

Influence of additives in the transesterification of crude coconut oil catalyzed by *Burkholderia cepacia* lipase immobilized onto agro-industrial waste fibers**Influência de aditivos na transesterificação de óleo de coco bruto catalisado por lipase de *Burkholderia cepacia* imobilizada em fibras de resíduo agroindustrial**

DOI:10.34117/bjdv6n7-444

Recebimento dos originais: 10/06/2020

Aceitação para publicação: 17/07/2020

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ABSTRACT

In this study was verified the influence of different additives in the transesterification reaction of the crude coconut oil catalyzed with lipase from *Burkholderia cepacia* immobilized by physical adsorption onto palm fiber originated from agroindustrial wastes. Reactions was performed under the conditions: molar ratio 1:7 (oil:alcohol), 10 % biocatalyst immobilized for 96 h at 40 °C in the presence in different concentrations of additives (water, *tert*-butanol, molecular sieve and protic ionic liquids). The results showed that the use of all the additives in this study did not increase conversion to ethyl esters. The maximum conversion in the absence of the additives was 72 %, in the presence of water 45 ± 2 %, molecular sieve 53 ± 2 % and *tert*-butanol 59 ± 2 %, respectively, all in the lowest concentrations. For ionic liquids, those with higher alkyl chains had the highest conversions, but lower than in their absence.

Keywords: Additives, transesterification, lipase, immobilization, agroindustrial wastes

RESUMO

Neste estudo foi verificada a influência de diferentes aditivos na reação de transesterificação do óleo de coco bruto catalisado por lipase de *Burkholderia cepacia* imobilizada por adsorção física em fibras de dendê originadas de resíduos agroindustriais. As reações foram realizadas nas condições: razão molar 1:7 (óleo:álcool), 10% de biocatalisador imobilizado por 96 h à 40°C na presença de diferentes aditivos (água, *tert*-butanol, peneira molecular e líquidos iônicos próticos), em diferentes concentrações. Os resultados mostraram que o uso de todos os aditivos neste estudo não aumentou a conversão do óleo de coco bruto em ésteres etílicos. A conversão máxima na ausência dos aditivos foi de 72%, na presença de água 45 ± 2 %, peneira molecular 53 ± 2 % e *tert*-butanol 59 ± 2 %, respectivamente, todos nas concentrações mais baixas. Para líquidos iônicos, aqueles com cadeias alquílicas mais altas tiveram as maiores conversões, mas menores do que na sua ausência.

Palavras-chave: Aditivos, transesterificação, lipase, imobilização, resíduo agroindustrial

1 INTRODUÇÃO

Enzymes are highly efficient biocatalysts in different processes. During a reaction several products can be synthesized by enzymatic catalysis, further increasing the importance of continuous studies that lead to a significant improvement in the properties of the enzyme, mainly in the reaction medium (BARBOSA *et al.*, 2020; BARBOSA *et al.*, 2019; SÁNCHEZ; TONETTO; FERREIRA, 2018). Biocatalysis has advantages such as obtaining the desired products in high proportions due to

the specificity and selectivity of some enzymes. Furthermore, it needs a low purification condition due to little or no generation of by-products, thus making an ecologically viable process (BARBOSA *et al.*, 2014; BARBOSA *et al.*, 2016b; BARBOSA *et al.*, 2019; MOTA *et al.*, 2020; SANTANA *et al.*, 2020).

Lipases (E.C. 3.1.1.3), while having many advantages in protecting the environment, are easily inactivated by substrates and/or byproducts during the reaction (SU *et al.*, 2016). To try to mitigate these types of problems the researchers see developing techniques, such as the use of additives, to somehow stabilize the enzyme and still increase its catalytic activity during the reaction (BABAKI *et al.*, 2017; BARBOSA *et al.*, 2014; BARBOSA *et al.*, 2016b; CAO *et al.*, 2017; CARVALHO *et al.*, 2018; LI, Ying Xia; DONG, 2016; NAVARRO LÓPEZ *et al.*, 2016).

The catalytic activity of the enzymes and their stability have strong influences on the presence of water, which is essential to keep the enzyme active in organic solvents (BABAKI; YOUSEFI; HABIBI; MOHAMMADI; *et al.*, 2015; LI, YING XIA; DONG, 2016). In addition, some lipases need a certain amount of water to maintain their own conformation (LI *et al.*, 2012). Another important aspect is that, like *Burkholderia cepacia* lipase (BCL), some have the catalytic site protected by a protein structure, the lid or lid, which may be closed rendering the enzyme inactive, but in the presence of the oil-water interface, the lid opens and leaves exposed the active site so that catalysis occurs (BARBOSA *et al.*, 2020; NIGAM *et al.*, 2014).

