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HAL Id: hal-01608788

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Gerard, Doriane^{ORCID} and Currie, Fiona^{ORCID} and Medina-Gonzalez, Yaocihuatl^{ORCID} and Camy, Séverine^{ORCID} and Marty, Alain^{ORCID} and Condoret, Jean-Stéphane^{ORCID} *Resolution of 2-bromo-arylacetic acid ester by Yarrowia lipolytica lipase in water/supercritical CO₂ two-phase systems.* (2017) *The Journal of Supercritical Fluids*, 121. 96-104. ISSN 0896-8446

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Resolution of 2-bromo-arylacetic acid ester by *Yarrowia lipolytica* lipase in water/supercritical CO₂ two-phase systems

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A B S T R A C T

A mutated lipase from *Yarrowia lipolytica* was used in *aqueous phase/scCO₂* two-phase systems to perform the enzymatic resolution of (*R, S*) 2-bromophenyl acetic octyl ester. A solution of phosphate salt (1000 mmol/L) was added to buffer the aqueous phase in contact with CO₂ and resulting pH values around 6 were measured in a high pressure cell using a solvatochromic probe. Thus, an acceptable conversion rate and good enantioselectivity could be obtained but kinetics were shown to remain slower compared to an *aqueous phase/decane* two-phase system. Moreover, increasing pressure was shown to further slow-down the kinetics. This was hypothesized to be related to the mechanism of opening of the active site of the lipase which requires interfacial contact with a hydrophobic solvent phase. This condition is suspected not to be met in the case of scCO₂ in contact with an aqueous phase because the amount of water dissolved in the supercritical phase diminishes its hydrophobicity.

Keywords:

Supercritical carbon dioxide

Two-phase systems

Lipase

Yarrowia lipolytica

Enzymatic enantiomeric resolution

1. Introduction

α -substituted aryl and alkyl carboxylic acids are important intermediates encountered in synthetic pathways of numerous drugs, such as prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts. All these molecules contain an asymmetric carbon at the α position of the carboxylic function, leading to the co-existence of two enantiomers. In most cases, only one enantiomer exerts the required biological activity. Recently, enzymatic resolution of 2-bromophenyl acetic octyl ester was proposed, using variant V232S of the lipase Lip2 from *Yarrowia lipolytica*, taking advantage of the enzyme enantioselectivity [1–3]. Lipases are biocatalysts that are frequently used in liquid two-phase systems of the *aqueous phase/organic solvent* type, in this case decane as the organic phase. But such a process is impeded by the use of an organic solvent which contradicts the environmental benefit of using “natural” catalyst like enzymes. Therefore the replacement of the organic solvent by supercritical CO₂ (scCO₂) to propose a greener processes and to take advantage of the facilitated

post reaction separation is an attractive option. The usual procedure for using enzymes in scCO₂ has been to immobilize the enzyme on a porous solid support. In such cases, the reaction is carried out in what will be called here a single-phase system, *i.e.*, without an aqueous phase to solubilize the enzyme. In these systems, it was demonstrated that the control of the water activity in the system is crucial for the activity of the enzyme during synthesis reactions (esterification, transesterification, amidation. . .) [4–6]. Conversely, the use of scCO₂ in an *aqueous phase/scCO₂* two-phase system has been rarely proposed [7,8], even though it allows the direct use of the enzyme and therefore eliminates the immobilization step. These studies [7,8] proved that the enzymatic reactions are possible in this kind of two-phase systems but they did not provide comparison with the conventional *aqueous phase/organic* two-phase system, which prevented assessment of the advantages or disadvantage of using scCO₂ in replacement of an organic solvent. Note that use of *water/scCO₂* microemulsions was also described by in [9]. However, in this case surfactants were used and the reaction was studied for only 10 min, which was too short to assess a possible effect of the enzyme stability.

A specificity of the *water/CO₂* two-phase system stands in the generation of carbonic acid in the aqueous phase, due to CO₂ partial solubilisation in water and the resulting chemical equilibria.

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Indeed, at high pressure (from 50 bar), the pH of aqueous phase is expected to decrease to around 3.2 at 32 °C [10]. This pH decrease is usually an important drawback for using enzymes because most of them are only active in a narrow range of pH values, generally close to neutral. Use of a buffered aqueous phase by addition of sodium bicarbonate was shown to improve the activity in the case of the asymmetric reduction of ketones catalysed by an alcohol dehydrogenase from *Geotrichum candidum*, where the yield of the reaction was improved when the pH was maintained to neutral [8].

The objective of this work is to achieve the enzymatic resolution of 2-bromophenyl acetic octyl ester (belonging to the 2-halogeno-carboxylic acids family) using *aqueous phase/scCO₂* two-phase systems. In previous works, the *Yarrowia lipolytica* lipase was shown to be efficient for the resolution of this substrate and its enantioselectivity was improved using site-directed mutagenesis [2,3,11,12]. The mono-substituted variant V232S of the lipase from *Yarrowia lipolytica* (Lip2 V232S) was shown to exhibit enantioselectivity higher than 200, and thus provides purity compatible with pharmaceutical legislation, and was therefore used in this study. Indeed, only the (*S*) enantiomer is hydrolysed by the lipase, whereas the (*R*) enantiomer remains as an ester (Fig. 1). The resulting acid and ester can then be easily separated because their hydrophobicities are very different.

