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FULL PAPER

Thermal and mechanical stability of immobilized *Candida antarctica* Lipase B: an approximation to mechanochemical energetics in enzyme catalysis.

Mario Pérez-Venegas,*[a] Miriam M. Tellez-Cruz,[a] Omar Solorza-Feria,[a] Agustín López-Munguía,[b] Edmundo Castillo,*[b] Eusebio Juaristi,*[a, c]

Abstract: Very recently, several successful enzymatic processes carried out with mechanical activation have been disclosed; that is, despite the mechanical stress caused by High-Speed Ball-Milling, immobilized enzymes can retain activity. In the present study, the effect of thermal and mechanical stress was examined as potential inducers of enzymatic denaturation, when using either free, immobilized, or ground immobilized enzyme. The recorded observations show a remarkable stability of ground immobilized enzyme. Moreover, ground biocatalyst turns out to exhibit an increase of one order of magnitude in the efficiency of the catalytic process, maintaining excellent enantiodiscrimination, without significant activity loss even after four milling cycles. These observations rule out enzyme inactivation as direct consequence of the milling process. Additionally, boosted enzyme efficiency was used to optimize a relatively inefficient chiral amine resolution reaction, achieving a 25% faster biotransformation (in 45 min) and yielding essentially enantiopure products (*ee* > 99%, *E* > 500).

Introduction

Enzymes are potent biocatalysts that are able to carry out numerous chemical transformations with remarkable chemo-, regio- and stereo-selectivity.[1-5] The intrinsic sustainability of enzymatic processes along with the use of green reaction conditions such as absence of potentially toxic solvents, and activation with non-conventional sources of energy has expanded the use of biocatalysis in both chemical industries and in academic institutions, focusing in drug discovery and in the synthesis of molecules with relevant biological activity.[6-8] Recent developments in protein engineering and enzyme directed evolution,[9, 10] together with the availability of efficient methods for the production of recombinant molecules, have led to rapidly expanding new fields in biocatalysis.[11, 12] Nevertheless, there are still several major issues to overcome in order to generalize the use of enzymes on an industrial scale, especially in the area of organic synthesis. Salient obstacles include the normally high cost and limited commercial availability of enzymes. In some cases this is directly related to a limited industrial production of bioactive pure proteins.[13] Furthermore, the recent developments in enzyme engineering[9, 10] and in novel methods for protein production and purification have contributed to increase the availability of biocatalysts at more affordable prices.[11, 12] Additional limitations in the use of biocatalysts in organic synthesis come from severe operational requirements of enzymatic processes such as the use of non-aqueous solvent media and prevention of high temperatures in order to avoid denaturation of the protein and, as a consequence, a reduction of reaction efficiency.[14-16]

Indeed, biocatalyst inactivation provoked by harsh chemical reaction conditions that contrast with the mild natural media of enzymes,[16] is the most common challenge when sensible proteins are used in synthetic chemistry. Normally, enzyme inactivation arises from partial or complete protein unfolding, that is physical loss of the native structure. This process could lead to changes in the tertiary or quaternary structure of the enzyme's active site that hamper the predicted bioconversion.[17] In this regard, several attempts to stabilize the enzymes have been described.[18] These efforts include the use of non-conventional reaction media,[19, 20] appropriate chemical modification of the enzyme,[21] and the design of biocatalysts through enzyme immobilization on inert supports, which offer substantial benefits when compared with the use of free enzymes in solution.[22] The easy handling and convenient recovery, reduced protein contamination as well as compatibility with continuous flow procedures have intensified the application of immobilized biocatalysts, especially those produced with broadly used enzymes in industry such as isomerases and lipases.[23, 24]

In this context, *Candida antarctica* Lipase B (CALB, commercially available as Novozym 435® [N435]) is a widely used enzyme, supported on acrylic resin, that has exhibit multiple pharmaceutical applications.[25] Particularly relevant is the extensive use of CALB to promote highly efficient bioconversions using non-aqueous media at relatively high temperatures.[26] Furthermore, CALB as N435 has shown remarkable stability and recyclability under rough chemical conditions.[27, 28]

[a] M. Pérez-Venegas, M. M. Tellez-Cruz, O. Solorza-Feria and E. Juaristi
Department of Chemistry
Centro de Investigación y de Estudios Avanzados
Av. IPN 2508, 07360 Ciudad de México, Mexico
E-mail: mperezv@cinvestav.mx; juaristi@relaq.mx
[b] A. López-Munguía, E. Castillo
Department of cellular engineering and biocatalysis
Universidad Nacional Autónoma de México
Av. Universidad 2001, Col. Chamilpa, 62210 Cuernavaca, Morelos, Mexico
E-mail: edmundo@ibt.unam.mx
[c] E. Juaristi
El Colegio Nacional
Luis Gonzáles Obregón 23, Centro Histórico, 06020 Ciudad de México, Mexico.
E-mail: juaristi@relaq.mx

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Recently, as part of our interest in the development of more sustainable synthetic methodologies,[29-32] and considering previous reports using proteins mechanically activated through the High-Speed Ball-Milling technique,[33, 34] we were able to carry out various stereoselective biotransformations.[35, 36] Gratifyingly, CALB partially retains activity after the harsh mechanochemical conditions provoked by the collisions inside the milling-jar, and proved to be stable enough to carry out its catalytic function, affording highly enantioenriched products.[35, 36] In this regard, the stability of enzymes under mechanical stress has been confirmed using continuous grinding processes or mechanochemical activation followed by static incubation (*RAging*) with excellent results.[37, 38] Nevertheless, no detailed information concerning the effect of milling on the degree of denaturation, the parameters and conditions affecting enzyme efficiency, or changes in the structure of CALB have been disclosed.

