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ABSTRACT

Enzymatic transesterification of triglycerides in a continuous way is always a great challenge with a large field of applications for biodiesel, bio-lubricant, bio-surfactant, etc. productions. The lipase B from *Candida antarctica* (CalB) is the most appreciated enzyme because of its high activity and its non-regio-selectivity toward positions of fatty acid residues on glycerol backbone of triglycerides. Nevertheless, in the field of heterogeneous catalysis, we demonstrated that the medium hydrophilic nature of the support used for its commercial form (Lewatit VPOC1600) is a limitation. Glycerol is adsorbed onto support inducing drastic decrease in enzyme activity. Glycerol would form a hydrophilic layer around the enzyme resulting in diffusional limitations during triglyceride transfer to the enzyme. Accurel MP, a very hydrophobic macroporous polymer of propylene, was found not to adsorb glycerol. Immobilization conditions using this support were optimized. The best support was Accurel MP1001 (particle size < 1000 μm) and a pre-treatment of the support with acetone instead of ethanol enables the adsorption rate and the immobilized enzyme quantity to be maximized.

An economical approach (maximization of the process net present value) was expanded in order to explore the impact of immobilization on development of an industrial packed bed reactor. The crucial ratio between the quantity of lipase and the quantity of support, taking into account enzyme, support and equipped packed bed reactor costs was optimized in this sense. The biocatalyst cost was found as largely the main cost centre (2–10 times higher than the investments for the reactor vessel). In consequence, optimal conditions for immobilization were a compromise between this immobilization yield (90% of lipase immobilized), biocatalyst activity, reactor volume and total investments.

1. Introduction

Alkyl esters synthesized from vegetable oils are molecules used in a wide range of industrial applications: bio-fuels, bio-surfactants, bio-lubricants, bio-solvents, hydraulic and drilling fluids, dispersing agents, cosmetics, etc. [1–3]. They represent an alternative to fossil-based products which are finite resources and are known to accelerate climate disorders due to greenhouse effect gas emissions. Fatty acid esters present a lower toxicity, a higher biodegradability and are renewable leading to a lower carbon balance [3–5].

Alkyl esters are the result of the reaction between a fatty acid either in its free form (esterification) or contained in a triacylglycerol (transesterification) and an alcohol; the by product of the reaction is respectively water or glycerol. The alkyl ester industrial production is usually performed by chemical alkaline or acidic processes. These chemical methods yield a high conversion ratio to alkyl esters in a short time (4–10 h) [6,7]. However, chemical catalysis could have some unavoidable disadvantages. This chemical synthesis often presents poor reaction selectivity, leading to undesirable side-reactions. Furthermore, these processes are often high energy consumers. The recovery of glycerol is often difficult and a large amount of alkaline waste water from catalysts is produced [6,7]. The use of triacylglycerol lipases (E.C. 3.1.1.3) as biocatalysts has been developed as an alternative route to the conventional chemical process, as it is considered as an effective way to overcome these drawbacks [8,9].

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Many studies have shown the ability of enzymes to catalyze either esterification of fatty acids or transesterification of triacylglycerols from oils [6,10,11]. In spite of the wide range of possibilities and the great interest for enzymatic catalysis, industrial processes involving lipase are relatively scarce and limited. The main issue regarding enzymatic processes is the relatively high cost of the catalyst [12,13]. The first way to reduce economical impact of enzyme costs is to be able to recover and reuse it thanks to immobilization process [14]. A proper immobilization strategy may improve enzyme thermostability and resistance towards the environment (pH, solvents) and to increase the observed synthesis activity compared with non-immobilized enzyme by spreading the enzyme on a large surface area [15–17]. The most commonly used lipase is the lipase B from *Candida antarctica* (CalB), physically adsorbed on a methyl-methacrylate resin (Lewatit VP OC 1600), also commercially known as Novozyme 435®. This biocatalyst, developed by Novozymes, Denmark, presents applications in a number of industrial processes such as the synthesis of optically active compounds in the pharmaceutical industry [18,19], in the synthesis of polymers [20], in the synthesis of esters used in the flavor industry [21] or in production of FAEE for the biodiesel industry [22,23]. The enzyme has been developed for synthesis of high added value products where the high cost of the catalyst has just a limited impact. Nevertheless, in many applications, the high costs of the lipase represent the first limitation, especially in biodiesel industry where lack of stability due to short alcohols [22] or to the production of hydrophilic glycerol [24] is observed. Development of less expensive immobilized enzymes would enable new processes to be developed.

Another way to decrease the operational enzyme cost is to increase the operational stability of the biocatalyst. It is crucial for its industrial applicability because it will directly influence the enzyme cost per kilogram of product [25,26].

The use of immobilized lipase in continuous reactors significantly reduces the incidence of the catalyst added cost on the product. Tubular packed bed reactors (PBRs) lead to a higher performance than stirred-tank reactors and avoid enzyme support attrition [23,27,28]. However, to develop an economically relevant process, operational stability of the enzyme has to remain very high, for several weeks or even several months without significant activity loss. In this field of heterogeneous catalysis, the crucial point is to be able to control the partition of the produced polar compounds (water or glycerol) between the reaction medium and the enzyme support [24,29,30]. Adsorption of these polar compounds onto the enzyme support leads to a hydrophilic hindrance around the enzyme resulting in diffusional limitations for hydrophobic substrates to migrate from the liquid phase to the enzyme active site. As shown by Marty et al. [29] and Dossat et al. [30] adsorption of water or glycerol has a dramatic effect, in terms of activity of the enzyme, upon esterification or transesterification reactions. Reactant partition depends on both reaction medium and enzymatic support polarities. On the one hand, a solvent with moderate polarity, such as 2-methyl-2-butanol or *tert*-butanol, is able to solubilize efficiently all the reactants [25]. On the other hand, enzymatic support polarity has to be chosen in order to avoid adsorption of hydrophilic compounds.

In the present study, we are reporting the strategy of reasoned immobilization in view of the development of an economically viable process. This PBR is developed with the aim of the transesterification of oil triglycerides with butanol according to Eq. (1).

$$\text{Triglycerides} + 3\text{butan-1-ol} \rightarrow 3\text{butylester} + \text{glycerol} \quad (1)$$

We chose to use high oleic sunflower oil as a model substrate. It mainly contains triolein as triglycerides. The transesterification is selected to be catalyzed by CalB with a butanol/triglycerides ratio of 5. *Tert*-butanol, a non-toxic solvent of relative low cost, was selected for its ability to preserve CalB (Novozyme 435) activity and to improve greatly its stability [31]. This solvent presents a low boiling point compared to other solvents of medium polarity, which facilitates its separation and recovery during down stream process.

We are proposing to use Accurel MP, a very hydrophobic polymer of polypropylene, as an immobilization support in order to avoid glycerol adsorption at high substrate concentrations because it was demonstrated as very efficient for several lipase immobilizations. Batisda et al. [32] suggested that hydrophobic supports mimic the interface created by natural substrates of lipases, fixing the open form of these enzymes. This leads in general to a hyperactivation of lipases. CalB was reported to be prone to this interfacial adsorption process [33,34]. A pre-treatment of the support with ethanol is widely proposed in order to enhance the penetration of aqueous solution into the hydrophobic pores [12,35–39].

