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Stability improvement of immobilized *Candida antarctica* lipase B in an organic medium under microwave radiation

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The influence of microwave heating on the stability of immobilized *Candida antarctica* lipase B was studied at 100 °C in an organic medium. The microwave radiation was carried out before enzymatic reaction (storage conditions) or during the enzymatic catalysis (use conditions). In both cases, enzymatic stability was higher under microwave heating than under conventional thermal heating, in strictly identical operating conditions. Furthermore, the gain of enzymatic stability under microwave heating appears to be higher in a more polar solvent, which interacts strongly with the microwave field. Our results suggest that microwave radiation has an effect, not related to temperature, on the process of enzymatic inactivation.

Introduction

Microwave radiation as an energy source to heat is today widely used in organic chemistry. Indeed, an electromagnetic field of high frequency (2.45 GHz) induces molecular rotation of dipolar species, which is accompanied by intermolecular friction and subsequent dissipation of energy by heating in the core. Reductions in reaction times, enhancements in conversions and sometimes in selectivity have been reported concerning especially solvent-free reactions conducted under a microwave field.[1a–c]

In enzymatic synthesis, the use of microwave radiation remains limited. Literature on this subject is still poor and often controversial. First studies carried out in aqueous solutions with various enzymes did not demonstrate any effect of microwave field on enzymatic activity and stability.[2,3] More recently, still in an aqueous medium, it was reported that inactivation of a pectin methylesterase was faster in the microwave heating mode than in the conventional thermal heating mode, suggesting the presence of nonthermal effects under microwave radiation.[4] Similar results were obtained with thermophilic enzymes, on which microwave radiation has induced protein structural rearrangements not related to temperature.[5] On the other hand, kinetic and selectivity improvements of enzymatic reactions carried out under a microwave field in organic media have been reported,[6–9] but effects of microwave radiation on enzyme stability in non-conventional media have not been yet investigated. The aim of the present work was to study the stability of the Novozym®435 (immobilized preparation of the *Candida antarctica* lipase B widely used in enzymatic synthesis in non-conventional media) under microwave heating compared to conventional heating.

The model reaction chosen to test enzymatic activity after incubation at high temperature (100 °C) was an alcoholysis between ethyl butyrate and butanol, conducted in equimolar conditions (11/11 mmol) and without solvent addition (Scheme 1). In these conditions, the hydrolysis reaction (corresponding to butyric acid appearance) was so negligible (<0.5%) that butyl butyrate synthesis might be used to follow Novozym®435 activity.

Effects of microwave radiation on Novozym®435 stability have been studied in an organic medium when radiation was carried out before the reaction (storage conditions) or during the enzymatic catalysis (use conditions). The results obtained were compared with data observed under conventional thermal heating in strictly identical operating conditions.

Results

Kinetics of the butyl butyrate synthesis

In order to determine the kinetics of the alcoholysis model reaction, typical butyl butyrate syntheses were performed by adding different amounts of immobilized lipase to an equimolar mixture of substrates (ethyl butyrate/butanol, 11/11 mmol) at 100 °C. Experiments were carried out under conventional and microwave heating to detect a possible effect of the mode of heating on the reaction kinetic. Experiments were carried out in similar vessels and without internal agitation to be sure that differences observed were only consequent upon heating type.

The amount of butyl butyrate obtained after 15 min of reaction with various quantities of Novozym®435 is shown in Fig. 1. As expected, butyl butyrate production increases with the amount of enzyme. The linear behaviour for small quanti-

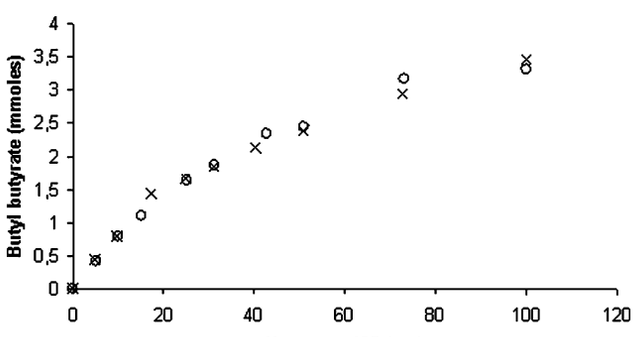
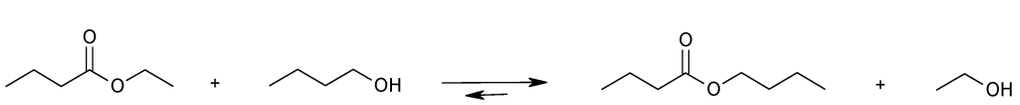


Fig. 1 Effect of the amount of Novozym®435 used for the butyl butyrate synthesis (○, conventional heating; ×, microwave heating).



