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Ultrasound technology and molecular sieves improve the thermodynamically controlled esterification of butyric acid mediated by immobilized lipase from *Rhizomucor miehei*

Lucas P. Fallavena,[a] Fábio H. F. Antunes,[a] Joana S. Alves,[a] Natalia Paludo,[a] Marco A. Z. Ayub,[a] Roberto Fernandez-Lafuente[b] and Rafael C. Rodrigues*[a]

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In this research, the effects of ultrasound stirring and the addition of molecular sieves on esterification reactions between butyric acid and several alcohols catalyzed by immobilized lipase from *Rhizomucor miehei* (Lipozyme RM-IM) were studied. Among the tested alcohols, 1-propanol and isobutanol allowed the highest activities, whereas Lipozyme RM-IM showed poor activities for esterification using secondary and tertiary alcohols. Different solvents were also tested and *n*-hexane was selected because of its reaction effects, besides being cheaper, available at low boiling point, and ease of recovery. Using the preselected alcohol and solvent, other reaction parameters (butyric acid concentration, temperature, substrate molar rate, and biocatalyst content) were studied to optimize the reaction conditions. Optimal conditions were acid concentration, 0.7 M; substrate molar ratio, 1 : 1 alcohol–acid; temperature 45 °C; biocatalyst content, 14% (by substrate mass). Under these conditions, it was possible to obtain a yield of 86% of butyl butyrate in 2.5 h. When molecular sieves (90 mg $mmol^{-1}$ butytic acid) were added to the reaction, the observed yield increased to 96%. The biocatalyst was used in 5 successive reaction cycles keeping 100% of its initial activity. The overall process productivity was improved 2-fold when compared to the traditional mechanical agitation, showing that ultrasound is a promising technology for application in biocatalysis.

1. Introduction

Esters formed by short chain carboxylic acids and alcohols can be used as flavors or fragrances, considered as natural flavors, with relevant applications in many different industrial sectors.[1] These natural flavors can be extracted from plants, which is characteristically a high-cost and low-yield process. Otherwise, these esters can be obtained through biotechnology process, produced using enzymes or cells, and they can be labeled as natural.[2]

Lipases are among the most used enzymes as catalyst for esterification reactions.[2,3] These enzymes catalyze *in vivo* the hydrolysis of oils and fats, however, because they present a broad specificity, they also catalyze reactions of esterification, transesterification, and acidolysis, being stable in very different reaction media[4] Moreover, there is a great variety of lipases with varying properties. One of the most used lipase, applied in chemical process and for oil and fats modification, is the one produced by the fungus *Rhizomucor miehei*. This enzyme is commercialized in soluble form as Palatase 20000 L and as an immobilized preparation commercially named Lipozyme RM-IM, both supplied by Novozymes.[5,6]

Esterification is a thermodynamically controlled synthesis (TCS), using unmodified acid and alcohol, forcing the reaction conditions to shift the equilibrium in the direction of the synthesis.[7] Esterification reactions are mainly achieved in low water activity systems because water, being a product of the reaction, can shift the equilibrium towards hydrolysis.[8] In this sense, the use of molecular sieves to control the excess of water in the reaction has been reported as a powerful tool to improve the enzyme performance, for example in the synthesis of isopropyl acetate in organic medium and in the synthesis of flavor esters in ionic liquids.[9,10]

Considering the use of immobilized lipases, the adsorption of water or substrates (carboxylic acid and alcohol) on the support is one of the most important reasons of biocatalyst inactivation. This might be solved by suitable washings to eliminate these compounds during successive batch operations.[11] Another important fact to keep the lipase stability and activity is to control the enzyme microenvironment conditions,

[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; Web: http://www.ufrgs.br/bbb. E-mail: rafaelcrodrigues@ufrgs.br; Fax: +55 51 3308 7048; Tel: +55 51 3308 7793*
[b]*Department of Biocatalysis, ICP – CSIC, Campus UAM-CSIC, Cantoblanco, ZC 28049, Madrid, Spain*

avoiding the formation of substrate or products phases in this environment. The use of proper mixing operations and an adequate stirring of the system may be very relevant to have a good performance of the biocatalyst, in most cases performed by traditional mechanical agitation.[9,12–15] More recently, some reports showing the advantages of the use of ultrasound technology as a better mixing strategy for lipase reaction systems appeared in the literature.[16–18] Ultrasound technology has the capability of increasing the interaction between phases by cavitation caused by the collapse of bubbles, and the ultrasonic jet, that disrupts the boundary phase and causes emulsification.[19,20] Furthermore, when ultrasound is applied to an aqueous solution or suspension, increasing in mixing, shearing and mass transfer are observed.[21,22] In spite of these possibilities, ultrasound technology has not been extensively used in biological reactions.[21,23,24]

