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Article

Optimization of Matrix Components for Improved Catalytic Activities of Cellulase Immobilized on Biochar-Chitosan Beads

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Abstract: Bioethanol is a renewable energy that is gaining popularity globally. It's biochemical production requires the use of enzyme, especially cellulase. Cellulase is an enzyme that catalyzes the degradation of cellulose and related polysaccharides which finds applications in food, textiles, detergents, biofuels etc. However, the worldwide use of cellulase is limited by its relatively high production costs and low biological activity. This study was design to locally produce biochar-chitosan beads at optimized conditions to immobilize cellulase for improved thermal and storage stability as well as ensure reusability of the enzyme so as to improve biological activity and avoid the continuous production of free cellulase thereby reducing the production cost. Biochar was produced by pyrolyzing sugarcane bagasse in a local airtight chamber for 1 hour. Beads were formed from different ratios of biochar and chitosan in varying concentrations of calcium chloride solution as generated by design expert software version 13. The beads were dried in an oven at 50 ^{0}C for 24 hours and functionalized in 25% glutaraldehyde (GDA). The beads were loaded with enzyme (10.06 µmole/min/mL) at room temperature (27 ± 3 ^{o}C). Enzyme activity, thermal stability, storage stability and reusability tests were carried out according to standard procedures. The half-life and activation energy were also evaluated. The result showed that the optimum activity of the loaded enzyme (2.63 µmole/min/mL) was obtained when 2.46 g of porous biochar was mixed with 2.48 g chitosan in 5 % Calcium chloride aqueous solution. The immobilized enzyme was able to maintain thermal stability between 30 ^{o}C and 70 ^{o}C while the activity for free enzyme started declining after 50 ^{o}C. Also, the activation energy for immobilized cellulase enzyme (23.17 KJ/mol) was lower than the activation energy (55.146 KJ/mol) for free cellulase. The half-life, when stored at ambient Temperature (27 ± 3 ^{o}C), for free enzyme was 0.4 days while the half-life for immobilized enzyme was 3.59 days. Therefore, cellulase immobilized on support locally produced at optimal conditions had improved catalytic properties when compared to the free enzyme. Hence, more indigenous materials and practices may be employed for a cost effective and cheaper industrial processes.

Keywords: cellulase; sugarcane bagasse; pyrolysis; local chamber; composite beads

1. Introduction

Cellulase is an enzyme that catalyzes the degradation of cellulose and related polysaccharides. It is mostly produced by fungi, bacteria, and protozoans [1]. The breakdown of cellulose is incredibly significant commercially since it is a crucial component of plants that is available for consumption and usage in chemical processes. Because cellulose molecules are securely bonded together, cellulolysis is more difficult than the breakdown of other polysaccharides like starch [2]. Cellulase has become effective catalysts for the conversion of lignocellulosic materials to bioethanol [3]. Cellulase enzymes are mostly used as free enzymes in industries,

being free, operational cost will increase and applications may be limited. It has been demonstrated previously that immobilization on a solid support can increase the stability of enzyme and makes it simpler to recover from the reaction medium [4]. Immobilization can also be a mean to increase the stability and reusability of enzymes for a solid substrate.

Currently, materials for enzyme immobilizations are expensive and may not easily be sourced. There is therefore need to produce renewable and eco-friendly immobilization matrix which will make industrial operations cheaper and cost effective. Such matrix when developed locally can also create local jobs and enhance economic earnings for local farmers [5]. Support matrices for enzyme immobilization include a variety of materials, such as organic, inorganic, and hybrid materials [6]. Interest on the usage of environmentally friendly materials such as biochar for support has also grown recently. Biochar is described as "a heterogeneous substance rich in aromatic carbon and minerals" by the European Biochar certificate (EBC). It is created through the regulated, clean pyrolysis of harvested biomass for any use that does not involve its quick conversion to CO_2. Thus, biochar serves as a carbon storage medium, and the production of biochar as a whole is a carbon-negative process that contributes to reducing atmospheric carbon dioxide levels [7]. Due to its high carbon concentration, biochar has potential uses in a variety of industries.

Biochar is gradually being established as a viable immobilization support for enzymes due to its many favorable characteristics [4,5]. However, the modern process involved in biochar production is complex and capital intensive. Therefore, the production of biochar using local chamber may contribute to cheaper industrial process. Also, biochar is limited in its ability to solely immobilize enzymes due to insufficient hydrophilic groups as well as difficulty in recovery from reaction media. Studies have shown that, chitosan when combined with biochar has given rise to a better catalysis because chitosan contains amino and hydroxyl group needed for enzyme binding and also binds with biochar to form a recoverable solid from reaction medium. Biochar-chitosan composites are a promising material for enzyme immobilization due to their high surface area, biocompatibility, and mechanical strength [6]. The composite matrix has been shown to retain and improve the immobilized enzyme properties.

Calcium chloride is a cross-linking agent that has been reported to improve the stability and reusability of immobilized enzymes [8,9]. The use of biochar-chitosan composites in different concentrations of calcium chloride for enzyme immobilization has been studied for a variety of enzymes, including cellulases, xylanases, and laccases [8]. In general, it has been found that the optimal concentration of calcium chloride for enzyme immobilization depends on the specific enzyme and the desired properties of the immobilized enzyme. However, there are scarce reports on the optimization of the concentrations of biochar, chitosan and calcium chloride needed for the production of matrix to immobilize cellulase. Therefore, this study was designed to locally produce biochar and as well optimize the conditions for the production of biochar/chitosan beads in calcium chloride to immobilize cellulase for improved reusability, thermal and storage stability.

