Short Communication

Improved production of butyl butyrate with lipase from Thermomyces lanuginosus immobilized on styrene–divinylbenzene beads

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HIGHLIGHTS

► TLL was immobilized on styrene-divinylbenzene beads (MCI-TLL).
► MCI-TLL was compared to Lipozyme TL-IM in butyl butyrate synthesis.
► MCI-TLL presented two-times more protein than Lipozyme TL-IM.
► The prepared biocatalyst was stable to higher concentration of butyric acid.
► MCI-TLL showed productivity 4-times higher than Lipozyme TL-IM.

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ABSTRACT

Two immobilized preparations from Thermomyces lanuginosus lipase (TLL) were compared in the synthesis of butyl butyrate. The commercial Lipozyme TL-IM, and TLL immobilized on styrene–divinylbenzene beads (MCI-TLL) were tested in the esterification reaction using n-hexane as solvent. The variables temperature (30–60 °C), substrate molar ratio (1:1 to 5:1), added water (0–1%), and biocatalyst content (3–40%) were evaluated in terms of initial reaction rate for each biocatalyst. SDS–PAGE analysis revealed that MCI-TLL had an immobilized enzymatic load twice as high as Lipozyme TL-IM, but with an activity 3-fold higher. MCI-TLL presented high initial reaction rates up to 1.0 M butyric acid, while Lipozyme TL-IM showed a decrease in its activity above 0.5 M. Moreover, MCI-TLL allowed a productivity of 14.5 mmol g⁻¹ h⁻¹, while Lipozyme TL-IM 3.2 mmol g⁻¹ h⁻¹, both by mass of biocatalyst.

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1. Introduction

Short chain butyrate esters are additives for the food industry as flavoring compounds naturally present in pineapple, mango, and banana (Rajendran et al., 2009). Esterification is a simple process, and conventional catalysis is an efficient way of producing these esters by the use of a proper inorganic catalyst at 200–250 °C. However, these esters are considered as “artificial flavors”. If they are obtained through biological routes (e.g., using enzymes), the resulting esters are considered as natural flavors, with subsequent economic advantages (Groussin and Antoniotti, 2012). Thus, research efforts are being made to develop enzymatic processes for the production of butyl and other short chain esters (de Paula et al., 2010; Serra et al., 2005). Although the natural function of lipases is to catalyze the hydrolysis of oils and fats (Jaeger and Eggert, 2002), these enzymes have also been used into catalyze esterification involving long fatty acids (Zheng et al., 2012), and short chain alcohols and carboxylic acids, (Mahapatra et al., 2009). Recently, production of butyl butyrate has been reported using lipase from Thermomyces lanuginosus (TLL) (Mendes et al., 2012). This enzyme has been used for many reactions in fine chemistry and for the modification of fats and oils, but it has been scarcely studied for the production of short chain esters (Fernandez-Lafuente, 2010).
Lipase properties have been modulated via immobilization and, in some instances, higher specific activities and stabilities may be achieved when a proper immobilization protocol is employed (García-Galan et al., 2011; Rodrigues et al., 2013). It has been shown that the use of different supports, with different hydrophobicities or internal morphologies, greatly alters enzyme properties such as stability, activity, selectivity, or specificity (Fernández-Lorente et al., 2008). Recent reports have shown that a support based on styrene–divinylbenzene (MCI) greatly improves the properties of lipase B from Candida antarctica (Hernández et al., 2011), including enhanced synthesis of butyl acetate (Graebin et al., 2012) and ethyl butyrate (Friedrich et al., 2012). The protein load capacity per wet gram of support is high. In this new paper, it was extended the studies of the use this support for the first time to TLL. Based on these facts, the aim of this work was to produce butyl butyrate, a fruit flavor ester, and to compare the new MCI-TLL preparation, with the commercial Lipozyme TL-IM. MCI-TLL is based on the immobilization via interfacial activation on a hydrophobic support (Hernández et al., 2011), while Lipozyme TL-IM is based on the ionic exchange of the enzyme on the support (Fernández-Lafuente, 2010), thus very different immobilization mechanisms have been followed.

