Coffee Pulp Activated Carbon for Immobilizing Cellulase from *Aspergillus niger* ICP2: Enhancing Enzyme Stability, Activity, and Its Reusability

Rudju Winarsa[1], *Nadhea* Ayu Sukma[2], *Dina* Afriyanti[1], *Farah* Salma Elida[1], *Ramdhan* Putrasetya[1], *Azizah*[1], *Purwatiningsih*[1], *Siswoyo*[2], and *Kahar* Muzakhar[1*]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Jember University, Jalan Kalimantan 37 Kampus Tegal Boto 68121 Jember, Indonesia
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Jember University, Jalan Kalimantan 37 Kampus Tegal Boto 68121 Jember, Indonesia

Abstract. Cellulase from *Aspergillus niger* ICP2 was successfully immobilized on coffee pulp activated carbon using adsorption. Carbon was derived from coffee pulp via controlled carbonization at 200 °C for 2 hours, followed by activation with 3M $ZnCl_2$ for 150 minutes. Reusability analysis exhibited over 50% relative activity retention after four cycles. The immobilized cellulase demonstrated stability at pH 4.0-6.0, retaining over 80% relative activity after 4 hours at 37°C. This approach proves economical for enhancing cellulase stability, activity, and reusability.

1 Introduction

Cellulase is an enzyme complex capable of breaking down cellulose into glucose monomers. This complex comprises endocellulase, exocellulase, and β-glucosidase, which, in microorganisms, work synergistically to break down cellulose polymer chains [1, 2]. Cellulase has been widely utilized in various fields, including textile, animal feed, agriculture, pharmaceutical, detergent, bioconversion, paper, and pulp industries [3, 4].

Filamentous fungi have the capability to commercially produce cellulases due to their potential to utilize cheaper substrates such as coffee pulp waste as energy and carbon sources, thereby reducing the cost of industrial fermentation processes [5, 6]. The cellulase production by *A. niger* ICP2, which is capable of producing 0.33 U/mL, indicates that this isolate is a potential isolate because the production process can be carried out inexpensively by using only coffee pulp waste, without any additional nutrients. However, cellulase in the free state is highly sensitive to extreme environmental conditions, is challenging to use repeatedly, and has a short shelf life. This sensitivity poses a barrier to the large-scale application of cellulases due to the high cost of enzymes [7, 8]. Enzyme immobilization offers an alternative solution to this problem. It involves attaching an enzyme to an insoluble support material, enhancing its resistance to environmental changes, and enabling the enzyme to be reused [6, 7]. This

* Corresponding author: kaharmzk@unej.ac.id

process is highly efficient and widely employed in the industry for enzyme-catalyzed reactions.

The adsorption method is considered a simple and fast technique for immobilizing enzymes that does not alter the conformation of the enzyme, thus having no significant impact on its activity. Adsorption is the process of a material being absorbed by solids on its surface in an adhesive manner, without the inward absorption process [9]. Activated carbon is one of the solid materials used for adsorption, and these materials can be derived from organic waste [10, 11].

Arabica coffee pulp is an organic waste containing 65.99% carbon [12]. Activated carbon can be produced through physical or chemical activation [13]. This research utilizes the abundance of arabica coffee pulp, which is a cheap plantation by-product. The purpose of this study is to determine the immobilization potential of cellulase from *A. niger* ICP2 based on activated carbon derived from arabica coffee pulp and to investigate the pH stability of immobilized cellulase on activated carbon during repeated use.

2 Materials and methods

2.1 Maintaining of *A. niger* ICP2 stock culture

A. niger ICP2 was periodically maintained in potato dextrose agar (PDA). The culture is inoculated in one loop on new slanted PDA media. The culture was then incubated at 30 °C for 72 hours [14, 15].

2.2 Optimization of crude cellulase production

The optimization of cellulase production was conducted using the solid fermentation method with coffee pulp containing 76% moisture content, carried out within 7 days of harvesting. One mL spore suspension (1.33 x 10^8 spores/mL) was inoculated into 10 g of the solid fermentation medium (10% or 10g/100 mL) for cellulase production. Cultures were incubated at 30 °C for 7 days. The preparation stage involved adding 10 mL of a 0.01% sodium azide and 1% NaCl solution to the solid fermentation medium to be harvested, followed by incubation on a shaker for 12 hours. The crude cellulase was obtained through filtration employing filter paper, followed by subsequent separation of residual debris via centrifugation at 8000 rpm for 10 minutes. The supernatant, containing cellulase, was employed for the enzyme activity assay [16].