On the other hand, besides the gradual protein denaturation reported in experiments carried out at high temperatures or aggressive reaction conditions,[35, 36] one might also anticipate loss of the enzyme due to leakage from the commercial support, that could arise from the stressful mechanical methods employed in the ball milling process. Nevertheless, the studies reported so far using CALB together with mechanical force do not identify explicitly the possible denaturation effect of milling on enzymatic reactivity. This makes difficult to assign the loss in activity to either mechanical, thermal, or alternative inactivation mechanisms. Additionally, leaking of the enzyme from the supporting matrix could modify the protein's activity. Considering eventual physical changes of the biocatalyst exposed to the ball-mill, the assays were also performed with previously ground N435, aiming to understand the inactivation mechanism of CALB on both N435 and in the ground biocatalyst.

Results and discussion

The overall objective of the present work was to investigate potential inactivation mechanisms of N435 under grinding conditions in a ball mill. In this regard, it was deemed essential to understand the corresponding inactivation process on soluble (free) CALB enzyme in order to determine the consequences of immobilization on CALB stability, and afterwards, the consequences that milling might have on the performance of the biocatalyst. Additionally, considering current theories formulated for heat distribution in mechanochemical methods (i.e., the hot spot theory),[39] it was decided to confirm or discard thermally induced denaturation as a source of enzyme inactivation.

Free CALB (Lipozyme, Novozymes A/S Denmark) activity was assayed by means of a kinetic protocol using *p*-nitrophenyl butyrate as substrate, in order to establish the appropriate conditions to carry out the experimental measurements of thermal stability (for extensive detailed information see Experimental Section and Supporting Information [SI], Section 1), which were then conducted at temperatures above the optimal stable range of CALB. As shown in **Figure 1**, when free CALB was incubated at 65 °C, a typical first order decay was observed, the residual activity rapidly decreasing during the first part of the essay. Nevertheless, it is apparent that inactivation of the enzyme significantly slowed down at longer periods of time at high temperature exposure, probably due to an apparent stabilizing effect of CALB forms (labile [$_L$] and resistant [$_R$]). It is also likely that, aggregation of the free enzyme at high temperatures may also contributed to the loss of activity, indicating a most complex inactivation mechanism. A similar behavior was also observed when CALB was incubated at 75 °C and 85 °C (**Figure 1**).

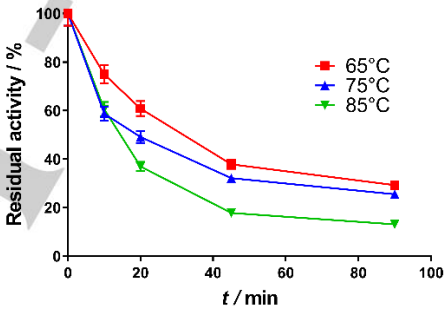


Figure 1. Residual activity of free CALB incubated at 65°C, 75°C and 85°C, showing a typical first order decay.

Complementary thermal inactivation studies were then carried out using N435 and previously ground N435, following a similar protocol than for the free CALB (see SI, Sections 2 and 3). Nevertheless, a key observation was made. The initial reaction rate of ground N435 substantially increased, reaching the highest value (up to one order of magnitude) when N435 was ground for 90 minutes (**Figure 2**). The increase of enzyme activity due to the milling process may be associated to diffusional effects in N435 that are overcome when the particle size of the biocatalyst is reduced. This leads to a higher effective concentration of available enzyme that increases the efficiency factor of the enzymatic reaction by approximately one order of magnitude. Thus, a thermal inactivation study was carried out using immobilized CALB; we found that N435 also exhibits a complex thermal inactivation mechanism following the same two-stage pattern exhibit by the free CALB that might be also affected by the diffusional effects present in the immobilized CALB (**Figure 3**).

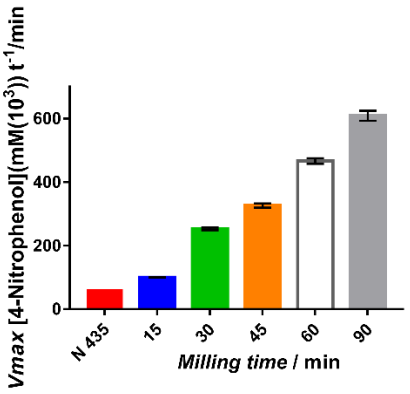


Figure 2. Enzyme activity as function of milling time [0.1mg of N435 or ground N435 / mL], using agate milling-jar and ball. Bar at 90 minutes of milling (Grey) shows the increase in activity of one order of magnitude due to the milling process

Nevertheless, it should be noted that CALB in N435 is more stable than in its free form as demonstrated by the higher residual activities found in the 65 °C to 75 °C temperature range (compare **Figure 3** with **Figure 1**). Indeed, thermal stability presented by the immobilized biocatalyst can be clearly appreciated at moderate temperatures (60 °C and 65 °C). It can also be observed that thermal stability provided by the enzyme's support becomes insufficient when the experiment is carried out at higher temperatures, as shown in the inactivation curve at 85 °C in **Figure 3**. This observation suggests that an abrupt inactivation of immobilized enzyme can take place upon exposure to high temperatures.

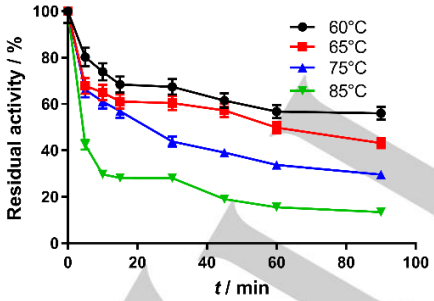


Figure 3. Thermal inactivation assay of N435 [0.1mg of N435 / mL] at 60°C, 65°C, 75°C and 85°C, showing a typical first order decay.