In this context, this work aimed at studying the application of ultrasound technology during the synthesis of esters from butyric acid and different alcohols in organic solvents using Lipozyme RM-IM as the biocatalyst. Furthermore, it was also evaluated the effect of the main parameters affecting the reaction yield (reaction temperature, biocatalyst content, acid concentration) using a central composite design and the response surface methodology (RSM), which is an effective statistical technique for the investigation of complex processes. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results, and it is a faster and less expensive method for gathering research data than the classical method.[25] It was also investigated the effect of using molecular sieves to improve the operational stability of the biocatalyst in repeated batches.

2. Materials and methods

2.1. Materials

Lipase from *R. miehei* immobilized in Duolite ES 562, a weak anion-exchange resin based on phenol–formaldehyde copolymers (Lipozyme RM-IM) was kindly provided by Novozymes Spain. Molecular sieves (3 Å, beads 4–8 mesh), butyric acid, ethanol, 1-propanol, 2-propanol, 1-butanol, isobutanol, *tert*-butanol, 1-pentanol, and other chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma. St, Louis, USA). Ultrasonic bath (Unique Inc., model USC 2880A, 40 kHz, 220 W, Brazil) with temperature control was used in all experiments.

2.2. Effects of alcohols on the enzyme performance

The different alcohols (ethanol, 1-propanol, 2-propanol, 1-butanol, isobutanol, *tert*-butanol, 1-pentanol) were mixed with butyric acid (0.1 M) at a molar ratio of 1 : 1 in 50 mL Erlenmeyer flasks (working volume of 10 mL), followed by the addition of enzyme (10%, by substrates mass), using *n*-hexane as solvent. The mixtures of butyric acid, alcohol, and Lipozyme RM-IM were placed in an ultrasonic bath (Unique Inc., model USC 2880A, 40 kHz, 220 W, Brazil) for 2 h at 40 °C.

2.3. Effects of organic solvents on the enzyme performance

n-Hexane, cyclohexane, *n*-heptane, and *n*-octane were tested as solvents in the reactions between butyric acid and 1-propanol or isobutanol. The mixtures of butyric acid (0.1 M), alcohol (0.1 M), and Lipozyme RM-IM (10%, by substrates mass) were placed in an ultrasonic bath for 2 h at 40 °C.

2.4. Effects of substrates concentrations on the enzyme performance

For this study, the concentrations of the reaction substrates were varied from 0.1 M to 1.0 M (of each substrate), and measuring the initial reaction rate. The mixtures of butyric acid, isobutanol (always keeping the molar ratio 1 : 1 alcohol–acid), and Lipozyme RM-IM (10%, by substrates mass) were placed in an ultrasonic bath at 40 °C.

2.5. Experimental design

A central composite design (CCD) with two variables (temperature and biocatalyst content) was carried out in order to obtain the optimal conditions for esterification reaction. The variables and their coded and uncoded values as well as the 12 treatments of the two variables, each at five levels are presented in Table 1. The design was constructed of 4 factorial points, 4 axial points (two axial points on the axis of design variable), and 4 replications at the central point. The mixtures of 0.7 M butyric acid, 0.7 M isobutanol, and Lipozyme RM-IM were placed in an ultrasonic bath for 1 h. The biocatalyst content and temperature were varied according to the CCD. In each case, the percentage of conversion for esterification 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 the variables.

Table 1 Coded levels, real values, and the yields of esterification obtained in the CCD

Run	Temperature (°C)	Biocatalyst content (%)	Yield (%)
1	−1 (34.5)	−1 (3.8)	9.88
2	−1 (34.5)	1 (17.2)	61.67
3	1 (55.5)	−1 (3.8)	21.59
4	1 (55.5)	1 (17.2)	59.53
5	−1.41 (30)	0 (10.5)	46.02
6	1.41 (60)	0 (10.5)	66.02
7	0 (45)	−1.41 (1)	0.89
8	0 (45)	1.41 (20)	58.44
9	0 (45)	0 (10.5)	42.34
10	0 (45)	0 (10.5)	46.25
11	0 (45)	0 (10.5)	31.42
12	0 (45)	0 (10.5)	52.93

2.6. Effects of the presence of molecular sieves on the enzyme performance

The effect of molecular sieves on the reaction was evaluated by varying the amounts of sieves from 0 to 120 mg of molecular sieves per mmol of butyric acid. The mixtures of 0.7 M butyric acid, 0.7 M isobutanol, and Lipozyme RM-IM (14%, by substrates mass) were placed in the ultrasonic bath at 45 °C for 2.5 h.