2. Materials and Methods

2.1. Materials

Cellulase used for this study was produced by A. *niger* as reported in our previous study [10]. Other chemicals such as Potassium hydroxide (KOH), Hydrochloric acid (HCL), Chitosan (CS), Acetic acid (HAc), Sodium acetate (NaAc), glutaraldehyde (GA, 25% *v/v*), and Carboxymethyl Cellulose (CMC) were of analytical grade and obtained from Biochemistry Laboratory, Federal University of Technology Minna, Niger State, Nigeria. Clay pot used for the pyrolysis was obtained from central market in Minna, Niger State.

2.2. Sample Collection

The Sugarcane Bagasse used for biochar production was collected from Kasuwan Gwari in Minna, Niger State, Nigeria.

2.3. Sample Preparation

The sugarcane bagasse was separated from dirt, thoroughly washed with clean water and then dried until a constant weight was obtained. This was used for further studies.

2.4. Production of Biochar from Sugarcane Bagasse

The production of porous biochar from sugarcane bagasse is shown in Figure 1. The pyrolysis of sugarcane bagasse was done using a local clay pot tightly covered with a foil paper guided by a solid plank cover to regulate the penetration of oxygen during pyrolysis. After the sugarcane bagasse has been pyrolyzed for 1 hour, the black residue obtained was then blended into powdered form after which 1.5M of HCl was added to remove ash from the biochar and then it was dried at 50 ^{0}C in the oven [11].

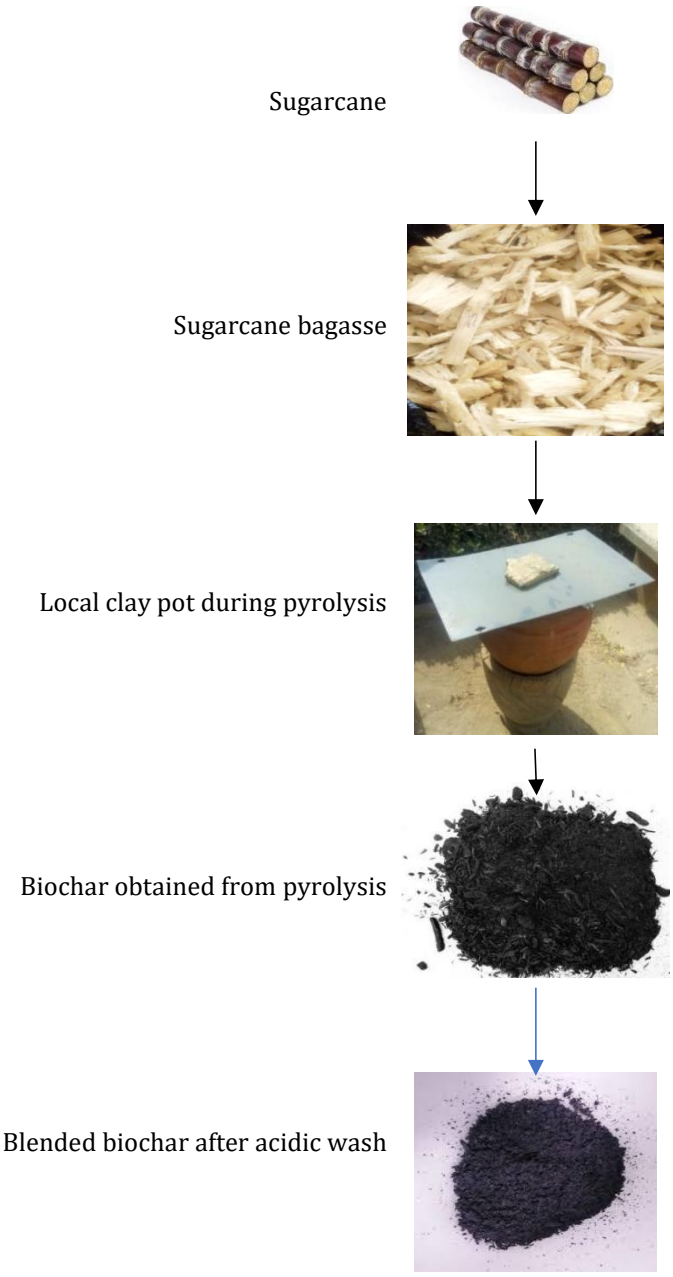


Figure 1. Production of Biochar from Sugarcane Bagasse.

