Journal of Molecular Catalysis B: Enzymatic 105 (2014) 18–25

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Comparison of the performance of commercial immobilized lipases in the synthesis of different flavor esters

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ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 10 March 2014
Accepted 26 March 2014
Available online 4 April 2014

Keywords:
Esterification
Flavor esters
Lipase
Immobilized enzyme
Enzyme reuse

ABSTRACT

In this work, it is compared the performance of three commercial lipase preparations (Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM) in the synthesis of flavor esters obtained by esterification of acetic, propionic, and butyric acids using ethanol, isopropyl alcohol, butanol, or pentanol. A comprehensive comparison was performed verifying activities of these three enzyme preparations versus the different couples of substrates, checking the obtained yields. In general, the longer the acid chain, the higher the reaction yields. Novozym 435 was the most efficient enzyme in most cases, and only Lipozyme RM-IM offered better results than Novozym 435 in the production of ethyl butyrate. Reactions with butyric acid showed the highest conversion rates using all biocatalysts. Using optimal substrates, the reactions catalyzed by the three enzymes were optimized using the response surface methodology, and the catalytic performance of the biocatalysts in repeated batches was assessed. After optimization, yields higher than 90% were obtained for all three enzymes, but Lipozyme TL-IM needed four-times more biocatalyst content than the other two preparations. Novozym 435 kept over 80% of its activity when reused in 9 successive batches, whereas Lipozyme RM-IM can be reused 5 times and Lipozyme TL-IM only 3 times. In general, Novozym 435 showed to be more suitable for these reactions than the other two enzyme preparations.

1. Introduction

Many commercially important fruity notes are esters formed by short chain alcohols and carboxylic acids [1]. The extraction of these notes from natural products is expensive and, as a consequence, their chemical synthesis has become the standard industrial practice, i.e., the reaction using inorganic catalysts and elevated temperatures (200–250 °C) [2], and the obtained esters are classified as "artificial flavors". In contrast, the enzymatic syntheses of flavor esters catalyzed by lipases allow their classification as "natural flavors", with immediate economical advantages [3]. Lipases are well-studied enzymes, showing hydrolytic activities, but also catalyzing reactions of esterification, transesterification, and alcoholysis [3–6]. Several researches reported on esterifications catalyzed by lipases, especially those using a short chain alcohol and a long chain fatty acid [2,7–11]. However, reactions using short carboxylic acids have been the subject of fewer studies [12]. The enzymatic syntheses of flavor esters require very low water contents in order to shift the thermodynamic equilibrium toward synthesis, thus non-aqueous media are required. Solvent-free systems [13,14], ionic liquids [15,16] or supercritical fluids [17] have been used as media for lipase catalyzed reactions. However, still most of the examples that can be found in the literature use the most traditional organic solvents [13,16,18].

Although this is a very common reaction, and much effort has been expended to find an ideal biocatalyst, a comparison of the activity of the most used lipases has not been reported to date. This may be interesting to understand the behavior and specificities on the synthesis of flavor esters, and, for first time, this has been performed in this manuscript. Thus, the main objective of this work was to evaluate the performance of three of the most used commercially available biocatalysts (Novozym 435; Lipozyme TL-IM; and Lipozyme RM-IM) for the synthesis of esters of different short chain acids (acetic, propionic, and butyric acids), and

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http://dx.doi.org/10.1016/j.molcatb.2014.03.021
1381-1177/

alcohols (ethanol, butanol, and pentanol) on organic solvents to comprehend the enzymes activities and specificities against these substrates. A secondary alcohol (2-propanol) has also been tested in order to allow us a better understanding of the enzymes specificities. The resulting esters are among the most interesting fruit flavors, such as pineapple, banana, peach, apricot, mango, among others.

Novozym 435 is possibly the most used biocatalyst [19]. This immobilized preparation of lipase B from *Candida antarctica* (CALB) is obtained by immobilization via interfacial activation of the enzyme on a moderately hydrophobic resin, Lewatit VP OC 1600 [20].

The second used immobilized lipase biocatalyst was that from *Thermomyces lanuginosus* (TLL). It is immobilized on a cationic silicate via anion exchange (Lipozyme TL IM®) [21]. This enzyme has been used in multiple reactions [22] and is produced by a genetically modified strain of *Aspergillus oryzae* [23]. Its structure is also well known and the enzyme has a large lid, able to fully seclude the active center from the reaction medium in the closed form [24].

The third biocatalyst used in this study is the immobilized lipase from *Rhizomucor miehei* (RML), commercially available from Novozymes as immobilized form (Lipozyme RM-IM). The support of this immobilized enzyme is Duolite ES 562, a weak anion-exchange resin based on phenol–formaldehyde copolymers [25]. The enzyme has been used on many processes [26,27] and its structure shows a typical lid that isolates the active center of the enzyme from the medium when closed [28]. Therefore, these three biocatalysts differ not only about the enzyme source, but also in the nature of the matrix and on the mechanism of immobilization of the enzyme and significant differences on enzyme activity, specificity, and stability may be expected [29].

We also studied the enzyme performance on some of the esterification reactions using the response surface methodology, and tested the biocatalysts activities during reuse in several batches.