2. Methods

2.1. Materials

TLL free and immobilized on a silicate support (Lipozyme TL-IM), were kindly donated by Novozyymes (Spain). The styrene–divinylbenzene MCI GEL CHP20P porous support and p-nitrophenyl butyrate (pNPB) were purchased from Sigma. Butyric acid, n-butanol, and other chemicals were of analytical grade.

2.2. Immobilization of TLL

Firstly, the support was wetted as previously described (Hernández et al., 2011). 10 g of MCI GEL CHP20P support were re-suspended in 500 mL of an enzyme solution containing 1200 mg of protein in 10 mM sodium phosphate at pH 7 and 20 °C for 24 h. Activity of supernatant was followed using the pNPB assay, showing an immobilization yield higher than 95%. The immobilized preparation was designated MCI-TLL.

2.3. SDS–PAGE

SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (Laemmli, 1970) using a SE 250–Mighty small II electrophoretic unit (Hoefer Co.); 15% (concentration fraction) running gel in a separation zone of 9 × 6 cm, and a stacking gel of 5% (concentration fraction) polyacrylamide. Samples of 100 mg of the immobilized enzyme were re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol, volume fractions), boiled for 5 min and 20 μL aliquots of the supernatant were used in the experiments. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from GE Healthcare were used (14000–97000 Da).

2.4. Synthesis of butyl butyrate via enzymatic esterification

The standard reactions were carried out into 50 mL Erlenmeyer flasks (working volume of 15 mL) using n-hexane as solvent and 0.7 M of butyric acid, in an orbital shaker (200 rpm). Previously to the reactions, the immobilized enzyme was dried for 24 h at 40 °C to remove any adsorbed water. Substrate molar ratio varied in the range of 1:1 to 5:1 (n-butanol:butyric acid). Biocatalyst content (dried immobilized TLL) and added water varied from 1% to 40% and 0% to 1%, respectively in relation to the substrate mass fraction (considering in the calculations the mass of both substrates in stoichiometric ratio). Reaction temperature varied from 30 to 60 °C. All variables were evaluated individually, and the best condition obtained in the previous parameter was used to study the next one. Substrates were mixed with the solvent and the initial added water, and when reached the temperature the desired amount of biocatalyst was added to start the reaction. The progress of esterification was monitored by determination of the residual acid content by titration as previously described (Friedrich et al., 2012; Graebin et al., 2012).

![Image](image-url) Fig. 1. SDS–PAGE of the TLL derivatives. Lane 1: molecular weight markers (kDa); lane 2: MCI-TLL; lane 3: Lipozyme TL-IM.
3. Results and discussion

3.1. Preliminary comparison of Lipozyme TL-IM and MCI-TLL

In a first approach, the mass of lipase on both dried derivatives was semi-quantitatively compared. For this, the immobilized preparations were boiled in SDS to desorb the enzymes from the support, and the supernatants were resolved in a SDS–PAGE gel. As Lipozyme TL-IM was adsorbed by ion-exchange, NaCl 1 M was added to rupture buffer to avoid the re-immobilization of the enzyme, which could be caused due to the high cationic charge of the support and anionic charge of the enzyme coated with SDS. The results show, qualitatively, that there was a higher amount of enzyme on MCI-TLL than in the Lipozyme TL-IM (Fig. 1). Thus, MCI GEL CHP20P support presents a higher load capacity to immobilize the TLL compared with the commercial preparation. In contrast, in an early report (Graebin et al., 2012), it has been shown that the commercial immobilized lipase B from Candida antarctica (Novozym 435) presented a higher enzymatic load than CALB immobilized on MCI GEL CHP20P. It was verified in preliminary tests that, for Lipozyme TL-IM, it was necessary around 30% (by substrate mass) of biocatalyst to obtain high yield conversions in short time. Using MCI-TLL, the same yield conversions were obtained with approximately 10% (by substrate mass) of biocatalyst content. Although the SDS–PAGE showed that MCI-TLL had apparently 2 times more enzyme than Lipozyme TL-IM, its activity was 3-fold that of the commercial preparation, meaning that the specific activity of the enzyme immobilized in the new support was around 50% higher than that of the commercial preparation under these conditions. Therefore, for the following experiments it was used a content of biocatalyst of 30% for Lipozyme TL-IM and 10% for MCI-TLL.