2.7. Operational stability of Lipozyme RM-IM over repeated batches

Lipozyme RM-IM was tested in repeated batches under the optimal reaction conditions defined in our study. After the esterification reaction, the immobilized enzyme was separated from the reaction medium by vacuum filtration using a sintered glass funnel. The recovered biocatalyst was then used in a new batch reaction without any treatment.

2.8. Reaction analysis

The progress of esterification was monitored by determining the residual acid content 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 was calculated as being equivalent to the amount of consumed acid. A calibration curve was constructed to ensure the reliability of this acid determination using laboratory-made mixtures of acid, alcohol, and commercial ester, and confirmed by GC analysis in some samples.

2.9. 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 (2D).

3. Results and discussion

3.1. Effects of alcohols on the enzyme performance

In order to verify the effect of alcohol in the esterification of butyric acid catalyzed by Lipozyme RM-IM under ultrasound energy, 7 different alcohols were tested, and the results are presented in Fig. 1. The enzyme presented good activity in the esterification reactions with most alcohols, except for 2-propanol and *tert*-butanol, secondary and tertiary alcohols, respectively. Lipozyme RM-IM presented a negligible esterification activity with these alcohols. The ester conversion improved from ethanol to 1-propanol, and then decreased with the alcohol chain increase (*n*-butanol and pentanol). A similar behavior was reported by Mahapatra *et al.*,[13] who showed that the yields of *n*-propyl acetate were higher than *n*-butyl acetate in the reaction catalyzed by the lipase from *Rhizopus oligosporus*. Isobutanol allowed a slightly higher activity than *n*-butanol, and lower than the obtained using 1-propanol, suggesting than the short branches has not a relevant effect in the enzyme recognition. These results suggest that the length and the ramification of the aliphatic chain, in the case of secondary and tertiary alcohols, are both important factors for the enzyme activity. From these results, 1-propanol and isobutanol were selected for the next experiments.

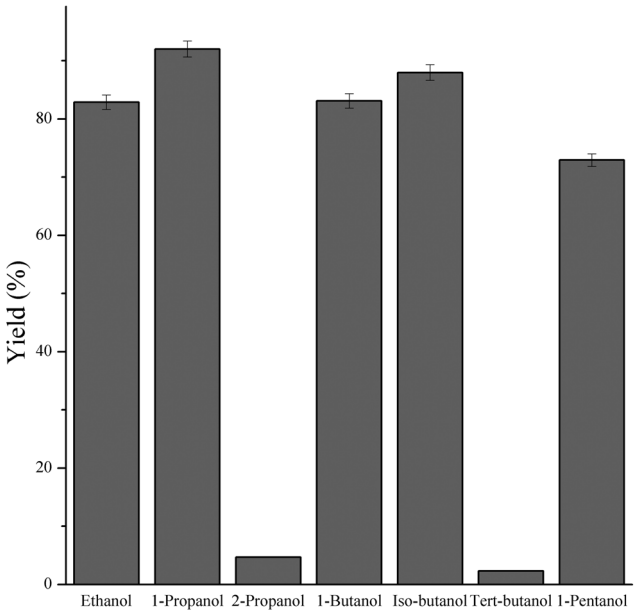

Fig. 1 Effects of the nature of alcohol on the ultrasound-assisted esterification of butyric acid catalyzed by Lipozyme RM-IM. Reaction conditions: butyric acid, 0.1 M; substrate molar ratio, 1 : 1 (alcohol–acid); biocatalyst content, 10% (by substrate mass); 40 °C, 2 h.

3.2. Effects of organic solvents on the enzyme performance

Esterification reactions are usually performed in low water activity medium, and organic solvents, ionic liquids, or supercritical fluids are used for this purpose.[10,26,27] In the case of organic solvents, it is convenient to use the one producing the lowest deleterious effects on enzyme activity and stability. In this work, four different organic solvents were evaluated for the esterification reactions catalyzed by Lipozyme RM-IM under ultrasound energy. The results of esterification yields using each solvent as well as their melting points and log P are presented in Table 2. Good enzyme activities were obtained using anyone of the solvents, for both alcohols, except for cyclohexane when isobutanol was used as substrate. According to Laane *et al.*[28] the higher the log P (log $P > 4.0$), the better the performance of the biocatalysts in organic solvents. Since there were no important differences among the solvents, *n*-hexane was chosen for the following experiments because it is the cheapest and it has a lower boiling point than the other solvents and butyl butyrate (165 °C). Concerning the ester, isobutyl butyrate is more important as flavor ester in food industries than propyl butyrate, thus, it was chosen for the sequence of the work.