The main objective of this paper is to couple experimental results with an economical analysis of the global process. The economical impact of the immobilization process on the continuous packed bed reactor cost is analyzed in order to define conditions offering the best process cost-effectiveness.

2. Material and methods

2.1. Materials

Commercial liquid form of lipase B from *C. antarctica*, namely lipozyme CalB-L, was a gift from Novozymes (Bagsvaerd, Denmark), Lewatit VP OC 1600 was kindly provided by Lanxess (Leverkusen, Germany). Accurel MP1000 (particle size under 1500 μm) and MP1001 (particle size under 1000 μm) were purchased from Membrana GmbH (Wuppertal, Germany). Pore radius are distributed in two groups varying from mesoporous to macroporous scales: one, around 10 nm and the second, around 8 μm. The specific surface area was measured at 78.92 cm^2/g and the total cumulative volume at 1.955 cm^3/g [39].

90% oleic acid, 99.5% glycerol, pure *p*-nitrophenyl butyrate and molecular sieve 3 Å were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents (butanol, *tert*-butanol) were of the highest purity and were purchased from Acros Organics (Geel, Belgium).

2.2. Glycerol and butanol adsorptions on enzymatic support

Batch glycerol and butanol sorption experiments were carried out in 15 mL conical flasks at 37 °C on a rotational shaker for 24 h. Dried Lewatit VP OC 1600 and Accurel MP1001 (0.25, 0.5, 0.75 and 1 g) was thoroughly mixed with 10 mL of solutions containing 250 mM of glycerol and/or 1000 mM of butanol and/or 1500 mM of butyl-oleate. *Tert*-butanol was chosen as a solvent. After shaking the flasks for 24 h, the reaction mixture was analyzed to measure the residual glycerol and butanol concentrations. The solvent was evaporated under a flow of nitrogen and diluted for analysis. With samples containing butyl-oleate, it was necessary to extract glycerol with three volumes of water.

Glycerol adsorption on Novozyme 435® was obtained by adding 10 mL of solutions of glycerol in methanol with concentration ranging from 50 g L^{-1} to 0 g L^{-1} to 0.5 g of Novozyme 435®. The methanol was slowly evaporated at room temperature using a flow of nitrogen.

2.3. Enzyme immobilization on Accurel MP

The Accurel particles were first suspended in 10 mL of ethanol (protocols A and B) or 10 mL of acetone (protocol C). The particles are mixed for 30 min at room temperature. 10 mL 100 mM phosphate buffer 100 mM NaCl and 10 mL of lipozyme CALB-L were directly added. Protocol A requires a second pre-wash with 10 mL 50% ethanol–H_2O (v/v). After 30 min it was given a gentle stir at room temperature, the mixture was replaced with 10 mL deionised water, decanted then washed with 10 mL fresh 100 mM phosphate buffer pH 7.2, 100 mM NaCl. 10 mL of Lipozyme CalB-L were directly added to the support.

The Accurel MP in contact with enzyme was moderately shaken horizontally at room temperature for 48 h. Then, the support particles loaded with lipase were filtered from enzymatic solution, quickly washed three times with 20 mL of 100 mM phosphate buffer pH 7.2, 100 mM NaCl to remove loosely bound lipase from the carrier.

The adsorbates were dried using a two-step procedure: the first step was done in a closed chamber crossed by an air flow at room temperature until end of evaporation. In a second step, water activity was adjusted in a closed chamber containing activated molecular sieve. The immobilized lipase was stored at 4 °C, ready for the assay of immobilized lipase activity.

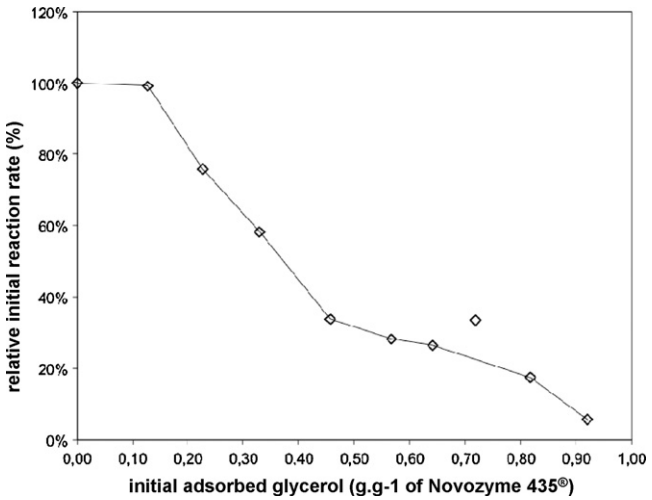

Fig. 1. Relative initial reaction rates of esterification in presence of Novozyme 435® pre-treated with glycerol. Reaction conditions: substrate concentrations: 200 mM oleic acid and 600 mM butanol in *n*-decane; Novozyme 435®: 500 mg; reaction temperature: 60 °C.

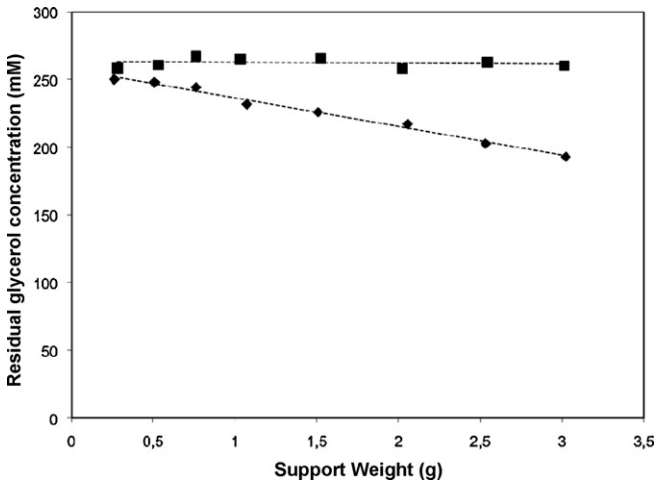

Fig. 2. Residual glycerol concentrations (mM) after contact of different quantities (g) of Accurel (■), Lewatit VP OC 1600 (♦). Initial conditions: glycerol concentration: 260 mM in *tert*-butanol.

2.4. Assay of free lipase activity

The lipase activity of free enzyme (immobilization supernatant) was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*NPB) to butyrate and *p*-nitrophenol [40,41]. 2-Methyl-butan-2-ol (2M2B) was used to solubilize *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates with 20 μL of the immobilization supernatant containing the lipase, 175 μL of a 100 mM phosphate buffer pH 7.2, 100 mM NaCl and 5 μL of *p*NPB 40 mM in 2M2B. Activity was measured by monitoring absorbance at 405 nm at 30 °C for 10 min using the VersaMax tunable microplate reader apparatus (Molecular Devices, Rennes, France). One unit of free lipase activity was defined as the amount of enzyme releasing 1 μmol of butyric acid per min at 30 °C and pH 7.2.