3.2. Effect of acid concentration on the enzyme activity

The effect of butyric acid concentration on the initial reaction rate is presented in Fig. 2a for Lipozyme TL-IM and MCI-TLL. There is a linear increment in the enzyme activity as the initial reaction rate increased up to 0.5 M butyric acid for Lipozyme TL-IM, while MCI-TLL presented a linear increase up to 0.7 M. Lipozyme TL-IM showed a slightly decrease from 0.5 to 0.7 M and a sharp decrease from 0.7 to 1.0 M butyric acid, whereas MCI-TLL presented a mild reduction in its initial reaction rate at 1.0 M butyric acid. Nevertheless, the optimal acid concentration found in this work is higher than previously reported for TLL in esterification reactions, with butyric acid, around 0.1 M (Mendes et al., 2011, 2012). Thus, it was selected the concentration of 0.7 M for the subsequent experiments for both preparations, even with MCI-TLL showing good results up to 1.0 M butyric acid.

In order to verify whether the loss of enzyme activity would be caused by a distortion of the enzyme molecule, a low value of pH, inhibition by substrate concentration, or enzyme inactivation, the reactions using 0.5 and 1 M of butyric acid and butanol were analyzed (Fig. 2b). Results show that an increment in the concentration of reactants produced a clear decrease in the activities of TL-IM and MCI-TLL, but the reaction courses are linear both with 0.5 M and 1 M of acid, suggesting that the enzymes are not inactivated during reaction, but possibly inhibited by high substrate concentration.

3.3. Effect of substrate molar ratio on the enzyme activity

Experiments were performed using 0.7 M of butyric acid and varying the substrate molar ratio from 1:1 to 4:1 (n-butanol:butyric acid). In many studies of the influence of substrate molar ratio in esterification, the mass of biocatalyst is considered in relation to the mass of all substrates (alcohol and acid). This makes to increase the substrate molar ratio at the same time that the biocatalyst content, and can drive to misunderstandings. In this work, the biocatalyst content was calculated in relation to the mass of the stoichiometric ratio (1:1). The biocatalyst content was fixed at 10% for MCI-CALB and 30% Lipozyme TL-IM; the added water at 0%, and temperature at 30 °C. The results are presented in Fig. 3a. Lipozyme TL-IM showed an increase in its initial reaction rates from 1:1 to 2:1, decreasing after this for all studied concentrations of n-butanol, while MCI-TLL presented maximal activities at stoichiometric ratio. This may be due to a better partition of the relatively hydrophobic alcohol in the more hydrophobic MCI-TLL, or due to the fact the enzyme has the open form stabilized by adsorption on the hydrophobic support (Fernandez-Lorente et al., 2012).
et al., 2008), while Lipozyme TL-IM active center may be not in the open conformation. Thus, for the following experiments, substrate molar ratio was fixed at the best level tested for each biocatalyst, 2:1 for Lipozyme TL-IM and 1:1 for MCI-TLL.

3.4. Effect of initial added water on the enzyme activity

Water is one of the products of the esterification reaction and excessive water content in the reaction mixture can negatively affect the yield conversion. However, some initial water activity is needed to allow lipases to retain their active three-dimensional conformational form and mobility (Yahya et al., 1998). Thus, it was verified the influence of initial added water in the reaction rate (Fig. 3b). Added water was tested varying its concentration from 0% to 1% (in relation to the stoichiometric substrate mass fraction). Substrate molar ratio was fixed at the levels selected in the previous experiments, biocatalyst content was 30% for Lipozyme TL-IM and 10% for MCI-TLL and the assays were carried out at 30 °C. For MCI-TLL, the initial reaction rate was not affected by added water in the tested range, while for Lipozyme TL-IM initial reaction rate presented a slightly increase from 0% to 0.25%, remaining constant until 0.75% and afterward decreasing. This could be due to the lower water retention by the very hydrophobic MCI GEL CHP20P when compared to Lipozyme TL-IM. Thus, for the next step of this study, it was selected the addition of 0.25% water for Lipozyme TL-IM and no addition of water for MCI-TLL.