The objective of the study is thus to assess the feasibility of performing the resolution of 2-bromo phenyl acetic octyl ester in *aqueous/scCO₂* two-phase systems. Also, the merits of using an *aqueous phase/scCO₂* two phase system will be identified by comparison with the conventional *aqueous phase/decane* two-phase system.

2. Materials and methods

2.1. Chemical reagents and enzyme

Construction of Lip2 V232S by site-directed mutagenesis and its production were previously described [3]. The culture supernatant of *Yarrowia lipolytica*, containing the mutant of the lipase Lip2 V232S, is directly used as the catalytic phase for the resolution of the ester, with or without addition of adequate masses of salts (Na₂HPO₄ and KH₂PO₄) to buffer this aqueous phase. So this aqueous mixture containing the lipase Lip2 V232S will be referred here as “supernatant”. Peptone, tryptone and yeast extract necessary for the culture were purchased from Difco (Paris, France) and oleic acid was obtained from Prolabo (Fontenay sous Bois, France). All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and used as received. Carbon dioxide was supplied by Air Liquid (purity around 99.98 %). Because only the racemic (*R, S*) 2-bromophenyl acetic acid was commercially available (Sigma-Aldrich, St. Louis, MO), it was necessary to synthesize the corresponding racemic octyl ester. The procedure for the preparation of the 2-bromophenyl acetic octyl ester was previously described [13].

2.2. Enzymatic hydrolysis of (*R, S*) 2-bromophenyl acetic octyl in buffered aqueous phase/decane two-phase systems performed in Eppendorf tubes

When there is no need to compare with *buffered aqueous phase/scCO₂*, the experiments with decane were done in Eppendorf tubes. In this case 750 µL of culture supernatant containing the enzyme and 750 µL of racemic (*R, S*) 2-bromophenyl acetic octyl ester (50 mmol/L in decane) were mixed in a 2 mL Eppendorf tube. The tube was stirred in a vortex Genie 2 (D. Dutscher, Brumat, France) at 22 °C for at least 24 h. At regular time intervals (every 15 min, total of 6 samples of 50 µL), the progress of the reaction was

monitored by analyzing the organic phase composition after phase separation by centrifugation (dilution 10 fold in hexane) using HPLC as described in section 2.6.

2.3. Effect of the pH

In order to obtain a given value of the pH of the aqueous phase and to study effect of the pH on the enzyme activity, the culture supernatant of *Y. lipolytica* was 10 fold concentrated using centrifugal filter units (size of the filter 10 kD by Amicon® Ultra Centrifugal Filters, Merck Millipore). Then the concentrated enzyme was diluted by 10 using the adequate buffer. pH values of 3, 4, 5 and 6 were obtained using phosphate citrate buffer solution (500 mmol/L), pH values of 7 and 8 using phosphate buffer solution and pH values of 9 and 10 using carbonate buffer solution.

2.4. Enzymatic hydrolysis of (*R, S*) 2-bromophenyl acetic octyl ester in buffered aqueous phase/scCO₂ two-phase systems

In a 90 mL high pressure reactor (Top Industrie, France) (Fig. 2), 45 mL of culture supernatant containing the enzyme and 0.8 g of (*R, S*) 2-bromophenyl acetic octyl ester were added. Then scCO₂ was injected to the desired pressure, 80, 120 or 250 bar depending on the experiment, using a syringe pump (Teledyne ISCO 260D). Using the high pressure visualisation cell presented in the section 2.5, it was preliminarily checked that this amount of ester is totally soluble in scCO₂ at the conditions used in the reaction experiments. Reactor was stirred with several kinds of stirring devices (magnetic barrel, double rushton turbine, inclined blade impeller and gas dispersing turbine) at different rotation speeds (500 or 1600 rpm) at 35 °C for at least 24 h. Every hour, the progress of the reaction was monitored by analyzing the composition of the supercritical carbon dioxide phase after sampling of this phase (at least 6 samples of 0.5 mL per experiment) using a high pressure sampler (Top Industrie, France). 0.5 mL samples were taken and scCO₂ is re-injected using the high pressure pump (Teledyne ISCO 260D) to recover the initial value of the pressure which slightly decreased after sampling (a few bar).

The contents of the sampler were recovered and diluted using decane before HPLC analysis (Section 2.6). For comparison, in the conventional *buffered aqueous phase/decane* two-phase system, the same 90 mL reactor and same concentrations were used. The experiments were replicated twice.

To increase the concentration of the phosphate buffer (PO₄) (from 100 mmol/L to 1000 mmol/L) adequate amounts of salts Na₂HPO₄ and KH₂PO₄ were added to the culture supernatant that already contained 100 mmol/L of phosphate buffer.

2.5. Measurement of the pH of the aqueous phase/scCO₂ two-phase systems

The bromocresol purple coloured indicator was used to determine the pH of the aqueous phase in contact with high pressure CO₂. A spectroscopic method was developed to accurately evaluate the colour change of the indicator in the aqueous phase as a function of CO₂ pressure. For these measurements, a StellarNet Incorporation EPP2000 light source and an Ocean Optics DH2000 spectrophotometer were coupled to a high-pressure view cell by optical fibres as shown in Fig. 3.

The absorption spectrum of the bromocresol dye at various pH is shown in Fig. 4 where it can be seen that the bromocresol purple is composed of two forms, one acid and one basic. These two forms absorb at different wavelengths (430 nm for the acid form and 590 nm for the basic form) [14].

