140251. DOI: https://doi.org/10.1016/j.ijbiomac.2025.140251

[8] Pezzana, L., Emanuele, A., Sesana, R., et al., 2023. Cationic UV-curing of isosorbide-based epoxy coating reinforced with macadamia nut shell powder. Progress in Organic Coatings. 185, 107949. DOI: https://doi.org/10.1016/j.porgcoat.2023.107949

[9] Zhou, J., He, Y., Huang, L., et al., 2024. Preparation of magnetic biochar from macadamia nutshell pretreated by $FeCl_3$-assisted mechanochemical activation for adsorption of heavy metals. Journal of Environmental Chemical Engineering. 12(4), 113122. DOI: https://doi.org/10.1016/j.jece.2024.113122

[10] Shi, R., Tao, L., Tu, X., et al., 2022. Metabolite profiling and transcriptome analyses provide insight into phenolic and flavonoid biosynthesis in the nutshell of Macadamia ternifolia. Frontiers in Genetics. 12, 809986. DOI: https://doi.org/10.3389/fgene.2021.809986

[11] Sjoholm, K.H., Cooney, M., Minteer, S.D., 2009. Effects of degree of deacetylation on enzyme immobilization in hydrophobically modified chitosan. Carbohydrate Polymers. 77(2), 420-424. DOI: https://doi.org/10.1016/j.carbpol.2009.02.006

[12] Bai, Y., Jing, Z., Ma, R., et al., 2023. A critical review of enzymes immobilized on chitosan composites: characterization and applications. Bioprocess and Biosystems Engineering. 46(11), 1539-1567. DOI: https://doi.org/10.1007/s00449-023-02914-0

[13] Silva, N.G.S., Cortat, L.I.C.O., Mulinari, D.R., 2021. Effect of alkaline treatment and coupling agent on thermal and mechanical properties of macadamia nutshell residues based PP composites. Journal of Polymers and the Environment. 29(10), 3271-3287. DOI: https://doi.org/10.1007/s10924-021-02112-7

[14] Maria de Medeiros Dantas, J., Sousa da Silva, N., Eduardo de Araújo Padilha, C., et al., 2020. Enhancing chitosan hydrolysis aiming chitooligosaccharides production by using immobilized chitosanolytic enzymes. Biocatalysis and Agricultural Biotechnology. 28, 101759. DOI: https://doi.org/10.1016/j.bcab.2020.101759

[15] Yandri, Y., Ropingi, H., Suhartati, T., et al., 2022. The effect of zeolite/chitosan hybrid matrix for thermal-stabilization enhancement on the immobilization of Aspergillus fumigatus α-amylase. Emerging Science Journal. 6(3), 505-518. DOI: https://doi.org/10.28991/ESJ-2022-06-03-06

[16] Kaushal, J., Seema, Singh, G., et al., 2018. Immobilization of catalase onto chitosan and chitosan-bentonite complex: a comparative study. Biotechnology Reports. 18, e00258. DOI: https://doi.org/10.1016/j.btre.2018.e00258

[17] Winarsa, R., Sukma, N.A., Afriyanti, D., et al., 2024. Coffee pulp activated carbon for immobilizing cellulase from Aspergillus niger ICP2: enhancing enzyme stability, activity, and its reusability. BIO Web of Conferences. 101, 01002. DOI: https://doi.org/10.1051/bioconf/202410101002

[18] Visvanathan, R., Qader, M., Jayathilake, C., et al., 2020. Critical review on conventional spectroscopic α-amylase activity detection methods: merits, demerits, and future prospects. Journal of the Science of Food and Agriculture. 100(7), 2836-2847. DOI: https://doi.org/10.1002/jsfa.10315

[19] Nolasco-Soria, H., 2021. Amylase quantification in aquaculture fish studies: a revision of most used procedures and presentation of a new practical protocol for its assessment. Aquaculture. 538, 736536. DOI: https://doi.org/10.1016/j.aquaculture.2021.736536

[20] Caturla, F., Molina-Sabio, M., Rodríguez-Reinoso, F., 1991. Preparation of activated carbon by chemical activation with $ZnCl_2$. Carbon. 29(7), 999-1007. DOI: https://doi.org/10.1016/0008-6223(91)90179-M