The ideal concentration of water is a very important factor in a transesterification reaction catalyzed by enzymes, since it has a compromise between minimizing the hydrolysis and maximizing the enzymatic activity (YÜCEL, 2013). Thus, its addition or removal through the use of adsorbents, such as silica gel blue, molecular sieve, among others, can effectively control their concentration in the reaction medium, and thus result in an improved ester yield (BABAKI; YOUSEFI; HABIBI; BRASK; *et al.*, 2015). Tertiary alcohols are studied in some works and demonstrate to be good solvents for conversion of oils to esters in lipase-catalyzed transesterification reaction, among them t-amyl alcohol and t-butyl alcohol (LI; ZONG; WU, 2009).

Another alternative of additives for the production of biodiesel are the ionic liquids (IL) that are organic salts composed entirely of ions, among them voluminous asymmetric cations and small inorganic anions (SU *et al.*, 2016). They have characteristics that have attracted many researchers, such as insignificant vapor pressure, recyclability, low combustibility, high thermal stability and the possibility of existence in the liquid state at room temperature and because they have these characteristics are considered potential green solvents for various applications (QIN *et al.*, 2016; SU *et al.*, 2016).

Few studies report the use of crude oils for the production of biodiesel. The use of biocatalysts immobilized in substrates derived from agroindustrial waste is still very scarce but of great importance because it has low cost and to provide a more environmental awareness which makes necessary new technologies for the value added in this residue to obtain products of interest industrial. Therefore, the objective of this study was to evaluate the influence of additives at different concentrations on the transesterification reaction of the crude coconut oil catalyzed with lipase from *Burkholderia cepacia* immobilized by physical adsorption onto palm fiber originated from agroindustrial wastes.

2 MATERIALS AND METHODS

2.1 MATERIALS

The lipase (E.C.3.1.1.3) used in the present study was from *Burkholderia cepacia* – BCL (Amano Lipase) and was purchased from Sigma Aldrich (Japan). Palm fibers acquired in a palm oil industry in Costa do Dendê (Bahia, Brazil). Crude coconut oil purchased from a local market. Ethanol (99 %), *tert*-butanol (99 %), molecular sieve UOP type 3 Å (form rod, and size 1=16 in) and ionic liquids (acetate, propanoate, butanoate and pentanoate of N-methylmonoetanolamine designated by C₂, C₃, C₄ and C₅, respectively). All other chemicals were of analytical grade from various suppliers.

2.2 LIPASE IMMOBILIZATION ONTO PALM FIBERS

The immobilization process of the lipase onto palm fibers consists in 1g of fibers in contact with 10 mL of hexane maintained for 15 minutes in agitation. Thereafter the aqueous enzyme solution (300 mg of enzyme solubilized in 10 ml of phosphate buffer, pH 7.0 at 100 mM) was added and stirred for 3 hours. Subsequently, the system is left at rest for a period of 24 hours at 4 °C. Finally washed with distilled water and stored in a refrigerator.

2.3 TRANSESTERIFICATION REACTION

Immobilized lipases used for ethyl esters production by transesterification of crude coconut oil and ethanol with different additives and concentrations. Reactions was performed under the best conditions in according by Barbosa *et al.* (2016a): the molar ratio (oil: alcohol) of 1:7, temperature of 40 °C for a period of 96 h under constant agitation of 250 rpm and immobilized biocatalyst concentration of 10 % (m/m). Water concentrations of 1 to 10 % (v/v), molecular sieves of 2.5 to 10 %, *tert*-butanol of 10 to 60 % (m/v) and ionic liquids in a concentration from 1 to 10 % (v/v). The samples were then removed and purified for quantification of the ethyl esters.