It is possible to correlate the quotient of the two peak heights to the pH. A calibration curve was thus obtained by recording the

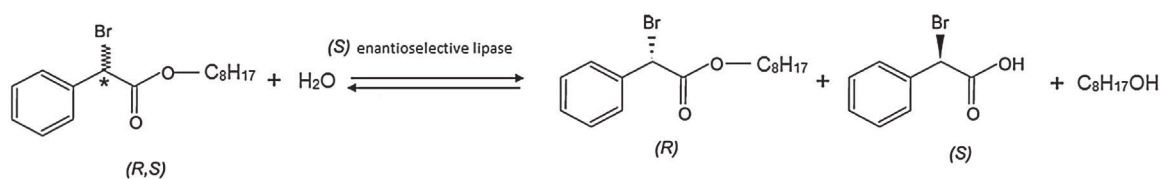


Fig. 1. Reaction scheme for the enzymatic resolution of 2-bromophenyl acetic octyl ester by enantioselective lipase hydrolysis.

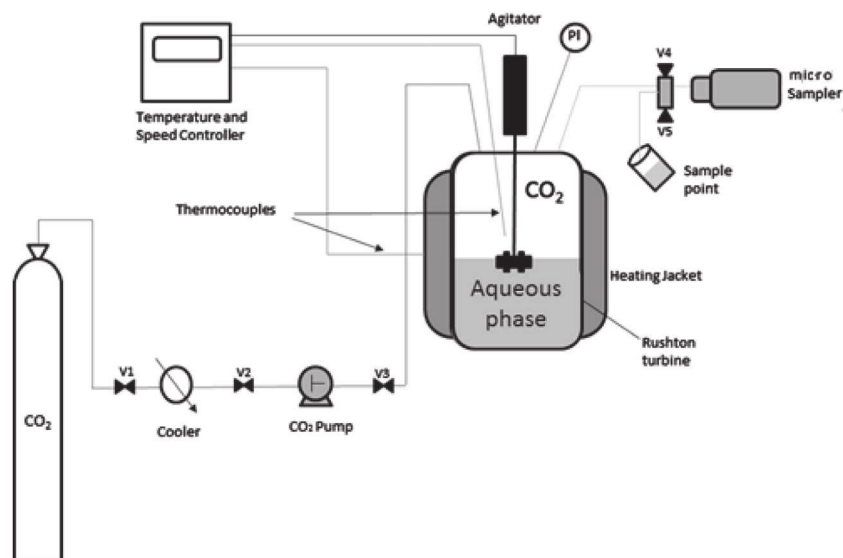


Fig. 2. Buffered aqueous phase/scCO₂ high pressure reactor.

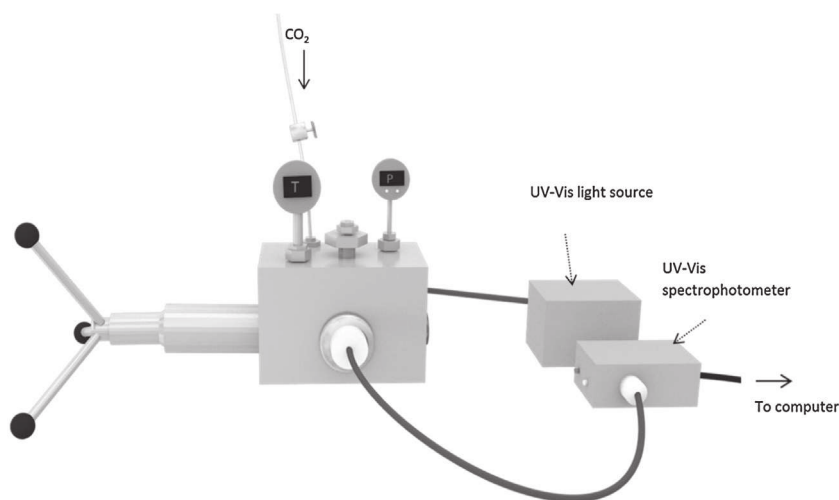


Fig. 3. Experimental set-up of spectroscopic apparatus combined with a high pressure cell.

absorbance of a solution of bromocresol purple in water at different pH values, the ration of the heights of the peaks obtained in these conditions were correlated to the pH (Fig. 5). This calibration curve was then used in pH measurements of *aqueous phase/scCO₂* two-phase systems at different pressures both, in presence and in absence of buffer (section 3.2).

2.6. Chromatographic analysis

The HPLC device (Dionex, Ultimate 3000) was equipped with a chiral column: Chiralcel OJ-H (25 cm x 4.6 mm) (Chiral technologies Europe, Daicel group) connected to a UV detector (at 254 nm). The mobile phase was composed of a *n*-hexane/isopropanol mixture

[80:20 v/v] at a flow rate of 1.0 mL/min. Retention times were 6 and 7 min for the (*S*) and (*R*) enantiomers of the 2-bromophenyl acetic octyl ester, respectively.