2.3 Cellulase activity assay

Cellulase activity was determined through reducing sugar testing using the modified Somogyi-Nelson method [15, 17, 18]. In a test tube, 500 µL of cellulase was mixed with 500 µL of 0.5 % CMC in 20 mM acetate buffer at pH 5. After 2 hours of incubation, 500 µL of Somogyi reagent was added to the solution, followed by boiling for 15 minutes. Once the solution cooled, 500 µL of Nelson's reagent was added, and the mixture was centrifuged at 8000 rpm for 10 minutes. The absorbance value of the supernatant was measured with a spectrophotometer at a wavelength of 500 nm. Cellulase activity was calculated based on the reducing sugar value obtained using the formula in equation (1).

$$\text{Cellulase Activity } \left(\frac{U}{mL}\right) = \frac{Y \text{ x } DF}{\text{Glucose MW x t x V}} \quad (1)$$

Where:

Y	=	reducing sugar value (µg/mL)
DF	=	dilution factor
Glucose MW	=	glucose molecular weight (g/mol)
t	=	incubation time (minute)
V	=	enzyme volume (mL)

2.4 Enzyme dialysis

The cellulase dialysis was conducted using 50 and 10 kDa columns. Crude enzyme (23 mL) was introduced to the exterior of the column, and it flowed into the interior of the column along with 250 mL of 20 mM acetate buffer at pH 5. The buffer flow was maintained in a loop at a speed of 7.5 mL/min. The dialysis process lasted for 24 hours, during which the buffer was replaced 5 times. Dialysis results were measured for enzyme activity using the Somogyi-Nelson method and for protein content.

2.5 Carbonization of the coffee pulp

The arabica coffee pulp was obtained from the Blawan Plantation, Bondowoso, East Java. The dried coffee pulp is carbonized using a furnace at 400℃ for 1 hour. The resulting crushed carbon is then washed with distilled water and dried in an oven at 120℃ for 12 hours.

2.6 Carbon activation

The carbon 5 g was activated using 50 mL of $ZnCl_2$ 3M for 2 hours at 80 ℃. The activated carbon was filtered and dried at 120 ℃ for 12 hours. Subsequently, the carbon was immersed in 50 mL of 0.5 M HCl for 12 hours on a shaker. Afterward, the carbon was washed with distilled water until the washing water reached a pH of 6-7. Finally, it was dried in the oven for 12 hours at 120 ℃.

2.7 Immobilization of cellulase

One point five grams (5 mL) of coffee pulp activated carbon was loaded into a glass column with a diameter of 1 cm and a length of 5 cm. The activated carbon was washed by eluting with 50 mL of distilled water using the Pharmacia Pump P-500 at a speed of 200 mL/hour for 10 minutes. Subsequently, it was equilibrated with 100 mL of 20 mM acetate buffer at pH 5, with a speed of 100 mL/hour. In a volume of 100 mL, purified cellulase with a concentration of 100 units/mL was pumped into a column at a rate of 2 mL/minute. Subsequently, the cellulase immobilization process was carried out through continuous pumping cycles for 6 hours at a pump rate of 50 mL/hour. Every 30 minutes, the residual cellulase not absorbed by the activated carbon was measured using a spectrophotometer at a wavelength of 280 nm. The unabsorbed cellulase in the immobilization process was also assessed based on its relative enzymatic activity. The cellulase immobilization process will be terminated when the remaining unabsorbed cellulase reaches a constant quantity.

2.8 Activity immobilized cellulase and analysis of its reusability

This analysis involved repeated hydrolysis, initiated by washing immobilized cellulase with 50 mL of 20 mM acetate buffer at pH 5, at a flow rate of 150 mL/hour. Subsequently, 60 mL

of 0.5 % CMC substrate in 20 mM acetate buffer at pH 5 was introduced into the column at a flow rate of 100 mL/hour. The substrate was replaced every 2 hours, with a total of 7 substrate changes. The activity of immobilized cellulase was quantified by reducing sugars produced using based on the Somogy-Nelson method during the hydrolysis process of the CMC substrate flowing in the column of immobilized cellulase. This analysis was conducted by sampling 20 mL of hydrolysate every 30 minutes.