2.4 GAS CHROMATOGRAPHY ANALYSIS

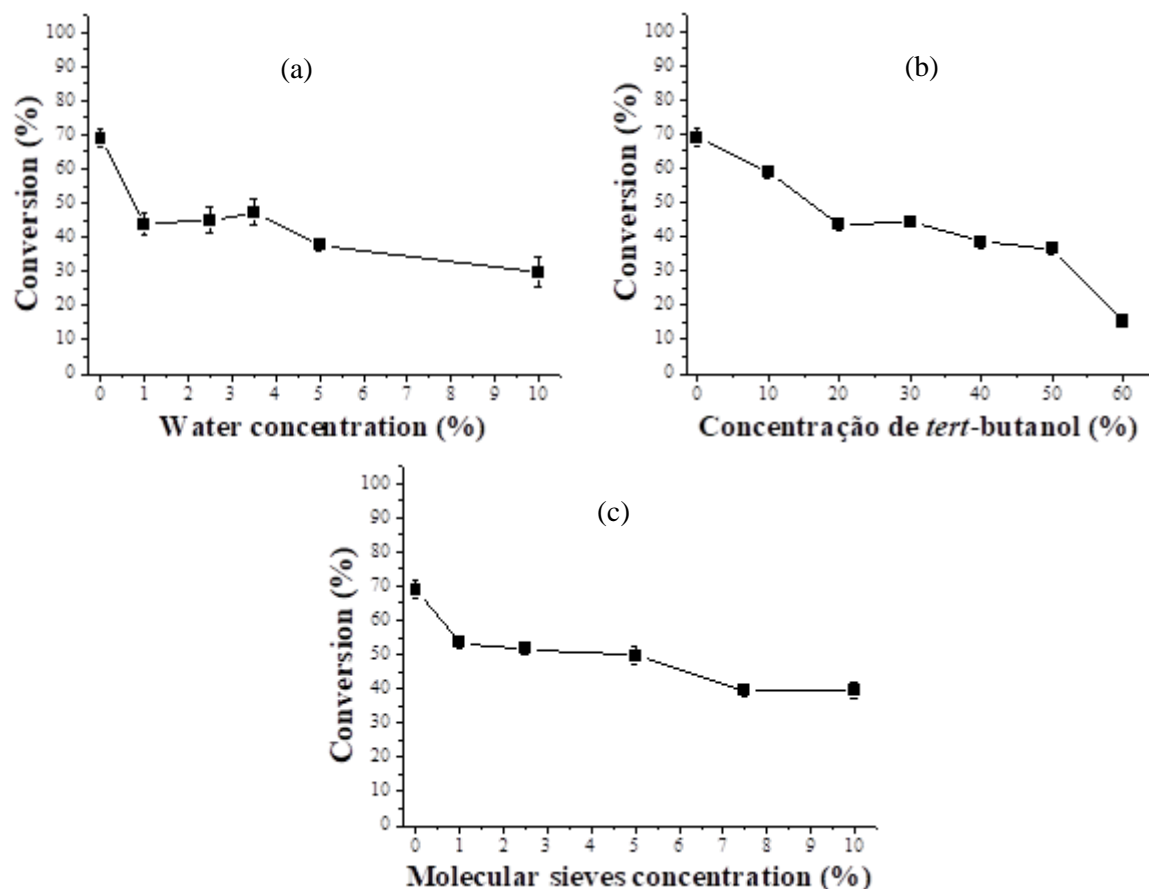
The FAEE (fatty acid ethyl esters) contents in the reaction mixture were quantified using a Agilent Technologies GC System 7820A model equipped with a mass spectrography detector (Agilent Technologies Series MSD 5975 model) and a capillary column (Supelcowax10, 30 m length \times 0.25 mm ID \times 0.25 μ m film thickness). The injector and detector temperatures were set at 250°C. Helio used as the carrier gas at a constant flow of 1 mL/min. The column temperature was held at 130 °C for 2 min, then heated to 220 °C at 20 °C·min⁻¹, after to 222 °C at 0.5 °C·min⁻¹, 250 °C at 20 °C·min⁻¹ and then maintained at this temperature for 3 min. The injection volume was 1 μ L. Methyl heptadecanoate was used as internal standard for GC analysis. All GC measurements performed in triplicate.

3 RESULTS AND DISCUSSION

In order to optimize and improve the results obtained in previous studies (BARBOSA *et.al.*, 2016a) in the conversion to ethyl esters from the crude coconut oil catalyzed with *Burkholderia cepacia* lipase immobilized onto palm fiber by physical adsorption (ADS) in the transesterification reaction, The influence of some additives such as water, *tert*-butanol, molecular sieve (Figure 1) and some protic ionic liquids of different sizes of alkyl chains (Figure 2).