2.5. Assay of immobilized lipase activity

The enzymatic activity of Novozyme 435® with adsorbed glycerol was measured in a 500 mL glass bottle containing oleic acid (200 mM), butanol (600 mM), and the immobilized enzyme (500 mg) with *n*-decane previously dried with 3 Å molecular sieve as a solvent. The enzymatic activity of immobilized CalB was measured in a 10 mL glass tube containing oleic acid (200 mM), butanol (600 mM), and the immobilized CalB (20 mg) with *tert*-butanol previously dried with 3 Å molecular sieve as a solvent. The reaction mixture was incubated at 60 °C and magnetically stirred. Butyl-oleate synthesis was monitored by withdrawing samples at regular intervals for analysis. The reaction was stopped by centrifugation at 13,500 rpm for 2 min with an Eppendorf 5415D microtube centrifuge.

2.6. Analytical methods

Butanol and butyl-oleate analysis were done using an Agilent 6890N gas–liquid chromatograph equipped with an HP5 capillary column (230 m by 0.32 mm by 0.25 μm film thickness), a split/splitless mode injector and a flame ionization detector. The split mode injection was adopted with a split ratio of 200. The oven temperature was 200 °C, maintained for 3 min, raised to 280 °C at rate of 10 °C/min, and maintained at 280 °C for 1 min, while the injector and detector temperature were set at 250 °C and 270 °C, respectively. The sample size was 1 μL. The carrier gas (helium) was controlled at 2 mL min^{-1}.

Glycerol analysis was realized thanks to a Dionex Ultimate3000 HPLC system, equipped with a Shodex RI-101 refractive index detector and a Biorad Aminex HPX-87K (300 mm × 7.8 mm) column. Elution was carried out at 65 °C isocratically with deionised water as mobile phase and a flow rate of 0.6 mL min^{-1}. 10 μL samples were analyzed.

3. Results and discussion

3.1. Choice of the enzyme support

Novozyme 435® is the most efficient non-regio-specific lipase, called CalB. This enzyme is commercially immobilized on Lewatit VP OC 1600 support, a material which presents moderate polar character, and is susceptible to be adsorbed by glycerol [24]. Fig. 1 presents relative residual activity of this catalyst after adsorption of increasing glycerol quantities. Effect of glycerol adsorption into enzymatic activity was estimated using a simple model reaction, esterification of oleic acid with butan-1-ol in order to avoid the complexity of a transesterification. A very hydrophobic solvent, *n*-decane, was chosen to prevent glycerol desorption. The absence of monoacylglycerol, diacyglycerol or triacylglycerol synthesis was verified during reaction through TLC analysis.

Low quantities of adsorbed glycerol (below 0.1 g/g) do not affect the initial velocity. Up to this value, a rapid decrease in activity is observed, leading to a nearly complete inactivity at 0.9 g/g of adsorbed glycerol. This phenomenon was already observed by Dossat et al. [30] during the transesterification of sunflower oil by Lipozyme IM (*Rhizomucor miehei* lipase immobilized on Duolite A568). These authors postulated that the glycerol forms a hydrophilic layer around the enzyme impeding the mass transfer of hydrophobic substrates.

Therefore, during the transesterification reaction of triglycerides in a continuous process, a drastic loss of reactor performances versus time is expected, due to produced glycerol adsorption. To prevent this from happening, it would be of interest to adapt the enzymatic support polarity to avoid glycerol adsorption. The first objective was consequently to optimize support polarity for CalB immobilization. In the literature the most used hydrophobic support is Accurel MP. Its performances were then compared with ones observed using Lewatit VP OC 1600.

Fig. 2 presents the residual glycerol concentration after contact with different quantities of the two enzymatic supports in a 250 mM solution of glycerol in *tert*-butanol as solvent. A moderate adsorption of glycerol is reached for Lewatit VP OC 1600. Glycerol disappears linearly when the quantity of support is increased. If the conventional Langmuir approach for adsorption isotherm is considered [42], the adsorption phenomenon is in the linear zone of the isotherm, far from the saturation of the support. Interestingly enough, for Accurel MP1001, glycerol adsorption is completely avoided in these conditions.

As the partition of glycerol is also function of the medium polarity, it appears necessary to mimic the reaction medium. As a model reaction, the transesterification of 0.5 M of triglycerides (high oleic sunflower oil) with 2.5 M of butan-1-ol (ratio 5) was chosen. The reaction medium considered for the partition study was the final composition obtained at complete conversion (1.5 M butylester, 1 M butan-1-ol).

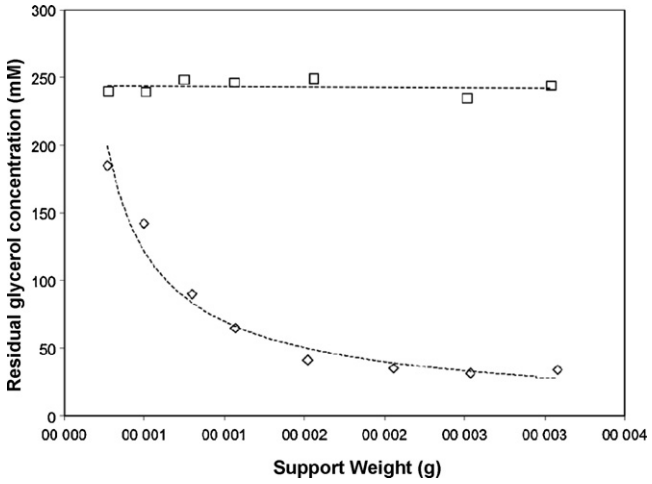

Fig. 3. Residual glycerol concentrations (mM) after contact with different quantities (g) of Accurel (□), Lewatit VP OC 1600 (◊) with a butyl-oleate and butanol containing solution of glycerol at room temperature. Initial conditions: glycerol concentration: 235 mM; butyl-oleate concentration: 1500 mM; butanol: 1000 mM in *tert*-butanol.

Butan-1-ol is not adsorbed whatever the support (data not shown).

The quantity of glycerol adsorbed on Lewatit VP OC 1600 is largely increased in this medium. This result is in accordance with the increase in log(*P*) (partition coefficient between octanol and water) calculated using Cosmotherm software (Cosmologic Germany) [43]. The log(*P*) of pure *tert*-butanol was estimated at 1 whereas there is a 3-fold increase in the considered reaction medium. According to the Langmuir equation [42], saturation of the Lewatit support is reached at a value of 1.9 g of adsorbed glycerol per g of support (Fig. 3).

On the most hydrophobic support tested, Accurel MP1001, no glycerol adsorption was observed, and therefore, this support would enable perfect stability of a continuous reactor to be obtained. As a consequence, the immobilization optimization will be achieved with this support.

3.2. Optimization of CalB immobilization on Accurel MP

Pre-treatment of Accurel MP is needed to improve the penetration of the aqueous lipase solution into hydrophobic Accurel MP pores. In the literature, two protocols were described: protocol A consists in wetting the support with, sequentially: ethanol, aqueous ethanol solution and finally with water, with intermediary filtration [35,36,44]; protocol B consists in a single wetting with ethanol and then a direct contact with the enzyme solution without removing ethanol [12,38,39]. Ethanol improves the immobilization process by inducing a better penetration of the enzyme solution inside the very hydrophobic Accurel [37] and by reducing the enzyme thermodynamic activity, thus forcing the adsorption process. CalB being well-known for its strong tolerance to high solvent quantities, a third method (protocol C) is proposed consisting in the replacement of ethanol in protocol B by a more polar solvent, acetone. It was checked that no loss of enzyme activity occurred when acetone or ethanol was added to the lipase solution in the same conditions as during immobilization process over 48 h at room temperature (data not shown).