2.5. Preparation of Coagulation Medium and Formation of Biochar-Chitosan Beads

The coagulation medium was prepared by first dissolving varying masses of Calcium Chloride ($CaCl_2$) in 10 mL of water (H_2O) to form different concentrations generated by design expert software version 13, as shown in Table 1, to form solution A. The second solution (B) was prepared by adding 1 g of Sodium Hydroxide (NaOH) in 10 mL of 2.6% Ethanol. Finally, solution A and B were combined and used as the coagulation medium to form the

beads [8,12,13]. The beads were formed in different categories by using varying amount of biochar and chitosan paste (Table 1). The paste was dropped with a 10 mL syringe into a coagulating medium to form fresh beads as shown in Figure 2. The beads produced were washed thrice (3 times) with 0.1 M Acetate buffer (pH 5) and then rinsed with distilled water (DH_2O) until the water became neutral. After which the beads were dried in an oven at 50 °C.

Table 1. Three-level factorial Box-Behnken experimental design for the optimization of bead (matrix) concentration.

Run	Factor 1 A: Porous Biochar	Factor 2 B: Chitosan	Factor 3 C: Calcium Chloride
	Gram	Gram	%
1	1.5	1.5	7.5
2	1.5	2.5	5
3	2.5	0.5	7.5
4	2.5	1.5	10
5	2.5	2.5	7.5
6	0.5	2.5	7.5
7	1.5	0.5	10
8	0.5	1.5	10
9	1.5	0.5	5
10	0.5	0.5	7.5
11	0.5	1.5	5
12	2.5	1.5	5
13	1.5	2.5	10

Figure 2. Production of biochar-chitosan beads.

2.6. Activation and Loading of Biochar-Chitosan Beads

Biochar-Chitosan Beads (5 g) produced in different categories following the runs generated by design expert software version 13 were place in 10 mL of 25% Glutaraldehyde (GDA), then it was placed on an orbital shaker at 150 rpm for 4 hours. The GDA was then decanted and beads were washed with distilled water to remove excess GDA until the water became neutral. Activated biochar-chitosan Beads were added into 5 mL of

enzyme (10.062 μmole/min/mL) and 2 mL of citrate buffer (pH 5.0) then placed on an orbital shaker for 16 hours (overnight) and the beads were removed for further studies [4].

The activity (μmole/mL/min) of cellulase in the loaded biochar-chitosan beads was determined by the use of the formula in Equation (1).

$$\frac{(\text{mg/ml } of\ glucose\ released) \times (dilution\ factor) \times 1000}{incubation\ time\ \times\ volume\ of\ enzyme\ x\ molecular\ weight\ of\ product} \quad (1)$$

Porous biochar concentration, chitosan concentration, and Calcium chloride concentration were optimized to get the optimum cellulase activity based on the model developed by design expert software version 13.

2.7. Reusability Assay for Immobilized Cellulase

Biochar-chitosan beads (1 g) was added to 1 mL of 1% carboxymethyl cellulose (CMC) in citrate buffer (pH 5.0) as and incubated in water bath at for 10 minutes, after which the enzyme activity was determined [14]. At the end of each batch, the biochar-chitosan beads were separated by mesh and washed with water, followed by reusing the biochar-chitosan beads in a new reaction cycle. This process was repeated using fresh substrate for each cycle. The operational stability (%) was evaluated by the percentage of residual enzyme activity from each cycle by the formular shown in Equation (2). The enzyme activity in the first cycle for the immobilized enzyme was taken as the control and correspond to 100% activity.

$$\frac{\text{Activity in nth order}}{\text{Activity in the first order}} \times 100 \quad (2)$$

2.8. Thermal Stability of Immobilized Cellulase

The free and immobilized enzymes were incubated in citrate buffer (pH 5.0) at varying temperatures between 40 °C and 90 °C for 3 hours without substrate to determine the thermal stability as described by reference [15]. Samples were taken at 30 minutes interval to determine the residual enzyme activity by DNS method using carboxyl methyl cellulose as substrate. Activation energy (E_a) of free and immobilized enzymes was also determined from the slope of Arrhenius plot using Equation (3).

$$E_a = -R\ slope \quad (3)$$

where R = Universal gas constant = 8.314 J $mol^{-1}K^{-1}$.

Arrhenius plot is a graph of natural logarithm (ln) of rate constant (K) versus inverse of reaction temperatures in Kelvin.

2.9. Determination of Storage Stability of Free and Immobilized Cellulase

Biochar-chitosan beads (1 g) was added to 1 mL of 1% carboxymethyl cellulose (CMC), incubated in citrate buffer (pH 5.0) at ambient Temperature (27 ± 3 °C). The enzyme was stored for 5 days and enzyme assay repeated at 24 hours intervals [16].

3. Results and Discussion

3.1. Variable Factors and Responses for the Determination of Cellulase Activity

The actual values obtained for cellulase activity at different variable concentrations during the experiment is shown in Table 2. Table 2 also includes predicted responses for cellulase activities as generated by design expert software version 13. Thirteen runs were generated by design expert software for the present study as shown in Table 1. However, some combinations (runs 9, 10, and 11) could not form a solid bead. This could be as a result of very weak bonds formed between the chitosan and porous biochar combination. A comparison of the predicted values with the responses obtained from the experiment shows a little deviation, which mean that there is high extent of correspondence between the predicted and the actual cellulase activities. Porous biochar