2.9 Analysis pH stability of immobilized cellulase

Immobilized cellulase was analyzed for pH stability in the range of pH 3 to 8. A total of 0.3 g of activated carbon containing immobilized cellulase was suspended in 500 µL of 20 mM buffer in a 1.5 mL Eppendorf tube. Acetate and phosphate buffers were used for pH 3 to 5 and pH 6, 7, and 8 respectively. The mixtures were then incubated in a water bath for 4 hours at a temperature of 37℃. Controls were boiled for 15 minutes. Subsequently, each vial was added to 500 µL of 0.5% CMC substrate and incubated at 37℃ for 2 hours in a water bath. The vials were then shaken at 150 rpm for 30 minutes. For the analysis of cellulase activity stability, a 500 µL sample was taken, and the reducing sugar produced was measured. This analysis was performed in duplicate.

3 Results and discussion

Optimization of cellulase production was carried out based on incubation time. Incubation time affects the cellulase production by *A. niger* ICP2, as depicted in Fig.1. Cellulase production continued to increase with the length of incubation time and reached its maximum value on the 7th day of incubation, with an activity of 1.011 ± 0.018 U/mL. Fig.1 illustrates that the lowest cellulase production occurs on days 1 to 4, suggesting that *A. niger* ICP2 utilizes nutrients in the media for its growth [19]. According to [20], *A. niger* ICP2 produced the highest cellulase on day 4 with a cellulase activity of 0.33 U/mL. Based on the results of the research by [21], cellulase production increased until day 5 and then decreased thereafter. This observation suggests that, in addition to being influenced by biomass, cellulase production is also influenced by the growth phase of the microorganism. Cellulases, as primary metabolites, are typically produced during the exponential growth phase in solid state fermentation.

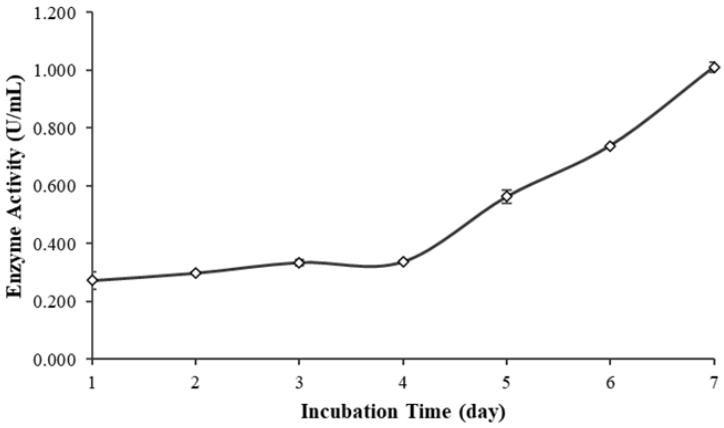

Fig. 1. Optimization of incubation time for cellulase production from *A. niger* ICP2

The crude cellulase produced by *A. niger ICP2* was harvested after 7 days of incubation and had activity 1.135 ± 0.018 U/mL. The cellulase purification steps are presented in Table 1. The crude cellulase that has been dialyzed using a hollow fiber membrane 50 kDa shows an activity of 0.932 U/mL. However, when the crude cellulase was dialyzed on a hollow fiber membrane 10 kDa, cellulase activity increased at 43.14 U/mL, resulting in a purity 41.5 times higher than that of crude cellulase. An increase in purity was observed when cellulase was purified via 10 kDa dialysis, indicating the presence of proteins and other contaminants in cellulase derived from crude products, thus necessitating a purification step [22]. Based on the results of purification through 50 and 10 kDa dialysis, it can be observed that the cellulase from *A. niger* ICP2 falls within the size range of 10-50 kDa. At each purification stage, there is a decrease in total cellulase activity but an increase in specific activity [22].

