



Article

Development of Carbon-Based Support Using Biochar from Guava Seeds for Lipase Immobilization

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Abstract: Enzymes are promising tools for achieving an environmentally benign process. However, enzymes are required to be immobilized on economically competent supports to be reusable and maintain their activity. In this work, the aim was to evaluate the application of the biochar for immobilization of *Burkholderia cepacia* lipase (BCL) by physical adsorption (PA) and covalent binding (CB). Additionally, it was observed that regarding the biochemical properties, the optimal pH was 4.0 for the BCL immobilized by PA and pH 7.0 for the BCL free and immobilized by CB. Among the kinetic parameters, the maximal velocity ($V_{\max}$) for the free enzyme was $2500 \mu\text{mol g}^{-1} \cdot \text{min}^{-1}$, and for the PA- and CB-immobilized biocatalyst the values of $V_{\max}$ were 2000 and $3333 \mu\text{mol g}^{-1} \cdot \text{min}^{-1}$, respectively. The Michaelis-Menten constant (K_m) value for the free lipase was 665 mM and for the biocatalysts immobilized by PA and CB the K_m values were 219 and 369 mM, respectively. Immobilized LBC exhibited superior thermal stability. The reusability tests showed that the LBC immobilized by PA preserved 50% of the initial activity after 16 cycles. Thus, biochar is a by-product of a renewable source; therefore, it is a promising alternative for lipase immobilization demonstrating its potential for use in a wide range of greener industrial processes.

Keywords: guava seeds; biochar; lipase; immobilization; biocatalyst immobilized



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1. Introduction

The biomass-derived biochar emerges as a sustainable alternative for the development of carbon-based materials and transforms biomass residues into value-added materials [1]. Brazil is responsible for the production of 43.6 million tons of fruits per year, which makes the country stand out in the generation of fruit and food residues, and most is destined for inappropriate places. Guavas are a fruit of relevant impact in Brazil, as about 0.6 million tons are produced [2]. In the processing stages of the guava industry, there is waste generation, which constitutes up to 30% of residue (seeds and the pulp and peel) [3].

According to previous research, seeds are a suitable raw material for bio-oil production due to the good properties and optimum pyrolysis temperatures between 400 and 600 °C [3–5]. Pyrolysis can convert biomass into three kinds of pyrolysis products such as bio-oil, biochar and bio-gas [6]. Thus, the operating conditions such as pyrolysis temperature, pyrolysis time and heating rate influence the yield and quality of pyrolysis product. Low pyrolysis temperature is conducive to the production of biochar [6–8].

Biochar is a kind of pyrolysis product which has already been investigated. The most used applications for biomass-derived biochar are for the adsorption of organic compounds,

soil fertilization and immobilization of metals and enzymes [9–11]. Biochar is a solid product obtained by pyrolysis of biomass under high temperatures and conditions. Biochar offers advantages such as high surface area, inertness, pore structure and physicochemical resistance [11].

Currently, there is a growing number of studies on the use of Biochar as a support for enzyme immobilization [11–15]. Among the enzymes with industrial potential, lipases are of great interest due to their versatility of applications in several sectors (food, pharmaceuticals, detergents, leather, textiles, cosmetics, and paper industries) [16]. To widen their application in synthesis reactions, efficient immobilization methods are being investigated to reduce the cost of enzymes in industrial processes [11,17]. Some advantages of the use of enzymes as biocatalysts in industrial processes have been highlighted, such as mild reaction conditions and reduction in unwanted by-products [17,18].

However, the use of free enzymes is unusual in large-scale industrial processes due to limitations on reuse. The use of immobilized enzymes in biocatalysts is an alternative because these catalysts are easily separated from the product, allowing them to be applied in a large variety of industrial operations [19,20]. Several studies have been carried out seeking to enhance immobilization techniques on biochar supports aiming for reduced costs and increases in the thermal and operation stability of various immobilized biocatalysts [11–15]. Gonzalez et al. [12] evaluated the use of biochar derived from cascades of oats and pine nuts at 300 and 500 °C for the immobilization of *Candida rugosa* lipase by physical adsorption, which verified that the pyrolysis temperature and the raw materials used influence the structure and the chemical properties of biochar. Our group studied the use of biochar derived from guava seed for the immobilization by physical adsorption of *Burkholderia cepacia* lipase (BCL) and evaluated the effects of conventional (muffle) and non-conventional (ultrasound and microwave) treatments with dichloromethane (CH₂Cl₂), potassium hydroxide (KOH) and phosphoric acid (H₃PO₄) for modification of biochar [21]. The results show that the treatments performed on the biochar produced modifications in its morphological structure with promising results for lipase's immobilization.

Therefore, it is necessary to choose the appropriate method of immobilization for each type of support and enzyme, since the biochemical, mechanical, and kinetic properties of the immobilized biocatalyst can be improved according to the type of interaction between the enzyme and the support [22]. In order to obtain new alternatives for the sustainable development of the biocatalysts field, the goal of this work was to evaluate the use of biochar from guava seeds as a support for the immobilization of *Burkholderia cepacia* lipase (BCL) by physical adsorption (PA) and covalent binding (CB). The analysis was carried out in two stages: immobilization conditions (enzyme concentration), biochemical characterization of the immobilized biocatalysts (optimal pH, optimal temperature, kinetic parameters, thermal and operational stability), and their morphological and physical—chemical properties.

2. Materials and Methods

2.1. Materials and Reagents

Lipase from *Burkholderia cepacia* (BCL) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nominal lipase activity was 2500 U/g. The guava seeds sample was kindly provided by the fruit pulp industry (POMAR), located in the city of Aracaju (Brazil). Hexane and acetone were obtained from Isotar (Rio de Janeiro, Brazil); 95% ethanol was obtained from Vetec (Rio de Janeiro, Brazil); Arabic gum was obtained from Cromoline (São Paulo, Brazil); olive oil was purchased at a local market (Aracaju, Brazil). Other chemicals were of analytical grade and used as received.

2.2. Biochar Production

The production of biochar was carried in Laboratory-scale pyrolysis equipment as described in detail by Almeida et al. [21]. The conditions of the pyrolysis were used based on previous studies carried out in our research group and showed the better results in the

production of the bio-oil. The conditions: temperature = 500 °C, heating rate = 30 °C min^{−1}. The biochar was treatment with dichloromethane.

2.3. Determination of Hydrolytic Activity

For the determination of hydrolytic activities of free and immobilized lipase by the olive oil emulsion method according to the Soares et al. [22], one unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of free fatty acid per min of reaction. Analyses of hydrolytic activities performed on free and immobilized lipase were used to determine the immobilization yield (%) according to Equation (1).

$$\text{RI (\%)} = \frac{U_S}{U_0} \times 100 \quad (1)$$

in which U_S corresponds to the total enzyme activity recovered on the support and U_0 represents the enzyme units offered for immobilization.