Scheme 1 Scheme of the model reaction.

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ties of enzyme confirms that diffusion at 100 °C was sufficient to obtain reproducible experiments without internal agitation. For all immobilized lipase quantities tested, the same initial rate was observed under conventional and microwave heating (in our conditions, Novozym®435 has an activity of 6.3 μmol min^{-1} mg^{-1} for both heating modes).

Butyl butyrate synthesis was conducted until equilibrium with 10 mg (limiting quantity, linear part of the curve Fig. 1) or 25 mg of Novozym®435 under conventional and microwave heating to determine the equilibrium constant of the reaction (Fig. 2). For both enzyme quantities tested, the same equilibrium constant (K_{eq} = 2.25) was obtained under conventional and microwave heating.

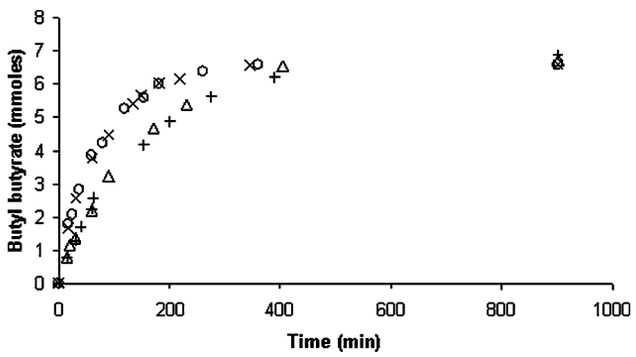

Fig. 2 Reaction kinetics obtained under microwave heating compared to that provided under conventional heating (○, conventional heating, 25 mg Novozym®435; ×, microwave heating, 25 mg Novozym®435; Δ, conventional heating, 10 mg Novozym®435; +, microwave heating, 10 mg Novozym®435).

Butyl butyrate synthesis was conducted in the same conditions with substrates and biocatalyst of which the thermodynamic activity of water was fixed at 0 before reaction. Identical enzymatic activity and equilibrium constant were found again, for both heating types.

These results show that in our conditions, the heating mode has no effect on the initial rate and conversion rate of the reaction.

Enzymatic stability in storage conditions

In order to study the real impact of the mode of heating on the enzymatic stability, 25 mg of Novozym®435 were preincubated in butanol or ethyl butyrate at 100 °C for various times, under conventional or microwave heating. Residual enzymatic activities obtained after preincubation are shown in Fig. 3. Under conventional heating, as expected in an organic medium,[10] enzymatic stability was found to be higher in ethyl butyrate, which is less polar than butanol: after 30 min of preincubation at 100 °C in ethyl butyrate, the enzyme maintains 39% of its activity against 13% when it was preincubated in butanol. Under microwave heating, different behaviour was observed: after 30 min of preincubation in butanol or ethyl butyrate, the same residual activity (45%) was obtained. So, Novozym®435 appears to be more stable under microwave heating than under conventional heating in both organic substrates. These results show a difference between conventional and microwave heating, modifying the enzymatic inactivation process. Contrary to stability studies previously cited, the temperature rise, which is normally different according to heating type, has no influence in our results. Indeed, in our experiments, the temperature of the preincubation medium was fixed at 100 °C before rapidly adding the biocatalyst.

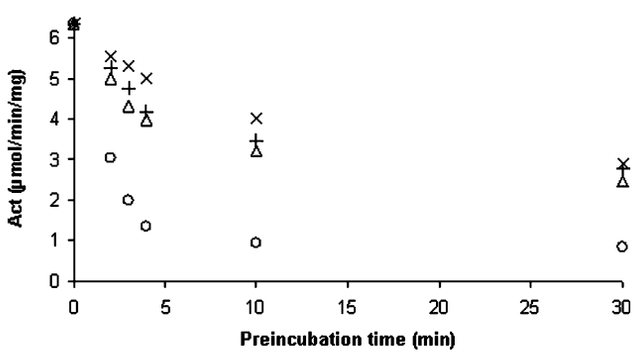

Fig. 3 Residual activity after preincubation in organic substrates (○, preincubation in butanol, conventional heating; Δ, preincubation in ethyl butyrate, conventional heating; ×, preincubation in butanol, microwave heating; +, preincubation in ethyl butyrate, microwave heating).