The shapes of the experimental adsorption curves suggest a Langmuir type adsorption. Justification and demonstration of this adsorption behaviour is out of the scope of this study but, for the sake of convenience, we will use a Langmuir type equation to model our results.

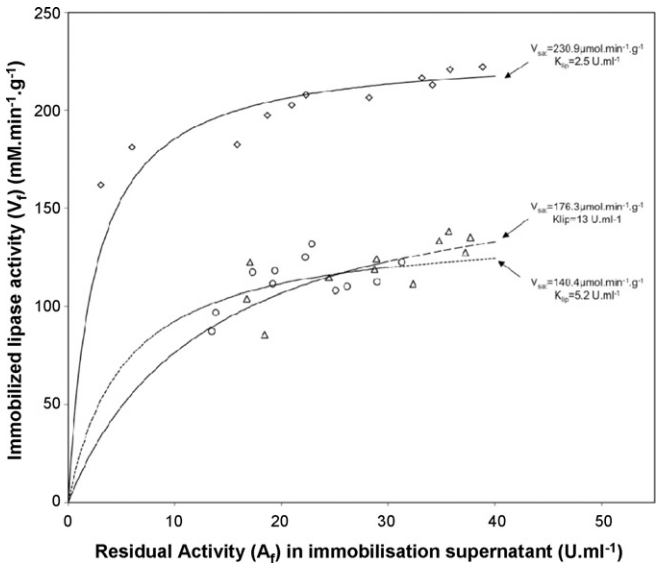

Fig. 4. Comparison of adsorption curves for the adsorption of CalB on Accurel MP1000 with protocol A (○), protocol B (△) and protocol C (◊) at room temperature. The dashed, dotted and solid curves are the Langmuir isotherms calculated with the parameters fitting the adsorption data obtained with protocols A, B and C, respectively. Reaction conditions: substrate concentrations: 200 mM oleic acid and 600 mM butanol in *tert*-butanol; immobilized CalB: 20 mg; reaction temperature: 60 °C.

A typical Langmuir behaviour for protein adsorption had been already observed by Gitlesen et al. [38] and Al-Duri and Yong [36] for the immobilization of lipases from *Candida rugosa*, *Humicola lanuginosa* and *Pseudomonas fluorescens* on Accurel MP.

Protein content determination methods are largely perturbed by salts, solvents, etc. To get round this problem, adsorption curves were represented by the plotting of immobilized lipase activity (V_f, $\mu mol\,min^{-1}\,g_{sup}{}^{-1}$) versus the residual free lipase activity in the supernatant (A_f, $U\,mL^{-1}$). This is based on the hypothesis that lipase activities are directly proportional to protein concentrations in the bulk phase for the free lipase and to the quantity of lipase adsorbed into the support for the immobilized lipase. In addition, this approach enables better conditions for enzyme activity optimization to be easily identified. So, in this case, the Langmuir equation describing isotherm adsorption [42] is modified in Eq. (2):

$$V_f = \frac{V_{sat} A_f}{K_{lip} + A_f} \quad (2)$$

with V_{sat} ($\mu mol\,min^{-1}\,g_{sup}{}^{-1}$) the velocity at saturation and K_{lip} ($U\,mL^{-1}$) a constant of adsorption of CalB on Accurel.

For practical reasons (higher reaction rates, analytical facilities, etc.), the PBR development was mimicked using a simpler reaction of esterification of oleic acid with butan-1-ol presented in Eq. (3). Even if several parameters may play a role, the reaction optima would be likely to be very similar.

$$\text{Oleic acid} + \text{butan-1-ol} \rightarrow \text{butyl-oleate} + H_2O \quad (3)$$

Fig. 4 presents the isotherm equilibrium of CalB on Accurel MP obtained for the three immobilization methods. Both the adsorption velocity and the maximal capacity of adsorption appear to be drastically dependent on the immobilization methods.

Protocols A and B led to similar maximal adsorption (V_{sat} of 140 $mmol\,min^{-1}\,g^{-1}$ and 176 $mmol\,min^{-1}\,g^{-1}$, respectively), whereas with protocol C the maximal adsorption was increased to 231 $mmol\,min^{-1}\,g^{-1}$.

Mei et al. [45] have shown that for a Novozyme 435® with particle size of 600 μm, CalB was exclusively located in the external shell of the bead, with a thickness of 80–100 μm. The same kind

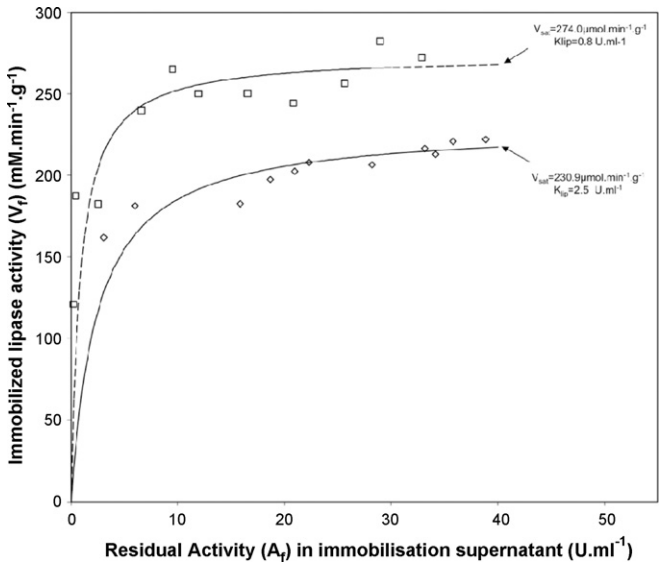

Fig. 5. Comparison of adsorption curves for the adsorption of CalB on Accurel MP1000 (◊) and on Accurel MP1001 (□) pre-wetted with acetone (protocol C) at room temperature. The solid and dotted curves are the Langmuir isotherms calculated with the parameters fitting the adsorption data obtained with Accurel MP1000 and MP1001, respectively. Reaction conditions: substrate concentrations: 200 mM oleic acid and 600 mM butanol in *tert*-butanol; immobilized CalB: 20 mg; reaction temperature: 60 °C.

of distribution of CalB in an external shell of the Accurel particle is indeed expected, with a thickness depending on the method of immobilization. Compared to the two other methods, the use of acetone was thought to induce a deeper penetration within the support, giving access to a larger area for lipase adsorption and an extended potential maximal activity.

Moreover, the affinity between enzyme and support is favored by the presence of acetone (5.2, 13.0 and 2.5 U mL^{-1} for methods A, B and C, respectively). Acetone, which is an aprotic solvent, decreases the solubility and the thermodynamic activity of protein in solutions more efficiently [46].