2.4. Lipase Immobilization on Biochar by Physical Adsorption (PA)

The immobilization by physical adsorption (PA) of Lipase from *Burkholderia cepacia* on biochar was accorded the methodology of Soares et al. [22] with little modifications. A total of 1 g dry wt of biochar was added to 10 mL hexane in agitation at room temperature for 15 min, then 10 mL enzymatic solutions (a mass of the enzyme solubilized in 10 mL 0.1 M sodium phosphate buffer pH 7.0) was added and agitated for 3 h, followed by an additional period of incubating for 24 h at 4 °C. Immobilized lipase was recovered by vacuum filtration coupled with repeated washes with hexane, then stored at 4 °C until use.

2.5. Lipase Immobilization on Biochar by Covalent Binding (CB)

The *Burkholderia cepacia* lipase immobilization was carried out as described by Simões et al. [23]. The support was activated with a solution of epichlorohydrin 2.5% (v/v) in potassium phosphate buffer (0.1 M and pH 7.5), at a ratio of 1 g of support for every 10 mL of solution. After homogenization, the mixture was kept under agitation for 1 h at room temperature and then submitted to vacuum filtration. During filtration the support was exhaustively rinsed with distilled water and phosphate buffer solution, and then incubated for 24 h at 60 °C to remove excess moisture. The activated support was then immersed in hexane at a proportion of 1:10 (solid: liquid) and kept under agitation for 2 h. Afterwards, for each gram of the support, the enzyme solution—with respect to the proportions described in the next topic—was added together with PEG-1500 at a fixed amount (100 µL of aqueous solution containing 5 mg of PEG-1500/mL). The suspension containing enzyme, support, and PEG—1500 was homogenized by soft stirring, followed by static contact for an additional period of 18 h at 4 °C. The immobilized biocatalyst was recovered by vacuum filtration followed by successive rinsing with hexane and finally drying in a desiccator at room temperature for 24 h.

2.6. Determination of Optimal Enzyme Concentration for Support

Different concentrations of g enzyme/g support (0.15, 0.225, 0.3, 0.375, and 0.45) on immobilization by PA and CB were tested to determine the optimal amount of lipase on support [24].

2.7. Morphological and Physicochemical Properties

Specific surface area (Brunauer–Emmett–Teller, BET) and pore volume (BJH) were determined using a NOVA 1200-Quantachrome by adsorption and desorption of nitrogen at 77 K. Morphology was analyzed using scanning electron microscopy (SEM, Hitachi S-3000N). Sample weight loss upon heating was determined using a thermogravimetric analysis (TGA) apparatus (Shimadzu TGA-60 thermogravimetric analyzer) over the temperature range of 30–1000 °C. Samples with mass variation of 2–6 mg were put in a sealable platinum pan and analyzed under a nitrogen atmosphere with a heating rate of 10 °C·min^{−1}.

Samples of free lipase, support and immobilized derivative were submitted to FTIR analysis (IR Prestige 21, Shimadzu). Spectra were obtained in the 400 to 4000 cm^{-1} wavenumber range to evaluate the immobilization procedure. Energy dispersive X-ray analysis (EDX) (Shimadzu Rayny EDX 700) was used as the biochar and immobilized derivative.

2.8. Effect of pH and Temperature on Activity

The effect of pH on the activity of free and immobilized lipase was determined in buffer of values between pH 2.0 to 10. The buffers used were 0.1 M citric acid-sodium citrate (pH 2.0–5.0), 0.1 M potassium phosphate (pH 6.0–8.0), and 0.1 M bicarbonate-carbonate (pH 9.0–10). The optimal temperature for the activity of free and immobilized lipase was assayed in the 25–80 °C range in the same 0.1 M potassium phosphate buffer (pH 7.0) [24].

2.9. Thermal Stability

The thermal stability of free and immobilized lipase was determined by incubating the biocatalyst in sodium phosphate buffer solutions (0.1 M, pH 7.0) for 4 h (with sampling each 1 h) at 60 °C. The thermal inactivation constant (K_d) and half-life ($t_{1/2}$) for immobilized BCL were calculated using Equations (2) and (3), respectively, according to the literature [25,26].

$$A = A_0 \exp(K_d \cdot t) \quad (2)$$

$$t_{1/2} = \ln(0,5) / -K_d \quad (3)$$

where A is the residual activity after heat treatment for a period of incubation (U), A_0 is the initial enzyme activity (U), K_d is the inactivation constant (h^{-1}), and $t_{1/2}$ is the half-life (h).

2.10. Determination of Kinetic Constants

The Kinetic constants of Michaelis–Menten (K_m and $V_{\max}$) were determined by different fatty acids concentrations between 37 and 2232 mM on reaction systems. This was obtained from emulsions containing different proportions of olive oil (1–60%) and aqueous solution of gum Arabic (7% w/v). The apparent values of K_m and $V_{\max}$ were calculated according to the linearization methods of Lineweaver-Burk [24].

2.11. Operational Stability

The operational stability of the immobilized systems was assayed by running hydrolysis reactions in consecutive batches using the same biocatalyst. The time of each hydrolysis reactions was 10 min at a temperature of 60 °C and pH of 7.0. After each reaction, the biocatalyst was rinsed once with hexane and reused for the next cycle of hydrolysis [24].

2.12. Desorption Test of Immobilized Lipase by Covalent Binding

The test was performed according to Palomo et al. [27] with some modifications. Immobilized lipase by covalent binding was incubated in the presence of Triton X-100 (0.5%) for 3 h. Posteriorly, was washed it with water and the enzymatic activity of the treated immobilized biocatalyst was determined.

3. Results and Discussion

The immobilized lipase behavior depends on the physical structure of the support, the chemical physical properties of the enzyme and the immobilization technique used. To evaluate the behavior of the immobilized BCL by physical adsorption and covalent binding in the guava seed biochar, the effects of the enzyme concentration on the relative activity of immobilized biocatalyst were studied. Additionally, their biochemical, physic-chemical, and morphological characteristics of the immobilized biocatalysts were studied for the different immobilization techniques.

3.1. Lipase Immobilization in Biochar

To determine the best enzyme/support concentration, the results obtained were calculated according to relative activity. Our group studied the use of biochar derived from guava seed for the immobilization by physical adsorption, and the maximal relative hydrolytic activity of the BCL immobilized by PA was obtained with a $0.15 \text{ g}_{\text{enzyme}}/\text{g}_{\text{support}}$ with around 54% immobilization yield and 258 U/g hydrolytic activity [28]. However, for the biocatalysts immobilized by CB, the maximal hydrolytic activity of the BCL was obtained using $0.3 \text{ g}_{\text{enzyme}}/\text{g}_{\text{support}}$, with around 89% immobilization yield and 833 U/g hydrolytic activity.