Table 2 Effects of the nature of the organic solvents on the ultrasound-assisted synthesis of isobutyl butyrate

Solvent	log *P*	Boiling point (°C)	Yield (%)	
			1-Propanol	Isobutanol
Cyclohexane	3.2	81.0	80.3	57.6
n-Hexane	3.5	69.0	87.7	84.4
n-Heptane	4.0	98.0	85.2	83.4
Isooctane	4.5	99.0	82.7	83.9

3.3. Effects of substrates concentrations on the enzyme performance

In order to assess the effect of butyric acid and isobutyl alcohol on the enzymatic activity, experiments were carried out varying the substrates concentrations from 0.1 to 1.0 M, but keeping the molar ratio of 1 : 1 constant (Fig. 2). An increase in the initial reaction rate was observed up to 0.7 M of substrates, above that remaining constant up to 1.0 M. Comparatively, in a previous study using mechanical agitation,[29] the initial reaction rate increased linearly up to 1.0 M, but was 3 times lower than that obtained in this work. Therefore, ultrasound technology produced much higher yield in shorter reaction times. The maximum substrate concentration of 0.7 M found in this work is higher than that reported in many studies using this enzyme and carboxylic acid,[30–32] and was selected in the following experiments. The possibility of using high butyric acid concentration may be interesting, since it might increase the industrial feasibility of the process.

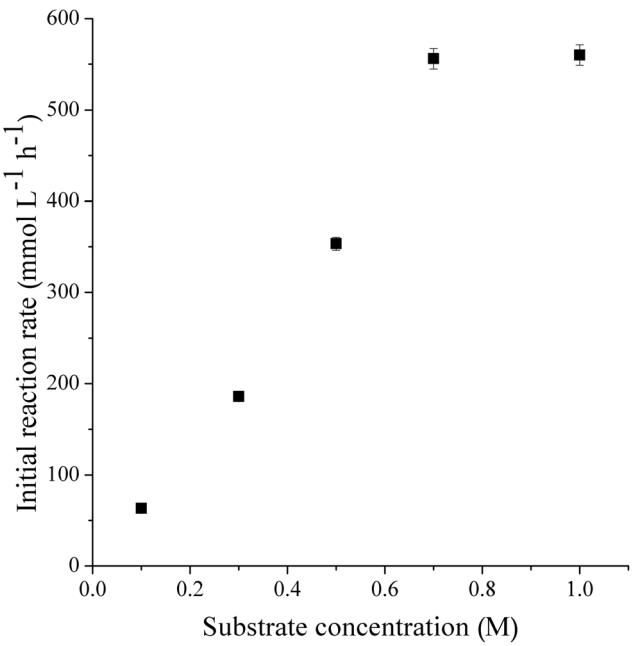

Fig. 2 Effects of butyric acid concentration on the initial reaction rate. Reaction conditions: butyric acid, 0.1 M; substrate molar ratio, 1 : 1 (alcohol–acid); biocatalyst content, 10% (by substrate mass); 40 °C.

3.4. Experimental design and model fitting

After defining the target product, the organic solvent and the substrates concentration, it was performed an experimental design to optimize the temperature and biocatalyst content of the reaction, both important variables in esterification reactions.[33–36] Experimental data obtained for the ultrasound-assisted lipase-catalyzed synthesis of isobutyl butyrate are shown in Table 1. The highest yield of conversion in 1 h was 66% in treatment 6, the highest temperature, while the less effective, with yields lower than 1%, was the treatment 7, the lowest biocatalyst content. In general, using ultrasound energy, most of treatments presented yields of conversion higher than 40% in only 1 h of reaction, much faster than using mechanical agitation. For instance, during the optimization of butyl butyrate catalyzed by Lipozyme RM-IM, the yields were around 60% after 24 h of reaction.[29]

Statistical testing of the model was done using Fisher's statistical test for ANOVA. The computed F value (30.62) was highly significant ($p = 0.0009$). The determination coefficient ($R^2 = 0.9683$) implies that the sample variation of 96.83% for ultrasound-assisted isobutyl butyrate synthesis is attributed to the independent variables and can be explained by the model. The correlation coefficient ($R = 0.9840$) suggests a highly satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation. The experimental data have been adjusted to the proposed model by the second-order polynomial eqn (1). and the second-order polynomial model is presented in eqn (2):

$$Y = 47.19 + 21.42X_2 - 9.98X_2^2 \quad (2)$$

where Y is the percentage yield, X_1 and X_2, are the coded values of biocatalyst content.