Once the pattern of thermal inactivation on both free and in N435 was established, we proceeded to evaluate the enzyme's inactivation using the previously ground biocatalyst, knowing that diffusional limitations (**Figure 2**) may interfere in the measurement of the observed denaturation rate. Thus, N435 was ground in an agate milling-jar using one agate ball (For additional details see the Experimental Section). Residual activity of the ground supported biocatalyst (**Figure 4**) shows an apparently higher stability of the enzyme relative to the previous studies carried out employing free CALB and N435 (except for experiments carried out at 85°C), suggesting a high stability of N435 to the harsh conditions of the mechanical ball milling procedure, and indicating that the difference in particle size and the concomitant increase in enzyme activity due to the grinding process resulted in an apparent higher thermal stability of CALB in ground N435.

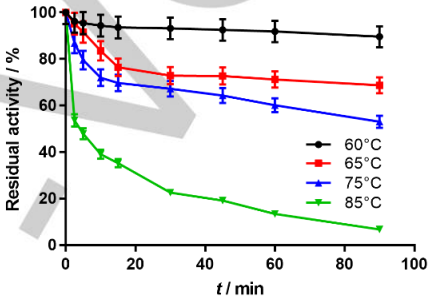


Figure 4. Thermal inactivation assay of previously ground N435 [0.1mg of ground N435 / mL] at 60°C, 65°C, 75°C and 85°C, showing a typical first order decay.

Moreover, potential leaking of CALB from the milled support was evaluated; nevertheless, no leaked protein was detected. Thus, it can be pointed out that the enzyme´s inactivation can be induced by thermal energy as indicated in **Figures 1**, **3** and **4**; however, immobilization provides substantial resistance to the harmful thermal effects; additionally, enzyme stability seems not to be affected by the mechanical process as indicated in previous reports.[33]

Ground biocatalyst demonstrates an important diffusional limitation of N435 for this reaction which is demonstrated by the increase in the observed initial reaction rate. Indeed, it appears that mechanical stress overcomes this limitation and increases the biocatalyst efficiency. As it can be appreciated in **Figure 2**, the apparent enzyme activity of the ground N435 milled for 90 minutes is one order of magnitude higher than the apparent enzyme activity of N435 (compare the red and grey bars in **Figure 2**). In this regard, grinding apparently leads to an increase in the apparent activity of N435 (0.1mg N435/mL) from 7.4 IU/mL to 98.4 IU/mL on the previously ground biocatalyst (0.1mg of ground N435 / mL).

The recorded progressive increase in enzyme activity and in the apparent available enzyme associated to the milling process are in line with observations (higher enzyme activity) made in previous mechanoenzymatic protocols, in which the

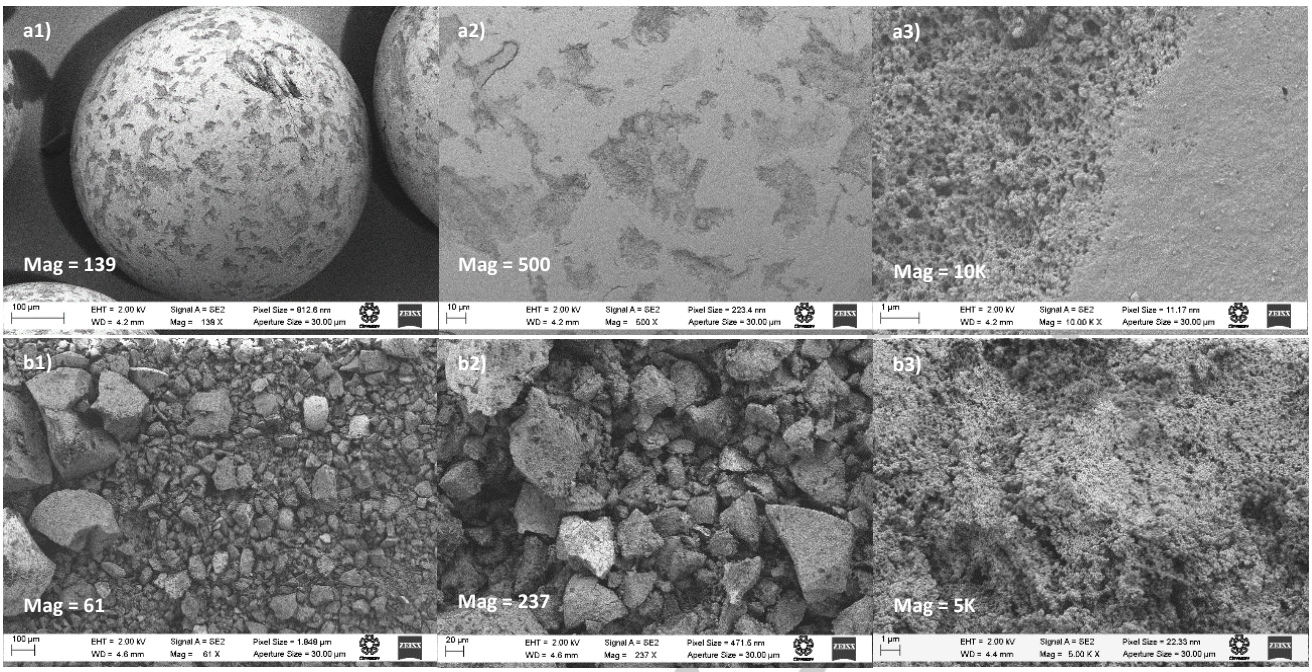


Figure 5. SEM images of commercial supported (**a**, Novozym 435) and ground CALB (**b**, 10 Hz, 90 min), showing the irregular distribution of channels in N435 [a2)]. This irregular distribution, as well as variations in particle size, decrease as the biocatalyst is ground [b3)]. A complete gallery of SEM images is presented in SI, Section 4.