Table 1. Cellulase purification from *A. niger* ICP2

Enzyme Fraction	Volume (mL)	Unit Activity (U/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude	300	1.135	340.52	13995	0.024	100	1.0
Dialysis 50 kDa	23	0.041	0.93	11.7	0.079	0.27	3.3
Dialysis 10 kDa	23	1.876	43.14	42.7	1.009	12.67	41.5

The technique for immobilizing enzymes in carbon was initiated with the activation process of the carbon to expand the surface pores and enhance adsorption capacity [13]. According to [23], the use of $ZnCl_2$ as an activator is highly efficient for activated carbon production as it increases porosity and surface area. Cellulase immobilization was carried out using 0.5 g (about 5 mL volume) of activated carbon previously activated with 3 M $ZnCl_2$. The enzyme immobilization process involved circulating 100 mL of cellulase at a rate of 50 mL/hour. At this rate, it can be observed that 100 mL of cellulase would completely pass through the carbon in 2 hours (120 minutes). Residual cellulase activity and un-immobilized protein were measured every 30 minutes for 6 hours (Fig.2). Based on the previously described speed assumptions and research results, it is evident that at 120 minutes, cellulase passes through activated carbon for 1 cycle, and the adsorption of activated carbon on cellulase is not maximized (Fig.2a). This is indicated by the high residual cellulase activity at 0.318 ± 0.006 U/mL. The highest cellulase adsorption occurs at 150 minutes, with cellulase activity of 0.281 ± 0.001 U/mL, indicating that cellulase has passed through activated carbon for 2 cycles. This is evident from the decrease in residual cellulase activity compared to the previous 30-minute measurement at 120 minutes, which was 0.318 ± 0.006 U/mL. The 3rd cycle, at 270 minutes, shows a stable trend, interpreted as cellulase being adsorbed on the activated carbon. Residual cellulase activity displays a negative correlation with the adsorption ability of activated carbon on cellulase. Higher residual cellulase activity corresponds to a lower adsorption capacity of activated carbon on cellulase [7, 24]. Fig.2b illustrates the protein residues immobilized on activated carbon. Protein levels are measured using a UV spectrophotometer at a wavelength of 280 nm, ensuring that the measured protein encompasses both the target protein and non-target protein [25]. According to the figure, the protein amount decreases during cycle 1 (120 minutes), indicating protein adsorption on activated carbon. However, upon entering the second cycle, protein levels increase. It is suspected that OD 280 also measures non-target protein. These results are reflected in enzyme activity because the activity measurement is focused on the target protein (cellulase).

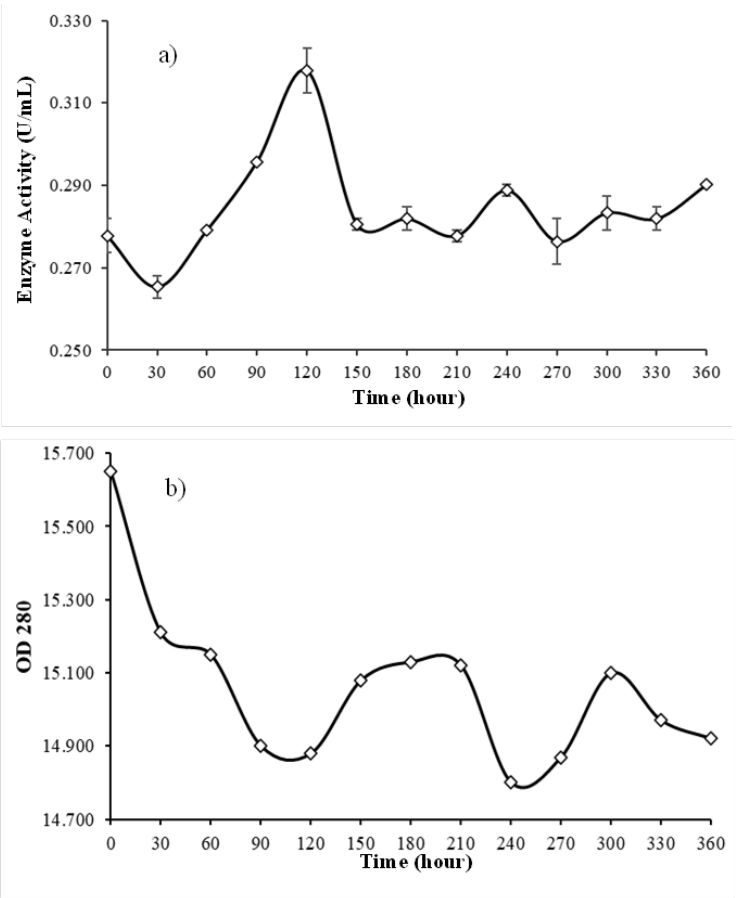

Fig. 2. Residual activity of immobilized cellulase adsorbed in activated carbon coffee pulp based (a) and remaining cellulase residues during immobilization (b)