2.7. Determination of the conversion rate

Conversion of the (*S*) enantiomer, which corresponds to the percentage of hydrolysis, is given by the following equation where *i* stands for initial time and *t* for final time.

$$\% \text{Hydrolysis} = 100 - \frac{[\text{concentration (S) ester}]_t}{[\text{concentration (S) ester}]_i} * 100$$

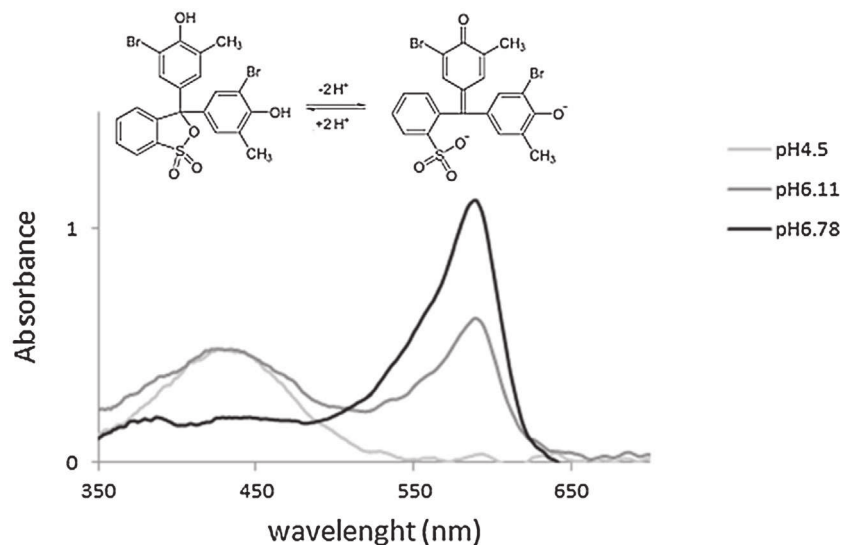


Fig. 4. UV/visible spectra of bromocresol purple at various pH in water at 35 °C.

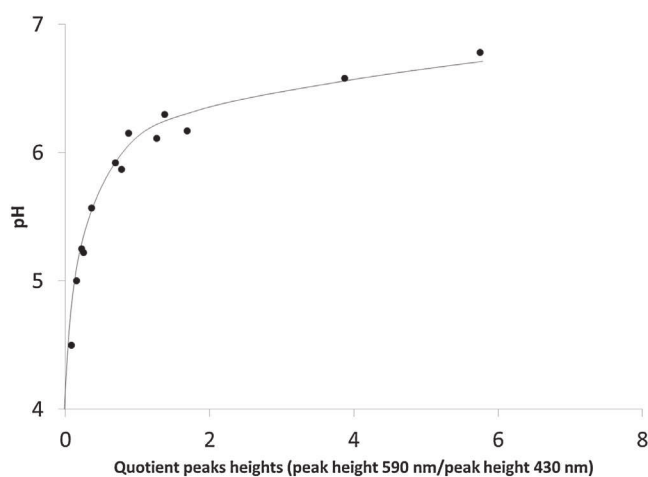


Fig. 5. Calibration curve of pH correlated to the quotient peaks heights of coloured indicator (Bromocresol purple).

3. Results and discussion

3.1. Influence of the pH on the activity of Lip2 from *Yarrowia lipolytica*

It is well known that for a given enzyme there is corresponding optimal pH range. The optimal conditions for Lip2 from *Yarrowia lipolytica* were already described in previous studies [15–17] and were shown to lie between 5 and 9, depending on the authors and the lipase studied. It can be considered that the optimal pH conditions for the mono-mutant V232S are similar. To investigate the influence of the pH on enzyme activity, preliminary experiments were done in order to evaluate the initial velocity of the hydrolysis of 2-bromophenyl acetyl octyl ester (dissolved in decane as the organic phase) using an aqueous phase containing the enzyme at different pH values. Initial velocity is evaluated as the initial slope of the curve % hydrolysis versus time and results are reported in Fig. 6. The initial velocity is clearly affected by the pH of the aqueous phase. Indeed, at pH = 5, the initial velocity is three fold lower than its optimum value at pH 7–8. These results demonstrate that the optimal pH for Lip2 V232S is comprised between 7 and 8 which is in agreement with the results given by Fickers et al. [15].

Table 1

Experimental values of the pH of the aqueous phase in contact with scCO₂, using different concentrations of phosphate buffer at 100 bar, 35 °C.

Phosphate buffer concentration in water (mmol/L)	Measured pH
100 (equivalent to broth supernatant)	<5
400	5
500	5.4
1000	5.9
1500	6.1

3.2. Determination of the pH in the buffered aqueous phase/scCO₂ two-phase systems

As stated previously, the pH of an aqueous phase in contact with CO₂ is around 3.2 as soon as the pressure is greater than 50 bar [10] because of the formation of carbonic acid, as a consequence of the solubilisation of CO₂ in the aqueous phase. Because Lip2 V232S activity is highly affected at this pH value, it is necessary to buffer the aqueous phase to maintain a pH value around 7–8. The method and apparatus presented in section 2.5 were used to determine the value of pH in the aqueous phase, with and without salts at different concentrations, when in contact with scCO₂; Values are given in Table 1. The Na₂HPO₄ and KH₂PO₄ salts (phosphate buffer) were chosen to buffer the aqueous phase because they were already used for the culture of the yeast *Yarrowia lipolytica*. Thus, after the culture growth, the phosphate buffer concentration of the supernatant containing the enzyme (Lip2 V232S) is about 100 mmol/L. However, it can be observed in Table 1 that, at this buffer concentration, the pH of the aqueous phase in contact with CO₂ at 100 bar and 35 °C was still lower than 5. A concentration of phosphate buffer of 1000 mmol/L was determined necessary to obtain pH values close to 6. Higher buffer concentrations did not lead to a significant increase in pH (6.1 at 1500 mmol/L). Stabilization of the pH in the buffered aqueous phase/scCO₂ two-phase system has already been studied [18–21] and improvement for the hydrolysis in biphasic water/scCO₂ system using sodium bicarbonate was proved [8]. Unfortunately these publications are not useful here because the phosphate buffer (Na₂HPO₄ and KH₂PO₄) was not chosen.