3.5. Effect of parameters and optimal conditions

Only biocatalyst content presented a statistically significant effect on the reaction performance (95% confidence level), 42.84, considering the two variables. These effects and their interactions can be better understood observing the contour plot in Fig. 3. The effect for reaction temperature was positive (9.47) but not statistically significant. The biocatalyst content, however, positively affected reaction rate up to 14%, with yields remaining constant above this value. Therefore, we propose the optimal conditions for ultrasound-assisted isobutyl butyrate synthesis catalyzed by Lipozyme RM-IM as temperature of 45 °C and biocatalyst content of 14% (by substrate mass). Under these conditions, the theoretical value for the yield of reaction, predicted by the model, was 55.4%. In order to validate the model, an experiment was carried out under the optimal conditions and the kinetics of ultrasound-assisted isobutyl butyrate synthesis is presented in Fig. 4 (filled squares). After 1 h, the reaction yield was 60.9%, which is close to the theoretical value, showing a good correlation between the model and the experimental data. After 2.5 h, a yield of 86% was reached.

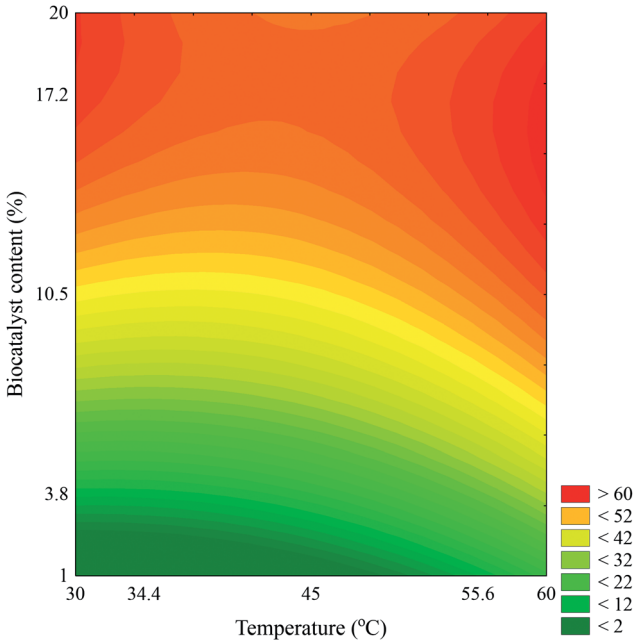

Fig. 3 Contour plots of yields (%) of isobutyl butyrate synthesis as a function of biocatalyst content (%) and temperature (°C). *Reaction conditions*: butyric acid, 0.7 M; substrate molar ratio, 1 : 1 (alcohol–acid), 1 h.

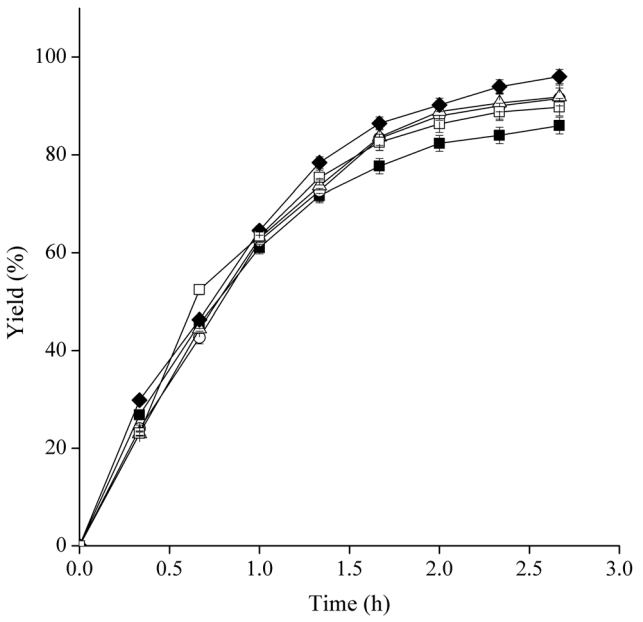

Fig. 4 Effect of the addition of molecular sieves (mg $mmol^{-1}$ butyric acid) in the ultrasound-assisted isobutyl butyrate synthesis catalyzed by Lipozyme RM-IM. (■) 0; (○) 30; (△) 60; (◆) 90; (□) 120 mg $mmol^{-1}$. *Reaction conditions*: butyric acid, 0.7 M; substrate molar ratio, 1 : 1 (alcohol–acid); biocatalyst content, 14% (by substrate mass); 45 °C.

