Engineering Coffee Cherry Pulp-derived Activated Carbon for Cellulase Immobilization and its Role in Regulating Non-Sterile Coffee Fermentation

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Abstract. During non-sterile coffee fermentation, microbial activity and substrate availability change as the mucilage is degraded. Activated carbon prepared from Arabica coffee cherry pulp by carbonization and $ZnCl_2$ activation was used as a support for cellulase immobilization. Cellulase produced by *Cytobacillus* sp. isolated from civet (*Paradoxurus hermaphroditus*) feces was immobilized by adsorption and used as part of the fermentation starter system. The immobilization efficiency was 80% and the immobilized cellulase had a specific activity of 613.22 U/mg. During fermentation, cellulase activity decreased from 2.45 U/mL at 0 h to 1.71 U/mL at 72 h, with approximately 70% of the initial activity remaining. At 48 h, the reducing sugar concentration was 10.7 mg/mL in the fermentation with immobilized cellulase, compared with 6.1 mg/mL in the control. The microbial growth rate was 0.132 h^{-1} with immobilized cellulase and 0.084 h^{-1} in the control. Pearson correlation coefficients were 0.93 between cellulase activity and reducing sugar concentration, 0.91 between reducing sugar concentration and microbial growth, and 0.88 between cellulase activity and microbial growth rate. Immobilized cellulase was associated with changes in sugar availability and microbial growth during non-sterile coffee fermentation.

Keywords: *activated carbon; cellulase immobilization; coffee fermentation; microbial growth; reducing sugar.*

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1 Introduction

Coffee fermentation helps remove the mucilage surrounding the beans and may also influence coffee quality. In many coffee-producing regions, the process is carried out under non-sterile conditions, so microorganisms naturally present in the coffee material can grow during fermentation [1–3]. Their activity changes as the fermentation progresses, together with changes in the substrates available in the mucilage. These changes can affect the rate of polysaccharide degradation and sugar release [4–5].

Microorganisms involved in fermentation produce several hydrolytic enzymes that contribute to mucilage degradation. Cellulase is involved in the breakdown of polysaccharide components and may therefore contribute to the release of soluble sugars during fermentation [6]. In spontaneous fermentation, however, cellulase activity can vary with the microorganisms present and with the fermentation conditions. During fermentation, free cellulase may lose activity or become diluted. This leaves less active enzyme available for further hydrolysis [7].

Immobilizing cellulase on a solid support can help retain the enzyme during fermentation. In adsorption immobilization, cellulase binds to the surface of the support and remains available to interact with the substrate. Activated carbon has potential as a support for enzyme immobilization. Its adsorption properties and chemical stability make it suitable for this purpose. Coffee cherry pulp, which is generated during coffee processing, can also be converted into activated carbon and used as a value-added material [9]. In this study, activated carbon prepared from Arabica coffee cherry pulp was used to immobilize cellulase produced by *Cytobacillus* sp. The performance of immobilized cellulase during non-sterile coffee fermentation was assessed by measuring cellulase activity, reducing sugar concentration, and microbial growth.

2 Materials and Methods

2.1 Materials and Enzyme Source

Arabica coffee cherry pulp from the Kalisat–Jampit Ijen Coffee Plantation, Bondowoso Regency, East Java, Indonesia, was used as the precursor for activated carbon. Cellulase was produced using *Cytobacillus* sp. isolated from civet (*Paradoxurus hermaphroditus*) feces. All chemicals used in the study were of analytical grade.

2.2 Preparation of Crude–Partially Purified Cellulase

Cellulase was produced by fermenting *Cytobacillus* sp. in a 5-liter Erlenmeyer flask containing 2.5 liters of medium supplemented with 10% (w/v) fresh coffee pulp as the sole carbon and nitrogen source. The culture was incubated at 37 °C and 120 rpm for 72 h. After fermentation, crude cellulase was extracted by adding NaCl to the culture broth to a final concentration of 1% (w/v), followed by agitation for 12 h. The culture broth was filtered through a 40-µm filter and centrifuged at 4,000 × g for 10 min to remove coarse particles, residual cells, and insoluble debris. The supernatant was collected as the crude enzyme extract. The protein concentration was measured using the Bradford method with BSA as the standard. Specific activity was expressed as U/mg protein.