Finally, compared to the protocol A, the two protocols B and C could be easily transposable to an *in situ* immobilization process by a simple circulation of first, the solvent and then, the lipase solution directly added to the solvent through a reactor packed with the support.

Different kinds of Accurel MP supports are available. Accurel MP1000 presents particle size below 1500 μm, Accurel MP1001 below 1000 μm and Accurel MP1004 below 400 μm. The latest was discarded for practical reasons: it is a fine powder which can induce high pressure drop in a continuous PBR and its low density induces difficulties for the contact between phases. Fig. 5 shows the adsorption curves of CalB on Accurel MP1000 and MP1001.

Oliveira et al. [47] have shown that the layer thickness formed by CalB within a Novozyme 435® bead is constant whatever the particle size, when the immobilization conditions are similar. As a matter of fact, they demonstrated that the smaller particle size have the highest specific enzyme content. Our results are in accordance with this effect of dilution of CalB within the support. This effect was also observed by Al-Duri and Yong [35] who have demonstrated the enhancement of lipase PS and lipolase 100 L activity immobilized on Accurel EP100 when the particle size was decreased from 2500 μM to 750 μm. Sabbani et al. [48] have also reported an improved activity rate of esterification of 2-methylhexanoic acid when decreasing Accurel particle sizes were used to adsorb *C. rugosa* lipase.

The maximal immobilized activity, calculated for the couple Accurel MP1001/protocol C, is improved by 18.7%, 55.4% and 95.1%, compared to the ones obtained with the couples Accurel MP1000/protocol C, Accurel MP1000/protocol B and Accurel MP1000/protocol A, respectively; therefore, the couple Accurel MP1001/protocol C with Novozyme 435® as a reference will be used for the following demonstration.

The absence of glycerol adsorption onto Accurel support after immobilization was verified using a medium containing *tert*-butanol or a mixture of *tert*-butanol and hexane (60/40) (adsorption of glycerol was not feasible in presence of esters to avoid synthesis reactions). In every case, no glycerol adsorption was measured whatever the level of protein loading onto the Accurel support (data not shown). Therefore, it could be concluded that CalB immobilization does not affect the positive effect of the high hydrophobicity of the support. Moreover it would suggest that glycerol accumulation principally occurs onto the matrix of hydrophilic supports.

3.3. Enzyme cost analysis

Most of immobilization studies in the literature provide immobilization optimization in terms of activity, i.e., the best immobilized enzyme presents the highest activity. This is generally obtained with the highest enzyme loading on the support. It often corresponds to the use of a high lipase/support quantity ratio which is often directly related to high cost [12].

The cost of the immobilized CalB on Accurel MP is dependent on four different factors: (i) the cost of the support, (ii) the cost of the lipase, (iii) the cost of additive products (acetone, ethanol, etc.) and (iv) the operating costs. In our procedure, volumes of lipase and additives were kept constant. So the main factor influencing the cost calculation is the cost of the support and by extension the ratio between support and lipase quantities. The general cost calculation is given in Eq. (4).

$$\text{Cost}_{\text{immobilized lipase}} = \frac{m_{\text{support}} \times \text{cost}_{\text{support}} + m_{\text{lipase}} \times \text{cost}_{\text{lipase}} + \text{cost}_{\text{aditives}} + \text{operating costs}}{m_{\text{immobilzed lipase}}} \quad (4)$$

where the m_x (kg) are the mass of the considered elements and the cost$_x$ (€/kg) are the specific costs of the considered elements. It was considered to carry out the adsorption of lipase directly in the packed bed reactor. The unitary operation of CalB immobilization can be simplified to the circulation of the different components through the packed Accurel, using a system in adequacy to the proportion of volume defined in the lab conditions and including a loop for recycling of immobilization medium in order to approach the external mass transfer conditions of an agitated batch. As a consequence, operating costs were supposed to be identical whatever the immobilization conditions. The pumping costs to ensure the immobilization directly in the PBR were thought to be negligible with respect to the costs of support and lipase, and they were not included into the immobilized lipase production cost calculation.

The adsorption of lipase on a support is led by thermodynamics through partition phenomena between the solid phase and the reaction medium. These phenomena are highly dependent on the medium. So, reproducible immobilizations are only obtained when the reaction medium and more precisely the proportions of each component are completely controlled.

In the cost calculation, it is important to discuss about the treatment of the quantity of non-adsorbed enzyme after immobilization. This quantity can be considered as lost, or can be recycled in a new immobilization process. The main choice criterion is the percentage of residual enzyme after immobilization. The lower this percentage,

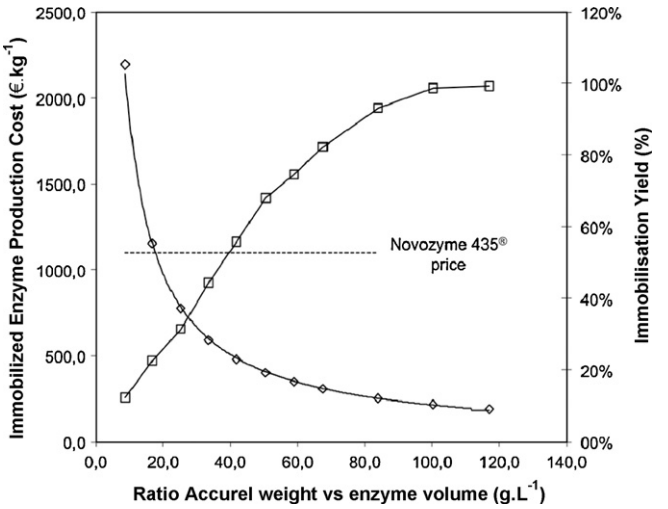

Fig. 6. Comparison between the yield of *Candida antarctica* lipase B immobilization on Accurel MP1001 using Protocol C (□/right axis) and the resulting production cost of the immobilized lipase (◊/left axis) Dotted line represents the Novozyme 435® commercial price.

the easier the residual enzyme can be considered as lost. Moreover, the recycling of enzyme leads to problems in the reproducibility of the immobilization process. The commercial preparation of CalB contains stabilisers (sorbitol and glycerol) and preservatives (sodium benzoate and potassium sorbate). These compounds, as well as all components directly added to the enzyme for the immobilization process (acetone, ethanol or buffer) can play an important role in the adsorption process. In the present procedure, after immobilization, concentrations of each of these components have inevitably varied: the lipase is adsorbed and its concentration is reduced while glycerol and sorbitol are not and their concentrations are supposed to remain identical (see Section 3.1). The recycling of the unloaded lipase is thus synonymous of restoration of initial proportions for all these components. If this adjustment were not done, the recycling of the non-loaded lipase would lead to the production of non-homogeneous immobilized lipase batches, which is highly undesirable in an industrial process. Therefore, the non-loaded lipase was considered as lost after immobilization in our experiments. This hypothesis will be discussed again later.

Fig. 6 presents the calculated cost of immobilized CalB on Accurel MP1001. It appears that the ratio between the support and the lipase quantities in contact has a drastic effect on the resulting production cost. The higher the ratio between support and lipase, the lower the production cost of immobilized enzyme. As expected, the least expensive enzyme is obtained for the highest immobilization yield with a minimal loss of enzyme. Compared to Novozyme 435® commercial price, the production cost of CalB immobilized on Accurel MP1001 can be up to six times less expensive.