and chitosan are the major components of the immobilization bead. However, the amount of calcium ions which diffuse through the composite binds the unoccupied site of the chitosan, facilitating the development of the bead shell. Since the driving force of the calcium diffusion from the droplet to the outside solution is a concentration gradient, an increase in $CaCl_2$ concentration would result in the increase of biochar-chitosan bead shell thickness, as well as a degree of crosslinking with cellulase [17]. In the present study, Table 2 shows that, all the experimental runs with high calcium ion concentrations (10%) have low cellulase activity except run 13 which higher cellulase activity may be enhanced by the high concentration of chitosan. This corroborated the report by [18] that a high concentration greater than 5% (>0.4 M) of Ca^{2+} concentration led to a decrease in the activity of enzyme immobilized on alginate beads as a result of alteration in pH and distortion in the active site which may lead to reduced catalytic activities at microenvironment of the beads. The author reported that, at a concentration of 0.4 M (5%) or lower, the biochar-chitosan bead has an improved shell thickness that is capable of an increase of alginate concentration might result in the increase of the amount of carboxyl group in the alginate shell, which facilitated the reaction between the negatively charged carboxyl groups of alginates and the protonated amino groups of (3-Aminopropyl) triethoxysilane (APTES). This suggests that optimum Ca^{2+} concentration has the capacity to improve the cellulolytic activity in the immobilized state, thus supporting the study by reference [19].

Table 2. Three-level factorial Box-Behnken experimental design for the optimization matrix concentration and experimental responses.

	Factor 1	Factor 2	Factor 3	Response	
Run	**A: Porous Biochar**	**B: Chitosan**	**C: Calcium Chloride**	**Actual Cellulase Activity**	**Predicted Cellulase Activity**
	Gram	Gram	%	U/mL	U/mL
1	1.5	1.5	7.5	1.56005	1.56
2	1.5	2.5	5	2.30354	2.16
3	2.5	0.5	7.5	2.23047	2.27
4	2.5	1.5	10	1.40754	1.59
5	2.5	2.5	7.5	2.10655	2.37
6	0.5	2.5	7.5	2.50371	2.30
7	1.5	0.5	10	1.89049	2.09
8	0.5	1.5	10	1.79835	1.70
12	2.5	1.5	5	2.54502	2.34
13	1.5	2.5	10	1.94451	1.86

3.2. Optimized Conditions for Variable Factors and Immobilized Cellulase Activity

Optimization study was done using design expert software version 13. The optimum cellulase activity of 2.61 μmole/min/mL was observed when 2.5-gram chitosan reacted with 2.5 gram of porous biochar in 5% Calcium chloride solution.

3.3. Statistics Relevance of Developed Model

The relevance of the model developed is shown in Table 3. The model developed for the present study was statistically significant ($p < 0.05$). The predicted R^2 of 0.8332 in reasonable agreement with the adjusted R^2 of 0.9403 (the difference is less than 0.2). Also, R^2 value of 97.01% is an indication that the model satisfactorily represents the relationship between the independent variables (porous biochar concentration, chitosan

concentration and Calcium chloride concentration) and the response (cellulase activity). Adequate Precision which measures the signal to noise ratio has a high value of 14.489, which indicates an adequate signal with little or no noise. Therefore, this model can be used to navigate the design space. This is consistent with the model developed by reference [20], who obtained an R^2 value of 0.953 which is an indication of high model significance when sugarcane molasses was used to produce bioethanol. Equations (4) and (5) were generated by design expert software for futuristic predictions of the responses outside the current design space. Equation (4) is the coded equation which was first generated by the software for the software to interpret the variables. The actual equation was generated from the coded equation.

Table 3. Statistics relevance of developed model.

Std. Dev.	0.2318
Mean	1.56
C.V.%	14.85
R^2	0.9701
Adjusted R^2	0.9403
Predicted R^2	0.8332
Adequate Precision	14.4890

Coded Equation (4):

$$\boldsymbol{Y} = 1.56079 + 0.42139 \times A + 0.450381 \times B + 0.415829 \times C + (-0.733958 \times AB) + 0.811008 \times AC + 0.562382 \times BC \quad (4)$$

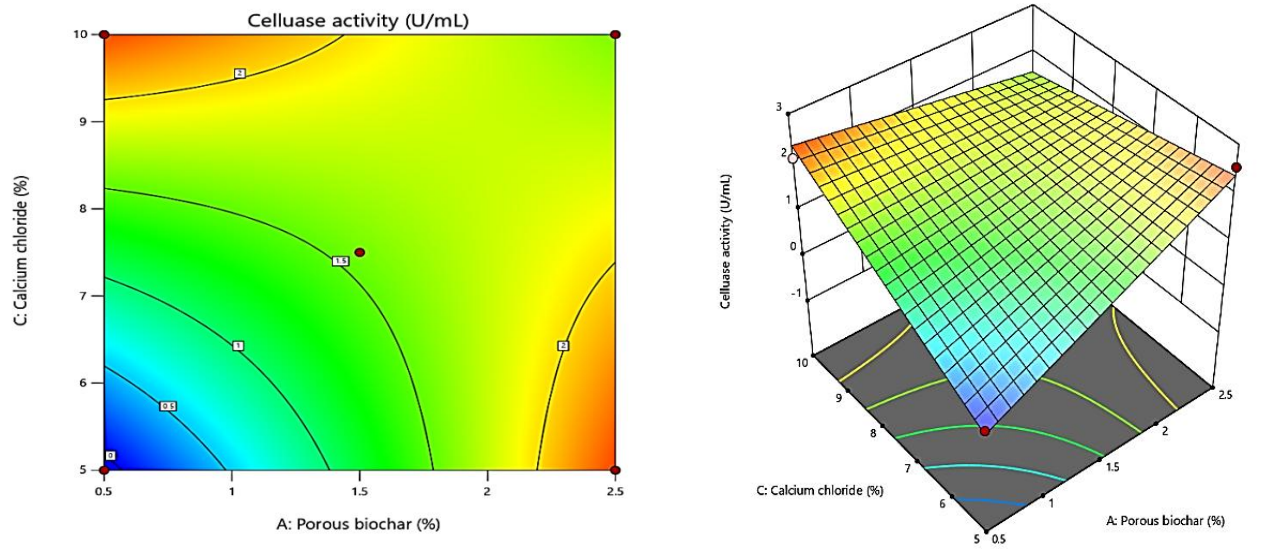
Actual Equation (5):