The conversion of crude coconut oil to ethyl esters in the absence of additives was 72 %, according to the work done by Barbosa *et al.* (2016a). The influence of water on the transesterification of crude coconut oil showed that with the increase of water concentration in the reaction medium there was a decrease in the conversion of ethyl esters (Figure 1a). Similar results were found by Babaki *et al.* (2015) when the reaction was catalyzed with *Candida antarctica* lipase (CALB) immobilized onto silica activated with epoxy groups, which in the absence of water obtained a conversion of 46 % and with the addition of 10 to 30 % of water the conversion was between 1 and 5 %. This behavior may be associated to the increase of the hydrolysis reaction that converts the oil to fatty acids, due to the high water content that increases the available interfacial area, resulting in a significant decrease in ester production (LI; DONG, 2016).

Figure 1. Influence of additives in the transesterification reaction of crude coconut oil on conversion to ethyl esters under the conditions: molar ratio 1:7 (oil:alcohol), 10% biocatalyst immobilized for 96 h at 40 °C. Additives: water (a); *tert*-butanol (b); molecular sieves (c). Results are the mean of triplicates.



The presence of water may also reduce catalytic activity in the reaction due to enzyme aggregation in hydrophobic media and at the same time it has a negative effect on enzyme stability (BABAKI; YOUSEFI; HABIBI; BRASK; *et al.*, 2015). Another important factor is that as coconut oil is crude and did not undergo any type of treatment the amount of water presented in the reaction medium (in coconut oil) was sufficient for lipase activity and there was no need to add more water as another component. Similar results were found by Nikpour & Pazouki (2016) when using residual cooking oil as a substrate.

The addition of other solvents to transesterification reactions is often used to improve conversion (BABAKI; YOUSEFI; HABIBI; BRASK; *et al.*, 2015). However, the results found for the transesterification of crude lipid catalyzed ethyl alcohol and ADS showed an opposite result with a reduction in conversion to ethyl esters as the concentration of *tert*-butanol reducing from 72 to 15 % in the absence of the additive and at the highest concentration studied, respectively (Figure 1b). This result can be explained by the sensitivity of lipases to polar solvents, such as hexane and *tert*-butanol, which interact with the amount of water that is indispensable for the acquisition and maintenance of the catalytic conformation of the enzyme, resulting in any alteration or sometimes distortion of the

catalytic conformation and consequently result in the inactivation of the enzyme (CAO *et al.*, 2017; NARA; HARJANI; SALUNKHE, 2002; NAVARRO LÓPEZ *et al.*, 2016). Moreover, extremely polar solvents due to their hydrophilicity may interact with the secondary structure of the functional protein through multiple hydrogen bonds or other strong interactions that may also lead to unfolding, leading to their inactivation (NARA; HARJANI; SALUNKHE, 2002).

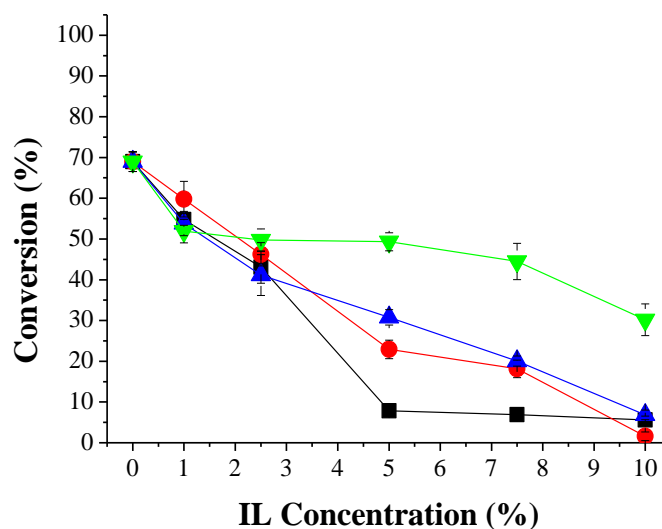
The increased polar solvent concentration also reduced conversion of methyl esters from lipids of microalgae from 81 to about 65 and 51 to approximately 44 % using *tert*-butanol and hexane respectively, catalyzed by *Rhizopus oryzae* lipase in soluble form (NAVARRO LÓPEZ *et al.*, 2016). In the transesterification of residual cooking oil using *Rhizomucor miehei* (RML) and *Candida antarctica* (CALB) lipases immobilized on silica functionalized with epoxy groups, the increase of the *tert*-butanol concentration in the reaction medium also caused a reduction in the production of methyl esters of 75 to 35 % (BABAKI *et al.*, 2017). Another explanation for this behavior is that when more than 1.5 molar equivalents of alcohol are used the enzymatic activity has decreased because the alcohols which are hydrophilic become insoluble in the oils and tend to remove the hydration layer from the lipase water (TAHER; AL-ZUHAIR, 2017).