biocatalytic reaction was faster relative to procedures using conventional heat and stirring conditions.[35-36, 38] In fact, this explains the apparent higher stability showed by the previously ground biocatalyst (**Figure 3**) when compared with N435, as the activity of the un-milled biocatalyst is controlled by a low diffusional rate. In this regard, it appears that the increase of available enzyme may be the consequence of a decrease in the diffusional barrier due to the reduction of N435 particle size (**Figure 2**) as a result of mechanical beating.[40]

To gather information that could shed light on this proposal, a Brunauer-Emmett-Teller (BET) analysis of the total specific area and pore volume was carried out. As expected, no considerable increase in specific surface area was found after the milling process ($81 m^2 g^{-1}$ and $83 m^2 g^{-1}$ for N435 and ground N435 respectively, see SI, Section 4). However, it is likely that the reduction in particle size (from 600 µm in N435 to 0.5 µm in the ground biocatalyst) induced by the milling process is sufficient to explain the higher activity observed in the treated biocatalyst. This phenomenon clearly facilitates the substrate diffusion and in consequence increases the apparent activity of the N435 after the grinding process.

Additionally, N435 and the previously ground biocatalyst were examined through scanning electron microscopy (SEM) as shown in **Figure 5**. N435 is constituted by 330 µm to 580 µm diameter spheres (**Figure 5a1**, see also SI, Section 4) with a rather polished continuous surface where no channels are observed (**Figure 5a3**)

In contrast, the ground N435 shows a distribution of different sizes and shapes after the mechanical process (**Figure 5b1**) varying from 0.5 µm to 20 µm diameter, in the most homogenous milling process, presenting a higher channel distribution (**Figure 5b3**). These observations demonstrate that for this particular reaction system diffusional limitations control the observed reaction rate in N435 as also found in previous reports. [40] Nevertheless, this limitation may be overcome with the ground biocatalyst.

On the other hand, even when **Figures 1**, **3**, and **4** present similarities, an appropriate evaluation of several inactivation parameters such as the inactivation energy (E_a), the time required to reduce the activity of the biocatalyst by 10% (D), and the temperature needed to diminish D in one logarithmic unit (z) for both labile ($_L$) and resistant ($_R$) forms of the free CALB, N435 and ground N435 was necessary for a better understanding of this process, especially in the CALB and ground N435. The above parameters were calculated employing the equations for nonlinear Arrhenius behavior and are summarized in **Table 1**. (For details of the calculations see SI, Section 5).[41, 42]

The dependence of the enzymatic stability with respect to the temperature of the reaction is shown in **Table 1** (*cf*. decrease

Table 1. Inactivation parameters for free, Novozym 435® and ground Novozym 435®.

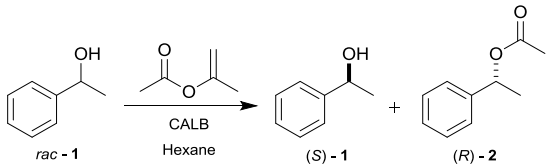
T (°C)	k_R (1/sec)	k_L (1/sec)	E_{aR} (Kcal/mol)	E_{aL} (Kcal/mol)	D_R-value (min)	D_L-value (min)	z_R (°C)	z_L (°C)
				Free CALB				
65	0.414±0.014	0.063±0.000	-11.6±0.3	4.46±0.18	5.555	36.380	47.72±2.5	119.87±6.5
75	0.258±0.048	0.078±0.003			8.920	28.403		
85	0.158±0.048	0.092±0.004			14.580	25.125		
				Novozym 435®				
60	0.517±0.048	0.517±0.014	6.68±0.36	2.75±0.09	1.932	1.623	31.64±2.6	113.63±3.3
65	0.630±0.035	0.630±0.016			1.588	0.870		
75	0.862±0.117	0.862±0.132			1.160	0.723		
85	1.046±0.114	1.046±0.186			0.955	0.692		
				Ground Novozym 435®				
60	0.138±0.014	0.244±0.020	19.25±1.22	18.33±0.12	7.249	4.104	4.11±0.3	6.72±0.6
65	0.217±0.037	0.268±0.027			4.609	3.730		
75	0.486±0.043	0.633±0.036			2.056	1.580		
85	1.058±0.208	1.567±0.274			0.945	0.640		

k_R and k_L = rate constant for the resistant and labile component in CALB. E_a = activation energy. D = time required to decreased 10% the enzyme's activity. z = temperature needed to diminish D in one logarithmic unit

in D value, expect for free enzyme). Additionally, high thermal stability (high z value) and exceptional resilience to temperature changes (low E_a value) confirm the high stability for N435 in contrast with the data for free CALB. On the other hand, the thermal parameters of the previously ground biocatalyst (**Table 1**) suggest a sensitizing process that could arise from the grinding procedure that gives rise to amorphous biocatalyst particles.

Thermal parameters of N435 and previously ground N435 can be used to estimate the relative thermal stability of the biocatalyst in the two different presentations. Nevertheless, relevant experimental parameters, such as the actual temperature during the mechanochemical process, as well as relative biocatalyst enantioselectivity, which would provide complementary information about the extent of enzyme inactivation due to mechanical energy, could not be evaluated in the present thermal study.