Figure 1 shows the concentration profile of BCL immobilized by CB, presenting enzymatic saturation on the support at a higher lipase concentration compared with PA. This behavior is probably related to the activation of the support with spacing arms providing functional groups for enzyme immobilization, as well as the influence of PEG-1500 additive in the formation of enzyme aggregates. The formation of these aggregates gave the support a layer with a whitened aspect, and with the increase in enzyme concentration a higher concentration of this layer was also seen, as shown in Figure 2.

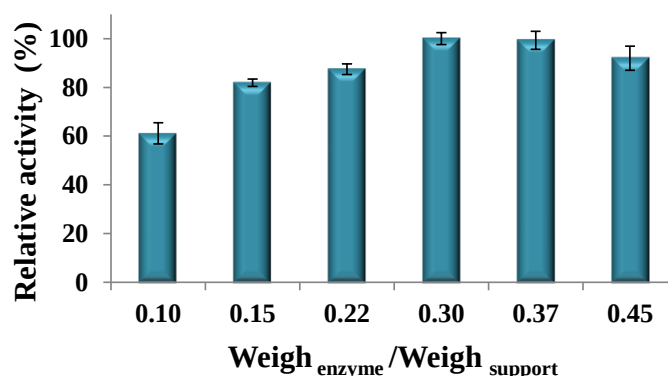


Figure 1. Relative activity (%) as a function of enzyme/support ratio during immobilization of the BCL on biochar by CB.

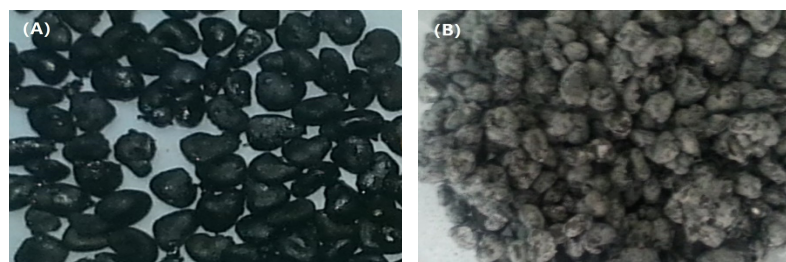


Figure 2. Macroscopic images show (A) Biochar surface after BCL immobilization by physical adsorption and (B) covalent binding. Image obtained using a 48 megapixels digital camera.

The system immobilized by CB presented a superior immobilization yield to that described for PA, possibly due to the stabilizing effect of the PEG-1500 on the enzyme which results in a considerable increase in the catalytic activity [29]. In order to verify the influence of the PEG-1500 additive on the formation of enzymatic aggregates, the CB immobilization process was performed without the presence of PEG, using the same concentration that was observed in the formation of the aggregates in the presence of peg ($0.3 \text{ g}_{\text{enzyme}}/\text{g}_{\text{support}}$). The immobilized biocatalyst did not present whitened aspect and showed a low immobilization yield of about 12% with hydrolytic activity of 120 U/g. The effect of additives on the immobilization process is not yet well understood regarding the aggregates that were observed just in the presence of PEG. The combination of the characteristics, as such, had various effects including enzyme protection from inactivation during the immobilization step [27] and retention of a water layer around the catalyst

(aggregates), and furthermore a greater immobilization yield and enzyme activity for the CB immobilization process with the presence of PEG.

3.2. pH and Temperature Effects

According to Abdulla and Ravindra [30] the surface of an enzyme contains a great number of acid and basic groups and depending on the pH of the medium the charges on these groups may vary and alter the enzyme activity, structural stability, and solubility. The effect of pH on the lipase activity of free and immobilized BCL is presented in Figure 3, and the statistical analysis demonstrated that the pH also significantly influenced the lipase activity of free and immobilized BCL, displayed in supporting information (Figure S1). It is possible to note that lipase both free and immobilized by covalent binding (CB) presented an optimal pH of 7.0, indicating that this method of immobilization did not alter the optimal pH of the enzyme. However, for the BCL immobilized by PA, the pH optimum was changed to a more acidic value (pH 4.0). The result agrees with other studies which reported the pH effect on activity profile of free and immobilized lipase. A shift in optimal pH for immobilized enzymes is reported for different sources of lipase and types of support [31]. Naghdi et al. [32] evaluated the stability of immobilized laccase on functionalized nanobiochar, showing higher stability in acidic region (5 to 30% activity loss in pH 3–6). In the study of Carvalho et al. [33] the lipase of *Burkholderia cepacia* immobilized on xerogel silica showed an optimum pH shift to 3.0, while the optimum pH of the free lipase was 7.0. This change was attributed to the characteristics of the support and the method of immobilization. Usually, lipases immobilized on polycationic supports tend to shift their optimal pH to more acidic values [34]. Pereira et al. [35] reported for *Candida rugosa* lipase immobilized by PA on chitosan an optimal pH 6.0, slightly lower than the one seen for free *Candida rugosa* lipase, (optimal pH of 7.0), which was attributed to the unbalancing of the microenvironment of the immobilized enzyme due to electrostatic interactions with the support.

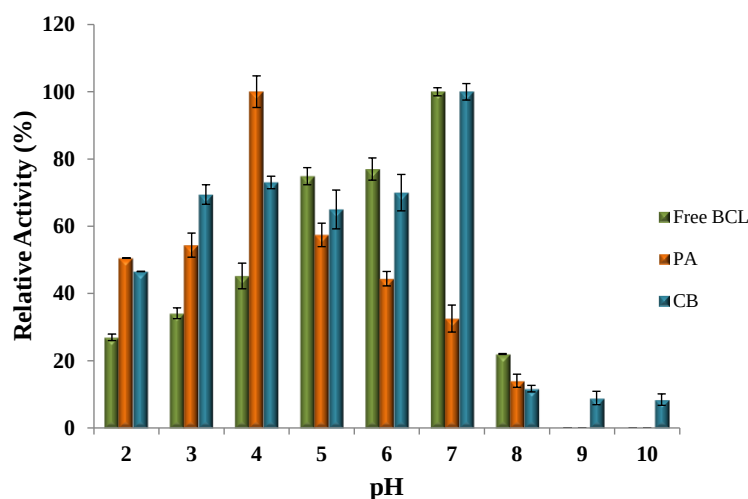


Figure 3. pH effect on the activity of free BCL and immobilized by physical adsorption (PA), and covalent binding (CB).