$$Cellulase\ activity = -8.82601 + 3.95535 \times Porous\ biochar + 3.23846 \times Chitosan + 0.990365 \times Calcium\ chloride - 0.733957 \times Porous\ biochar \times Chitosan - 0.324403 \times Porous\ biochar \times Calcium\ chloride - 0.224953 \times Porous\ biochar \times Calcium\ Chloride \quad (5)$$

3.4. Visual Representation of the Interaction between the Independent Variables

Response surface curves were plotted to examine the effect of the interaction between the independent variables and to determine the optimum levels of the variables in relation to the response. To determine the optimal levels of the independent variables affecting cellulase activity, three-dimensional (3D) response surface and contour plots were constructed according to the regression model. The 3D plots were generated by keeping one factor at its optimum point and varying the other two factors within their experimental ranges. The plots show how porous biochar concentration, chitosan concentration and Calcium chloride concentration affects cellulase activity. The contour plot may be rising ridges, saddle point, elliptical or circular plot. A circular or elliptical plot indicates that there exists a significant interaction between the operating parameters [20].

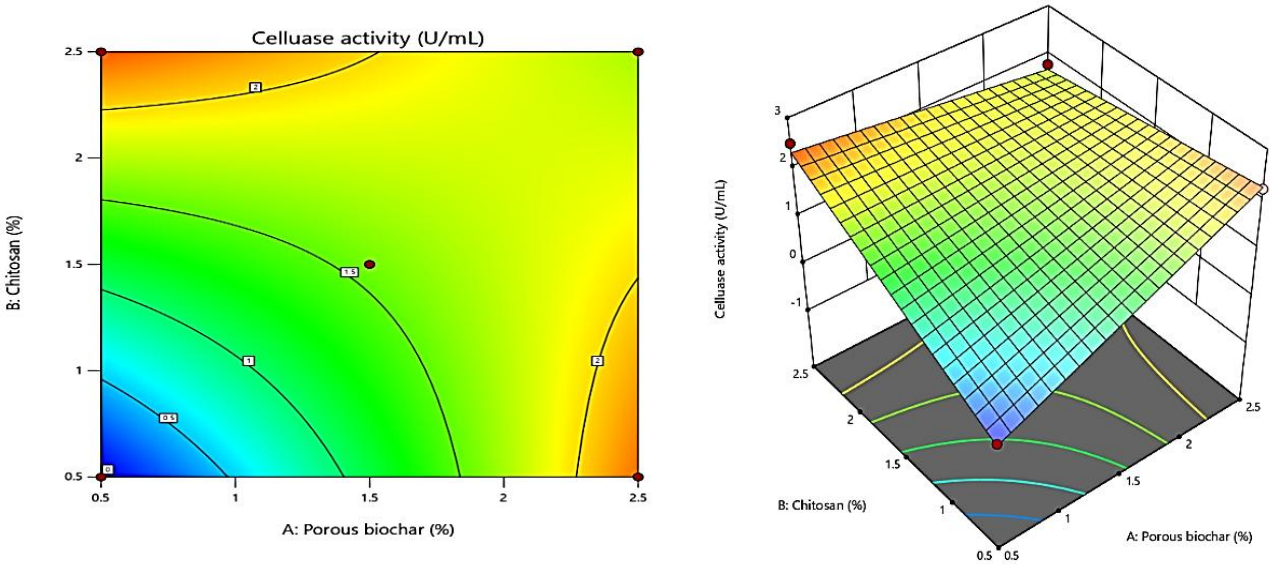
The effect of porous biochar concentration and Calcium chloride on cellulase activity when chitosan was kept constant at 0.5% is shown in Figure 3. It was observed that the highest cellulase activity (2.30 μmol/min/mL) was obtained when Calcium chloride concentration was 5% and porous biochar was 2.48%. However, the lowest cellulase activity (0.04 μmol/min/mL) was obtained when the concentration of porous biochar was reduced to 0.57%. This is an indication that the low enzyme activity could be as a result of insufficient functional groups for enzyme binding due to low concentrations of porous biochar and chitosan.



(**a**) (**b**)

Figure 3. Contour plot and corresponding 3-D response surface plot for interactive effect of the concentration of calcium chloride and porous biochar on cellulase activity. (**a**) Contour plot; (**b**) 3-D response surface plot.