Crude cellulase was partially purified and concentrated by ammonium sulfate precipitation at 60% saturation. Solid ammonium sulfate was gradually added to the crude enzyme extract under continuous stirring until 60% saturation was reached. The mixture was maintained at 4 °C for 12 h to allow the proteins to precipitate. The precipitated proteins were recovered by centrifugation at 12,000 × g for 20 min and resuspended in 20 mM phosphate buffer (pH 6.2). Residual ammonium sulfate was removed by dialysis against the same buffer using a 10-kDa molecular weight cut-off (MWCO) membrane at 4 °C for 24 h. The dialysis buffer was replaced every 8 h, with three replacements in total. The dialyzed enzyme preparation was used for immobilization and enzymatic activity assays.

Table 1 Purification steps.

Steps	Volume (mL)	Enzyme Activity (U/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold	Yield (%)
Crude enzyme	2,480	0.99	2,455.2	126.8	19.36	1	100
$(NH_4)_2SO_4$ precipitation	9.6	146	1,401.6	2.19	640.0	33.06	1.70
Dialysis	9.94	155	1,540.7	2.01	766.52	39.59	1.59

Table 1 presents the cellulase purification results. The crude enzyme preparation had a volume of 2,480 mL, an enzyme activity of 0.99 U/mL, and 126.8 mg of total protein. Its specific activity was 19.36 U/mg, which was taken as the reference value for the purification factor (1-fold) and yield (100%). Following ammonium sulfate precipitation, the enzyme volume decreased to 9.6 mL. At this stage, enzyme activity reached 146 U/mL, whereas total protein decreased to 2.19 mg. Specific activity increased to 640.0 U/mg, corresponding to a 33.06-fold purification factor, while the yield was 1.70%. After dialysis, enzyme activity reached 155 U/mL and total protein was 2.01 mg. Specific activity increased

further to 766.52 U/mg, giving a purification factor of 39.59-fold. The overall yield was 1.59%.

2.3 Preparation of Coffee Cherry Pulp-derived Activated Carbon

Dried Arabica coffee cherry pulp was carbonized at 400 °C for 1 h under oxygen-limited conditions to produce biochar. The biochar was impregnated with 1 M $ZnCl_2$ solution at 80 °C for 2 h to allow the activating agent to enter the carbon matrix. The impregnated material was thermally activated at 400 °C for 1 h under oxygen-limited conditions, following the procedure reported for 1 M $ZnCl_2$-assisted activation of lignocellulosic biomass-derived carbon [10–11]. After activation, the material was leached with 0.5 M HCl under continuous agitation to remove residual zinc species and inorganic impurities. It was washed repeatedly with distilled water until the pH reached 6–7 and then dried in an oven at 120 °C for 12 h. The resulting activated carbon served as the immobilization matrix.

2.4 Immobilization of Cellulase

Cellulase was immobilized by physical adsorption onto $ZnCl_2$-activated carbon. Activated carbon (0.04 g) was suspended in 500 µL of 20 mM acetate buffer (pH 5.0) and mixed with 1 mL of purified cellulase with a specific activity of 766.52 U/mg in a microvial. A control vial was prepared in parallel using 1 mL of 20 mM acetate buffer (pH 5.0) and the same amount of cellulase previously inactivated by boiling at 100 °C for 20 min. The inactivated enzyme was used as negative control. Both test and control vials were incubated on an orbital shaker at 150 rpm for 1 h at room temperature.

The immobilization efficiency of cellulase on coffee cherry pulp-derived activated carbon was determined based on the reduction of enzymatic activity in the supernatant before and after the immobilization process. Cellulase activity of the initial enzyme solution (A_0) was measured prior to contact with the activated carbon. Following adsorption immobilization, the enzyme–carbon complex was separated and the residual cellulase activity in the supernatant (A_r) was measured using the Somogyi–Nelson method under the same assay conditions. Immobilization efficiency (IE, %) was calculated according to Equation (1):

$$IE(\%) = \left(\frac{Ao - Ar}{Ao}\right) X100 \quad (1)$$

where A_0 is the initial cellulase activity (U/mL) and A_r is the residual cellulase activity (U/mL) in the supernatant after immobilization. All measurements were performed in triplicate and the results are reported as mean ± standard deviation.