[21] Premalatha, A., Vijayalakshmi, K., Shanmugavel, M., et al., 2023. Optimization of culture conditions for enhanced production of extracellular α-amylase using solid-state and submerged fermentation from Aspergillus tamarii MTCC5152. Biotechnology and Applied Biochemistry. 70(2), 835-845. DOI: https://doi.org/10.1002/bab.2403

[22] Raul, D., Biswas, T., Mukhopadhyay, S., et al., 2014. Production and partial purification of alpha amylase from Bacillus subtilis (MTCC 121) using solid state fermentation. Biochemistry Research International. 2014, 1-5. DOI: https://doi.org/10.1155/2014/568141

[23] Gois, I.M., Santos, A.M., Silva, C.F., 2020. Amylase from Bacillus sp. produced by solid state fermentation using cassava bagasse as starch source. Brazilian Archives of Biology and Technology. 63, e20170521. DOI: https://doi.org/10.1590/1678-4324-2020170521

[24] Kunamneni, A., Permaul, K., Singh, S., 2005. Amylase production in solid state fermentation by the thermophilic fungus Thermomyces lanuginosus. Journal of Bioscience and Bioengineering. 100(2), 168-171. DOI: https://doi.org/10.1263/jbb.100.168

[25] Sahnoun, M., Kriaa, M., Elgharbi, F., et al., 2015. Aspergillus oryzae S2 alpha-amylase production under solid state fermentation: Optimization of culture conditions. International Journal of Biological Macromolecules. 75, 73-80. DOI: https://doi.org/10.1016/j.ijbiomac.2015.01.026

[26] Ghimire, A., Trably, E., Frunzo, L., et al., 2018. Effect of total solids content on biohydrogen production and lactic acid accumulation during dark fermentation of organic waste biomass. Bioresource Technology. 248, 180-186. DOI: https://doi.org/10.1016/j.biortech.2017.07.062

[27] Manuel, C.R., Carlos, Q.F., Carmen, P.C., et al., 2022.

Fungal solid-state fermentation of food waste for biohydrogen production by dark fermentation. International Journal of Hydrogen Energy. 47(70), 30062-30073. DOI: https://doi.org/10.1016/j.ijhydene.2022.06.313

[28] Hashemi, M., Razavi, S.H., Shojaosadati, S.A., et al., 2010. Development of a solid-state fermentation process for production of an alpha amylase with potentially interesting properties. Journal of Bioscience and Bioengineering. 110(3), 333-337. DOI: https://doi.org/10.1016/j.jbiosc.2010.03.005

[29] Divya, A.S., Chee, C.Y.K., Jer, N.Y., et al., 2025. Optimisation of parameters for the extraction of β-amylase from sweet potato via liquid biphasic floatation. Cleaner Engineering and Technology. 24, 100841. DOI: https://doi.org/10.1016/j.clet.2024.100841

[30] Sun, J.L., Liang, X.H., Jia, Y.J., et al., 2011. Study on water-extracting technology of β-amylase from sweet potato. Advanced Materials Research. 396-398, 1563-1566. DOI: https://doi.org/10.4028/www.scientific.net/AMR.396-398.1563

[31] Yusree, F.I.F.M., Peter, A.P., Zulkifli, N.A., et al., 2022. Towards green recovery of β-amylase from slurry of sweet potato (Ipomoea batatas) of Vitato variety via liquid biphasic system. Sustainable Chemistry and Pharmacy. 25, 100579. DOI: https://doi.org/10.1016/j.scp.2021.100579

[32] Elida, F.S., Azizah, Wiyono, H.T., et al., 2020. Efficiency of cellulase production using coffee pulp waste under solid state fermentation by Aspergillus sp. VT12. Proceedings of The International Conference on Science and Applied Science (ICSAS2020); July 7, 2020; Surakarta, Indonesia. pp. 1-5. DOI: https://doi.org/10.1063/5.0030482

[33] Gasani, O.N., Azizah, A., Siswanto, S., et al., 2021. Pectinase production by using coffee pulp substrate as carbon and nitrogen source. Key Engineering Materials. 884, 165-170. DOI: https://doi.org/10.4028/www.scientific.net/KEM.884.165

[34] Sunarto, N.I., Azizah, A., Utarti, E., et al., 2021. Preliminary investigation of cellulase producer candidate isolate VT11 using coffee pulp waste under solid-state fermentation. Key Engineering Materials. 884, 234-240. DOI: https://doi.org/10.4028/www.scientific.net/KEM.884.234