Indeed, temperature analysis during mechanochemical reactions as well as the energy transfer mechanisms from the milling-jar and balls to the reactants are cutting-edge topics in the study of mechanochemical transformations.[43-45] In this context, the enantiopreference of a biocatalyst (E) in an enzymatic resolution is a temperature dependent process.[46] Thus, an adequate assessment of a stereoselective reaction carried out under conventional heating and stirring conditions *vis-a-vis* mechanical activation could lead to an estimation of the approximate temperature range for the process and could help to clarify the extend of inactivation due to mechanical stress. In this regard, we examined the efficiency of a kinetic resolution reaction employing α-methylbenzyl alcohol as racemic secondary alcohol and both, Novozym 435 and the ground biocatalyst (**Scheme 1**).



Scheme 1. Enzymatic kinetic resolution of α-methylbenzyl alcohol.

In particular, the extent of conversion and enantioselectivity under ball milling settings at various reaction temperatures

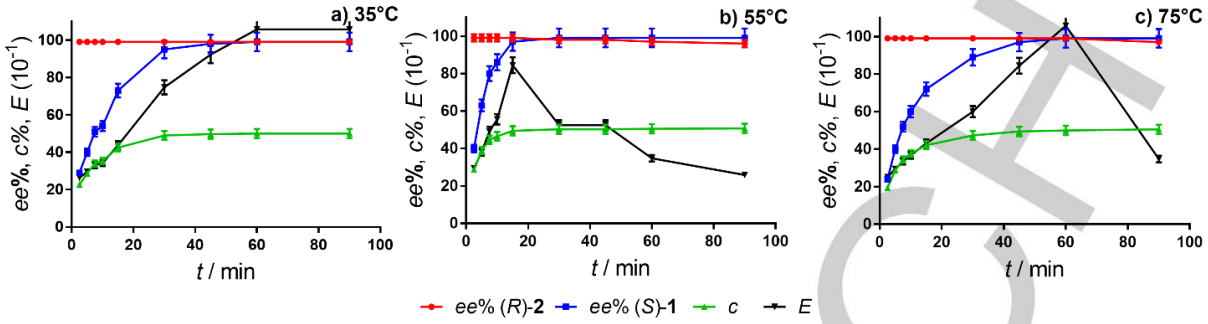


Figure 6. Enzyme kinetics of N435 for the resolution of α-methylbenzyl alcohol under conventional heat and stirring conditions.

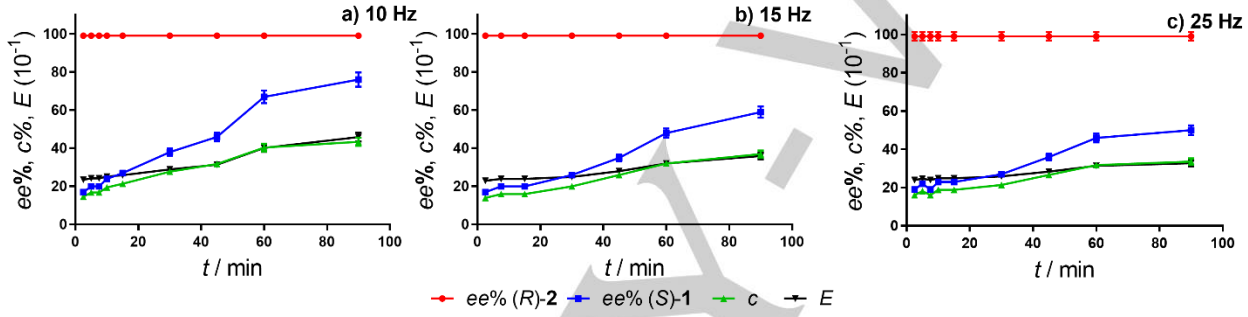


Figure 7. Enzyme kinetics of N435 for the resolution of α-methylbenzyl alcohol under mechanical activation.

was estimated. To this end, using conventional heat and stirring conditions in hexane solution,[47] the enzymatic kinetic resolution using N435 proceeded with high enantioselectivity and enantiopreference (**Figure 6a**). It is noteworthy that, a slight decrease in enantiomeric excess (as little as 1%) provokes a rapid decease in enantiopreference (*E*) as indicated in **Figure 6b** and **Figure 6c**. A similar behavior was observed with previously ground N435 in the kinetic resolution process under thermal activation (see SI, Section 6).

With the aim to compare the energetics of the process using conventional heat and stirring conditions with the process under mechanochemical activation, a similar protocol was carried out in a ball mill, employing agate components (milling-jars and balls) and N435, evaluating the effect of milling frequency in the process. Surprisingly, the kinetic resolution proceeds smoothly, even at high frequencies (**Figure 7c**), reaching a high *E* value (*E* >> 200) at long reaction times (90 min). High enantiomeric excess for the main product (*R*)-**2** (*ee* > 99%) as well as the gradual increase of enantiopurity of the non-transformed alcohol (*S*)-**1**, the extent of conversion (*c*) and the high *E* value (enantiopreference) along the mechanoenzymatic procedure, indicate the high stability of N435 exposed to the ball milling process, this is in accord to the aforementioned low effect of milling over the biocatalyst´s stability. Finally, ground biocatalyst was evaluated along with mechanical activation in the mechanoenzymatic procedure. No significant differences were observed relative to the same process started with non-milled N435 (see SI, Section 6), confirming the null effect of milling over the stability of the enzyme.