Thus, it is possible to obtain a pH value of 6 with a 1000 mmol/L phosphate buffer. At this pH, the enzyme activity is 89 % of the maximum enzyme activity at pH 8 (Section 3.1). However high concentrations of salt are likely to be detrimental to enzyme activity.

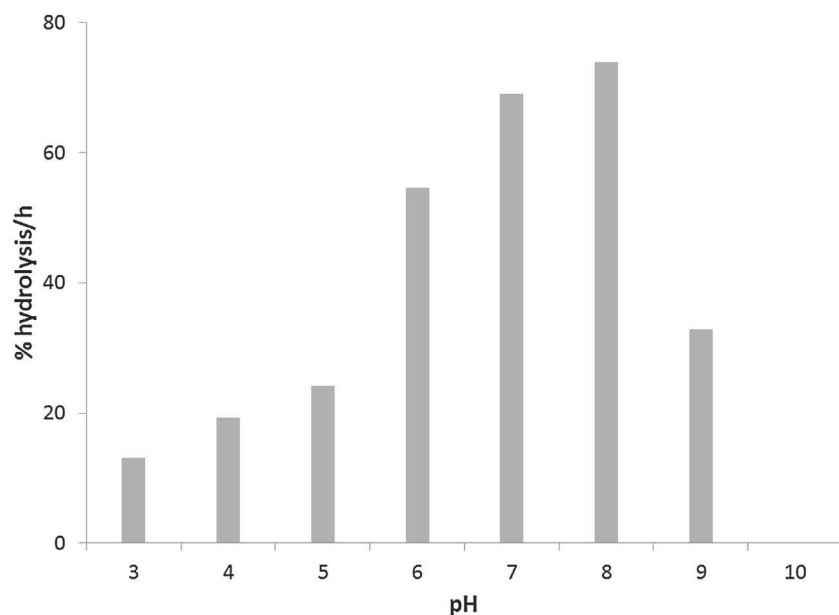


Fig. 6. Initial velocity of the hydrolysis of (S) 2-bromophenyl acetyl octyl ester using Lip2 V232S in the buffered aqueous phase/decane two-phase system (in same volumic ratio) obtained in Eppendorf tube (2 mL) at 22 °C.

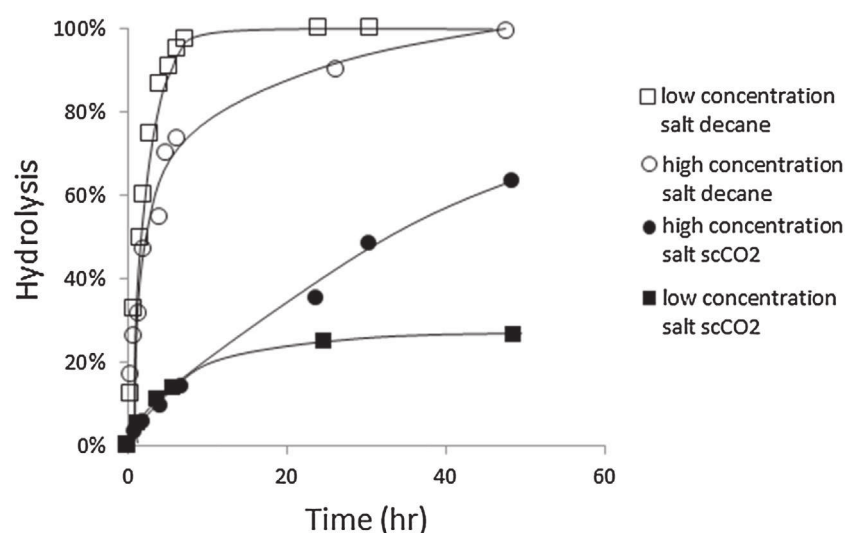


Fig. 7. Influence of the concentration of salt (phosphate buffer) in two-phase systems with decane (35 °C) or with scCO₂ (120 bar, 35 °C). High concentration corresponds to 1000 mmol/L of PO₄ buffer and low concentration to 100 mmol/L in the aqueous phase. Reaction is the hydrolysis of 2-bromophenyl acetyl octyl ester catalysed by Lip2 V232S from *Yarrowia lipolytica* using Rushton turbine at 1600 rpm.

Therefore influence of high salt concentrations on enzyme activity was investigated first.

3.3. Influence of the concentration of salts in two-phase systems

For comparison, the influence of salt concentration was investigated in the conventional buffered aqueous phase/decane two-phase system using the same 90 mL reactor and same concentrations (salts and reactants) and temperature (35 °C) as in the buffered aqueous phase/scCO₂ two-phase system. Results for both systems at low (100 mmol/L pH < 5) and high (1000 mmol/L pH around 6) salt concentrations are compared in Fig. 7. In buffered aqueous phase/decane two-phase systems, it appears that a high concentration of salt is not highly detrimental to enzyme activity. Indeed, only 25 % of activity are lost at 1000 mmol/L compared to the activity obtained at 100 mmol/L. In buffered aqueous phase/scCO₂

two-phase systems, it is confirmed that maintaining a pH around 6 (at 1000 mmol/L phosphate buffer) enables better kinetic performances to be obtained. At a low concentration of salt, and consequently low pH, it appears that the enzyme stability is reduced because the reaction stopped after 20 h. By comparing the initial slopes of the curves, initial kinetics are lower (10 fold) in scCO₂, than in decane, even at high concentrations of salt (1000 mmol/L in the aqueous phase). This decrease of activity cannot be fully explained by the difference of pH, 7 in decane versus 6 in scCO₂.