Some additives such as polymers (PEG) and protein (albumin) are included in the preparation of lipase for immobilization in order to stabilize the conformational modifications of lipase that occur during the immobilization process. It is thought that these polar compounds protect enzymes against the negative influence of the medium and favourably influence the distribution of water in the microenvironment of the enzyme. The use of PEG-1500 in the lipase immobilized by covalent binding (CB) helped to maintain the same pH as in free lipase. Distribution of water in the microenvironment of the enzyme can cause often-conformational changes, as observed as one slight increase in activity at pH 4.0,

a decrease and an optimum at pH 7.0. Additionally, in the physical adsorption there was significant change in the pH due the absence of polymers.

Temperature is a very important parameter for reaction speed, because with its increase a higher kinetic energy is observed in the reagent molecules, which promotes a higher number of productive collisions per unit time. However, in reactions catalyzed by enzymes, in order to keep the same catalytic activity, the tertiary and secondary enzyme structure needs to be kept intact. An increase in temperature can lead to absorption of excessive energy, provoking the rupture of this structure and consequently the denaturation of the enzyme, therefore it is important to evaluate the effect of temperature in the micro-environment of the immobilized biocatalyst [36].

The effect of temperature on the catalytic activity for the free and immobilized biocatalyst can be seen in Figure 4, and the statistical analysis demonstrated that the temperature also significantly influenced the lipase activity of free and immobilized BCL by physical adsorption (PA) and covalent binding (CB), displayed in supporting information (Figure S2). The maximum activity of the free BCL occurred at 50 °C, while for the immobilized biocatalysts this occurred at 60 °C, possibly due to the restricted mobility granted by the fixation of the lipase on the support which reduces the contact with the substrate, resulting in an apparent reduction in activity. Similar results were observed in works using inorganic supports for the immobilization of BCL supported on mesoporous silica, presenting a maximum activity at 60 °C [37]. Similarly, it was also seen in studies involving organic supports developed by Cabrera-Padilla et al. [24] which used a natural biopolymer poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) for the immobilization of *Candida rugosa* lipase, presenting an immobilized optimum temperature range of 37–45 °C, and a free enzyme optimum of 37 °C. The immobilized enzymes presented an increase in hydrolytic activity at higher temperatures compared with the free enzymes, which can be attributed to the more rigid conformation of the immobilized enzymes because of electrostatic interactions and hydrogen bonding between the enzyme and support [24].

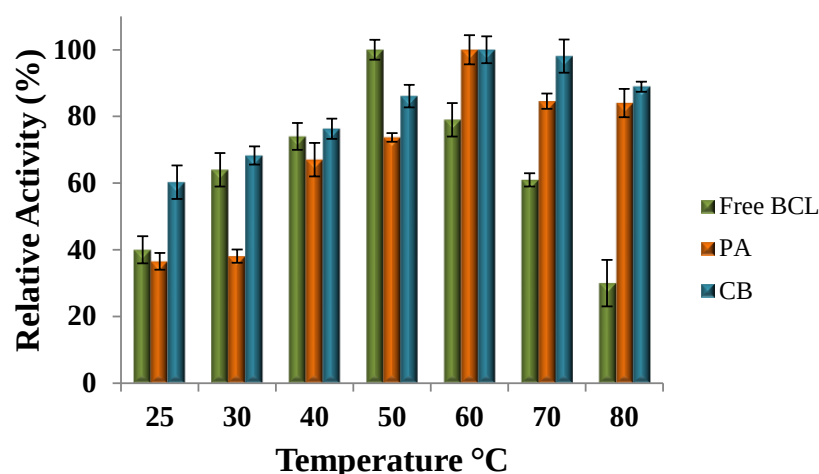


Figure 4. Temperature effect on the activity of the free and immobilized BCL, by physical adsorption (PA), and covalent binding (CB).

3.3. Determination of Kinetic Parameters

To measure the kinetics of the hydrolytic activity, values of K_m (mM) and V_{max} ($\mu\text{mol g}^{-1}\cdot\text{min}^{-1}$) were calculated using the Michaelis–Menten equation for the free enzyme and the immobilized biocatalyst. The values are presented in Table 1. The value of V_{max} for the free lipase was $3333 \mu\text{mol g}^{-1}\cdot\text{min}^{-1}$, and for the immobilized biocatalysts PA and CB, the V_{max} values were 2000 and $2500 \mu\text{mol g}^{-1}\cdot\text{min}^{-1}$, respectively. The value of V_{max} for the immobilized BCL (PA and CB) was reduced by around 1.33- and 1.66-fold, respectively, compared with the free BCL.

Table 1. Kinetic parameters ($V_{\max}$ and K_m) of the free and immobilized enzyme.

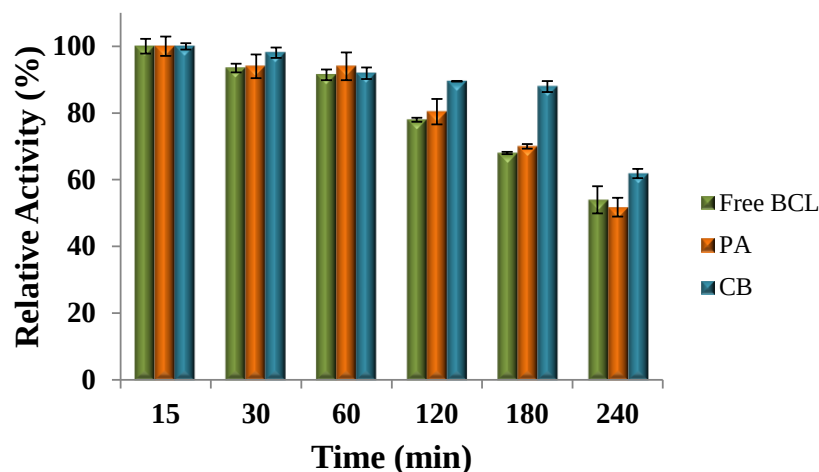
	K_m (mM)	$V_{\max}$ ($\mu\text{mol g}^{-1}\cdot\text{min}^{-1}$)
Free BCL	665	3333
Physical Adsorption (PA)	219	2000
Covalent binding (CB)	369	2500

The immobilization process cannot control the enzyme fixation on the support and inadequate fixation can induce the formation of an inactive conformation and/or alter the properties of the active sites, which can reduce the $V_{\max}$ value for immobilized enzymes. The K_m value for the free lipase was 665 mM and the K_m values for the lipase immobilized by PA and CB were 219 and 369 mM, respectively. The literature describes the reduction in K_m for an immobilized enzyme as an increase in affinity between the active sites and the substrate [38,39]. Furthermore, a difference between the K_m values for the biocatalysts immobilized by PA and CB was seen, indicating different affinities of the immobilized lipase for the substrate depending on their immobilization method. The higher affinity of lipase immobilized by physical adsorption to the substrate is associated with the conformational changes in the enzyme after adsorption in the biochar (hydrophobic); the lipases are adsorbed through the open active center, accessible to the substrate. According to Knežević et al. [40], the lipases recognize hydrophobic surfaces similar to those of their natural substrates and undergo interfacial activation during immobilization.