[35] Rusdianti, R., Azizah, A., Utarti, E., et al., 2021. Cheap cellulase production by Aspergillus sp. VTM1 through solid state fermentation of coffee pulp waste. Key Engineering Materials. 884, 159-164. DOI: https://doi.org/10.4028/www.scientific.net/KEM.884.159

[36] Hidayah, A.A., Azizah, Winarsa, R., et al., 2020. Utilization of coffee pulp as a substrate for pectinase production by Aspergillus sp. VTMS through solid state fermentation. Proceedings of The International Conference on Science and Applied Science (ICSAS2020); July 7, 2020; Surakarta, Indonesia. pp. 1-5. DOI: https://doi.org/10.1063/5.0030474

[37] Santos, J.C.S. dos Barbosa, O., Ortiz, C., et al., 2015. Importance of the support properties for immobilization or purification of enzymes. ChemCatChem. 7(16), 2413-2432. DOI: https://doi.org/10.1002/cctc.201500310

[38] Dhiman, S., Srivastava, B., Singh, G., et al., 2020. Immobilization of mannanase on sodium alginate-grafted-β-cyclodextrin: an easy and cost effective approach for the improvement of enzyme properties. International Journal of Biological Macromolecules. 156, 1347-1358. DOI: https://doi.org/10.1016/j.ijbiomac.2019.11.175

[39] Xu, H., Liang, H., 2022. Chitosan-regulated biomimetic hybrid nanoflower for efficiently immobilizing enzymes to enhance stability and by-product tolerance. International Journal of Biological Macromolecules. 220, 124-134. DOI: https://doi.org/10.1016/j.ijbiomac.2022.08.048

[40] Guisan, J.M., Fernandez-Lorente, G., Rocha-Martin, J., et al., 2022. Enzyme immobilization strategies for the design of robust and efficient biocatalysts. Current Opinion in Green and Sustainable Chemistry. 35, 100593. DOI: https://doi.org/10.1016/j.cogsc.2022.100593

[41] Xu, K., Chen, X., Zheng, R., et al., 2020. Immobilization of multi-enzymes on support materials for efficient biocatalysis. Frontiers in Bioengineering and Biotechnology. 8, 660. DOI: https://doi.org/10.3389/fbioe.2020.00660

[42] Ashkan, Z., Hemmati, R., Homaei, A., et al., 2021. Immobilization of enzymes on nanoinorganic support materials: an update. International Journal of Biological Macromolecules. 168, 708-721. DOI: https://doi.org/10.1016/j.ijbiomac.2020.11.127

[43] Olivares, A.O., Baker, T.A., Sauer, R.T., 2016. Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines. Nature Reviews Microbiology. 14(1), 33-44. DOI: https://doi.org/10.1038/nrmicro.2015.4

[44] Wei, L., Zhang, W., Lu, H., et al., 2010. Immobilization of enzyme on detonation nanodiamond for highly efficient proteolysis. Talanta. 80(3), 1298-1304. DOI: https://doi.org/10.1016/j.talanta.2009.09.029

[45] Wu, C., Cha, H.J., Valdes, J.J., et al., 2002. GFP-visualized immobilized enzymes: degradation of paraoxon via organophosphorus hydrolase in a packed column. Biotechnology and Bioengineering. 77(2), 212-218. DOI: https://doi.org/10.1002/bit.10065

[46] Siar, E.H., Abellanas-Perez, P., Rocha-Martin, J., et al., 2024. Support enzyme loading influences the effect of aldehyde dextran modification on the specificity of immobilized ficin for large proteins. Molecules. 29(15), 3674. DOI: https://doi.org/10.3390/molecules29153674

[47] Wu, K., Sun, W., Li, D., et al., 2022. Inhibition of

amyloid nucleation by steric hindrance. The Journal of Physical Chemistry B. 126(48), 10045-10054. DOI: https://doi.org/10.1021/acs.jpcb.2c06330

[48] Caparco, A.A., Dautel, D.R., Champion, J.A., 2022. Protein mediated enzyme immobilization. Small. 18(19). DOI: https://doi.org/10.1002/smll.202106425

[49] Morellon-Sterling, R., Siar, E.H., Braham, S.A., et al., 2021. Effect of amine length in the interference of the multipoint covalent immobilization of enzymes on glyoxyl agarose beads. Journal of Biotechnology. 329, 128-142. DOI: https://doi.org/10.1016/j.jbiotec.2021.02.005