Nevertheless, the immobilization conditions which minimize the biocatalyst production cost lead to a biocatalyst with the lowest relative specific activity. To obtain a given productivity, higher enzyme quantity will be necessary and consequently, the reaction volume will be higher. It is of importance, then, to calculate the cost of the global process.

3.4. Reactor design

The PBR is a column filled with a mass W_{ENZ} of enzymatic catalyst. The reactor is characterized by its volume V_{PBR} (L), diameter D (cm) and length L (cm). The PBR inner wall has a thickness t_{PBR} (mm).

The design was carried out according to the determination of the required quantity of each immobilized lipase needed to reach a fixed productivity. These weights were calculated by reference to the initial velocity obtained in batch reaction. In a batch reaction in a volume V_b (L) with a mass W_b (g) of enzyme, the reaction rate r (mol min^{-1} g^{-1}) is defined as the variation of substrate concentration dS (M) in a time lapse dt (min) (Eq. (5))[49].

$$\frac{dS}{dt} = \frac{W_b}{V_b} r \tag{5}$$

The time t_b (min) necessary to obtain the transformation of S_0 in S is obtained by the integration of Eq. (6) with S_0 (M) the initial substrate concentration.

$$\int_{S_0}^{S} \frac{dS}{r} = \frac{W_b}{V_b} t_b \tag{6}$$

The general equation of a PBR is given in Eq. (7) where t_s (min) is the residence time in an element of volume [49].

$$\frac{dS}{dt_s} = \frac{W_{ENZ}}{V_{PBR}} r \tag{7}$$

The mass of enzyme needed to transform S_0 in S is given by Eq. (8) after integration of Eq. (7), with T_s (min) the PBR residence time and with q the volumetric flow rate (L min^{-1})

$$W_{ENZ} = \frac{V_{PBR}}{T_s} \int_{S_0}^{S} \frac{dS}{r} = q \int_{S_0}^{S} \frac{dS}{r} \tag{8}$$

From Eqs. (6) to (8), the general Eq. (9) for calculation of the required mass in a PBR (W_{ENZ}) to ensure a fixed productivity and a fixed conversion rate is obtained. In our calculation, a productivity of 10,000 tons per year with a conversion rate X of 90% has been arbitrarily chosen, which corresponds to a flow rate q of 312 L min^{-1}.

$$W_{ENZ} = q \frac{W_b t_b}{V_b} \tag{9}$$

The different corresponding volumes of PBR are computed from immobilized CalB apparent density. The design of the PBR was achieved by adopting a ratio length to diameter equal to 8. Table 1 gives the characteristics of the PBR obtained for CalB immobilized on Accurel MP1001 and the corresponding enzyme expenses C_{ENZ} ($C_{ENZ} = W_{ENZ} \times \text{Cost}_{\text{immobilized lipase}}$). Because of the wide range of immobilized lipase production costs (see Section 3.3), the higher enzyme expenses correspond to the smaller reactors, which were obtained with the smallest ratio between Accurel, and lipase quantities put in contact for immobilization.

3.5. Reactor cost estimation

The average investment costs for the PBR, assimilated to a tubular vessel, were conventionally computed by using three different methods to make up for the low level of accuracy of these methods (around 25%) [50–52].

All the computations have been updated by using the Marshall and Swift Equipment Cost Index (MSECI) (1926 Index = 100) and the Chemical Engineering Plant Cost Index (CEPCI) presented in Table 2: a corrective factor corresponding to the ratio between index from year of publication and index from present was used for update. AS304 stainless steel was chosen as a PBR construction material.

Table 1
Characteristics of the PBR developed for different conditions of CalB immobilization on Accurel MP1001 using protocol C carrying out acetone for support pre-treatment.

Accurel used for immobilization (g/L immobilization medium)	Enzyme weight in PBR W_{ENZ} (kg)	PBR volume V_{PBR} (L)	PBR diameter D (cm)	PBR length L (cm)	Enzyme costs C_{ENZ} (k€)
8	206	1719	81.8	327.2	453
16	198	1657	80.8	323.3	229
25	219	1825	83.5	333.8	169
33	292	1915	84.8	339.2	136
42	224	1869	84.1	336.5	107
50	224	1870	84.1	336.6	90
58	211	1764	82.5	330.1	74
67	234	1951	85.3	341.3	72
84	299	2495	92.6	370.5	76
100	307	2563	93.5	373.8	66
117	463	3861	107.1	428.6	88
Novozyme 435®	130	1085	70.2	280.7	143

3.5.1. General methodology of capital cost estimate: Chauvel et al. [52], Peters et al. [51]

The methods, proposed both by Chauvel et al. and Peters et al., are based on the calculation of the reactor vessel weight. In order to cope with pressure forces applied on reactor vessel inner wall, it is necessary to calculate the minimal thickness to be used for the construction of the reactor vessel (Eq. (10)) from the dimensions of the reactor and the conditions of use (see Section 3.5),

$$t_{PBR} = \frac{PD}{2(\alpha t - 0.6P)} \tag{10}$$

where α is a coefficient of soldering (α equal to 1 in our case) and t is the maximal acceptable pressure for the chosen steel (t = 1195 bar for AS304 stainless steel). P was estimated by the calculation of pressure drops in the reactor according to Darcy's law (Reynolds number calculation applied to the different reactors ranging between 9 and 15). Because support particle size is large (1000 μm), P appears to be very low (between 1.2 and 1.4 bar). Consequently, a traditional 8 mm thickness was adopted for design and it can be considered that the same pump can feed all PBRs.

The total weight of the PBR is calculated by determining the mass W_{PBR} (kg) of the tubular vessels and their two circular ends presenting a surface area equal to the reactor vessel section and the same thickness (Eq. (11)). ρ_s is the volumetric mass for the considered material.

$$W_{PBR} = \pi t_{PBR} \rho_s \left[\frac{1}{2}(D + 2t_{PBR})^2 + L(D + t_{PBR})\right] \tag{11}$$

Chauvel et al. [52] have established relations between the reactor vessel diameter and the price (€/kg) of plant vessel. This abacus graph is given for ordinary steel materials and for a reactor vessel thickness of 1 mm. We have converted the abacus graph for vessel diameter ranging from 250 mm to 1250 mm with a correlation coefficient of 0.9828. The calculation of reactor cost (€) is given in Eq. (12).

$$C_{PBR} = f_f \times f_t \times f_m \times (14.3 \times D^{-0.18}) \times W_{PBR} \tag{12}$$

f_f is a form corrective factor (2.8 for reactor diameter below 1 m and 2.5 for diameter ranging between 1 m and 1.5 m), f_t is a corrective factor for thickness (1 in our case) and f_m is a material correction factor (2.3 for stainless steel 304).

Table 2
Marshall and Swift Equipment Cost Index (MSECI) (1926 Index = 100) and the Chemical Engineering Plant Cost Index (CEPCI) used in the reactor cost estimation.