The possible formation of aggregates on immobilized lipase by covalent binding (CB) is influenced by PEG addition in the formation of aggregates. In this case, the possibility to improve the value of the $V_{\max}$ and formation of aggregates influences mass transfer by values of the K_m . Therefore, these conformational changes were observed in the morphological sense with the formation of aggregates and biochemical characterization for immobilized lipase by covalent binding (CB) with a major decrease in K_m .

3.4. Thermal Stability of the Free and Immobilized BCL on Biochar

The thermal stability of the free BCL and the immobilized biocatalysts were investigated and compared by determination of their hydrolytic activity towards an olive oil emulsion at 60 °C for different reaction times. Figure 5 shows that the biocatalyst immobilized by covalent binding (CB) presented a higher thermal stability when compared to the free enzyme. For the biocatalyst immobilized by PA, the kinetic profile was very similar to that of the free enzyme. Statistical analysis showed a significant low influence on the lipase activity of free and immobilized BCL by physical adsorption (PA) and covalent bonding (CB), presented in the supporting information (Figure S3).

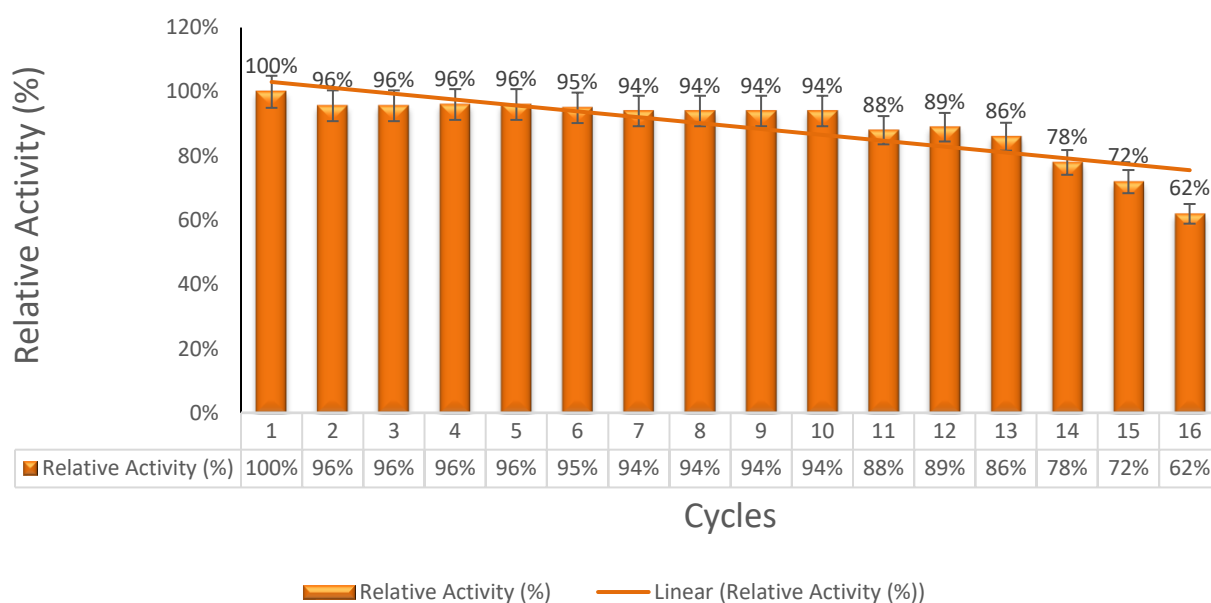
**Figure 5.** Thermal stability of the free and immobilized by PA and Covalent binding (CB) biocatalyst incubated at 60 °C.

Half-life for free BCL and for the immobilized biocatalyst showed the best performance for immobilization by covalent bond (CB); an increase was positive for the half-life of the enzyme immobilized in biochar by covalent bond (CB) of 7.2 h. Comparing the half-life of the free enzyme with the values obtained for the immobilized biocatalyst, it was noted that immobilization by PA does not significantly alter the half-life when compared to Free BCL (4.5 and 4.4 h, respectively). These results are in accordance with the activity recovery data, for which the biocatalysts immobilized by CB presented a higher recovery of activity due to stabilising effects of PEG 1500, which confers greater protection on the BCL when compared to the biocatalyst immobilized by PA.

The result is in agreement with other studies which reported that the thermal stability of immobilized enzymes was generally more stable than free enzyme. For example, according to Li et al. [41], the thermal stability of immobilized laccase on biochar is significantly higher than that of free laccase at 60 °C. At the end of the experiment, 30.3% of the initial activity was maintained for the immobilized laccase, whereas there was only 10.6% for the free laccase. Brígida et al. [42] reported on the immobilization of *Candida antarctica* lipase B by PA on coconut fibre. The immobilization by CB resulted in a higher thermal stability of *Candida antarctica* lipase B presenting a half-life time 1.4-fold higher than the immobilized by PA.

3.5. Operational Stability

Operational stability is one of the most important characteristics for the application of the biocatalyst on an industrial scale once the reuse capability of the immobilized enzyme has reduced the process cost. The operational stability of immobilized lipase on biochar was tested in a hydrolysis reaction of olive oil emulsion for 10 min at 60 °C, as seen in Figure 6. The BCL immobilized by PA can be reused 17 times keeping 50% of its initial activity. It was possible to observe that BCL immobilized by physical adsorption presented good results. This fact is possibly related to the hydrophobicity of the biochar, since the lipases have as a characteristic a cap on the catalytic site that changes its conformation depending on the environment in which it is found, open conformation at the water/oil interface and closed in aqueous media. As reported by Knežević et al. [40], the lipases recognize hydrophobic surfaces similar to those of their natural substrates and they undergo interfacial activation during immobilization creating an open active site accessible to the substrate. This results, in most lipases, in a large increase in activity when adsorbed on hydrophobic support.



(a)

Figure 6. Cont.