2.5 Changes in Cellulase Activity and Reducing Sugar during Fermentation

The cellulase assay was based on the reducing sugars released from carboxymethyl cellulose (CMC) following a modified Somogyi–Nelson method [12–13]. For the assay, 500 μL of enzyme solution or fermentation supernatant was mixed with 500 μL of 0.5% (w/v) CMC prepared in 20 mM sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 2 h. To stop the reaction, 500 μL of Somogyi reagent was added and the mixture was heated in a boiling water bath for 15 min. After cooling, 500 μL of Nelson reagent was added. The samples were centrifuged at 8,000 rpm for 10 min. The absorbance of the supernatant was measured at 500 nm using a spectrophotometer. The reducing sugar concentration was calculated from a D-glucose calibration curve.

Cellulase activity is reported in units (U). One unit represents the amount of enzyme that released 1 μmol of reducing sugar per minute under the assay conditions. During coffee fermentation, residual cellulase activity at each sampling point was calculated relative to the activity measured at 0 h and expressed as a percentage.

2.6 Coffee Fermentation and Experimental Design

Coffee fermentation was conducted using fresh coffee beans with intact mucilage under non-sterile conditions. Immobilized cellulase was added to the fermentation as part of the starter system. The control fermentation was conducted under the same conditions without cellulase addition. Fermentation was carried out at ambient temperature. Samples were aseptically collected at 0, 24, 48, and 72 h. Centrifugation at 8,000 × g for 10 min was used to separate the fermentation liquor from the solid material. The resulting supernatant was used to measure cellulase activity and reducing sugar concentration. Separate samples were collected for microbial analysis. Residual cellulase activity was calculated as a percentage of the activity measured at 0 h for each sampling time.

2.7 Microbial Growth Rate Determination

Microbial growth during fermentation was monitored by total viable count (TVC). Fermentation samples (1 mL) were diluted 10^2, 10^3, 10^4, and 10^5 times with sterile physiological saline (0.85% NaCl). From each dilution, 100 μL was spread onto plate count agar and incubated at 30 °C for 48 h. Microbial population density was reported as colony-forming units per milliliter (CFU/mL). The specific growth rate (μ) of the microbial population was calculated during the exponential growth phase using Equation (2):

$$\mu = \frac{\ln N_t - \ln N_0}{t - t_0} \quad (2)$$

where N_t and N_0 represent microbial counts (CFU/mL) at times t and t_0, respectively.

2.8 Data and Statistical Analysis

The relationships among cellulase activity, reducing sugar concentration, and microbial growth rate were evaluated using correlation analysis. Pearson's correlation analysis was used to examine two relationships during fermentation. The first was between cellulase activity and the reducing sugar concentration, the second was between the reducing sugar concentration and the microbial growth. All experiments were conducted three times and the data are presented as mean ± standard deviation.

3 Results and Discussion

3.1 Immobilization Efficiency of Cellulase on Coffee Cherry Pulp-derived Activated Carbon

The activated carbon showed an immobilization efficiency of 80%, compared with approximately 79% reported for a carbon-based immobilization matrix [14]. After immobilization, the cellulase showed a specific activity of 613.22 U/mg, corresponding to approximately 80% of the specific activity of the immobilized enzyme (752.66 U/mg). Cellulase was immobilized by adsorption without the use of a chemical crosslinking agent and the retained activity indicates that the enzyme remained active after adsorption onto the activated carbon [16]. The use of $ZnCl_2$ during activation may have contributed to enzyme adsorption by promoting pore development in the carbon material [15].

3.2 Cellulase Activity and Stability during Coffee Fermentation

The cellulase activity during coffee fermentation under non-sterile conditions is shown in Table 2. Fermentation with immobilized cellulase showed higher cellulase activity than the control throughout the fermentation period. The initial activity was 2.45 U/mL. It gradually decreased during fermentation and reached 1.71 U/mL after 72 h. This value corresponded to approximately 70% residual activity. Some activity was lost during fermentation, but the enzyme remained active after 72 h.