The effect of porous biochar concentration in Figure 4. It and chitosan concentration on cellulase activity when Calcium chloride is kept at 7.5% is shown was observed that when high concentrations of porous biochar (2.45%) and low concentration of chitosan (0.68%) were interacted the cellulase activity (2.34 μmol/min/mL) was high. Also, high enzyme activity (2.25 μmol/min/mL) was obtained when low concentration of porous biochar (0.63%) interacted with high concentration of chitosan (2.47%) However, the enzyme activity (0.15 μmol/min/mL) was lowest when the concentration of chitosan (1.06%) and porous biochar concentration (1.10%) were low. This implies that at constant calcium chloride concentration, the concentration of porous biochar and chitosan should be inversely proportional to obtain a high cellulase activity.

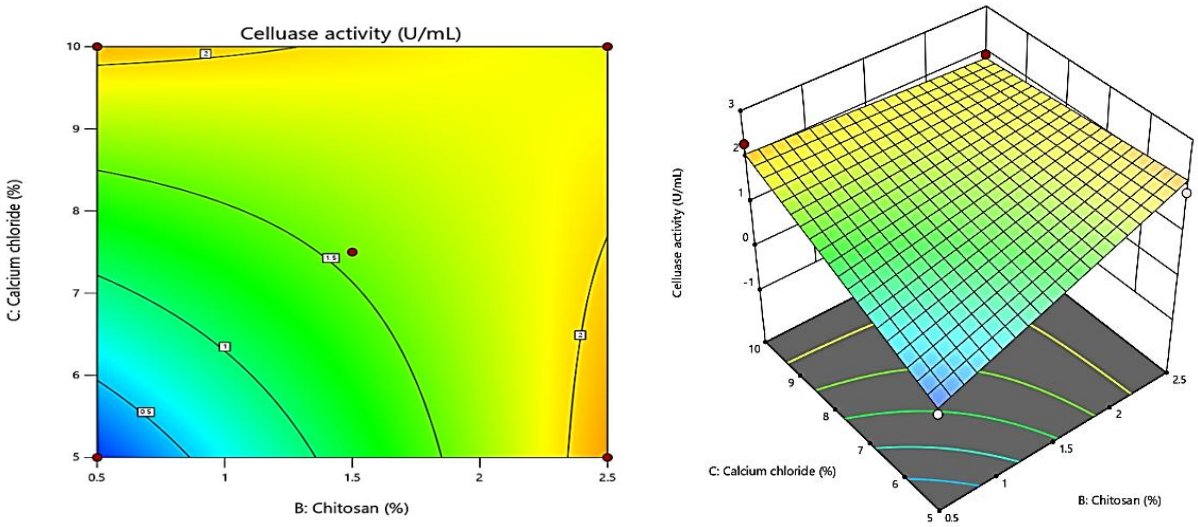


(**a**) (**b**)

Figure 4. Contour plot and corresponding 3-D response surface plot for interactive effect of the concentration of chitosan and porous biochar on cellulase activity. (**a**) Contour plot; (**b**) 3-D response surface plot.

The effect of concentration of Calcium chloride and chitosan on cellulase activity when porous biochar was kept constant at 1.5% is shown in Figure 5. It was observed that an increase in chitosan concentration (2.46%)

and a decrease in calcium chloride concentration (5%) resulted into increased cellulase activity (2.14 μmol/min/mL). However, the enzyme activity was lowest (0.1 μmol/min/mL) when chitosan concentration was low (0.53%) despite keeping the calcium chloride at the same 5%. This further confirms the need for optimum concentration of chitosan to provide adequate functional groups so that sufficient binding sites could be available for the enzyme.



(**a**) (**b**)

Figure 5. Contour plot and corresponding 3-D response surface plot for interactive effect of the concentration of calcium chloride and chitosan on cellulase activity. (**a**) Contour plot; (**b**) 3-D response surface plot.

3.5. Validation of Statistical Model

In order to confirm the validity of the statistical model, three confirmation experimental runs were performed at the chosen optimum cellulase activity conditions. The result (Table 4) shows that the maximum experimental cellulase activity of 2.52 μmol/min/mL obtained was close to the predicted value of 2.57 μmol/min/mL The excellent correlation between the predicted and measured values of these experiments shows the validity of the statistical model.

Table 4. Confirmation of statistical model developed for immobilized cellulase activity.

Analysis	Predicted Mean	Predicted Median	Observed	Std Dev	N	SE Pred	95% PI low	Data Mean	95% PI high
Cellulase Activity	2.571	2.571	2.573	0.232	3	0.265	1.923	2.517	3.220

3.6. Thermal Stability and Activation Energy of Free and Immobilized Cellulase

The thermal stability of free and immobilized enzymes is as shown in Figure 6 Immobilized enzyme maintained a stable catalytic activity up to 70 °C after which a decline in activities were observed. However, a catalytic activity of over 30% of the initial activities were retained even at 90 °C for 90 minutes. Meanwhile, despite the initial high activity of free cellulase at the reaction temperatures, there was a rapid decline of activity after 50 °C and the enzyme virtually lost its activity at 90 °C for 90 minutes. Studies have shown that Immobilize enzyme have greater resistance to thermal denaturation which implies that they can maintain their activity at higher temperature, extending their use in processes where temperature fluctuations are common [21,22].