The reusability analysis of immobilized cellulase was conducted by assessing the capability of the CMC hydrolysis process. The reusability test was performed over several cycles, and the relative cellulase activity was measured in each cycle. After each reaction cycle is completed, it will be followed by the next hydrolysis cycle with the first replacing the new CMS substrate. Results from seven test cycles indicate that immobilized cellulase remains effective for up to 4 reuse cycles, maintaining a minimum of 50% relative activity. Research by [26] demonstrated that immobilized laccase could be utilized for up to 6 cycles while retaining 60% relative activity. Repeated use of immobilized cellulase from cycles 5 to 7 resulted in a decline in relative activity from 31% to 0% (Fig.3a). The decrease in relative activity with each reuse is associated with the reduction in the adsorption capacity of immobilized cellulase. According to [10], this decline is attributed to the release of Van Der Waals bonds between hydrophobic enzymes and activated carbon. Research by [6] showed that after 10 cycles, immobilized enzyme activity remained above 50% of its initial activity, but almost no activity was observed after 15 cycles due to the weak binding of immobilized enzymes to carbon. Fig.3b illustrates the activity of immobilized cellulase measured every 30 minutes for 2 hours during each cycle change. 0.5 g (5 mL) of immobilized cellulase was diluted with 60 mL of substrate at a rate of 100 mL/hour. Based on this rate, the time required for the substrate to pass through the immobilized cellulase completely is approximately 2 hours (120 minutes). Fig.3b indicates that the highest activity of immobilized cellulase

against repeated use was achieved at the 120th minute. Cellulases immobilized on substrates 1-4 retained 50% of their relative activity. The time at 120 minutes signifies that 60 mL of the substrate had completely passed through immobilized cellulase, completing one cycle and resulting in a high enzyme product. Immobilized cellulase exhibited relatively low activity at the 5 cycles of substrate replacement, followed by a further decline in activity at the 6th cycle, and by the 7th cycle, its activity could be considered negligible (0%) [6], [8].