The control fermentation showed only low cellulase activity, which may have been associated with enzymes produced by native microorganisms [17–18]. Cellulase activity was higher in the fermentation containing immobilized

cellulase. The added enzyme may have contributed to the higher activity during fermentation. The residual activity was approximately 70% after 72 h. Activity decreased from 2.45 to 1.71 U/mL during fermentation. Despite this decrease, immobilized cellulase remained active at the end of the 72 h period.

Table 2 Cellulase activity during coffee fermentation.

Time (h)	Control (no cellulase) (U/mL)	Immobilized cellulase (U/mL)
0	0.05 ± 0.01	2.45 ± 0.08
24	0.08 ± 0.02	2.21 ± 0.07
48	0.10 ± 0.02	1.92 ± 0.06
72	0.12 ± 0.03	1.71 ± 0.05

3.3 Efficiency of Cellulase on Coffee Cherry Pulp-derived Activated Carbon

Changes in reducing sugar concentration during fermentation are shown in Table 3. In the presence of immobilized cellulase, the reducing sugar concentration increased from 4.3 mg/mL at 0 h to 10.7 mg/mL at 48 h, followed by a slight decrease to 9.9 mg/mL at 72 h. In the control fermentation, the concentration increased from 4.2 mg/mL at 0 h to 6.1 mg/mL at 48 h and then decreased slightly to 5.8 mg/mL at 72 h. At 48 h, the reducing sugar concentration was higher in the cellulase-treated fermentation (10.7 mg/mL) than in the control (6.1 mg/mL). This difference suggests that the addition of immobilized cellulase contributed to sugar release during fermentation. Previous work has shown that cellulase treatment can increase soluble solids extraction in coffee processing [19].

Table 3 Reducing sugar concentration during coffee fermentation.

Time (h)	Control (mg/mL)	Immobilized cellulase (mg/mL)
0	4.2 ± 0.3	4.3 ± 0.2
24	5.6 ± 0.4	8.9 ± 0.5
48	6.1 ± 0.5	10.7 ± 0.6
72	5.8 ± 0.4	9.9 ± 0.5

The decrease in reducing sugar concentration after 48 h occurred in both treatments, with a smaller decrease in the cellulase-treated fermentation (10.7 to 9.9 mg/mL) than in the control (6.1 to 5.8 mg/mL). The reduction suggests that the sugars released during fermentation were also being consumed at this stage. Despite the decrease, the cellulase-treated fermentation maintained a higher reducing sugar concentration than the control at 72 h.

3.4 Microbial Population Dynamics and Growth Rate

Microbial population changes during fermentation are shown in Table 4. At 0 h, the microbial counts were similar in the control (2.1×10^4 CFU/mL) and immobilized cellulase treatment (2.0×10^4 CFU/mL). The population increased in both treatments during fermentation, but the increase was greater in the presence of immobilized cellulase. At 24 h, the microbial count reached 2.3×10^6 CFU/mL in the cellulase-treated fermentation, compared with 8.5×10^5 CFU/mL in the control. The difference became larger at 48 h, when the counts reached 6.4×10^6 and 1.9×10^6 CFU/mL, respectively. At 72 h, the counts were 6.1×10^6 CFU/mL with immobilized cellulase and 2.2×10^6 CFU/mL in the control.

Table 4 Microbial population dynamics during coffee fermentation.

Time (h)	Control (CFU/mL)	Immobilized cellulase (CFU/mL)
0	2.1×10^4	2.0×10^4
24	8.5×10^5	2.3×10^6
48	1.9×10^6	6.4×10^6
72	2.2×10^6	6.1×10^6

The higher microbial population observed in the cellulase-treated fermentation was also reflected in the specific growth rate. The growth rate was 0.132 h^{-1} with immobilized cellulase, compared with 0.084 h^{-1} in the control. The difference in growth rate is consistent with the population data in Table 4, where the microbial count in the cellulase-treated fermentation increased more rapidly during the first 48 h and reached 6.4×10^6 CFU/mL, compared with 1.9×10^6 CFU/mL in the control. The higher growth rate and larger microbial population in the cellulase-treated fermentation were associated with the greater availability of reducing sugars. The higher sugar concentration may have provided additional substrate for microbial growth, particularly at 48 h (Table 3).

Table 5 Specific microbial growth rate (μ).