Arrhenius graphs (Figure 7) were plotted for free and immobilize cellulase using the values of cellulase activity at different Temperatures. These graphs were used to calculate the activation energy (Ea) of free and immobilized enzyme. The amount of energy required for a reaction to begin is referred to as activation energy [23]. Activation energy of free cellulase was 55.12 KJ/mol while the activation energy of immobilized cellulase

was 23.17 KJ/mol. This means that the reaction catalyzed by immobilized cellulase requires less energy to be initiated than reactions catalyzed by free cellulase. A similar increase in thermal stability following multi enzyme immobilization was also reported by [24,25] also reported that covalent immobilization of inulinase reduced the Ea from 28.41 to 16.216 KJ/mol, resulting in increased catalytic efficiency of the immobilized inulinase.

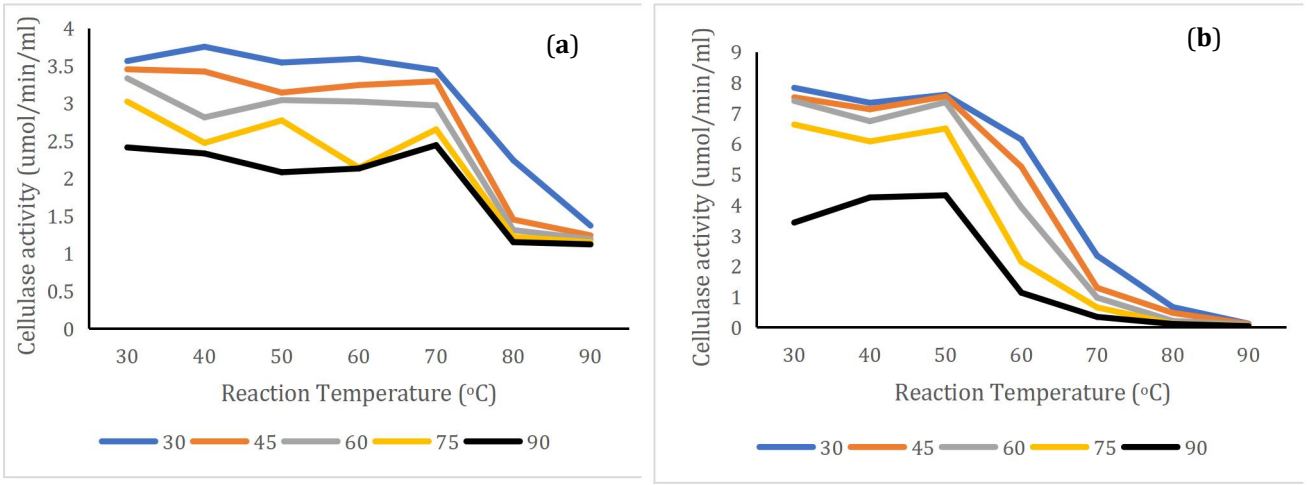


Figure 6. Effect of temperature on (**a**) immobilized cellulase; (**b**) free cellulase.

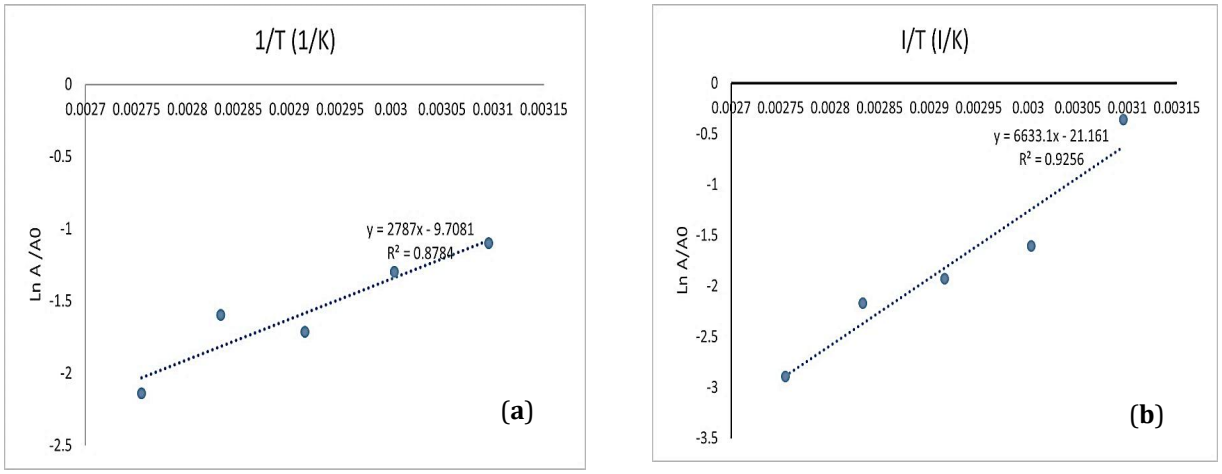


Figure 7. Arrhenius plots for (**a**) immobilized cellulase; (**b**) free cellulase.