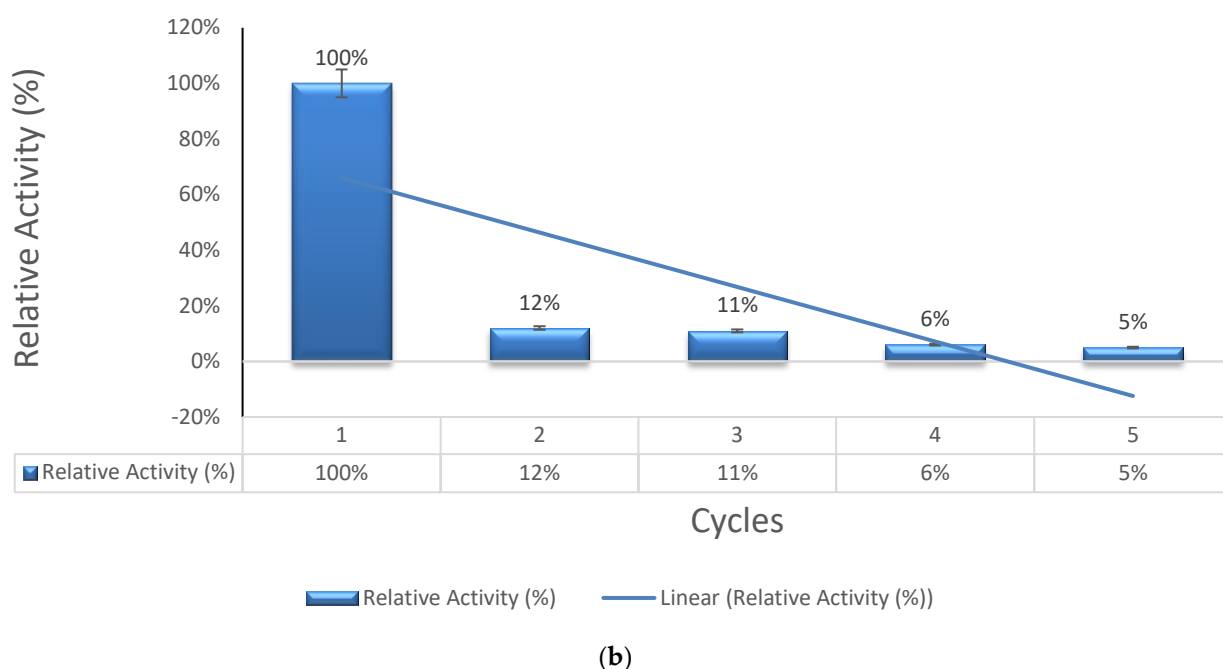


Figure 6. Operational stability of the immobilized lipase by PA (a) and CB (b) on biochar.

Additionally, the immobilization of lipases by non-covalent adsorption has shown to be promising, since immobilization by physical adsorption occurs through direct interactions between the enzyme and the support (mainly van der Waals, hydrogen bonds and hydrophobic interactions). For this reason, it often allows the regeneration of the immobilized biocatalyst, and because of the simplicity and the low cost of the adsorption method. Other authors [24,43] reported good operational stability when studying immobilization of enzyme by physical adsorption. In the study by Cabrera-Padilla et al. [24] the operational stability of PHBV-immobilized rugose *Candida* lipase was such that the immobilized biocatalyst could be reused up to 12 times, retaining 50% of its initial activity. Cristóvão et al. [43] found that, for commercial immobilized PA laccase on coconut fibre in the oxidation of diammonium salt of 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) salt, the enzyme can be reused up to 13 times, maintaining 55% of its initial activity.

However, the BCL immobilized by CB presented a low operational stability, losing more than 50% of its initial activity in the first cycle. From the results obtained, it is possible to suggest a significant loss in activity of the CB immobilization due to a leaching process during the cycles, possibly related to the formation of aggregates of the enzyme on the support. Based on the results obtained from the morphological analysis of the immobilized biocatalysts, the presence of the lipase aggregates on the BCL immobilized by CB was confirmed, which can be called the covalent binding (CB) with formation of the multilayered enzyme-biochar. Therefore, the results obtained show a loss of over 90% of the initial activity on the first cycle, when CB immobilization caused the formation of lipase aggregates adsorbed by weak bonding on the surface of the support after reuse verified the loss of the support enzyme. Thus, the study of desorption of immobilized lipase by covalent binding by methodology of the Palomo et al. was necessary [27]. In this test, only available lipases not immobilized by CB immobilization on supports can be absorbed. In the course of our studies were realized the desorption tests in the presence of Triton X-100 for BCL immobilized by CB with and without PEG-1500 and complete desorption of the lipase of the support with activity more than 50% in the supernatant was verified. The same behaviour was observed for operational stability of CB, indicating that amino or acid groups on the surface of the enzyme cannot react with the support forming covalent bonds. As explained before, this suggests that the formation of multi-layered enzyme-biochar on the biocatalyst obtained by covalent binding was the likeliest to be responsible of this effect.

In the study carried out by Brígida et al. [42], it was verified that *Candida antarctica* lipase B immobilized by PA presented 50% of its initial hydrolytic activity after the third cycle of reaction in methyl butyrate hydrolysis (aqueous medium). Meanwhile, the reaction in organic medium showed a good operational stability, maintaining 80% of its initial activity after the sixth reaction cycle. Brígida et al. [44] evaluated the operational stability of *Candida antarctica* lipase B immobilized by CB on coconut fibre with a treatment of nitric acid by means of two reactions: methyl butyrate hydrolysis (aqueous medium) and butyl butyrate synthesis (organic medium). After five reuses of the biocatalysts prepared in pH 7.0 (CALB-7), an activity of around 55% was found in aqueous medium (hydrolysis) and 75% in organic medium (synthesis), respectively, presenting similar behaviour for the biocatalyst prepared at pH 10 in aqueous medium. Around 55% of the residual hydrolytic activity was found, but presenting a lower result for the organic medium, around 45% of the residual activity.

Therefore, the guava seed biochar can be considered as a potential support for enzyme immobilization, and the BCL immobilized by physical adsorption can be reused for sixteen more cycles without significant loss of activity, demonstrating its potential for use in a wide range of industrial processes.

3.6. Morphological and Physical-Chemical Properties

3.6.1. Morphological Analysis (B.E.T.)

The study of morphological properties of porous solid materials is conveniently based on gaseous nitrogen adsorption, using volumetric measurements of the amount of gas adsorbed. The specific superficial area, volume and median pore size for biochar and the immobilized biocatalysts are summarised in Table 2.

Table 2. Specific superficial area, volume and pore diameter of biochar and immobilized biocatalysts.