Treatment	μ (h^{-1})
Control	0.084
Immobilized cellulase	0.132

3.5 Relationships among Enzyme Activity, Sugar Release, and Microbial Growth

Cellulase activity and reducing sugar concentration were strongly correlated ($r = 0.93$; Table 6). During fermentation, the reducing sugar concentration increased as cellulase activity gradually decreased from its initial value, although the enzyme remained active throughout the 72 h period. The correlation between the

two parameters is consistent with the role of cellulase in cellulose hydrolysis and soluble sugar release [20].

Table 6 Pearson correlation coefficients among fermentation parameters.

Parameter comparison	r value
Cellulase activity vs reducing sugar	0.93
Reducing sugar vs microbial growth	0.91
Cellulase activity vs growth rate (μ)	0.88

A similar relationship was observed between the reducing sugar concentration and the microbial growth ($r = 0.91$; Table 6). During the first 48 h, the cellulase-treated fermentation showed both a higher reducing sugar concentration and a larger microbial population. At 48 h, reducing sugar reached 10.7 mg/mL and the microbial population reached 6.4×10^6 CFU/mL, compared with 6.1 mg/mL and 1.9×10^6 CFU/mL, respectively, in the control. The higher sugar concentration coincided with greater microbial growth during this period [21].

The relationship between cellulase activity and microbial growth rate was also observed ($r = 0.88$; Table 6). The growth rate was 0.132 h^{-1} in the fermentation with immobilized cellulase and 0.084 h^{-1} in the control. Changes in cellulase activity were accompanied by changes in microbial growth rate during fermentation [22].

The use of immobilized cellulase is relevant to non-sterile coffee fermentation because the enzyme remained associated with the activated carbon during the process. In this study, cellulase was adsorbed onto activated carbon prepared from coffee cherry pulp. The enzyme remained active in the fermentation system after immobilization. Previous studies have also reported the stabilization of immobilized hydrolytic enzymes [23].

3.6 Implications for Coffee Fermentation and Quality

Sensory analysis was not conducted in this study, so the effect of the treatment on cup quality could not be assessed directly. The fermentation data showed differences between the control and the cellulase-treated fermentations, particularly in reducing sugar concentration and microbial growth. These differences were observed during the fermentation process associated with mucilage degradation. Previous studies have reported that fermentation conditions and microbial activity can affect the biochemical and sensory characteristics of coffee [24].

The effect of fermentation conditions on coffee quality has also been reported for different fermentation practices. In Conilon coffee, yeast-driven fermentation for

a 48 h produced higher sensory scores for acidity and aromatic characteristics than shorter or unfermented treatments [25]. Other work using self-induced anaerobic fermentation reported changes in microbial and enzymatic activity together with differences in sensory characteristics [26]. These reports provide a basis for considering fermentation behavior when discussing possible effects on coffee quality, although such effects were not measured directly in the present study.

In the present study, the addition of immobilized cellulase changed the fermentation conditions, as shown by the differences in reducing sugar concentration and microbial growth. The enzyme remained active throughout the fermentation period, while the microbial population was higher in the cellulase-treated fermentation. These results show that immobilized cellulase affected the fermentation environment, but they did not reveal whether the resulting coffee had better sensory characteristics. This distinction is important because microbial metabolites formed during fermentation may contribute to attributes such as sweetness, fruitiness, and aroma [26].

The results also indicate a possible application of the immobilized enzyme in coffee fermentation. Immobilized cellulase could be incorporated into a starter system to provide cellulase activity during fermentation. In addition, activated carbon prepared from Arabica coffee cherry pulp was used as the carrier for the enzyme, giving the coffee-processing residue a direct use in the immobilization process. The use of coffee pulp in cellulase-related applications has also been reported previously [27].

Conclusion

The immobilized cellulase prepared using activated carbon from Arabica coffee cherry pulp remained active during the 72 h non-sterile coffee fermentation. The immobilization efficiency was 80% and about 70% of the initial cellulase activity remained after 72 h. Compared with the control, the fermentation with immobilized cellulase showed higher reducing sugar concentrations and faster microbial growth. The differences were most apparent during the first 48 h. These findings support the use of coffee cherry pulp-derived activated carbon as a carrier for cellulase in a fermentation starter system.

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