Year	MSECI all industries	CEPCI
1975	444.3	182.4
1984	780.4	322.7
1990	915.1	357.6
2001	1093.9	394.3
2009	1487.2	539.7

Peters et al. [51] have directly described the costs of reactor vessels in US dollars as a function of their mass. From this abacus, the following Eq. (13) has been deduced with a correlation coefficient of 0.9968 over a range of weight from 250 kg to 1250 kg.

$$C_{PBR} = \frac{577.6 \times {W_{PBR}}^{0.67}}{R_\$} \tag{13}$$

where an average exchange rate $R_\$$ of 1.471 [53] between dollar and euro for 2009 was used.

3.5.2. Methodology described by Turton et al. [50]

Turton et al., 2001, suggest a relation for the procurement cost C_p^0 of equipment operating at ambient pressure and using carbon steel construction (Eq. (14)), expressed as.

$$\log C_p^0 = K_1 + K_2 \log(A) + K_3 [\log(A)]^2 \tag{14}$$

A is the capacity or size parameter for the equipment. In the case of PBR, A is equivalent to the volume of the vessels (between 0.3 and 520 m^3). K_1, K_2 and K_3 are data given by abacus and are equal to 3.4974, 0.448 and 0.1074, respectively, in the present case.

The cost of the desired equipment C_{PBR} is obtained by giving a material factor and bare module factors F_{BM} to the calculated C_p^0 as given in Eq. (15).

$$C_{PBR} = C_p^0 F_{BM} = C_p^0 (B_1 + B_2 F_M F_P) \tag{15}$$

B_1 and B_2 are constant depending on the type of vessels and are given in abacus graphs (equal to 2.25 and 1.82, respectively in our case). F_M is a material factor (F_M = 1.8 in our case). F_P is a pressure factor equal to 1 in the present case (low pressure).

3.5.3. Average capital cost estimate

The three methods provide similar trends, exhibiting a similar order of magnitude. The variation between the three methods was found under the 25% of the method general accuracy. Therefore, the three capital cost estimations can be averaged in Fig. 7. Contrary to the cost of immobilized enzyme which can vary greatly, the average PBR costs are slowly increasing from 22k€ to 33.3k€. The reactor cost estimated for Novozyme 435® is about 17.8k€.

The costs presented in Fig. 7 are the procurement costs of the developed PBR and of the respective content of lipase. It does not take into account its use in production conditions (reactor depreciation, lipase renewal, etc.) which is dealt with in the next Section 3.6. Nevertheless, the difference between these unit costs has to be highlighted, the purchase costs for the lipase being substantially higher than for the corresponding PBR. The difference can vary to a great extent, from one immobilization condition to another. For instance, a factor 10 is observed when 20 g of Accurel per L of immobilization medium were used and a factor 2 is obtained for 85 g of Accurel per L.

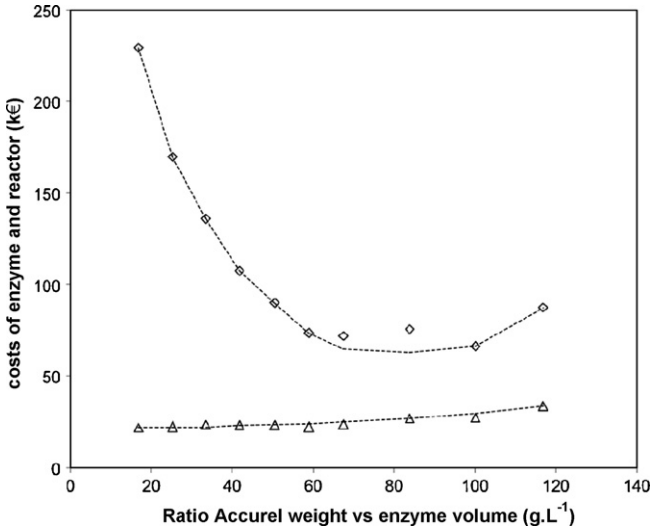

Fig. 7. Comparison between the costs of packed bed reactor designed for different conditions of immobilization of CalB on Accurel MP1001 (◊) and the cost of corresponding volumes of immobilized enzyme (△).

3.6. Net present value

The adequate choice of the immobilized lipase is carried out by maximizing the process net present value (NPV) [50–52,54,55]. It represents the difference between the global incomes and the global expenses (investments, maintenance and operating costs) (Eq. (16)). The NPV is generally calculated over the process lifetime. In our calculation, it was assimilated to the process depreciation time (t_D) which was commonly fixed to 10 years. The general calculation is given in relation 16, considering the country tax rate a (33% for France) and a price deflator i (fixed at 10% over 10 years) to cope with inflation over the process lifetime.

$$\mathrm{NPV} = -I_{\mathrm{process}} + \sum_{p=1}^{t_d} \frac{(R_{\mathrm{process}} - E_{\mathrm{process}} - D_{\mathrm{process}})(1-a) + D_{\mathrm{process}}}{(1+i)^p} - \sum_{p=0}^{E(t_d/t_{\mathrm{ENZ}})} \frac{nC_{\mathrm{ENZ}}}{(1+i)^{pt_{\mathrm{ENZ}}}} \quad (16)$$

I_{process} represents the battery limits investment. It involves the costs of every process component (reactor, pump, heat exchanger, process lagging up, upstream and downstream process). E_{process} represents the process operating costs (raw materials, utilities, labour, etc.). The costs due to the catalyst C_{ENZ} have to be treated separately from others. As usual, these costs have been taken into account at year 0 and are added to the operating costs, according to the own enzyme lifetime t_{ENZ}. Consequently, an additional term is considered only at the year corresponding to enzyme replacement The number of replacement is defined by the entire part of the ratio between the process and enzyme lifetimes $E(t_d/t_{\mathrm{ENZ}})$. If the immobilized lipase stability is under 1 year, a factor n is necessary to increase the catalyst costs by the number of change per year. For a lipase stability up to 1 year, the factor n is equal to 1.

D_{process} are the process depreciation charges and are considered as linear over the depreciation time ($D_{\mathrm{process}} = I_{\mathrm{process}}/t_d$).

R_{process} represents the global incomes associated with the process operating. R_{process} directly depends on the productivity and on the manufactured product selling price. Considering a fixed production for the different PBR, R_{process} is constant whatever the considered immobilized lipase. Thereby, the NPV is maximized when the global expenses E_{total} are minimized (Eq. (17)).