Assay	Specific Superficial Area ($\text{m}^2 \cdot \text{g}^{-1}$)	Pore Volume ($\text{cm}^3 \cdot \text{g}^{-1}$)	Pore Diameter (Å)
Biochar	77 ± 2	0.052 ± 0.002	18 ± 1
Physical Adsorption (PA)	60 ± 1	0.040 ± 0.001	18 ± 1
Covalent Binding (CB)	63 ± 1	0.041 ± 0.001	18 ± 1

For the biocatalysts immobilized by PA and CB, a reduction in superficial area and pore volume compared to biochar was seen, probably related to saturation of the support surface with the enzyme molecules. The reduction in the superficial area of the immobilized biocatalyst with PA and CB was 1.29- and 1.23-fold, respectively. Similar results were obtained by Rodrigues et al. [45] on the immobilization of *Candida antarctica* lipase type B with activated coal, obtaining a reduction of 1.4-fold in the superficial area after immobilization, indicating that the enzyme immobilization occurred on the surface of the activated coal.

3.6.2. Scanning Electronic Microscopy (SEM)

Scanning electronic microscopy (SEM) was used to characterise the morphology of the support (biochar) and of the immobilized biocatalysts, and the resulting micrographs are shown in Figure 7. It can be seen that biochar presented a porous superficial structure (Figure 3a).

In Figure 7B, a partial filling of the biochar pores possibly by simple layers of lipase on the biocatalyst immobilized by physical adsorption is seen. Figure 7C,D corresponds to the biocatalyst immobilized by CB where it is possible to observe the formation of lipase aggregates on the surface of the support similar to the results of Schoevaart et al. [46] for reticulated enzymatic aggregates of the *Candida rugosa* lipase. The layer with a whitened aspect indicated in Figure 7C and the possible formation of aggregates shown in Figure 7d highlight the influence of PEG addition in the formation of aggregates; this is the formation of the multi-layered enzyme-biochar.

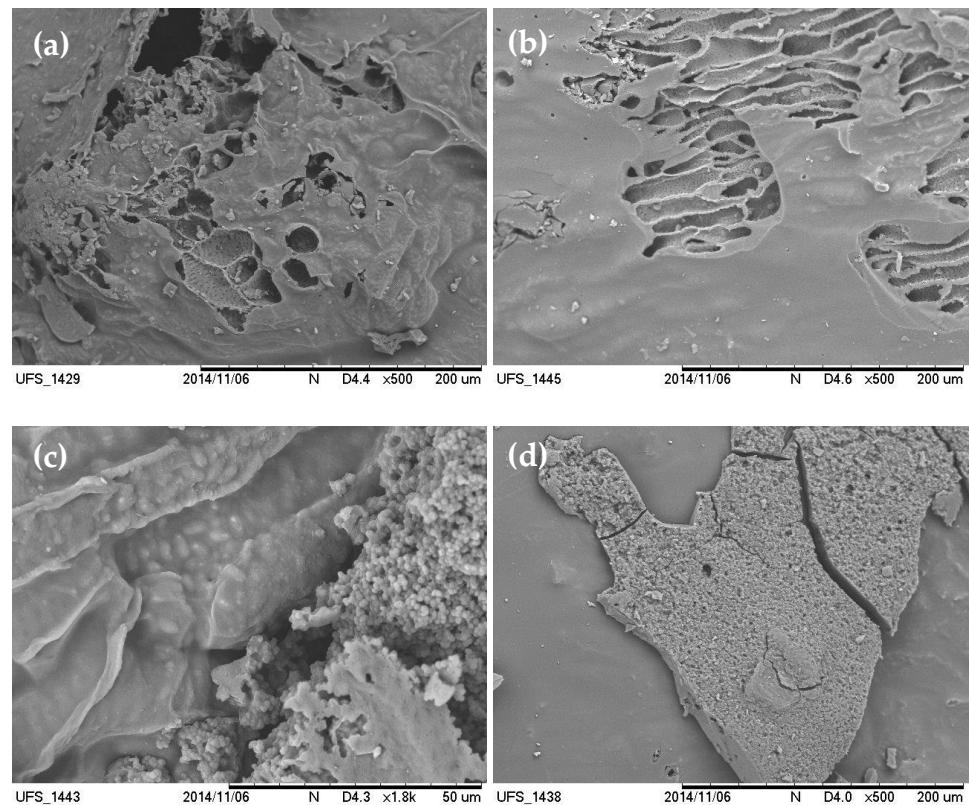


Figure 7. SEM of biochar and biocatalyst immobilized by different techniques: (a) biochar, (b) immobilized lipase by physical adsorption (PA) and (c,d) immobilized lipase by covalent binding (c,b).

3.6.3. Thermogravimetric Analysis (TG)

Thermogravimetric analysis for biochar and immobilized biocatalyst is present in Figure 8. The same profile was found for both systems, where the mass loss curves were divided into three regions: region I (25–200 °C), region II (200–600 °C), and region III (600–1000 °C).

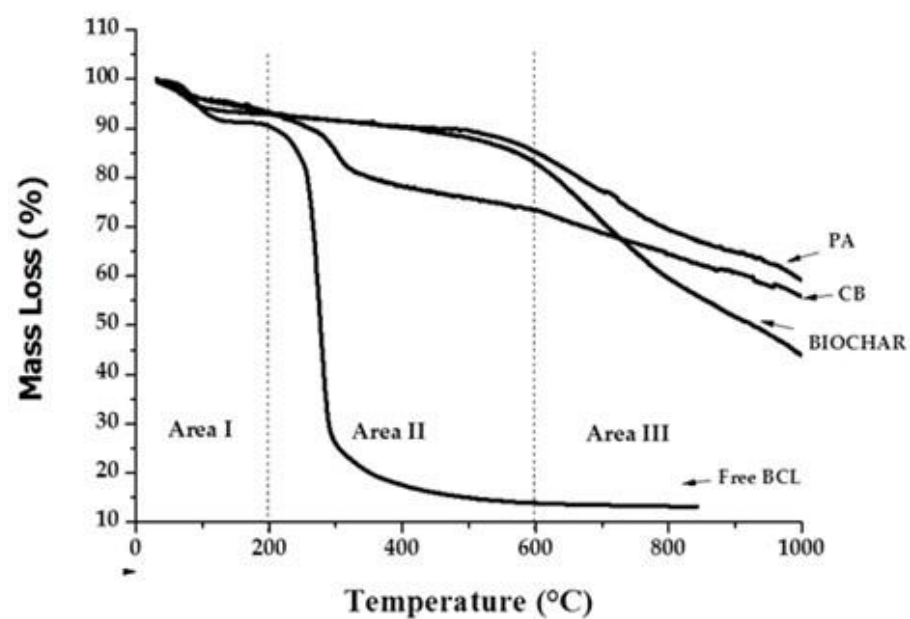


Figure 8. Thermogravimetric curves of the lipase of free BCL, biochar and biocatalysts immobilized by PA and CB.