The increasing enantiopreference observed during the enzymatic resolution of the α-methylbenzyl alcohol under mechanical activation (**Figure 7c**) at high frequencies and using conventional heat and stirring conditions (**Figure 6a**), indicates that the transferred kinetic energy in the ball milling procedure is necessarily lower than the energy provided by the system using thermal activation at 35°C. Therefore, comparing the kinetics in **Figure 6** and **7**, we can conclude that the temperature inside the ball milling reactor is similar to the thermal energy provided by the reaction under conventional heat and stirring conditions between 25°C (room temperature) and 35°C, when agate components were used. With the aim to obtain a more efficient mechanoenzymatic biocatalytic process, several reactor and balls materials were used, in order to evaluate the effect of the material of the milling-jar employed in the process.

Surprisingly, when Teflon® milling-jar and ball were applied in the mechanoenzymatic procedure a faster and complete resolution was reached after 30 min of reaction without loss in enantiopreference (**Figure 8**), even at long mechanical exposure times (90 min). This is in contrast with processes using thermal activation (see SI, Section 6)[48] in which the enantiopreference diminished at long time exposures. Moreover, to the best of our knowledge, this mechanoenzymatic resolution using Teflon® components is the most enantioselective and fastest resolution reported so far.[33]

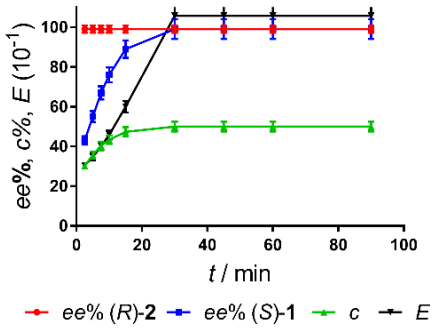


Figure 8. Enzyme kinetics of N435 under mechanochemical conditions using Teflon® milling-jar and ball, showing a swift (30 min) and enantioselective (*ee* > 99%, *E* >> 500) process.

Therefore, the kinetic resolution under thermal activation at several temperatures in the range of 35°C to 85°C were used to calculate the change in *Gibbs* free energy ($\Delta\Delta G^{\ddagger}$) for the process using N435 and ground biocatalyst. In this regard, Eyring transition state theory was applied by considering the rate constants of the enzymatic process to derive a modified equation (**eq. 1**) implicating a temperature dependence of the system throughout the kinetic resolution process.[46]

$$\ln E = -(\Delta\Delta G^{\ddagger}/RT) \ldots (\mathbf{eq.\ 1})$$

In this equation *R* is the ideal gas constant and $\Delta\Delta G^{\ddagger}$ corresponds to the Gibbs free energy difference for the activation of the two enantiomers. Such dependence on reaction temperature can be evaluated by means of a plot of *R*ln*E* *vs* 1/T that for the kinetic resolution under thermal activation after 90 minutes of incubation allowed the estimation of the Gibbs free energy (**Figure 9**).

For the kinetic resolution using N435 and ground N435 (GN435), $\Delta\Delta G^{\ddagger}_{N435}$ = 8.41 ± 0.79 kcal/mol and $\Delta\Delta G^{\ddagger}_{GN435}$ = 12.20 ± 0.91 kcal/mol, respectively. These results indicate a rather large energy difference, and consequently a substantial preference for the transitions state involving the (*R*)-isomer of the alcohol when ground N435 was used. This result agrees with previous observations and supported the positive effect of milling over the biocatalyst,[49, 50] moreover, helps to understand the high enantiopreference during the mechanoenzymatic process (**Figure 8**).

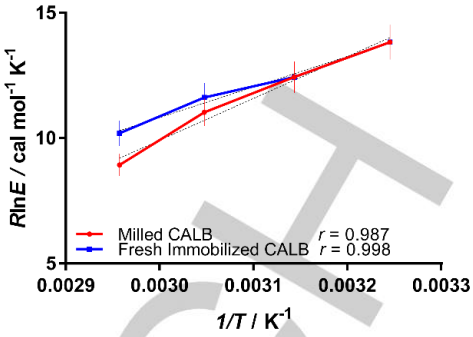


Figure 9. Eyring equation plot for the resolution of α-Methylbenzyl alcohol used to estimate the $\Delta\Delta G^{\ddagger}$ for both process.

The results obtained so far (*vide supra*) demonstrate the high stability of the N435 to milling conditions. However, as pointed out in previous reports,[35-36] sequential milling cycles usually reduce the activity and the enantiopreference of mechanoenzymatic reactions. To examine this potential drawback, commercial immobilized CALB was milled up to four times in an agate reactor as indicate in the Experimental section, and the resulting fine powder was tested in the mechanoenzymatic resolution of α-methylbenzyl alcohol (**Scheme 1**).

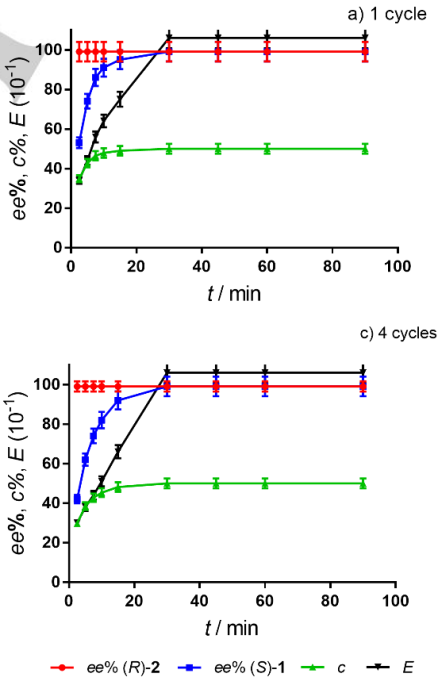


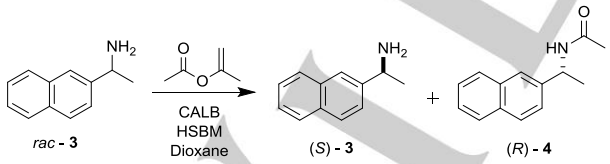
Figure 10. Enzyme kinetics for the resolution of α-Methylbenzyl alcohol after 4 grinding cycles, indicating the insignificant effect of milling on enzyme stability.