3.4. Influence of stirring conditions

In such two-phase liquid/liquid or liquid/fluid systems, using catalysts acting at the aqueous-organic interface, as is the case for lipases, the interfacial area and mass transfer between phases are

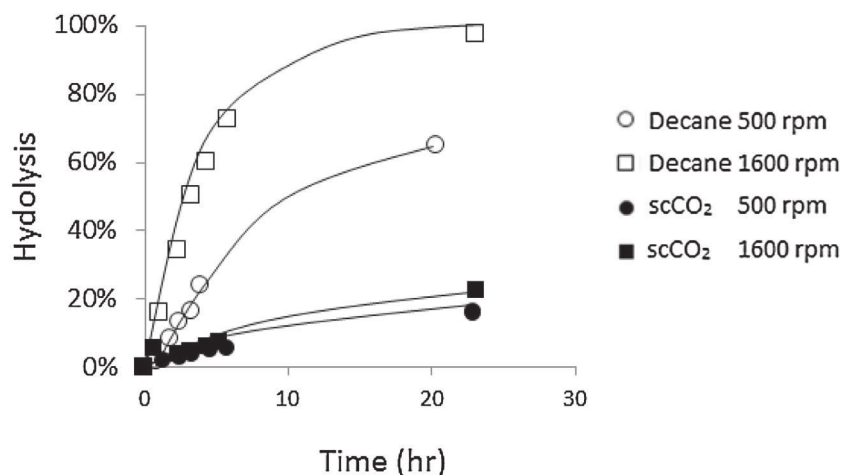


Fig. 8. Effect of the stirring speed (500 rpm or 1600 rpm) in buffered aqueous phase/decane (35 °C, 1000 mmol/L phosphate buffer) system or buffered aqueous phase/scCO₂ (35 °C, 120 bar, 1000 mmol/L phosphate buffer) system on the hydrolysis of (S) 2-bromophenyl acetic octyl ester by *Y. lipolytica* Lip2 V232S using a Rushton turbine.

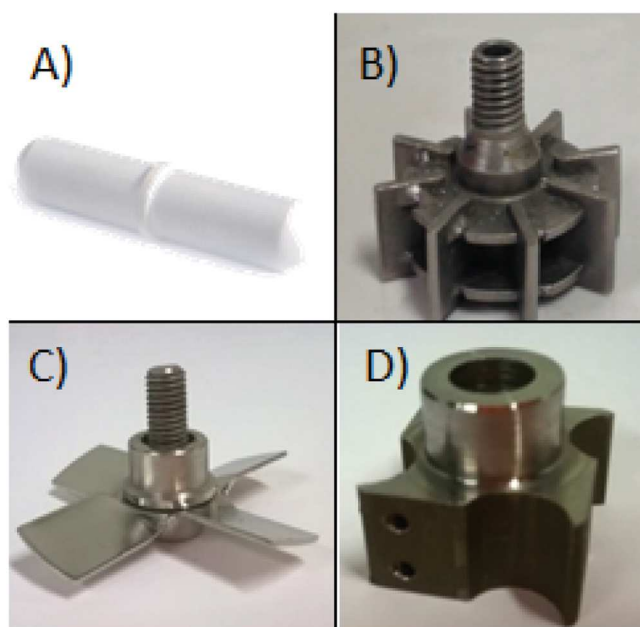


Fig. 9. Different stirring devices used in this work. A) magnetic barrel B) double Rushton turbine C) inclined blade impeller D) gas dispersing turbine.

expected to be a limiting parameters for the kinetics [22]. With the type of agitated reactor used in this study, impeller type and stirring speed are expected to have a significant effect on reaction performances. As a default stirring mobile, a double disk Rushton-type turbine was used to disperse organic phase (decane or scCO₂) in the aqueous phase. Influence of stirring speed (500 and 1600 rpm which corresponds to maximum speed of the set-up) was evaluated in the case of decane and scCO₂ and kinetics are reported in Fig. 8.

In the case of decane, the results show a clear influence of the stirring speed on the kinetics while this influence is almost negligible in the case of scCO₂. In the case of scCO₂, four different types of stirring devices were tested at 1600 rpm, (magnetic barrel, double Rushton turbine, inclined blade impeller, gas dispersing turbine) (Fig. 9) but no significant difference was observed (data not shown). This tends to indicate that limited mass transfer is not the cause of the slow kinetics in this case.

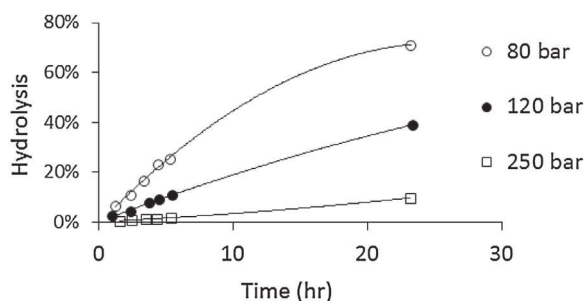


Fig. 10. Influence of the pressure on the conversion rate in buffered aqueous/scCO₂ two-phase system for the hydrolysis of (S) 2-bromophenyl acetic octyl ester using *Y. lipolytica* Lip2 V232S (at 35 °C, 1000 mmol/L of phosphate buffer, using magnetic barrel).