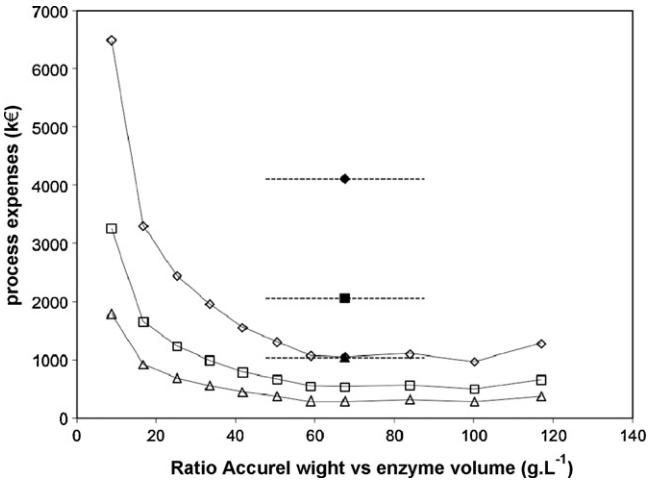

Fig. 8. Total reactor and enzyme cost optimization for a butyl-oleate production of 10,000 tons per year, for a process lifetime of 10 years with an enzyme renewal every 6 months (◊), 12 months (□) and 24 months (△). Filled symbols represent the expenses calculated for Novozyme 435® considering a stability of 6months (♦), 12months (■) and 24 months (▲).

$$E_{\mathrm{total}} = I_{\mathrm{process}} + \sum_{p=1}^{t_d} \frac{(E_{\mathrm{process}} + D_{\mathrm{process}})(1-a) - D_{\mathrm{process}}}{(1+i)^p} + \sum_{p=0}^{E(t_d/t_{\mathrm{ENZ}})} \frac{nC_{\mathrm{ENZ}}}{(1+i)^{pt_{\mathrm{ENZ}}}} \quad (17)$$

The process production being fixed, only the expenses directly dependent on the nominal lipase activity are variable. All the other expenses (heat exchanger and pumping system expenses, energy and labour costs, etc.) are constant and are not involved in the discrimination of the different immobilized lipases. Therefore, the minimization of E_{total} can be simplified by the minimization of the only investments made for PBR design and the only costs of catalyst spent over the reactor lifetime (Eq. (18)).

$$E = I_{\mathrm{PBR}} + \sum_{p=0}^{E(t_d/t_{\mathrm{ENZ}})} \frac{nC_{\mathrm{ENZ}}}{(1+i)^{pt_{\mathrm{ENZ}}}} + \sum_{p=1}^{t_d} \frac{-aD_{\mathrm{PBR}}}{(1+i)^p} \quad (18)$$

where I_{PBR} is PBR investment costs and D_{PBR} is the PBR depreciation charges.

The lipase total cost over the reactor lifetime is highly dependent on the stability of the lipase (lifetime t_{ENZ}) and thus on its renewal. For example, if a price deflator i of 10% and an enzyme lifetime of 1 year are considered, the lipase cost at termination (10 years) is increased by a factor of 7.1 as compared to the unit cost of one packing load (Fig. 7). This factor increases to 14.3 for a lipase renewal every 6 months.Fig. 8 presents the global costs as calculated with Eq. (17) for every quantity of Accurel used during immobilization. Several scenarios in terms of enzyme stability were applied to calculation, from 6 to 24 months. A packed bed reactor for butanolysis of sunflower oil at lab scale and pilot plant scale was perfectly stable during 3 months with no change in conversion and no modification in product profiles [2]. In view of such stability, a biocatalyst replacement time of 6 months (and more) was considered as reasonable. In all cases, the shape of the respective curves is identical: the expenses are drastically decreasing when the quantity of Accurel used for immobilization is increased from 8 to 60 g of Accurel/L of immobilization medium, even if the corresponding volume of PBR is increased (see Table 1). After this point (60 g/L), the decrease of lipase activity is not compensated for any longer by

the price decrease and the high volume increase leads to an additional cost increase. The use of 60 g of Accurel/L of immobilization medium in our conditions of immobilization turns out to be the best option to be considered in an industrial context. According to Fig. 6, the corresponding immobilization yield lies between 80% and 90%, which corresponds to a reasonable loss of lipase. Compared to Novozyme 435®, it represents a reduction of expenses of about 50% over 10 years, whatever the stability of the enzyme.

Thereby, the costs involved in the PBR only represent between less than 0.15% and 7% of the total costs. The design of an enzymatic process appears to depend almost only on the cost of the catalyst and the reactor costs have thus a very limited impact whatever its volume. Therefore, it is not necessary to develop the smallest reactor with the highest enzymatic activity but on the contrary, to find the enzyme allowing the lowest price whatever the reactor size.

If the principle of maximization of the process net present value is valid for any reactors, the three methodologies of capital cost estimate which were used in this study refer to range of reactor volumes developed for chemical industry, i.e., large volumes. For smaller productions (reactors smaller than 100 L), other methodologies have to be developed, notably in relationship with reactor manufacturers.

4. Conclusion

CalB, one of the lipases the most used in the industry, is only commercially immobilized on a medium hydrophobic support, the Lewatit VP OC 1600. For numerous applications, this support can be source of polar compound adsorption leading to a loss of lipase activity.

We have developed an immobilization system on a very hydrophobic support, the Accurel MP. An economic evaluation of the global costs enables the ratio between the quantity of support and the quantity of enzyme used for immobilization to be optimized. From an economic point of view, the protein adsorption yield has to be maximized, even if the catalyst is less active and the reactor volume higher. The new immobilized enzyme enables to avoid glycerol (and other polar compounds) adsorption and would permit long-lasting continuous transesterification reactions.

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Glossary

CalB: lipase B from *Candida antarctica*
PBR: packed bed reactor
pNPB: para-NITROPHenyl butyrate
2M2B: 2-methyl-2-butanol
V_f*:* immobilized lipase activity (μmol min^{-1} g$_{sup}$$^{-1}$)
V_{sat}*:* immobilized lipase activity at support saturation (μmol min^{-1} g$_{sup}$$^{-1}$)
A_f*:* residual free lipase activity in supernatant (U mL^{-1})
K_{lip}*:* lipase constant of adsorption (U mL^{-1})
m_x*:* mass of the considered element x (kg).
$Cost_x$*:* cost the considered element x (€/kg)
V_{PBR}*:* PBR volume (L)
V_b*:* batch reaction volume (L)
D: PBR diameter (cm)
L: PBR Length (cm)
P: pressure (bar)
t_{PBR}*:* PBR inner wall thickness (mm)
W_{ENZ}*:* mass of immobilized enzyme in PBR (g)
W_b*:* mass of immobilized enzyme in a batch reaction (g)
S_t*:* substrate concentration at instant t (M)
T_s*:* residence time in the PBR (s)
t_s*:* residence time in an element of volume (s)
q: process flow rate (L min^{-1})
MSECI: Marshall and Swift Equipment Cost Index
CEPCI: Chemical Engineering Plant Cost Index
α*:* coefficient of soldering
t: maximal acceptable pressure for the PBR material (bar)
ρ_s*:* volumetric mass for the PBR material (kg m^3)
C_{PBR}*:* PBR capital costs (€)
f_f*:* form corrective factor (Chauvel).
f_t*:* thickness corrective (Chauvel).
f_m*:* material correction factor (Chauvel).
$R_\$$*:* average exchange rate between dollar and euro for 2009
C_p^0*:* procurement cost of equipment operating at ambient pressure and using carbon steel construction (Turton et al.)
F_M*:* material factor (Turton et al.)
F_P*:* pressure factor (Turton et al.)
NPV: net present value (€)
a: country tax rate (%)
i: price deflator (%)
t_D*:* process depreciation time (year)
I_x*:* Investment costs of element x (€).
E_x*:* operating costs of element x (€)
$R_{process}$*:* process global incomes (€)
D_x*:* depreciation charges of element x (€)
t_{ENZ}*:* enzyme lifetime (year).