No significant differences (P << 0.05) in the kinetics were detected after each catalytic cycle (**Figure 10**, and see SI, Section 9).

Enzyme kinetics clearly indicate no damage in the structure of the biocatalyst as a consequence of mechanical stress, at least at moderate frequencies (10 Hz to 25 Hz), short

reaction times (up to 90 minutes) and material hardness. That is, decreased enzyme activity and loss in enantiopreference after the milling process in N435, must be associated to a different kind of inactivation, probably caused by contact with organic solvents (alcohols and ketones) during enzyme recovery following the mechanoenzymatic process. Indeed, enzyme inactivation due to contact with organic solvents has been well established.[51-53] Moreover, calculations also suggest a loss in the secondary structure of CALB due to the use of organic solvents,[49] which can perturb the enzyme's active site. With the aim to study the effect of mechanical force in other lipases, *Candida antarctica* lipase A (CALA) immobilized on immobead 150, and soluble porcine pancreatic lipase type II (PPL) were used in the kinetic resolution process under the mechanochemical conditions described in the general procedure. In the event, CALA shows a decrease in enantioselectivity and enzyme activity after the mechanochemical process, indicating a denaturation process that is not detected when N435 is used. This result might be the consequence of the different support matrix. Interestingly, while PPL shows no activity in hexane solution, when the reaction is carried out under mechanochemical conditions a significant but not enantioselective conversion to the acylated product was detected, indicating that the mechanical force can induce the desired bioconversion (see SI, Section 10). This observation paves the road for future studies of biocatalytic processes under mechanochemical conditions.

Finally, the finding of an increased efficiency of ground N435 as a result of the milling process can be used to improve inefficient enzymatic organic reactions. In this regard we decided to re-examine a well-known slow enzymatic procedure used in the resolution of racemic amines using ground biocatalyst (**Scheme 2**).[36] In this regard, CALB shows an extraordinary preference for the acylation of the (*R*)-enantiomer of racemic amine **3**, even at high temperatures (%$ee_{R,80\,°C}$ = 98%);[36] nevertheless, this kinetic resolution process is much slower than the corresponding alcohol deracemization.



Scheme 2. Enzyme kinetics resolution of racemic chiral amine *rac*-**3**.

The positive effect in the use or ground supported CALB was evidenced when comparing the conversion rate for the mechanochemical procedure using Novozym 435 in solution, achieving ca. 25% faster biocatalysis when ground supported lipase was used in the resolution process (see SI, Section 11).

Additionally, enantiopreference during resolution of *rac*-**3** using ground biocatalyst is substantially higher than the corresponding process using N435 under mechanical activation or conventional heat and stirring conditions (E_{GN435} >> 500, E_{N435} = 595 and $E_{80\,°C}$ = 317), leading to highly enantiopure compounds, higher than 99% for both (*S*)-**3** and (*R*)-**4**), and corroborating the aforementioned increase in $\Delta\Delta G^{\ddagger}$ when ground N435 is used (**Figure 9**).

Conclusions

The potential effect of mechanochemical activation on the efficiency of biocatalytic processes was evaluated. In particular, enzyme kinetics for commercial immobilized and previously ground N435 exhibited a very high activity of the biocatalyst during the milling process with no visible detrimental effect in enzyme activity due to mechanical stress as indicated by the high *E* value achieved in the mechanoenzymatic resolutions. The calculated thermal parameters also confirm a high mechanical stability for Novozym 435®. The observed increase of one order of magnitude in the enzyme activity in water as a result of the milling process can be used to accelerate slow enzymatic organic reactions as illustrated by the mechanoenzymatic resolution of racemic amines. The finding of more efficient enzymatic reactions under mechanochemical conditions can be explained in terms of a significant decreased of diffusional effects in the N435 that are eliminated when the supported material is broken, inducing a high value of apparent available enzyme of the biocatalyst without loss in enantiodiscrimination or stability. The enhanced activity of ground N435 is also accompanied by a more enantiopreferent process as determined from the kinetics of the resolution of α-methylbenzyl alcohol using ground biocatalyst, affording a high enantiopure process (*ee* > 99%, *E* >> 500) even over extended periods of time. This is in contrast with the same process under conventional heat and stirring conditions. Accordingly, previous reports of enzymatic inactivation under milling conditions cannot be ascribed to the mechanical process itself and must be reexamined considering the specific reaction in each case.

Experimental Section

^{1}H and ^{13}C spectra were recorded on a BRUKER DP300 (300MHz). High resolution mass spectra were recorded on a HPLC 1100 coupled to a MSD-TOF Agilent series HR-MSTOF model 1969 A. Chromatograms were acquired in a Dionex HPLC Ultimate 3000 with a UV/VIS detector, with a diode array, at 210 and 254 nm. High-Speed Ball-Milling reactions were carried out in a Retsch, Mixer Mill (MM200). Enzyme activity was measured in a Biotek Synergy H4 Hybrid Reader. All reagents were purchased from Sigma-Aldrich (Merck) and used as received. Free CALB was purchased from Novozymes, Lipozyme. Immobilized CALB was purchased from

Novozymes, Novozym 435® (Immobilized on acrylic resin, IU/g > 10000), *Candida antarctica* lipase A (CALA) immobilized on immobead 150 was purchased from Sigma-Aldrich (Merck) and soluble porcine pancreatic lipase type II was purchased from Sigma-Aldrich (Merck). For all the activity analysis, a 200 mM solution of *p*-nitrophenyl butyrate in 2-methyl-2-butanol was used as a substrate. Specific surface area of the immobilized and milled immobilized CALB was measured using Brunauer-Emmett-Teller (BET) analysis (Gemini equipment II-2370, Micrometrics). Samples were degassed at room temperature overnight in vacuum to prevent denaturation.