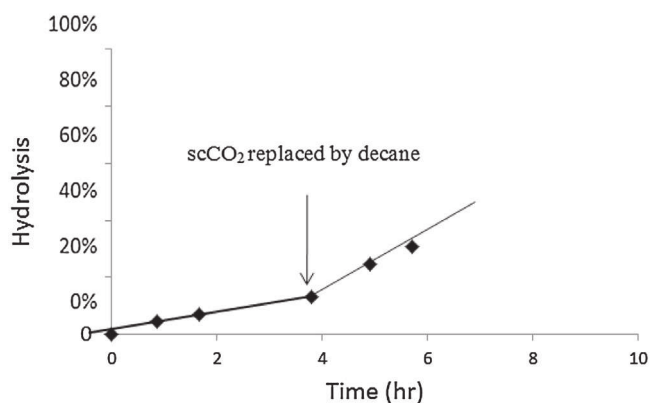


Fig. 11. Hydrolysis of (S) 2-bromophenyl acetic octyl ester by Lip2 V232S in buffered aqueous phase/scCO₂ (35 °C 120 bar) in a first stage and then replacement of scCO₂ by decane (35 °C) using a magnetic barrel.

3.5. Influence of the pressure

As usual in the case of supercritical fluid, it is interesting to assess the influence of the pressure. This was tested in the range 80–250 bar and results are presented in Fig. 10.

A noteworthy slowdown of the kinetics is observed in the system when the pressure is increased. The conversion after 24 h being decreased from 71 % at 80 bar to 10 % at 250 bar. It has been shown [10] that, in this range of pressure, the pH of the aqueous phase is only very weakly dependant of the CO₂ pressure, indicating that

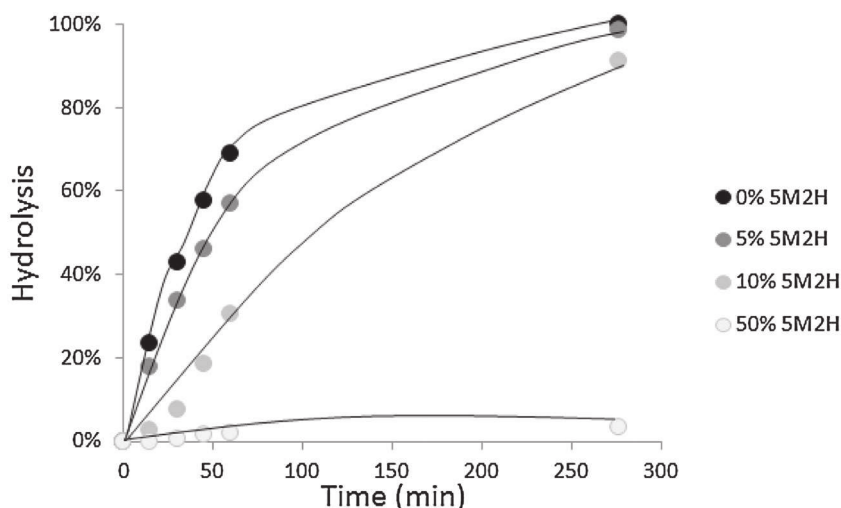


Fig. 12. Effect of various molar proportion of 5M2H in decane on the hydrolysis kinetics of 2-bromophenyl acetic octyl ester. In *aqueous phase/organic phase* two-phase systems by *Y. lipolytica* Lip2 V232S at 22 °C in Eppendorf tube (2 mL).

pH effect is not the cause. Although mass transfer in the fluid phase is likely to be disfavoured at higher pressure (due to higher viscosity and lower diffusivity of the CO₂ phase) the order of magnitude of the observed effect is too high to be attributed to mass transfer phenomena. Up to now, the effect of pressure on enzyme activity is not clearly understood [23]. Indeed, some authors reported that a large change of pressure, and in consequence of density, could significantly alter the interaction of CO₂ with the enzyme by formation of carbamates, from reaction of CO₂ with the free amine groups present on the surface of the enzyme [7,18,24–31]. These carbamates could be responsible for charge removal of the histidine residues present at the active site of the enzyme, thus resulting in a decrease of its activity or even in its complete inactivation [29,32,33]. Kamat et al. [28] studied the effect of carbon dioxide on subtilisin by providing direct evidence of the formation of carbamates structures by using laser desorption mass spectroscopy (LD-MS). Ikushima et al. [34] used Fourier transform infrared (FTIR) spectroscopy to monitor enzyme conformation in scCO₂ during esterification reaction. They showed a conformational change of the enzyme depending on the pressure.

Also, increase of pressure induces changes in the supercritical phase properties. Some authors reported the variation with pressure of the dielectric constant [18,27,35,36], or of the logP [31,37] in the case of sc-fluoroform or scCO₂ (log P is related to hydrophilicity of the organic phase). However there is weak influence of pressure on the dielectric constant in the case of scCO₂ [38] but an important influence on the density, which could increase the interaction of this fluid with the lipase, and have an effect on enzyme activity. Nevertheless, it is difficult to draw a conclusion around these possible causes.

Another hypothesis would be to relate the decrease of activity of the enzyme in respect to pressure with the state of active site of the enzyme. Indeed, it has been shown that the activity of most lipases is strongly correlated to the opening of a “molecular lid” located near the active site of the enzyme. The opening of the lid is a characteristic mechanism of lipases and is promoted in hydrophobic environments (organic phase) while its closing is induced in hydrophilic medium. This mechanism is responsible for the specific activation of most lipases at water-organic interfaces [39]. In monophasic system, where the detrimental effect of pressure was observed, the effect of CO₂ on the “lid” was also suggested [34,40]. Modification of supercritical phase hydrophobicity with the pressure could thus be suspected as an explanation for the observed

decrease of activity of the enzyme and is further investigated in section 3.7.