Control tests were also conducted to observe how Novozym®435 may react when it was irradiated alone, without organic substrate. In these conditions, the biocatalyst weakly interacts with the microwave field: after 30 min radiation at 300 W, the temperature of the immobilized lipase did not exceed 80 °C and only 20% of the enzymatic activity was lost (under conventional heating, Novozym®435 loses 17% of its activity after 30 min of incubation at 100 °C without solvent).

Enzymatic inactivation kinetics in storage conditions

The enzyme deactivation constant (k_d) has been calculated from the slopes of the best-fit curves obtained by linear regression when log ($A/A_0 \times 100$) was plotted against preincubation time (Fig. 4), where A was residual enzymatic activity obtained after preincubation and A_0 was enzymatic activity obtained without preincubation. The deactivation of Novozym®435 in butanol or ethyl butyrate obeyed first-order deactivation kinetics, for both heating modes, according to an irreversible deactivation mechanism in one step.

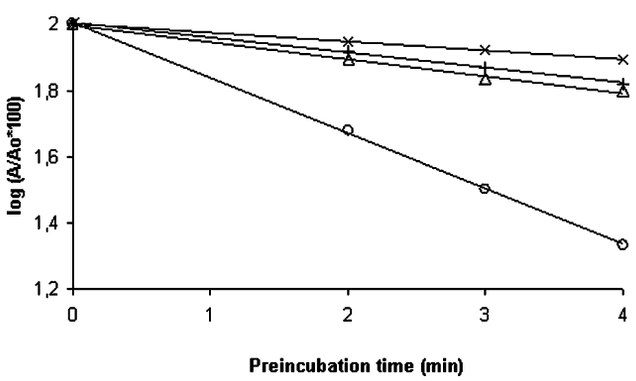

Fig. 4 Linear regression curves obtained from logarithmic representation of residual activity *versus* preincubation time (○, preincubation in butanol, conventional heating; Δ, preincubation in ethyl butyrate, conventional heating; ×, preincubation in butanol, microwave heating; +, preincubation in ethyl butyrate, microwave heating).

In addition (Table 1), we have calculated from the k_d, the half-life time ($t_{1/2}$) and the free energy of activation (ΔG_d) for the deactivation process. The half-life time was the time required for 50% loss of enzyme activity whereas ΔG_d was calculated from the following equation:

$$\Delta G_d = -RT \ln [k_d h / k_B T]$$

where k_d is the deactivation constant (h^{-1}), k_B Boltzmann's constant (1.38 × 10^{-23} J K^{-1}), h Planck's constant (1.84 × 10^{-37}), R the gas constant (8.314 J mol^{-1} K^{-1}) and T the temperature (K).

As shown in Table 1, the deactivation constant (k_d) in butanol is 6 times higher under conventional heating than under microwave heating, whereas the k_d in ethyl butyrate is nearly the same for both heating types. Similar results were obtained when a_w was not fixed at 0 before the experiment.

Table 1 Kinetic and thermodynamic parameters of Novozym®435 inactivation in storage conditions in organic substrates at 100 °C

Storage substrate	a_w	Heating mode	k_d (h^{-1})	$t_{1/2}$ (h)	ΔG_d (kJ mol^{-1})
Butanol	0	Conventional	10.04	0.030	110.29
Butanol	0	Microwave	1.59	0.189	116.00
Ethyl butyrate	0	Conventional	3.10	0.097	113.93
Ethyl butyrate	0	Microwave	2.71	0.111	114.35
Butanol	no fixed	Conventional	10.21	0.029	110.23
Butanol	no fixed	Microwave	3.14	0.096	113.89
Ethyl butyrate	no fixed	Conventional	2.36	0.128	114.78
Ethyl butyrate	no fixed	Microwave	2.36	0.128	114.78

Enzymatic inactivation under microwave heating is faster in the less polar substrate, ethyl butyrate. This result is in contrast with data obtained under conventional heating.

