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Short communication

Use of conventional or non-conventional treatments of biochar for lipase immobilization

Lays C. Almeida[a], Anderson S. Barbosa[a], Alini T. Fricks[a], Lisiane S. Freitas[b], Álvaro S. Lima[a], Cleide M.F. Soares[a,*]

[a] Institute of Technology and Research, Tiradentes University, Av. Murilo Dantas 300, 49032-490, Aracaju, Sergipe, Brazil
[b] Department of Chemistry, Federal University of Sergipe, Av. Marechal Rondon, s/n, Jd. Rosa Elze, 49100-000, São Cristóvão, Sergipe, Brazil

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ABSTRACT

The objective of this work was to evaluate the influence of dichloromethane (CH_2Cl_2), potassium hydroxide (KOH) and phosphoric acid (H_3PO_4) in the treatment of biochar from guava seeds on conventional and non-conventional techniques (ultrasound and microwave) for the immobilization of *Burkholderia cepacia* lipase (BCL) by physical adsorption. The effects of the different treatments on the physical and chemical properties of the biochar were evaluated by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The immobilization of the BCL on the biochar was evaluated in hydrolysis reactions of olive oil. The conventional treatment of the biochar with KOH showed increased modification on the surface of the biochar, which presented a highly porous surface, and a greater activity for the immobilized biocatalyst compared with the other treatments. The results revealed the potential of biochar as a support novelty for the immobilization of enzymes and for their application in biocatalysis.

1. Introduction

Biochar is a byproduct of the pyrolysis reaction (thermal degradation in the absence of oxygen) of lignocellulosic material, and has a high carbon content [1]. Recently biochar has been recognized as a multifunctional material due to its wide variety of applications, such as: the removal of organic and inorganic pollutants from aqueous solution [2]; the fertilization and pH correction of soils for agriculture [3,4]; and the immobilization of enzymes [5]. This has attracted the interest of the scientific community and prompted research into alternative methods to improve the morphological and physicochemical properties of biochar.

Several studies have reported the use of biochar from different lignocellulosic materials such as guava seed [6], sunflower seed [7], olive seed [8], bamboo [9,10], groundnut shell [11], pecan shells [12], rice [13], coconut [14], jackfruit [15], pineapple [16], sawdust [17] and wheat straw [18] as precursors for the production of activated carbon. Different activating agents (HCl, $ZnCl_2$, H_2SO_4, H_3PO_4, NaOH, K_2CO_3, CO_2 and KOH), as well as conventional (muffle) and non-conventional (microwave) heating techniques have been used. Most of the studies carried out in the preparation of activated carbon are aimed at the removal of organic and inorganic pollutants from aqueous solutions.

Multiple articles using activated carbon from lignocellulosic materials to immobilize enzymes are still scarce in the literature. Recently Brito et al. [19] evaluated the use of activated charcoal from yellow mombin fruit stones as a support for the immobilization of lipase from porcine pancreas type II produced by Sigma. Activated charcoal was prepared by chemical activation (impregnation of the activation agent in the material followed by carbonization) using conventional heating (muffle), which verified the influence of phosphoric acid and potassium hydroxide as an activation agent. The carbonization temperature and the physical and chemical properties of the activated carbon were evaluated. The results showed that both carbons produced good lipase adsorption capacity, which makes activated charcoal a promising alternative as a support for the immobilization of enzymes. However, the use of non-conventional treatments of biochar have not yet been reported.

Due to the search for green and sustainable industrial processes, enzymatic processes appear as a promising alternative to replace traditional chemical processes, because the enzymes are selective and act under mild reaction conditions (room temperature, atmospheric pressure and physiological pH). For commercial viability there is a need to immobilize the enzymes to improve their stability and increase the possibility of reuse of the biocatalyst in comparison with the free enzyme [20,21]. Among the methods used for immobilization, physical adsorption is considered the simplest and cheapest method when compared with covalent binding and encapsulation methods [22].

The objective of this work was to verify the influence on the

* Corresponding author.

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morphological and physico-chemical properties of guava seed biochar of conventional (muffle) and non-conventional (microwave and ultrasound) treatment with dichloromethane (CH_2Cl_2), potassium hydroxide (KOH) and phosphoric acid (H_3PO_4) for the purpose of immobilization of *Burkholderia cepacia* lipase by physical adsorption.

2. Materials and methods

2.1. Materials

Burkholderia cepacia lipase (BCL) was purchased from Sigma-Aldrich. (St. Louis, MO, USA), with an activity of 2500 U/g. The guava seeds were kindly provided by the fruit pulp industry (POMAR, Aracaju-Sergipe, Brazil), hexane P.A., potassium hydroxide (KOH), phosphoric acid (H_3PO_4), and dichloromethane (CH_2Cl_2) were obtained from Vetec (Rio de Janeiro, Brazil). The substrate used for the hydrolysis reactions consisted of commercial extra virgin olive oil with low acidity (Carbonell), and Arabic gum (Synth, Brazil). All other reagents used were of analytical grade.