2. Materials and methods

2.1. Chemicals

The enzymes used in this work were *Candida antarctica* lipase B immobilized in a macroporous resin (Novozym 435); *T. lanuginosus* lipase immobilized in a silicate support (Lipozyme TL-IM); and *R. miehei* lipase immobilized in an anion-exchange resin (Lipozyme RM-IM), all of them kindly donated by Novozymes (Spain). Acetic, propionic, and butyric acids; ethanol, 2-propanol, 1-butanol, 1-pentanol, and other chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma, St. Louis, USA).

2.2. Esterification reactions

2.2.1. Screening experiments

Esterification reactions were carried out using 0.1 M of each different acid and alcohol (1:1 alcohol:acid molar ratio) in the presence of *n*-hexane as solvent into 50 mL Erlenmeyer flasks (working volume of 10 mL), under agitation in an orbital shaker (200 rpm) for 2 h. Reaction temperature and the amount of each biocatalyst were set according to previous studies [30–32]: 10% (mass fraction of substrate) for Novozym 435 and Lipozyme RM-IM; 30% for Lipozyme TL-IM; 40 °C for Novozym 435 and Lipozyme RM-IM; and 50 °C for Lipozyme TL-IM.

2.2.2. Reaction optimization

From the preliminary screening, one combination of acid and alcohol was chosen for each enzyme. The optimization was performed by central composite design (CCD) and response surface methodology (RSM). The variables studied on the CCD were reaction temperature (30–60 °C), substrate molar ratio (1:1–5:1 alcohol:acid molar ratio), amount of added water (0–1%) and biocatalyst content (% considering in the calculations the mass of both substrates in stoichiometric ratio). The range for biocatalyst content was different for each enzyme according to their activity: 1–10% for Novozym 435 and Lipozyme RM-IM; 5–45% for Lipozyme TL-IM. Reactions were carried out by mixing 0.1 M of acid with different concentrations of alcohol into 50 mL Erlenmeyer flasks (working volume of 10 mL), followed by the addition of various amounts of water, immobilized enzyme (dried as described above), and *n*-hexane as solvent. The mixtures of acids, alcohol, and enzymes were incubated in an orbital shaker (200 rpm) at various reaction temperatures for 2 h for Novozym 435 and Lipozyme RM-IM, and 5 h for Lipozyme TL-IM. The conditions for each reaction were determined by the CCD, which is presented in Table 1.

Table 1
Experimental design and results of CCD.

Run	*X*1	*X*2	*X*3	*X*4	Novozym 435 (%)	Lipozyme RM-IL (%)	Lipozyme TL-IM (%)
1	−1	−1	−1	−1	25.67	30.17	43.24
2	−1	−1	−1	1	25.19	23.48	48.38
3	−1	−1	1	−1	54.43	60.00	77.62
4	−1	−1	1	1	59.71	62.18	81.71
5	−1	1	−1	−1	11.51	29.17	13.14
6	−1	1	−1	1	19.94	33.87	12.10
7	−1	1	1	−1	53.81	51.45	44.67
8	−1	1	1	1	47.58	42.06	30.29
9	1	−1	−1	−1	32.09	48.75	0.48
10	1	−1	−1	1	50.17	53.49	24.06
11	1	−1	1	−1	78.17	72.29	17.14
12	1	−1	1	1	75.37	67.47	24.48
13	1	1	−1	−1	43.58	29.76	28.86
14	1	1	−1	1	45.13	39.32	4.19
15	1	1	1	−1	69.64	78.47	45.62
16	1	1	1	1	67.94	74.62	37.62
17	−2	0	0	0	14.92	35.92	42.52
18	2	0	0	0	59.68	1.25	18.19
19	0	−2	0	0	45.57	44.35	68.86
20	0	2	0	0	52.47	40.17	0.76
21	0	0	−2	0	25.32	16.13	3.71
22	0	0	2	0	73.23	81.25	87.90
23	0	0	0	−2	49.57	62.77	55.52
24	0	0	0	2	38.09	53.97	46.86
25	0	0	0	0	48.60	43.86	60.10
26	0	0	0	0	36.44	46.67	55.00
27	0	0	0	0	47.94	55.29	58.76
28	0	0	0	0	44.13	48.62	48.38

*X*1: temperature; *X*2: substrate molar ratio; *X*3: biocatalyst content; *X*4: added water.

2.3. Hydrolytic activity

The hydrolytic activity of the immobilized enzymes was measured according to methodology previously developed [33]. A volume of 5 mmol of soybean oil was added into 50 mL Erlenmeyer flasks, followed by addition of 60 mmol of water (12:1 water:oil molar ratio), and 10% of biocatalyst (by oil mass). The mixtures of soybean oil, water, and lipases were stirred in an orbital shaker (200 rpm) for 1 h at 40 °C. The progress of hydrolysis was monitored by determination of the free fatty acid released by titration of 0.3 g samples using 0.01 M NaOH using phenolphthalein as pH indicator and 5 mL of ethanol as quenching agent. One unit (U) was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute at the experimental conditions.

2.4. Reaction analysis

The progress of esterification was monitored following the determination of the residual acid content by titration of 0.5 mL