3.6. Effect of molecular sieves on the esterification reaction

In order to improve the equilibrium yield, it was tested the effect of the addition of molecular sieves to the reaction mixture for removing the formed water, therefore shifting the equilibrium towards synthesis, also preventing the loss of enzyme activity caused by the accumulation of water in the enzyme environment.[11] The mass of molecular sieves (3 Å) varied from 0 to 120 mg per mmol of butyric acid, and the results for the reaction yields after 2.5 h are shown in Fig. 4. The yields were progressively improved with the addition of sieves from 86 to 96% up to 90 mg $mmol^{-1}$. The use of 120 mg $mmol^{-1}$ of molecular sieves reduced the observed yields, possibly because the capture of the water molecules necessary to keep the enzyme activity.[7] The positive effect of molecular sieves was also observed by other authors.[9,10,37] In our conditions, this was possible using 90 mg of sieves per mmol of carboxylic acid.

In order to compare and measure the effects of ultrasound and molecular sieves, an experiment was performed under the optimal conditions using traditional mechanical agitation and the ultrasound energy, in the presence and absence of 90 mg of sieves per mmol of carboxylic acid. The results presented in the Fig. 5 show that the use of molecular sieves improved the yield in both agitation methods and, the combined use of ultrasound and molecular sieves improved the yield in almost 1.5-times compared to mechanical agitation without sieves. It is important to remark that the conditions used for mechanical agitation here were different from that previously optimized.[29] In the previous study, lower temperature and biocatalyst content were used and yielded 90% of conversion only after 16 h.

3.7. Operational stability of Lipozyme RM-IM over repeated batches

For a cost-competitive application, immobilized enzymes must be reused several times. The downstream processing and

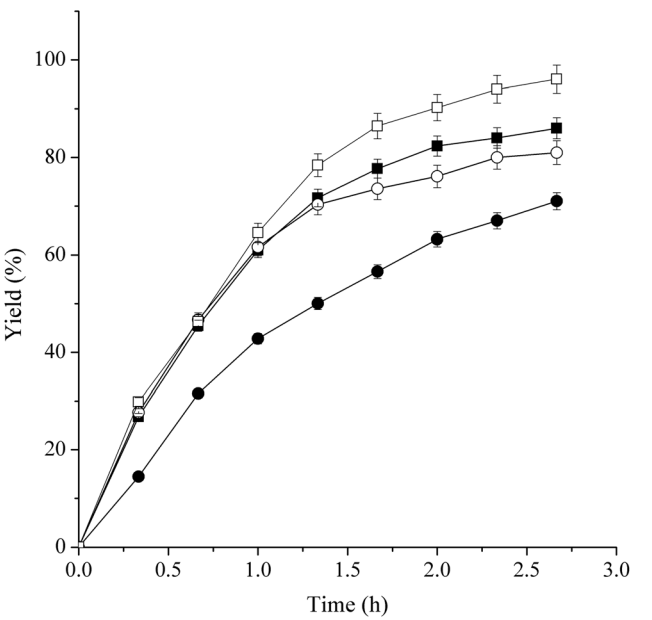

Fig. 5 Comparison of ultrasound and mechanical agitation in the isobutyl butyrate synthesis catalyzed by Lipozyme RM-IM. (●,○) mechanical agitation; (■,□) ultrasound energy. Closed symbols: without molecular sieves; open symbols: addition of 90 mg $mmol^{-1}$ of molecular sieves. *Reaction conditions*: butyric acid, 0.7 M; substrate molar ratio, 1 : 1 (alcohol–acid); biocatalyst content, 14% (by substrate mass); 45 °C.

product purification could be performed by a simple filtration and distillation to obtain a pure product.[38] In order to test the reuse of Lipozyme RM-IM, several batches were run at the optimal conditions, without any washing treatments in between them, and the results are shown in Fig. 6. It was possible to reuse the biocatalyst for 4 cycles keeping 100% of its initial activity, fast loosing activity in the following cycles. Considering that the molecular sieves were also reused, the result may be a consequence of the saturation of the molecular sieves with released water. In the synthesis of butyl butyrate using mechanical agitation, the recovery of activity was only possible by performing an *n*-hexane washing between every batch.[29] Even performing this washing, enzyme stability decreased since the first reuse, which did not happen using ultrasound energy. For industrial purposes, the elimination of this unit operation would be very important for reducing costs. Moreover, the process productivity was also improved using the ultrasound. Under mechanical agitation, a conversion yield of 96% was reached after 20 h, using 1.0 M butyric acid, which represents a productivity of 50 mmol L^{-1} h^{-1}.[29] In contrast, under ultrasound energy the conversion yield of 96% was reached in 2.5 h. using 0.7 M butyric acid, which represents a productivity of 250 mmol L^{-1} h^{-1}, 5-fold higher. If we compare both agitation methods under the same conditions, such as presented in Fig. 5, the overall productivity is 2-times higher using ultrasound and molecular sieves than using mechanical agitation. A similar effect was observed for the synthesis of butyl acetate catalyzed by Novozym 435, where reaction productivity and enzyme stability were improved by the use of ultrasound technology.[18]