3.6. Study of the reversibility of the effect of scCO₂ use on lipase activity

In order to understand the influence of scCO₂ on enzyme activity, the experiment consisted in starting the reaction of hydrolysis in an *buffered aqueous phase/scCO₂* two-phase system and then, after 4 h hydrolysis, to depressurize the system and replace scCO₂ by decane to run the reaction further. Clearly, (Fig. 11) an increase of the velocity is observed when the scCO₂ is replaced by decane, thus confirming an inhibiting effect of scCO₂ on the enzyme which is not permanent because activity is recovered after scCO₂ replacement. The effect of scCO₂ on immobilized *Yarrowia lipolytica* lipase was previously studied [32] in the case of esterification reaction of lauric acid. The authors evidenced that scCO₂ could affect this enzyme when in contact with scCO₂. To explain this effect of scCO₂ on the lipase activity, the authors proposed two hypothesis: the formation of carbamates or the stripping effect of scCO₂ which extracts the “indispensable” water on the support. The effect of stripping cannot be evoked in our case because water quantity is not limiting in an *aqueous phase/scCO₂* two-phase system.

3.7. Effect of the polarity of the solvent

As stated before in section 3.5, a possible explanation to the slowdown of kinetics when scCO₂ is used instead of decane could be a dysfunction of the “molecular lid” of the enzyme, due to the decrease of hydrophobic character of the CO₂ phase when pressure is increased. Indeed, because the supercritical phase is in contact with water, the thermodynamics of two-phase systems at equilibrium indicate that a pressure increase leads to a higher water content in that phase (at 32 °C, the solubility of water in CO₂ is changing from 1 g/L at 80 bar to 1.7 g/L at 250 bar [41,42]) inducing a change in the polarity of that phase. The influence of the change in polarity of the organic phase on the kinetics of hydrolysis were thus investigated by addition of several amounts (mole fraction ranging from 0 % to 100 %) of 5-methyl-2-hexanone (5M2H), an organic polar solvent, to decane.

Kinetic results are shown on Fig. 12 where the detrimental effect of increasing proportions of 5M2H in decane clearly appears. This result demonstrates that a polar organic phase is not favourable

to enzyme activity, which could explain poor hydrolysis performances obtained in scCO₂, especially when pressure increases.

4. Conclusion

This work attempted to bring deeper knowledge about the operation of two-phase enzymatic reactions using scCO₂ as the organic phase with enzyme dissolved in the aqueous phase. Use of two-phase systems for biocatalysis, especially with supercritical CO₂, is scarce in the literature while this configuration is convenient because it does not require immobilization of the lipase, which remains solubilized in the aqueous phase. On the other hand the hydrophobic ester remains in the scCO₂ phase and is easily separated. The tested reaction was targeted to perform enantiomeric resolution of a racemic mixture of 2-bromophenyl acetic octyl ester. This was done by hydrolysis using a specific lipase from *Y. lipolytica*. The reaction was effective with high yields in this system and good enantiomeric excess was obtained, similar to what was obtained in the same configuration using decane as the organic phase. One expected difference highlighted in this study was related to the decrease of the pH of the aqueous phase in contact with pressurized CO₂, because of the dissolution of this latter into the aqueous phase. As expected, use of a buffered aqueous phase was necessary to insure acceptable values of pH and to obtain good final conversion but, in all cases, kinetics were observed to be slower in the case of scCO₂, as compared to decane. Better agitation did not improve the kinetics indicating that mass transfer or interfacial area were probably not the cause of slower kinetics. So, one proposed hypothesis was related to the specific mechanism of the “opening of the molecular lid” above the active site. This is a characteristic mechanism of lipases (and of our specific *Yarrowia lipolytica* lipase) which requires interfacial contact with a hydrophobic solvent phase. This condition is not always met in the case of scCO₂ in contact with an aqueous phase because the small amount of water dissolved in the supercritical phase could diminish its hydrophobicity. This hypothesis is mainly supported by the negative effect of a pressure increase on kinetics. In this case hydration of CO₂ is increased because the solubility of water in scCO₂ increases with pressure, and therefore its hydrophobicity is decreased. Such *aqueous phase/organic* two-phase systems for enzymatic reaction were not an often studied on the point of view of kinetic performances for the design of the reactor, especially with supercritical CO₂. Significant work is still necessary to fully understand the specificity of the use of CO₂ in replacement of the organic solvent. Testing other types of reactions and other lipases would greatly help to draw generalizable conclusions. Nevertheless, one important objective of this work was to perform enzymatic enantiomeric resolution in an innovative configuration which potentially insures a production process meeting the principles of Green Chemistry. This objective was met because the enantiomeric performance of the chosen lipase was indeed maintained for the configuration using supercritical CO₂, demonstrating the feasibility of the process for enzymatic resolution of racemates. Finally, although kinetics in scCO₂ proved to be slower and that addition of salts was necessary, comparison with decane must include the expected simplification of the post reactional separation when using scCO₂, which was not considered here and which may give a decisive advantage to this type of process.

Acknowledgment

We thank the Pôle de Recherche et d'Enseignement Supérieur de l'Université de Toulouse and the Région Midi-Pyrénées (France) for PhD grant of Doriane Gerard.

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