3.5. Effect of reaction temperature on the enzyme activity

The effect of temperature on the initial rate of reaction was studied in the range 30–60 °C. Biocatalyst content was fixed at 30% for Lipozyme TL-IM and 10% for MCI-TLL. As it can be observed
3.6. Effect of biocatalyst content on the initial rate of reaction

The last step in the study of reaction parameters affecting the reaction rate of butyl butyrate synthesis is the effect of biocatalyst content. The biocatalyst content was varied from 3% to 15% (in relation to the stoichiometric substrate mass fraction) for MCI-TLL and from 5% to 40% to Lipozyme TL-IM, due to the difference on enzyme activity found along this paper. Substrate molar ratio, added water and temperature were fixed at the previous selected values. Whereas for MCI-TLL 10% of biocatalyst was enough to reach near maximal activity, the same initial rate was obtained with at least 40% of biocatalyst for Lipozyme TL-IM (Fig. 3d). Using MCI-TLL, initial reaction rates increased linearly from 3% to 10%, slowing down afterwards, while for Lipozyme TL-IM no activity was detected until 10%, slightly increasing from 30% to 40%. Thus, in order to use the smallest amount of biocatalyst possible, considering economical reasons in large scale, biocatalyst content was set at 30% for Lipozyme TL-IM and 10% for MCI-TLL.

After the analysis of all reaction parameters, it can be observed that the same lipase, immobilized in different supports, shows different optimal conditions for the synthesis of butyl butyrate. In synthesis, best conditions found in this research for the derivatives were: for Lipozyme TL-IM: substrate molar ratio of 2:1 n-butanol:butyric acid, biocatalyst content of 30%, added water of 0.25%, both in relation to the stoichiometric substrate mass fraction; and temperature of 50 °C; for MCI-TLL: substrate molar ratio of 1:1 n-butanol:butyric acid, biocatalyst content of 10%, no added water, and temperature of 50 °C.

3.7. Reaction courses of esterification under selected conditions

The courses of butyl butyrate synthesis under the best conditions tested for both biocatalysts are presented in Fig. 4. Although initial reaction rates were similar for both preparations, this was achieved using 3-times more mass of Lipozyme TL-IM than of MCI-TLL, meaning that the productivity of MCI-TLL is 4.5-times higher than Lipozyme TL-IM (14.5 mmol g\(^{-1}\) h\(^{-1}\) and 3.2 mmol g\(^{-1}\) h\(^{-1}\), respectively), regarding the mass of biocatalyst, and more than doubling regarding enzyme mass. The courses of the reaction catalyzed by both preparations showed linear behavior reaching at high yields of conversion (around 95%), although for MCI-TLL excess of alcohol was no used. The higher initial rate obtained for MCI-TLL suggests that the enzyme, immobilized in this support, was not submitted to inactivation, product inhibition, or substrate depletion. Possibly due to the hydrophobicity of the support, the reaction products, in special the water, migrate to the medium, averting the undesired effects of their presence in the reaction.

4. Conclusions

The performance of a TLL preparation with its open form stabilized versus a hydrophobic support has been compared with TLL immobilized via ionic exchange. MCI GEL CHP20P, a support based on styrene–divinylbenzene, allowed the immobilization of a higher protein load than in Lipozyme TL-IM. Moreover, it was possible to use higher concentrations of butyric acid, with an improved productivity of 4.5-fold using MCI-TLL compared to Lipozyme TL-IM. Therefore, the results obtained in this work are very promising for industrial scaling up, since a high concentration of butyric acid can be used and the reaction was fast using a small amount of biocatalyst.

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