Enzymatic stability in use conditions

Biocatalysts are generally more stable in use than in storage conditions, because substrate and product locations and interactions in the catalytic site stabilize the native enzymatic conformation. This could explain why no effect of heating mode has been observed when a typical synthesis was conducted at 100 °C without enzymatic preincubation in an organic medium (Fig. 2). To confirm this hypothesis, 25 mg of Novozym®435 were re-used for six successive runs carried out under conventional or microwave heating. The initial rate and conversion rate at 6 h obtained for each run and each heating mode are shown Figs. 5 and 6.

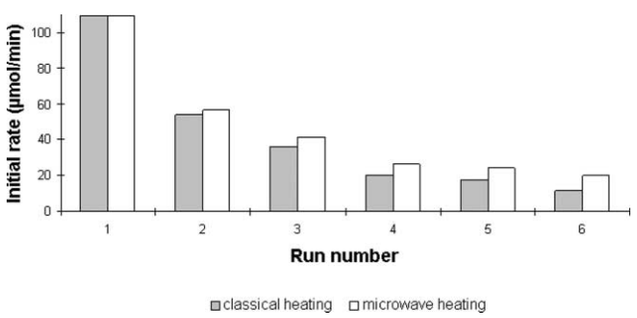

Fig. 5 Evolution of the initial rate with the number of biocatalyst re-uses.

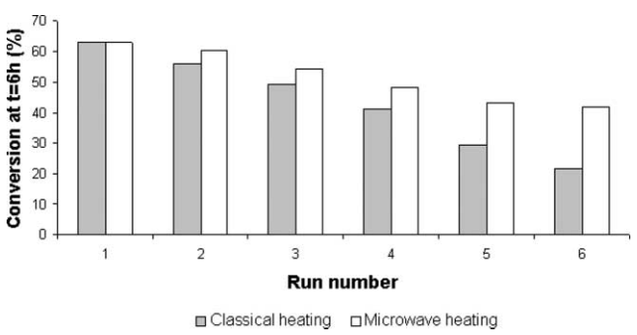

Fig. 6 Evolution of the conversion rate at $t = 6$ h with the number of biocatalyst re-uses.

For both heating types, the initial rate of the butyl butyrate synthesis decreases progressively when the number of runs increases (Fig. 5). As soon as the biocatalyst was re-used, the initial rate was higher under microwave heating compared to conventional heating. After 6 runs, a factor of 2 was reached between the remaining activity of the irradiated and conventionally heated biocatalyst. Concerning the conversion rate after 6 h of reaction, the same phenomenon was observed (Fig. 6). From the first re-use of Novozym®435, the thermodynamic equilibrium of the reaction was no longer reached for either heating type, but the conversion rate was even more important under microwave heating, until reaching a factor of 2 after 6 runs.

Discussion

At high temperature, enzymatic inactivation (thermodenaturation) consists of the loss of the catalytically active conformation. The stability of this active conformation is closely dependent on interactions between enzyme and its microenvironment. Indeed, different non-covalent forces such as hydrogen bonds, hydrophobic, ionic and Van der Waals interactions maintain enzymatic structure. At high temperature, these forces are reduced and enzyme molecules unfold. All the processes causing thermal inactivation of enzymes require water.[11,12] So, in an organic medium, where hydrophobic interactions are strongest, enzyme structural rigidity is increased, resulting in greatly enhanced thermal stability.[13] Consequently, enzymatic stability depends on the nature of the solvent and is higher in hydrophobic than in hydrophilic solvents.[10]

In our study, typical behaviour has been observed concerning Novozym®435 stability under conventional heating: enzyme deactivation constant (k_d) is more than 3 times higher in butanol than in ethyl butyrate which is more apolar.

On the other hand, surprising behaviour has been observed under microwave radiation: the enzyme deactivation constant is 1.7 times higher in ethyl butyrate than in butanol although the same residual enzymatic activity was obtained in both cases after 30 min preincubation at 100 °C.

Furthermore, Novozym®435 stability was enhanced under microwave heating compared to conventional heating, especially in the more polar solvent butanol: k_d is more than 6 times smaller, leading to a residual enzymatic activity 3 times greater after 30 min of preincubation at 100 °C. Under microwave heating, the polarity of solvent is an important parameter: the more polar the solvent is, the greater its ability to couple with the microwave energy.[1a,b,c] Thus, a 24 W power is enough in butanol to maintain the temperature at 100 °C although 210 W are required in ethyl butyrate.

