Ultrasonics Sonochemistry 22 (2015) 89–94

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

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

The combined use of ultrasound and molecular sieves improves the synthesis of ethyl butyrate catalyzed by immobilized *Thermomyces lanuginosus* lipase

Natalia Paludo [a], Joana S. Alves [a], Cintia Altmann [a], Marco A.Z. Ayub [a], Roberto Fernandez-Lafuente [b], Rafael C. Rodrigues [a,*]

[a] Biotechnology, Bioprocess and Biocatalysis Group, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil
[b] Department of Biocatalysis, ICP – CSIC, Campus UAM-CSIC, Cantoblanco, ZC 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 4 December 2013
Received in revised form 7 April 2014
Accepted 6 May 2014
Available online 13 May 2014

Keywords:
Esterification reactions
Ultrasound
Molecular sieves
Lipases
Lipozyme TL-IM
Operational stability

ABSTRACT

In this work, the combined use of ultrasound energy and molecular sieves was investigated for the synthesis of ethyl butyrate, ester with mango and banana notes, catalyzed by the immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL-IM). Initially, the best concentrations of biocatalysts (35%) and butyric acid (0.7 M) were tested using ultrasound as an alternative to mechanical agitation. The amount of acid in the reaction could be increased by 2-fold when compared to previous works where mechanical agitation was used. In the next step, substrate molar ratio and reaction temperature were optimized and the best conditions were at their lowest levels: 1:1 (acid:alcohol), and 30 °C, reaching 61% of conversion in 6 h. Molecular sieves (3 Å) were added to optimized reaction medium in order to remove the formed water and improve the maximum yield. The reaction yield increased 1.5 times, reaching 90% of conversion in 6 h, when 60 mg of molecular sieves per mmol of butyric acid was used. Finally, the reuse of Lipozyme TL-IM for the ultrasound-assisted synthesis of ethyl butyrate was verified for 10 batches, without any appreciable loss of activity, whereas in systems using mechanical agitation, the biocatalyst was completely inactivated after 5 batches. These results suggest that the combined use of ultrasound and molecular sieves greatly improve esterification reactions by stabilizing the enzyme and increasing yields.

1. Introduction

The production of short chain esters is an apparently simple, but very interesting reaction of a direct condensation of a short chain alcohol and a short chain carboxylic acid [1], being the resulting ester a volatile compound with flavor and fragrance properties [2]. These esters are industrially important for food, pharmaceutical and cosmetic products [2]. In many countries, the technical definition of natural flavors states that they must be prepared by either physical processes (extraction from natural sources), or via biotechnology (enzymatic or microbial processes) [3], therefore stimulating the research in the field of enzymatic production of these compounds. However, in order to turn enzymatic processes competitive, high enzyme stability, activity, and high reaction yields are required.

Lipases are among the most industrially important enzymes; they are versatile biocatalysts, whose natural function is the hydrolysis of oils and fats [4]. However, under suitable conditions, lipases are also capable of catalyzing esterification, transesterification, and interesterification reactions [5]. Lipases have been used in low water activity systems (organic solvents, ionic liquids, supercritical fluids), reaction conditions that are meant to reverse the equilibrium in the direction of the synthesis [6–8], which is important to increase productivity. Additionally, water plays an important role in biocatalytic reactions: catalytic activity usually increases with increasing water activity (a_w), due to improve enzyme mobility [9]. However, the raise in the water content also favors hydrolysis over synthesis. Thus, to maintain the yield, very low a_w values must be attained. A common practical approach has been the use molecular sieves [10,11].

Most of the studies on lipase-catalyzed esterification have been performed using standard mechanical stirring conditions [11–14]. However, ultrasound has emerged as an alternative source of homogenizing energy with potential applications in enzymatic reaction control. Ultrasound energy acts by increasing the

* Corresponding author. Tel.: +55 51 3308 7793; fax: +55 51 3308 7048.
E-mail address: rafaelcrodrigues@ufrgs.br (R.C. Rodrigues).
URL: http://www.ufrgs.br/bbb.

http://dx.doi.org/10.1016/j.ultsonch.2014.05.004
1350-4177/

interaction between phases in a system by cavitation caused by the collapse of bubbles, whereas the ultrasonic jet disrupts the boundary phase and causes emulsification [15–17]. When applied in aqueous solutions or suspensions, ultrasound increases mixing, shearing, and mass transfer rate of the system, reducing process time when compared with other conventional mixing techniques [18,19]. In biotechnological processes, ultrasound has been applied for some enzymatic reactions, wastewater treatment and biofuels production, with very good results [18,20–22]. While some reports have shown the positive influence of ultrasound on the kinetic parameters of esterification reactions catalyzed by enzymes [23–25], its application in this field remains scarcely explored, and few reports for lipase-catalyzed flavor esters syntheses are found in the literature. In a previous work of our group, the use of ultrasounds for the esterification of acetic acid and butanol catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435), allowed us to use higher acetic acid concentrations when compared to systems where mechanical agitation was employed, increasing the overall reaction productivity by 7.5-fold [26].