In the enzymatic production of biodiesel, during the reaction, free fatty acids with ethanol the water is also produced. And since the coconut oil used in this research is crude, there are a lot of free fatty acids, so the effect of the amount of water adsorbent, in this case the molecular sieve, has been investigated. The incremental addition of molecular sieve (1-10 % relative to the mass of the reaction medium) decreased the conversion of crude coconut oil to ethyl esters from 72 to 39 % (0 and 10 % sieve, respectively). The addition of the molecular sieve in the reactions may change the equilibrium of the reaction in the ester production by the removal of water, on the other hand, with the increase of the adsorbent amount the total conversion of the ethyl esters has decreased, this fact could have happened due to the limitation of the mass transfer (BABAKI; YOUSEFI; HABIBI; BRASK; *et al.*, 2015). In the studies developed by Babaki *et al.* (2015) the addition of the blue silica gel as adsorbent (15-70 % relative to the weight of the oil) in CALB catalyzed methanolysis immobilized on silica decreased the conversion of canola to esters from 85 to 67 % (at the concentration of 25 % and 55 % silica gel, respectively).

Lipase in specific ionic liquids (ILs) may undergo some changes in its tertiary structures, which would probably affect the active site of lipase, which may cause positive (hyperactivation and/or stabilization) or negative effects such as inactivation (SU *et al.*, 2016). In the transesterification of crude coconut oil and catalyzed ethanol with immobilized BC lipase and addition of ILs of different alkyl chain sizes (C₂ to C₅), no increase in conversion to ethyl esters was observed and the concentration of ILs increased Conversion was further reduced (Figure 2). It was also possible to

observe the behavior of the ionic liquids under study, that the higher the alkyl chain of IL (C₅) the better the conversion was in relation to the other lower alkyl chain IL (C₂).

Figure 2. Influence of ionic liquids as additives in the transesterification reaction of crude coconut oil on conversion to ethyl esters under the best conditions in the presence of ADS biocatalyst (molar ratio 1:7 oil:alcohol, 10% biocatalyst immobilized for 96 h at 40 °C). Ionic liquids: C₂ (■); C₃ (●); C₄ (▲); C₅ (▼). Results are the mean of triplicates.



The influence of the hydrophobicity of ILs attracts the attention of several researchers, in which the alkyl chain variation of the ILs and the concentration are studied, and it is possible to verify that in the majority of them, the greater the alkyl chain of IL, the more hydrophobic character it presents, and better efficiency in the enzymatic activity it provides (MARTINS *et al.*, 2016; QIN *et al.*, 2016; SU *et al.*, 2016).

The presence of the higher alkyl chain IL may have influenced the hydrophobicity of the microenvironment and thus influenced the lipase moisture level (MOHIDEM; BIN MAT, 2012). According to Nara *et al.* (2002) the polarity of the reaction medium greatly influences the catalytic activity of the enzymes and studies have shown that hydrophobic solvents favor faster reactions and that hydrophilic solvents prevent the rate of enzyme catalyzed reactions to some extent, independently of other parameters.

4 CONCLUSION

The results showed that the use of different additives did not demonstrate efficiency in a better conversion of crude coconut oil to ethyl esters catalyzed by *Burkholderia cepacia* lipase immobilized onto palm fiber. In this way we can conclude that studies of other additives are necessary in the reaction for optimization of the reaction. Considering the immobilized biocatalyst has a low cost

because the support was derived from agroindustrial wastes, in addition to application using the crude coconut oil as substrate and having undergone a considerable conversion of 72 % to ethyl esters.

ACKNOWLEDGEMENTS

The authors thank CNPq, CAPES (PDSE – Edital n° 19/2016, process n° 88881.133896/2016-01), FAPITEC/SE (PROMOB - Edital CAPES/FAPITEC/SE n° 01/2013) and UNIT for their support of this work, and for research scholarships.

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