3.7. Storage Stability Test for Free and Immobilized Cellulase

The potential of enzyme to be stored for future use is an important industrial requirement since enzyme may not be produced at every industrial process. The storage stability of immobiilized cellulase when compared to the free cellulase is shown in Figure 8. In the present study, free cellulase and immobilized stored at ambient condition (27 ± 3 °C) for 5 days were able to retain 35% and 65% respectively of their activities. Furthermore, the half-life of the enzymes was calculated as shown in Table 5 to determine how long (days) it will take for the enzymes to lose 50% of their initial activities. The half-lives of free cellulase and immobilized when stored at ambient Temperature was calculated as shown in Table 5. The immobilized enzymes had a longer half-life value of 3.59 days while the half-life of free enzyme was 0.4 days (6 hours). These findings suggest that the immobilization strategy employed in this study improved the storage stability of free cellulase. As a result, the immobilized enzymes could be stored at ambient temperature (27 ± 3 °C) for about 4 days while still retaining 50% of its activity.

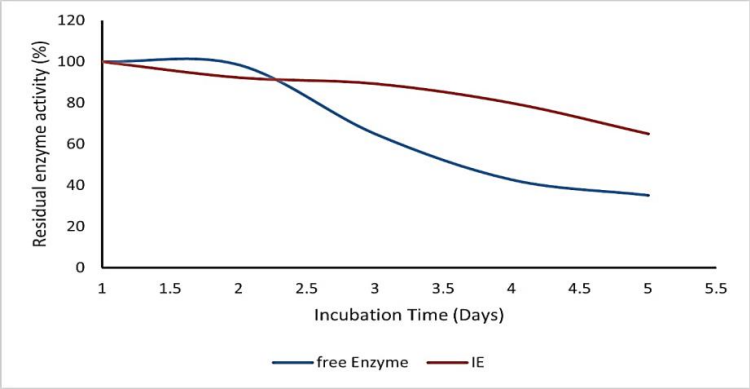


Figure 8. Effect of incubation time on the activity of free and immobilized cellulase.

Table 5. Half-life of free and immobilized cellulase.

Enzymes	Half-Life (Days)
Free cellulase	0.4
Immobilized cellulase	3.59

3.8. Reusability Test for Immobilized Cellulase

One important purpose of immobilizing enzyme is to facilitate catalyst recycling and reuse for the aim of reducing the cost. In the present study, the immobilized enzyme was able to retain more than 74.2% of its initial activity after five cycles of hydrolysis as shown in Table 6. This backs up the findings of reference [26], who found that the enzyme immobilized on biochar-chitosan composite beads retained approximately 60% of its initial activity after eight consecutive cycles. According to some reports, the observed decrease in catalytic properties of immobilized enzyme after repeated use is most likely due to factors such as product inhibition, structural modification of the enzyme, protein denaturation, and/or immobilized enzyme inactivation [27,28]. The ability of the immobilized enzymes prepared in this study to retain more than 50% activity after the 5th use suggests that a single batch of immobilized enzymes can be used for multiple hydrolysis using fresh substrate thereby avoiding the repeated production cost of producing free enzyme.

Table 6. The effect of number of usages on the activity of immobilized cellulase.

Runs	Activity (µmol/min/mL)	Residual Activity
1	2.21	100.0
2	2.07	93.7
3	1.98	89.6
4	1.83	82.8
5	1.64	74.2

4. Conclusions

This study investigated catalytic activities of cellulase immobilized on combination of locally produced sugarcane bagasse biochar and chitosan in calcium chloride at optimal concentrations. Optimal immobilized cellulase activity of 2.63 µmole/min/mL was obtained at optimal concentrations of 2.46 g for porous biochar, 2.48 g chitosan and 5% calcium chloride, The immobilized cellulase was stable up to 70 ^{0}C before losing its catalytic activity. The activation energy of free enzyme (55.12 KJ/mol) was higher than the activation energy of immobilized enzyme (23.17 KJ/mol) indicating that lesser energy was needed by the immobilized enzyme to initiate its catalytic reactions. Also, immobilized cellulase was able to retain 50% of its initial activity when stored in ambient condition (27 ± 3 ^{o}C) up to 4 days while free enzyme could only retain the 50% of its initial activity up to 0.4 days (6 hours) when stored in the same condition. Although, the initial activity of immobilized enzyme was low when compared to free enzyme but the ability of the immobilized enzyme to be re-used up to 5 times while still retaining over 70% of its activity gave it an edge over the free enzyme. It was clearly

demonstrated in the study that the biological activities, such as thermal and storage stability as well as activation energy of enzyme immobilized on locally produced matrix were improved when compared to free cellulase. Also, the ability of the immobilized enzyme to be re-used up to 5 times while still retaining over 70% of its activity, thereby avoiding the continuous production of free cellulase, may lead to reduction in production cost. Therefore, the production of matrix for immobilization using locally sourced material is a step toward a cost effective and cheaper industrial process such as production of bioethanol.

Author Contributions

The authors confirm their contributions to the paper as follows: Supervision, E.C.E.; Data collection, I.P.G.; Analysis and interpretation of result, I.P.G. and O.O.O.; Conceptualization, E.C.E. and O.O.O.; Writing—original draft preparation, I.P.G.; Writing—review and editing, E.C.E. and O.O.O. All authors have read and agreed to the published version of the manuscript.

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The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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