General method for the measurement of free enzyme activity.

The substrate (230 µL, see Experimental section) was placed in a 96 well-plate previously incubated at 45 °C (average stable temperature), before the addition of 20 µL of the free enzyme (as received or diluted with Tris buffer [10mM Tris HCl, pH 7, 2% Triton]). The plate progress was monitored at 405 nm in a Synergy H4 Hybrid reader for 20 minutes with readings in short time periods. Data were collected and plotted in order to determine the maximum conversion rate.

General method for the measurement of free CALB thermal inactivation.

Free enzyme (or diluted enzyme [10 mM Tris HCl, pH 7, 2% Triton]) was placed in a 1.5 mL Eppendorf tube and incubated in a Thermomixer at 300 rpm at various temperatures. Enzyme samples of 20 µL were taken at different periods of time. Enzyme activity was examined according to the *General method for the measurement of free enzyme activity*.

General method for the measurement of Novozym 435® and ground Novozym 435® thermal inactivation.

To a series of Eppendorf tubes of 1.5 mL containing 300 µg of N435 or ground N435 (Agate milling-jar and ball, 10 Hz for 1.5 hours) was added 1.4 mL of Tris buffer (10 mM Tris HCl, pH 7, 2% Triton) and the resulting mixture was incubated in a Thermomixer at 300 rpm at different temperatures. At the end of each incubation time, the content of the Eppendorf tube was transferred to a well of a 6 well-plate and treated with 1.6 mL of cold buffer. The plate was incubated at 45 °C for 5 minutes in a Synergy H4 Hybrid reader and then 20 µL of substrate was added. The plate was monitored at 405 nm for 20 minutes with short interval readings. Data were collected and plotted in order to determine the maximum rate of conversion.

General method for the kinetic resolution of α-methylbenzyl alcohol in solution.

To a solution of 500 µL of α-Methylbenzyl alcohol, hexane (10 mL) and isopropenyl acetate (450 µL), previously incubated at the desired temperature was added 300 mg of commercial supported CALB. Samples of 40 µL were taken in the initial time and after 2.5, 5, 7.5, 10, 15, 30, 45, 60 and 90 min, and were diluted with 1.5 mL of hexane HPLC grade. The resulting solution was filtered and analyzed by HPLC equipped with a chiral column. Both products (enantioenriched alcohol and acylated product) were analyzed simultaneously in an AD-H chiral column (hexane: IPA 95:5, Flow 0.5 mL/min, runtime 25 min). Enantiomeric excess (*ee*), conversion ($c = \%ee_s/(\%ee_s+\%ee_p)$) and enantiopreference ($E = \ln[1-c(1+\%ee_p)]/\ln[1-c(1-\%ee_p)]$) were calculated from the integration of peaks for each product (alcohol (*R*) 16.59 min, (*S*) 20.29 min, ester (*R*) 8.84 min, (*S*) 9.30 min). The peak for isopropenyl acetate appears at 6.57 min.

General method for the kinetic resolution of α-methylbenzyl alcohol under mechanical activation.

To a ball mill equipped with one agate milling-jar (12 mm of diameter, 4.6 mL capacity) and the corresponding ball (6 mm of diameter, 480 mg of weight); or stainless-steel milling-jar (15 mm of diameter, 4.6 mL of capacity) and the corresponding ball (8 mm of diameter, 1.5 g of weight); or Teflon® milling-jar (10 mm of diameter, 6.5 mL of capacity) and the corresponding ball (8 mm of diameter, 1.2 g of weight) were added 50 µL of α-Methylbenzyl alcohol, hexane (0.2 mL, HPLC grade), 41 µL of isopropenyl acetate, and 30 mg of commercial supported CALB. Reaction samples were collected at different times (2.5, 5, 7.5, 10, 15, 30, 45, 60 and 90 min), diluted with 1.3 mL of hexane HPLC grade, filtered and analysed by HPLC equipped with a chiral column (AD-H, hexane: IPA 95:5, Flow 0.5 mL/min, runtime 25 min).

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Conflicts of interest

There are no conflicts to declare.

Keywords: Mechanoenzymatic • Biocatalyst • CALB • Mechanostability • Sustainable

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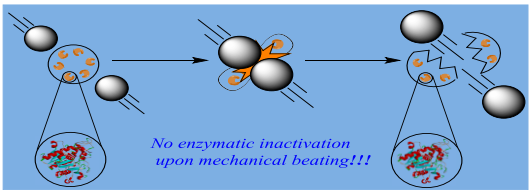

For the first time, the effect of grinding over enzyme activity and stability was systematically studied. The results indicate a high enzyme stability to the mechanochemical conditions and, unexpectedly, and increment in enzyme activity of one order of magnitude after the mechanochemical process without loss in enantiopreference of the enzyme.

M. Pérez-Venegas, M. M. Tellez-Cruz, O. Solorza-Feria, A. López-Munguía, E. Castillo,* E. Juaristi.**

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Thermal and mechanical stability of immobilized *Candida antarctica* Lipase B: an approximation to mechanochemical energetics in enzyme catalysis.