[50] Mehdi, W.A., Mehde, A.A., Özacar, M., et al., 2018. Characterization and immobilization of protease and lipase on chitin-starch material as a novel matrix. International Journal of Biological Macromolecules. 117, 947-958. DOI: https://doi.org/10.1016/j.ijbiomac.2018.04.195

[51] Bahri, S., Homaei, A., Mosaddegh, E., 2022. Zinc sulfide-chitosan hybrid nanoparticles as a robust surface for immobilization of Sillago sihama α-amylase. Colloids and Surfaces B: Biointerfaces. 218, 112754. DOI: https://doi.org/10.1016/j.colsurfb.2022.112754

[52] Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., et al., 2015. Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts. Biotechnology Advances. 33(5), 435-456. DOI: https://doi.org/10.1016/j.biotechadv.2015.03.006

[53] El-Shora, H.M., El-Sayyad, G.S., El-Zawawy, N.A., et al., 2024. Stability of immobilized L-arginine deiminase from Penicillium chrysogenum and evaluation of its anticancer activity. Scientific Reports. 14(1), 27216. DOI: https://doi.org/10.1038/s41598-024-77795-8

[54] Gao, F., Courjean, O., Mano, N., 2009. An improved glucose/O2 membrane-less biofuel cell through glucose oxidase purification. Biosensors and Bioelectronics. 25(2), 356-361. DOI: https://doi.org/10.1016/j.bios.2009.07.015

[55] Rahman, N.H.A., Jaafar, N.R., Annuar, N.A.S., et al., 2021. Efficient substrate accessibility of cross-linked levanase aggregates using dialdehyde starch as a macromolecular cross-linker. Carbohydrate Polymers. 267, 118159. DOI: https://doi.org/10.1016/j.carbpol.2021.118159

[56] Santos, M.P.F., Porfírio, M.C.P., Junior, E.C.S., et al., 2022. Pepsin immobilization: influence of carbon support functionalization. International Journal of Biological Macromolecules. 203, 67-79. DOI: https://doi.org/10.1016/j.ijbiomac.2022.01.135

[57] Jang, E., Choi, S.W., Lee, K.B., 2019. Effect of carbonization temperature on the physical properties and CO2 adsorption behavior of petroleum coke-derived porous carbon. Fuel. 248, 85-92. DOI: https://doi.org/10.1016/j.fuel.2019.03.051

[58] Borges, J.F., Nascimento, P.A., Alves, A.N., et al., 2024. Laccase immobilization on activated carbon from hydrothermal carbonization of corn cob. Waste and Biomass Valorization. 15(1), 501-520. DOI: https://doi.org/10.1007/s12649-023-02160-1

[59] Brito, M.J.P., Bauer, L.C., Santos, M.P.F., et al., 2020. Lipase immobilization on activated and functionalized carbon for the aroma ester synthesis. Microporous and Mesoporous Materials. 309, 110576. DOI: https://doi.org/10.1016/j.micromeso.2020.110576

[60] Brito, M.J.P., Veloso, C.M., Bonomo, R.C.F., et al., 2017. Activated carbons preparation from yellow mombin fruit stones for lipase immobilization. Fuel Processing Technology. 156, 421-428. DOI: https://doi.org/10.1016/j.fuproc.2016.10.003

[61] Santos, M.P.F., Ferreira, M.A., Junior, E.C.S., et al., 2023. Functionalized activated carbon as support for trypsin immobilization and its application in casein hydrolysis. Bioprocess and Biosystems Engineering. 46(11), 1651-1664. DOI: https://doi.org/10.1007/s00449-023-02927-9

[62] Prieto, L.M., Ricordi, R.G., Kuhn, R.C., et al., 2014. Evaluation of β-galactosidase adsorption into pretreated carbon. Biocatalysis and Agricultural Biotechnology. 3(3), 26-29. DOI: https://doi.org/10.1016/j.bcab.2013.12.008

[63] Yao, L.W., Khan, F.S.A., Mubarak, N.M., et al., 2022. Insight into immobilization efficiency of lipase enzyme as a biocatalyst on the graphene oxide for adsorption of azo dyes from industrial wastewater effluent. Journal of Molecular Liquids. 354, 118849. DOI: https://doi.org/10.1016/j.molliq.2022.118849