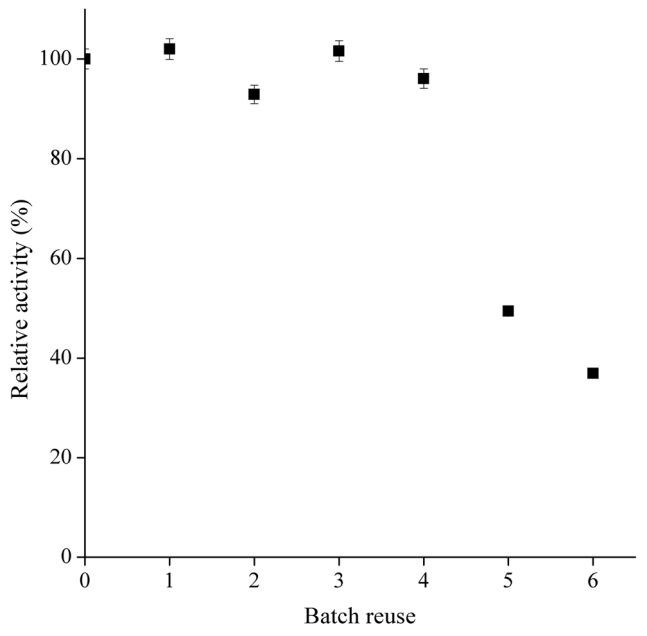

Fig. 6 Operational stability of Lipozyme RM-IM in repeated batches of ultrasound-assisted isobutyl butyrate synthesis. *Reaction conditions*: butyric acid, 0.7 M; substrate molar ratio, 1 : 1 (alcohol–acid); biocatalyst content, 14% (by substrate mass); 45 °C; molecular sieves, 90 mg $mmol^{-1}$ butyric acid.

Ultrasound mechanism is based on the high-energy waves that creates cavitation in the liquid solution, reducing the diffusion mass limitations of substrates and products in the porous matrix of enzyme supports.[19] Another advantage of using ultrasound is that it requires only 30 to 50% of the energy that is consumed during mechanical agitation.[20]

4. Conclusions

Ultrasound technology has shown to be very useful to improve the productivity of the esterification process catalyzed by Lipozyme RM-IM. Under the tested conditions, this biocatalyst exhibited good esterification activity using different primary alcohols. In addition, the use of molecular sieves, to capture the released water, increased the observed yield after 2.5 h from 86 to 96%. Moreover, the combined use of ultrasound technology and molecular sieves allowed improving the enzyme stability. Furthermore, the process productivity was improved by two factors: washing steps were avoided and enzyme stability was higher.

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References

1 R. G. Berger, *Biotechnol. Lett.*, 2009, **31**, 1651–1659.
2 K. P. Dhake, D. D. Thakare and B. M. Bhanage, *Flavour Fragrance J.*, 2013, **28**, 71–83.
3 S. Nagarajan, *Appl. Biochem. Biotechnol.*, 2012, **168**, 1163–1196.
4 U. T. Bornscheuer, *OCL: Oleagineux, Corps Gras, Lipides*, 2013, **20**, 45–49.
5 R. C. Rodrigues and R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2010, **64**, 1–22.
6 R. C. Rodrigues and R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2010, **66**, 15–32.
7 A. L. Paiva, V. M. Balcão and F. X. Malcata, *Enzyme Microb. Technol.*, 2000, **27**, 187–204.
8 S. Hari Krishna and N. G. Karanth, *Catal. Rev. Sci. Eng.*, 2002, **44**, 499–591.
9 M. L. Verma, W. Azmi and S. S. Kanwar, *Enzyme Res.*, 2011, **2011**, 919386.
10 P. Lozano, J. M. Bernal and A. Navarro, *Green Chem.*, 2012, **14**, 3026–3033.
11 A. B. Martins, N. G. Graebin, A. S. G. Lorenzoni, R. Fernandez-Lafuente, M. A. Z. Ayub and R. C. Rodrigues, *Process Biochem.*, 2011, **46**, 2311–2316.