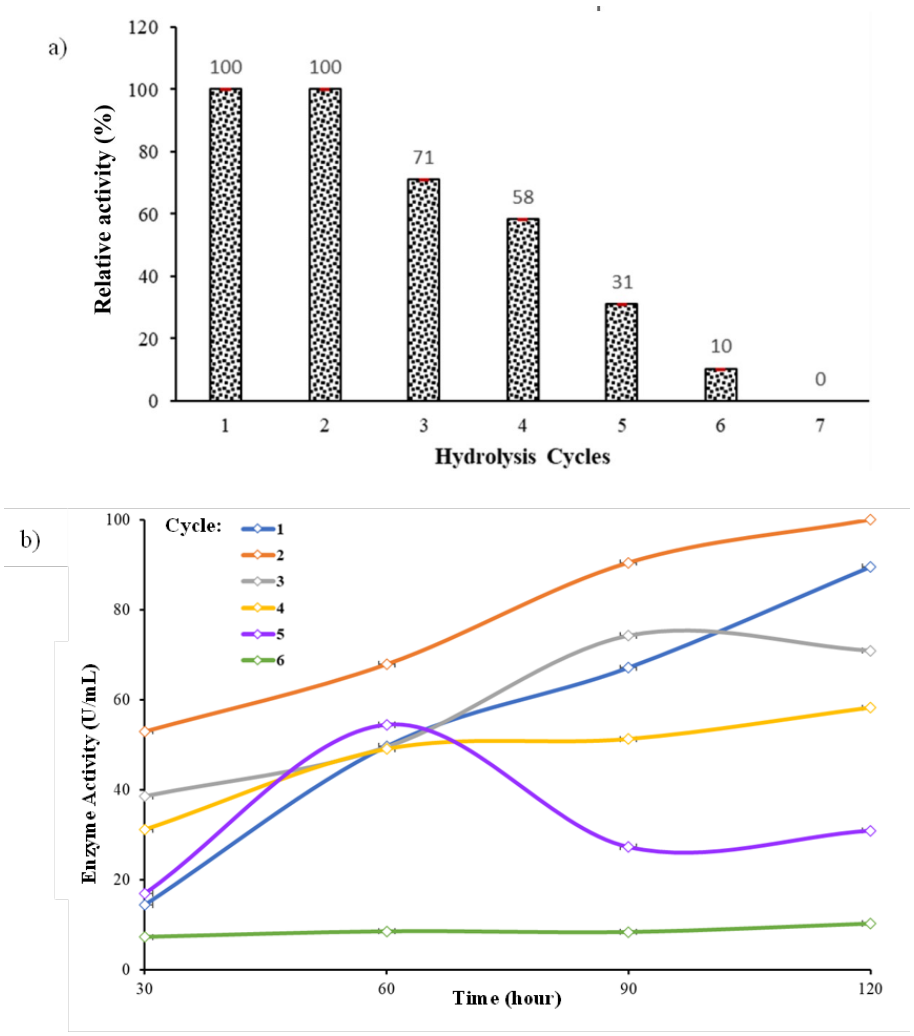

Fig. 3. Immobilized cellulase activity against repeated use a); immobilized cellulase activity against repeated use in the range of 30-120 minutes b)

The stability analysis of immobilized cellulase against pH revealed its stability within the pH range of 4-8, demonstrating its capability to hydrolyze the CMC substrate in a 50 mM acetate buffer, with limited maintenance of activity at 50% under acidic conditions (Fig.4). However, at pH 3, immobilized cellulase exhibited relative activity with a minimum capability of 29.1% (Fig.4). Stability and hydrolysis capability of the immobilized enzyme were notably favorable within the pH range of 4-6, as evidenced by the enzyme retaining over 80% of its initial activity after incubation in buffers with pH 4-6 for 4 hours at 37℃.

The activity of immobilized cellulase was found to be unstable at pH 3, remaining below 50%. The increase in H^+ ion concentration could potentially disrupt ionic bonds, thereby affecting the functional conformation of the enzyme's active site [22], resulting in a lower product yield. According to Huang et al. the pH stability of immobilized beta-glucosidase was achieved at pH 4.0-8.0 in a 20 mM Na_2HPO_4-$C_6H_8O_7$ buffer. Research by [27] demonstrated that immobilized lipase maintains pH stability within the range of 6.0-8.0. Research by [26] findings indicated that the pH stability of immobilized laccase was attained at pH 5.0-7.0. Furthermore, [28] observed that cellulase from *A. niger* NS-2 achieved pH stability within the range of 4.0-6.0 with a relative activity of 80%. This underscores that certain enzymes exhibit enhanced activity under acidic conditions.

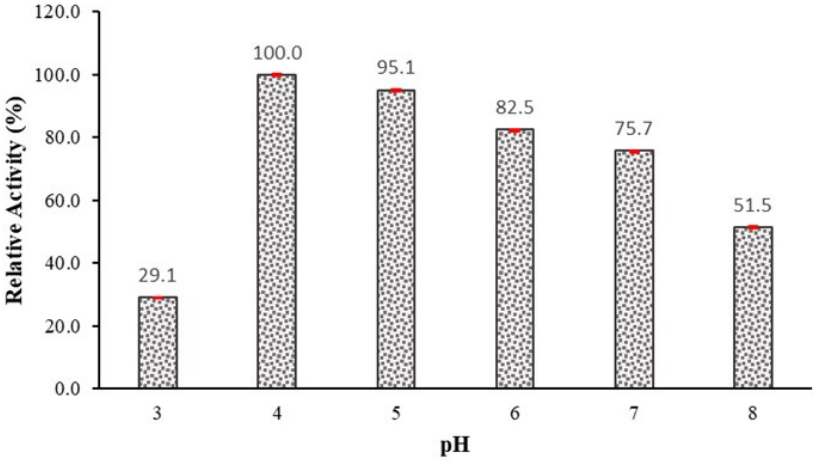

Fig. 4. pH stability of Immobilized cellulase

4 Conclusions

Activated carbon derived from coffee pulp is a promising and cost-effective material for use as an enzyme immobilizer. The cellulase enzyme from *A. niger* ICP2 demonstrates strong adsorption on this activated carbon and can be effectively immobilized. Testing reveals that the cellulase exhibits reusability for up to 4 cycles with a minimum relative activity of 50%. Analysis of the immobilized cellulase indicates its robust performance in the pH range of 4-6, maintaining a minimum relative activity of 80%. These results suggest that activated carbon from coffee pulp is highly suitable and holds great promise for use as an immobilizing material for cellulase enzymes.

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