[64] Mo, H., Qiu, J., Yang, C., et al., 2020. Preparation and characterization of magnetic polyporous biochar for cellulase immobilization by physical adsorption. Cellulose. 27(9), 4963-4973. DOI: https://doi.org/10.1007/s10570-020-03125-6

[65] Ashkan, Z., Hemmati, R., Homaei, A., et al., 2021. Immobilization of enzymes on nanoinorganic support materials: an update. International Journal of Biological Macromolecules. 168, 708-721. DOI: https://doi.org/10.1016/j.ijbiomac.2020.11.127

[66] Abbasi, Z., Shamsaei, E., Leong, S.K., et al., 2016. Effect of carbonization temperature on adsorption property of ZIF-8 derived nanoporous carbon for water treatment. Microporous and Mesoporous Materials. 236, 28-37. DOI: https://doi.org/10.1016/j.micromeso.2016.08.022

[67] Quan, C., Wang, H., Jia, X., et al., 2021. Effect of carbonization temperature on CO2 adsorption behavior of activated coal char. Journal of the Energy Institute. 97, 92-99. DOI: https://doi.org/10.1016/j.joei.2021.04.003

[68] Karimi, H., Heidari, M.A., Emrooz, H.B.M., et al., 2020. Carbonization temperature effects on adsorption performance of metal-organic framework derived nanoporous carbon for removal of methylene blue from

wastewater; experimental and spectrometry study. Diamond and Related Materials. 108, 107999. DOI: https://doi.org/10.1016/j.diamond.2020.107999

[69] Al-Najada, A.R., Almulaiky, Y.Q., Aldhahri, M., et al., 2019. Immobilisation of α-amylase on activated amidrazone acrylic fabric: a new approach for the enhancement of enzyme stability and reusability. Scientific Reports. 9(1), 12672. DOI: https://doi.org/10.1038/s41598-019-49206-w

[70] Defaei, M., Taheri-Kafrani, A., Miroliaei, M., et al., 2018. Improvement of stability and reusability of α-amylase immobilized on naringin functionalized magnetic nanoparticles: A robust nanobiocatalyst. International Journal of Biological Macromolecules. 113, 354-360. DOI: https://doi.org/10.1016/j.ijbiomac.2018.02.147

[71] Verma, N.K., Raghav, N., 2021. Comparative study of covalent and hydrophobic interactions for α-amylase immobilization on cellulose derivatives. International Journal of Biological Macromolecules. 174, 134-143. DOI: https://doi.org/10.1016/j.ijbiomac.2021.01.033

[72] Al-Harbi, S.A., Almulaiky, Y.Q., 2020. Purification and biochemical characterization of Arabian balsam α-amylase and enhancing the retention and reusability via encapsulation onto calcium alginate/Fe2O3 nanocomposite beads. International Journal of Biological Macromolecules. 160, 944-952. DOI: https://doi.org/10.1016/j.ijbiomac.2020.05.176

[73] Mulko, L., Pereyra, J.Y., Rivarola, C.R., et al., 2019. Improving the retention and reusability of alpha-amylase by immobilization in nanoporous polyacrylamide-graphene oxide nanocomposites. International Journal of Biological Macromolecules. 122, 1253-1261. DOI: https://doi.org/10.1016/j.ijbiomac.2018.09.078

[74] Kumari, A., Kaila, P., Tiwari, P., et al., 2018. Multiple thermostable enzyme hydrolases on magnetic nanoparticles: an immobilized enzyme-mediated approach to saccharification through simultaneous xylanase, cellulase and amylolytic glucanotransferase action. International Journal of Biological Macromolecules. 120, 1650-1658. DOI: https://doi.org/10.1016/j.ijbiomac.2018.09.106

[75] Atiroğlu, V., Atiroğlu, A., Özacar, M., 2021. Immobilization of α-amylase enzyme on a protein @metal-organic framework nanocomposite: a new strategy to develop the reusability and stability of the enzyme. Food Chemistry. 349, 129127. DOI: https://doi.org/10.1016/j.foodchem.2021.129127

[76] Primožič, M., Podrepšek, G.H., Pavlovič, I., et al., 2019. Enzyme immobilization onto biochar produced by the hydrothermal carbonization of biomass. Acta Chimica Slovenica. 66(3), 732-739. DOI: https://doi.org/10.17344/acsi.2019.5013