2.2. Biochar production

Biochar production was carried out at the laboratory scale using the pyrolysis equipment described in detail by Santos et al. [23]. The pyrolysis conditions of the guava seed were previously optimized for bio-oil production and the following conditions used: Temperature = 500 °C, heating rate = 30 °C min^{-1}, final time = 5 min and flow rate of N_2 = 1 mL min^{-1}. A quantity of guava seeds was (12 g) introduced into the quartz reactor in the pyrolysis furnace and the apparatus refluxed with a stream of nitrogen at 1 mL min^{-1} for 5 min. The sample was heated from 25 °C to 500 °C, at 30 °C min^{-1} and held at 500 °C for 60 min.

2.3. Biochar treatment

2.3.1. Conventional treatment (Muffle)

The guava seed biochar was incubated in three different chemical agents: CH_2Cl_2, KOH and H_3PO_4 in solution 10% (m/m), for a 24 h period with stirring at room temperature (25 °C). Biochar was muffled at a temperature of 500 °C for 30 min, then thoroughly washed at neutral pH and oven dried at 60 °C.

2.3.2. Non-conventional treatment (Microwave and ultrasound)

The microwave treatment was carried out in a Discovery CEM microwave reactor (Discovery & Explorer SP) using KOH and H_3PO_4 solutions at a concentration at 10% (m/m). 1 g of biochar was used with 5 mL of the solution, with a maximum power of 300 W and a constant temperature of 100 °C for 10 min. The biochar was then thoroughly washed to neutral pH and dried in an oven at 60 °C. Dichloromethane was not used in the Non-conventional treatment (microwave) because its properties render it invisible to the specific radiation used.

The ultrasound treatment, the guava seed biochar was submerged in three different chemical agents: CH_2Cl_2, KOH and H_3PO_4 in solution 10% (m/m). The mixture was maintained in an ultrasonic bath for 30 min at room temperature (25 °C), then thoroughly washed at neutral pH and oven dried at 60 °C.

2.4. Immobilization of lipase onto biochar

The immobilization procedure of *Burkholderia cepacia* lipase onto the biochar (support) by physical adsorption was performed as described by Soares et al. [24] with some modifications. The technique consists in contacting 1 g of the biochar in 10 mL of hexane for 15 min with vigorous stirring at room temperature (25 °C). Afterwards, 10 mL of enzymatic solution (0.15 g of BCL solubilized in 10 mL of 0.1 M sodium phosphate buffer pH 7.0) was added and kept under stirring for 3 h. The enzyme-support solution was maintained at 4 °C for 24 h. After this period, the immobilized biocatalyst was washed with hexane and dried in a desiccator at room temperature for 24 h.

2.5. Enzymatic activity assay

Enzymatic activities of both free and immobilized lipase samples were assayed by the olive oil hydrolysis method according to a modification used by Soares et al. [24]. The substrate was prepared by mixing 50 mL olive oil with 50 mL gum Arabic solution (7% w/v). the reaction was prepared with 5 mL of the emulsion, 2 mL 0.1 M sodium phosphate buffer (pH 7.0), and either free (100 mg) or immobilized (100 mg) enzyme was incubated for 5 min for free and 10 min for immobilized at 37 °C under stirring at 80 rpm. The reaction was stopped by addition of 2 mL acetone–ethanol–water solution (1:1:1). Liberated fatty acids were titrated with 0.01 M potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (*U*) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol·min^{-1}). Analyses of the hydrolytic activities of immobilized biocatalysts were used to determine the yield η(%) according to Eq. (1).

$$\eta\ (\%) = \frac{Us}{Uo}\ x\ 100 \quad (1)$$

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

2.6. Sample characterization

The morphology of guava seed biochar and the immobilized biocatalysts with the best results for hydrolytic activity was determined by scanning electron microscopy (SEM) (Hitachi S-3000N, operated at maximum accelerating voltages of 500 V at 30 kV and resolution of the 15A). The samples were also subjected to FTIR analysis (Agilent Technology CARY 630 FTIR spectrophotometer). Spectra were obtained in the wavelength range from 400 to 4000 cm^{-1}.

3. Results and discussion

Different treatments of biochar have been published for the removal of organic and inorganic pollutants and for other applications. However, for the use of biochar as a support for the immobilization of enzymes such as BCL by physical adsorption, other studies are necessary to improve the efficiency. This work first performed conventional and non-conventional treatment of guava seed biochar and then characterized the support and the immobilized biocatalyst.

3.1. Sample characterization

3.1.1. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to characterize the biochar morphology after conventional and non-conventional treatments. The resulting micrographs are shown in Fig. 1. They verified that untreated biochar has a porous surface structure with irregular cavities (Fig. 1a). Biochar after ultrasonic bath treatment is shown in Fig. 1b-d. There is an increase in the pores present on the surface of the biochar treated with dichloromethane (Fig. 1b), possibly related to the removal of bio-oil residues and ash present in the surface of the biochar, thus, unclogging the pores and making it accessible for adsorption of *Burkholderia cepacia* lipase (BCL) during the immobilization process. When KOH solution was used (Fig. 1c), it was possible to observe agglomerates on the surface possibly related to the KOH residues that were not totally removed during the washing process with distilled water at neutral pH. After treatment with H_3PO_4 (Fig. 1d), the surface of the biochar showed no obvious variations compared to the biochar