Table 3 shows that in region I, the mass loss was of around 7% for biochar and the immobilized biocatalysts, which can be attributed to water loss and volatile compounds. Region II is associated with the degradation of organic compounds, where the biocatalyst immobilized by CB presented a higher mass loss when compared with the PA, possibly influenced by the presence of multilayers of BCL on the surface (as suggested in the immobilization model proposed in the immobilized biocatalyst micrograph-Figure 7C,D). Region III shows a mass loss for biochar possibly associated with the degradation of aromatic groups ($C=C$; $C=O$) [24,46,47].

Table 3. Mass loss data (%) by TGA for the free lipase and immobilized biocatalysts.

Assay	Area I (25–200 °C)	Area II (200–600 °C)	Area III (600–1000 °C)
Free BCL	8.32	82.32	9.16
Biochar	7.2	11.4	47.74
Physical Adsorption (PA)	6.8	8.78	30.89
Covalent Binding (CB)	6.99	21.07	24.11

3.6.4. FT-IR Analysis

FT-IR spectra obtained for the biochar and the biocatalysts immobilized by PA and CB are shown in Figure 9. All spectra show bands in 1590 cm^{-1} which is associated with the vibration of $C=O$ bonds of aromatic groups. Furthermore, the characteristic vibration of the stretching of the $\gamma\text{-CH}$ bond at around 876 cm^{-1} was also seen [48,49]. In the spectra of the immobilized biocatalysts, bands were observed at 1383 cm^{-1} , which are associated with a ternary amide [50]. Only in the spectrum of the biocatalyst immobilized by CB spectrum was a low intensity band at 1110 cm^{-1} observed, which is associated with the vibration of $C\text{-O-C}$ bonds present in the PEG 1500 [51]. Therefore, it is possible to verify by physical—chemical analysis the presence of BCL on the surface of biochar, confirming the efficiency of the immobilization technique of the BCL by PA and CB.

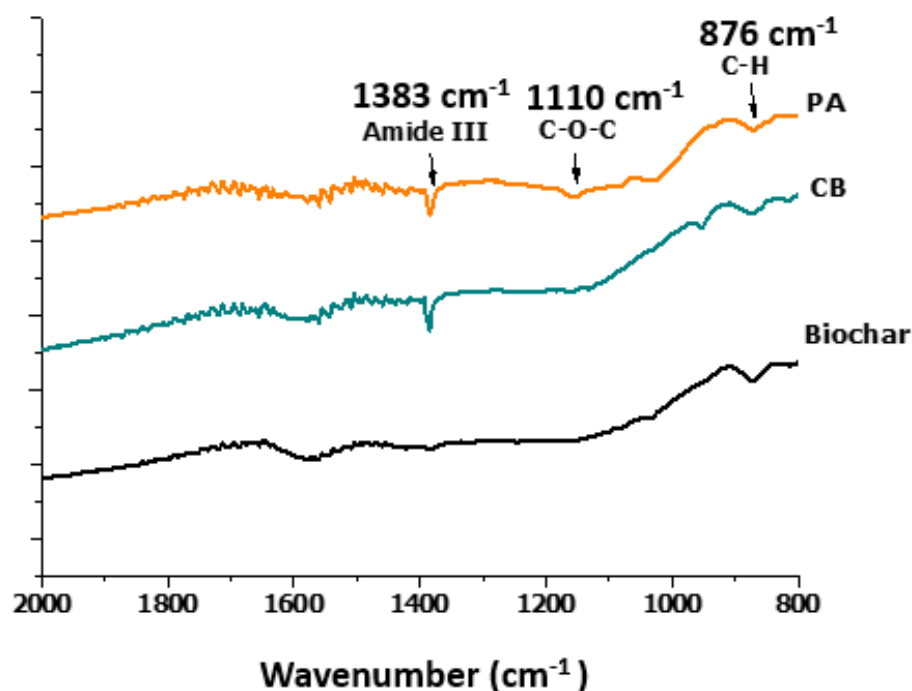


Figure 9. FTIR spectrum for biochar and biocatalysts immobilized by PA and CB.

3.6.5. EDX Elemental Analysis

The EDX analysis for the biochar and the biocatalysts immobilized by PA and CB are shown in Table 4. According to the result, the content of potassium in the immobilized biocatalysts is higher than biochar; this result was expected due to the use of the potassium phosphate buffer during the immobilization process. As expected, the activation process resulted in the presence of chlorine (Cl) attributed to the activation process with epichlorohydrin in the biocatalyst immobilized by covalent binding (CB), which suggests that the successful activation of the biochar could be confirmed.

Table 4. EDX elemental analysis for the Biochar and immobilized biocatalysts.

Elements (%)	Biochar	PA	CB
K	0.014 ± 0.006	0.222 ± 0.005	0.176 ± 0.006
Fe	0.009 ± 0.004	0.003 ± 0.002	ND (*)
Zn	0.002 ± 0.001	0.001 ± 0.001	ND (*)
Cu	0.002 ± 0.002	0.001 ± 0.001	ND (*)
Cl	ND	ND	0.065 ± 0.010

(*) ND—not determined.

4. Conclusions

The results shown in this paper show that the Biochar of guava seeds is a promising alternative in substitution of the traditional supports. Two different protocol immobilizations of *Burkholderia cepacia* lipase by physical adsorption and covalent binding were tests, and various characterization methods were used to study the immobilization efficiency in biochar. The protocol immobilization by physical adsorption showed that the best performance can be reused for sixteen cycles without significant loss of activity.

Future trends will unfold higher preferences for greener manufacturing processes becoming central for the development of effective strategies for enzyme immobilization-stabilization, and Biochar is a by-product of a renewable source; therefore, it is a promising alternative for lipase immobilization and for industrial level implementations of greener enzyme processes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/c8040064/s1>. Figure S1. pH effect on the activity of free BCL and immobilized by physical adsorption (PA), and covalent binding (CB). Bars represent the standard errors. For each response, bars with different letters indicate differences based on Tukey test ($p \leq 0.05$). Figure S2. Temperature effect on the activity of the free and immobilized BCL, by physical adsorption (PA), and covalent binding (CB). Bars represent the standard errors. For each response, bars with different letters indicate differences based on Tukey test ($p \leq 0.05$). Figure S3. Thermal stability of the free and immobilized by PA and Covalent binding (CB) biocatalyst incubated at 60 °C. Bars represent the standard errors. For each response, bars with different letters indicate differences based on Tukey test ($p \leq 0.05$).

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