In the present work, we investigate the influence of ultrasound energy on the esterification reaction between butyric acid and ethanol using hexane as solvent, catalyzed by the commercial immobilized preparation of the lipase from *Thermomyces lanuginosus*, using as support an ionic-exchange resin (Lipozyme TL-IM). Lipase from *T. lanuginosus* has been chosen because it is among the most used lipases, showing high stability [27]. It was also performed the optimization of the reaction as well as the analysis of the effect of the use of molecular sieves on the reaction performance and the possibilities of the reuse of the biocatalyst.

2. Material and methods

2.1. Materials

Commercial immobilized lipase from *T. lanuginosus* (Lipozyme TL-IM) was used in this work and it was kindly supplied by Novozymes (Spain). Molecular sieves (3 Å, beads 4–8 mesh), butyric acid, ethanol, and other chemicals were of analytical grade and purchased from Sigma–Aldrich (Sigma, St. Louis, USA).

2.2. Ultrasound-assisted esterification

The esterification reactions were carried out in an ultrasonic bath (Unique Inc., model USC 2880A, 40 kHz, 220 W, Brazil). The mixture of butyric acid, ethanol and enzymes were placed in the ultrasonic bath at the desired temperature for different times. The progress of esterification was monitored by determining the residual acid content of the reaction by titration of 0.5 mL samples with NaOH (0.01 M) using phenolphthalein as indicator and 5 mL of ethanol as quenching agent. The amount of ester produced was calculated as being equivalent to the amount of consumed acid.

2.3. Effect of substrates concentrations

In order to evaluate the effect of substrates concentrations on the reaction, butyric acid and ethanol concentrations (maintaining a molecular ratio of 1) were varied from 0.1 to 1.0 M, measuring the initial reaction rate. The mixtures of butyric acid, ethanol, and Lipozyme TL-IM (35%, by substrates mass) were placed in the ultrasonic bath at 40 °C.

2.4. Experimental design

A central composite design (CCD) with two variables, temperature and substrate molar ratio, was carried out in order to obtain the optimal conditions for esterification reaction. The variables and their coded and uncoded values are presented in Table 1, showing the 11 treatments of the two variables, each at five levels. The design was constructed of 4 factorial points, 4 axial points (two axial points on the axis of design variable), and 3 replications at the central point. The mixtures of butyric acid (0.7 M), ethanol, and Lipozyme TL-IM were placed in the ultrasonic bath for 5 h. The biocatalyst content and temperature were varied according to the CCD. In each case, the esterification percentage of conversion (or yield) was determined. The second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the response variable, β_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

2.5. Effect of molecular sieves

The effect of molecular sieves in the reaction was evaluated varying the concentration from 0 to 120 mg of molecular sieves per mmol of butyric acid. The mixtures of butyric acid and ethanol (both at 0.7 M), and Lipozyme TL-IM (35%, by substrates mass) were placed in the ultrasonic bath at 30 °C for 6 h.

2.6. Enzyme reuse

In order to test the possibility of reusing the biocatalyst several times, which is important for the economics of enzymatic reactions, repeated reaction batches were devised using the same preparation. After one esterification reaction, the immobilized enzyme was separated from the reaction medium by vacuum filtration using a sintered glass funnel and reused in a new fresh reaction without any further treatment.

2.7. Statistical analysis

Experiments were performed in triplicates and mean with the standard errors were plotted in the figures. The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, $p(t)$, were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots.

Table 1
Coded levels, real values (in the parenthesis) and results of CCD.

Run	Temperature (°C)	Substrate molar ratio (alcohol:acid)	Yield (%)
1	−1 (34.5)	−1 (1.3:1)	53.7
2	−1 (34.5)	1 (2.7:1)	34.9
3	1 (55.5)	−1 (1.3:1)	18.7
4	1 (55.5)	1 (2.7:1)	13.2
5	−1.41 (30)	0 (2:1)	62.5
6	1.41 (60)	0 (2:1)	15.1
7	0 (45)	−1.41 (1:1)	34.5
8	0 (45)	1.41 (3:1)	36.7
9	0 (45)	0 (2:1)	25.3
10	0 (45)	0 (2:1)	21.7
11	0 (45)	0 (2:1)	24.6