12 N. G. Graebin, A. B. Martins, A. S. G. Lorenzoni, C. Garcia-Galan, R. Fernandez-Lafuente, M. A. Z. Ayub and R. C. Rodrigues, *Biotechnol. Prog.*, 2012, **28**, 406–412.
13 P. Mahapatra, A. Kumari, V. Kumar Garlapati, R. Banerjee and A. Nag, *J. Mol. Catal. B: Enzym.*, 2009, **60**, 57–63.
14 A. B. Martins, J. L. R. Friedrich, J. C. Cavalheiro, C. Garcia-Galan, O. Barbosa, M. A. Z. Ayub, R. Fernandez-Lafuente and R. C. Rodrigues, *Bioresour. Technol.*, 2013, **134**, 417–422.
15 J. Sun, J. H. Chin, W. Zhou, B. Yu, P. Curran and S. Q. Liu, *J. Am. Oil Chem. Soc.*, 2012, **89**, 1991–1998.
16 K. M. Gonçalves, F. K. Sutili, S. G. F. Leite, R. O. M. A. De Souza and I. C. R. Leal, *Ultrason. Sonochem.*, 2012, **19**, 232–236.
17 R. Konwarh, S. Pramanik, D. Kalita, C. L. Mahanta and N. Karak, *Ultrason. Sonochem.*, 2012, **19**, 292–299.
18 A. B. Martins, M. F. Schein, J. L. R. Friedrich, R. Fernandez-Lafuente, M. A. Z. Ayub and R. C. Rodrigues, *Ultrason. Sonochem.*, 2013, **20**, 1155–1160.
19 H. Fazlena, S. Norsuraya and S. N. Nadiah, *Ultrasonic assisted enzymatic reaction: An overview on ultrasonic mechanism and stability-activity of the enzyme*, Langkawi, 2013.
20 V. B. Veljkovic, J. M. Avramovic and O. S. Stamenkovic, *Renewable Sustainable Energy Rev.*, 2012, **16**, 1193–1209.
21 B. Kwiatkowska, J. Bennett, J. Akunna, G. M. Walker and D. H. Bremner, *Biotechnol. Adv.*, 2011, **29**, 768–780.
22 M. M. Zheng, L. Wang, F. H. Huang, L. Dong, P. M. Guo, Q. C. Deng, W. L. Li and C. Zheng, *Ultrason. Sonochem.*, 2012, **19**, 1015–1020.
23 J. V. Sinisterra, *Ultrasonics*, 1992, **30**, 180–185.
24 L. Batistella, L. A. Lerin, P. Brugnerotto, A. J. Danielli, C. M. Trentin, A. Popiolski, H. Treichel, J. V. Oliveira and D. De Oliveira, *Ultrason. Sonochem.*, 2012, **19**, 452–458.
25 P. Xu, Z.-Y. Ding, Z. Qian, C.-X. Zhao and K.-C. Zhang, *Enzyme Microb. Technol.*, 2008, **42**, 325–331.
26 M. D. R. Diaz, J. M. Gómez, B. Díaz-Suelto and A. García-Sanz, *Eng. Life Sci.*, 2010, **10**, 171–176.
27 G. E. Jeromin and A. Zoor, *Biotechnol. Lett.*, 2008, **30**, 925–928.
28 C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, 1987, **30**, 81–87.
29 A. S. G. Lorenzoni, N. G. Graebin, A. B. Martins, R. Fernandez-Lafuente, M. A. Záchia Ayub and R. C. Rodrigues, *Flavour Fragrance J.*, 2012, **27**, 196–200.
30 G. Bayramoglu, B. Karagoz, B. Altintas, M. Yakup Arica and N. Bicak, *Bioprocess Biosyst. Eng.*, 2011, **34**, 735–746.
31 D. Bezbradica, D. Mijin, S. Siler-Marinkovic and Z. Knezevic, *J. Mol. Catal. B: Enzym.*, 2007, **45**, 97–101.
32 A. V. de Paula, G. F. M. Nunes, J. D. L. Silva, H. F. De Castro and J. C. Dos Santos, *Appl. Biochem. Biotechnol.*, 2010, **160**, 1146–1156.
33 S. W. Chang, J. F. Shaw and C. J. Shieh, *Food Technol. Biotechnol.*, 2003, **41**, 237–242.
34 H. C. Chen, Y. K. Twu, C. M. J. Chang, Y. C. Liu and C. J. Shieh, *Ind. Crops Prod.*, 2010, **32**, 522–526.
35 G. A. Macedo, G. M. Pastore and M. I. Rodrigues, *Process Biochem.*, 2004, **39**, 687–692.
36 J. C. Santos and H. F. De Castro, *World J. Microbiol. Biotechnol.*, 2006, **22**, 1007–1011.
37 H. Horchani, A. Bouaziz, Y. Gargouri and A. Sayari, *J. Mol. Catal. B: Enzym.*, 2012, **75**, 35–42.
38 L. Gubicza, A. Kabiri-Badr, E. Keoves and K. Belafi-Bako, *J. Biotechnol.*, 2000, **84**, 193–196.