The reasons for the increased Novozym®435 stability under microwave radiation are still unclear. Our results suggest that the microwave field changes the interactions between the enzyme and its microenvironment, preventing the enzyme thermodenaturation.

Conclusions

The ability to re-use a biocatalyst is a decisive parameter for the economic viability of a biocatalytic process. In this study, we have shown that the stability of immobilized *Candida antarctica* lipase in organic media could be enhanced by using microwave dielectric heating rather than conventional thermal heating. Furthermore, for the first time, the influence of microwave radiation on enzymatic stability in an organic medium has been studied and the existence of an effect of microwave field on the enzymatic inactivation process has been highlighted. The increase of enzymatic stability under a microwave field could explain enhancements in conversion rate observed for some enzymatic syntheses carried out under microwave heating. Enzymatic behaviour under microwave radiation is actually studied with various biocatalysts and solvents.

Experimental

Enzymatic and chemical materials

Candida antarctica lipase B immobilized on macro-porous polyacrylic resin beads (Novozym®435), water content 2%, activity 7000 PLU g^{-1} was procured from Novozymes A/S (Bagsvaerd, Denmark). Ethyl butyrate, butyl butyrate, butyric acid were purchased from Sigma Chemical Co. (USA). Butanol, ethanol and acetonitrile were obtained from Carlo Erba Reagenti (Italy). All substrates and solvents were of the highest purity (99% minimum).

Microwave equipment

Reactions were performed in a Synthewave S402 microwave oven (300 W monomode system; Prolabo, France) equipped with a variable speed rotation system and an infrared temperature detector. The temperature of the reaction mixture was controlled using an algorithm, which allows the temperature to be set at a given value by varying the power between 20 and 200 W to operate under the electromagnetic field over the reaction.[14,15]

Gas chromatographic analysis

The GC analysis was performed with a Hewlett Packard model 5890A instrument equipped with flame ionization detector (FID) and an OV 01 fused silica capillary column (Chrompack, France). The split ratio was 68/1.2. Injector and detector were kept at 220 and 250 °C respectively. Carrier gas was nitrogen and the flow rate in the column was 1.2 ml min^{-1}. Hydrogen and air were supplied to the FID at 45 and 350 ml min^{-1} respectively.

For the separation of the substrates and products, diluted in acetonitrile, the column temperature was programmed to increase from 60 to 190 °C, at 15 °C min^{-1}.

Typical butyl butyrate synthesis

Substrates, ethyl butyrate and butanol, were placed in equimolar amounts (11/11 mmol) in a 10 ml quartz vessel and heated to 100 °C in an oil bath (conventional thermal heating) or in the microwave oven (50–55 W). Novozym®435 (5–40 mg) was then rapidly added to the substrates solution and the mixture was maintained at 100 °C for 24 h. Periodically, 10 µl of the reaction medium were withdrawn, diluted in 400 µl acetonitrile and then analysed by GC.

When indicated in the text, the thermodynamic activity of water (a_w) was beforehand fixed at 0 by drying the substrate with $MgSO_4$ and drying Novozym®435 with P_2O_5 under vacuum.

Enzymatic stability in storage conditions in each organic substrate

For this study, the thermodynamic activity of water (a_w), of the substrates and of Novozym®435 were beforehand fixed at 0. One of the two substrates (11 mmol) was placed in the quartz vessel and heated to 100 °C under conventional or microwave heating. When the temperature of the substrate solution was stable, 25 mg of Novozym®435 were rapidly added and the mixture was incubated for various times at 100 °C under conventional or microwave heating (24 W in butanol, 210 W in ethyl butyrate). After the preincubation at 100 °C, the second substrate (11 mmol) was added to the mixture to test the residual activity of the lipase. This activity test was conducted for 3 min (initial rate conditions) under conventional heating in each case (100 °C), to be sure that only the influence of the heating mode on enzymatic stability was observed.

Enzymatic stability in use conditions

Typical butyl butyrate syntheses were carried out at 100 °C with 25 mg Novozym®435 under conventional or microwave heating for 6 h. After 6 h, the reaction medium was filtered in order to recover the biocatalyst. The enzyme so recovered was rinsed by 2 ml ethyl butyrate under vacuum before being re-used in another identical experiment.

Acknowledgements

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