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Burkholderia cepacia lipase immobilization for hydrolytic reactions and the kinetic resolution of the non-equimolar mixtures of isomeric alcohols

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ABSTRACT

The major drawbacks of native lipase applications in processes occurring in water or in organic solvents include: difficulties in catalyst recycling, low activity and operational instability. The immobilization of *Burkholderia cepacia* lipase by adsorption or covalent binding onto 5 differently functionalized carriers (silica, acrylic, cellulose-based) was performed to overcome this problem. The optimization of the reaction preparation in water-rich media was based on the hydrolytic reactivity of the preparations, as well as the thermal, operational and storage stabilities. Aminated silica carrier, activated with glutaraldehyde, was determined to be the carrier of choice. Regarding processes in water-restricted media, carrier selection was based on reactivity after drying and five preparations were chosen for the resolution of a non-equimolar isomer mixture (85:15 ratio of *R* to *S* isomers), treating the kinetic resolution of ((+)-(*S*/*R*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)] ethanol as a model. The resulting acetate of *R* configuration exhibits interesting sensory properties. The operational stability of the chosen catalysts was tested over 15 consecutive batch processes; the most beneficial results were obtained with lipase adsorbed on an acrylic carrier. Conversion increased gradually from 10 to 84% over the first five processes, which could be explained by the product sorption onto the carrier. Full kinetic resolution with maximal substrate conversion (approximately 84%) was achieved and remained stable during the next 10 runs, an excellent result, and thus, the proposed system might be regarded as an exceptionally attractive solution for the perfume and cosmetic industries.

1. Introduction

The broad substrate specificity of lipases, excellent regio- and stereospecificity, and the ability to retain activity in nonpolar organic solvents enable their wide implementation in various types of reactions to provide high-value compounds for cosmetics, surfactants, biofuels, and the production of chiral building blocks for the pharmaceutical and agrochemical industries [1–6].

As lipases are activated only when adsorbed to an oil/water interface, their activity can be facilitated by immobilization onto carriers that are insoluble in water or in organic solvent. Moreover, immobilization offers additional common advantages such as improved stability under operating conditions, facilitated the separation of reagents, and reusability in batch processes or use in continuous flow reactors. The feasibility of the successful implementation of immobilized lipases in industry has been widely discussed in the literature [7–9].

It is worth mentioning that lipase from *Burkholderia cepacia* (BCL, *Pseudomonas cepacia*) is known, among many industrial lipolytic enzymes, for its thermal stability and resistance towards many organic solvents, including alcohols [10]. These features have attracted the attention of the pharmaceutical industry. For example, Bristol-Myers Squibb utilized BCL in the production of Taxol (in an aqueous medium), thromboxane A_2 antagonist (in a biphasic system), hydroxymethylglutaryl coenzyme A (in toluene) and a tryptase inhibitor (in a biphasic system) [7]. Interestingly, the same enzyme was employed in all typical systems: water, biphasic, and water-restricted media.

The primary aim of this work was to test five types of carriers developed in our laboratory [11–14] for: (i) their feasibility for the adsorptive and covalent immobilization of PCL, (ii) the selection of a

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proper method for enzyme binding and/or (iii) the carrier's matrix suitability for reactions carried out in water or in organic solvent. As a model substrate for hydrolytic reaction, 4-nitrophenyl palmitate was chosen and four discrimination parameters were considered for the selection of a proper immobilized preparation, namely, (i) activity, (ii) thermal stability, and (iii) storage stability, with particular emphasis placed on (iv) operational stability in successive batch processes. For reaction in water-restricted medium, the transesterification reaction of ((+)-(*S/R*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)] ethanol was used as a model for kinetic resolution (KR), which is a common method for the preparation of optically pure secondary alcohols. Developments in the field of KR have progressed considerably in terms of substrate variety [15–17] and novel catalysts [18–20] but also from the perspective of environmental impact [21,22]. It is noteworthy that the selected substrate can be used for the production of *R*-acetate with known interesting sensory properties [13]. In this case, selective parameters were: (i) the activity of preparations after drying, (ii) activity towards the substrate and (iii) operational stability in successive batch processes. These studies indicated that the proposed system can be regarded as an attractive one for the perfume and cosmetic industries.

2. Results and discussion

2.1. Selection of suitable carriers for hydrolytic reactions in aqueous media or transesterifications in water-restricted media

For immobilization, 5 carriers of different material, porosity, and functionality were used for BCL binding by either adsorption or covalent attachment. This resulted in 11 enzyme-carrier preparations (Table 1). A relatively wide array of carrier materials and functionalities should make the proper selection of matrix more feasible. The yields of protein binding varied from 9 to 85% without a clear dependence on the superstructure of the carriers or on the pH of the coupling media. This result is in opposition to previous observations for other enzymes [12–14,23]. Most probably, the enzyme manufactured for industry is composed of many proteins (and stabilizers such as ballast proteins, diatomaceous earth, calcium chloride, dextran, etc.) with various affinities towards carriers, which results in a lack of predictable results. For that reason, the balance of lipase activity during immobilization was performed assuming the evaluation of only one protein response. One can calculate expected activity on the basis of activity provided for bonding and washed off enzymes, assuming that there were no inactivations, steric hindrances or the closed structures of lipase molecules. It was stated that BCL preferences towards carrier surfaces are as follows: G > A > ZS for both covalent and adsorptive attachments. Quite different results were obtained when lipase from *Candida rugosa* was bound to the same family of carriers where the observed preferences were: A > G > ZS [24]. This, again, indicates a lack of rules for a preliminary selection of carriers for particular enzyme immobilization.

Expected activity is a valuable parameter which allows for the calculation of the purification factor of lipase facilitated by interactions between the carrier surface and active/ballast proteins. This parameter is calculated as a ratio of expected activities based on the activity balance (Table 1) and the protein balance (bound protein multiplied by specific activity of native enzyme). Factors above 1.0 indicate the preferential binding of lipase among other proteins, whereas factors below 1.0 indicate preferential binding of ballasts. The order of the most advantageous matrices of carriers for PCL immobilization includes, as previously noted, adsorptive binding, but the purification factor of 10 upon adsorption on Granocel was not expected. The results clearly demonstrate the higher susceptibility of non-activated carriers to create a wider array of interactions, most likely hydrophobic, which are masked by carrier activators in other cases.

Measured activity is the most common factor applied to the selection of the best enzyme-carrier preparations, and the silica gel carrier functionalized with glycidyl groups is the optimal choice in this sense (No. 1). Generally, among the carriers tested, the silica family seems to be the best – this is in agreement with practical application, where such carriers are the most often used for lipase binding [25]. Concomitant high activity yields were noted along with the greatest extent of PCL bound to silicas; the highest immobilization efficiency especially reflects appealing conditions for activity expression. The last factor (the ratio of measured to expected activity) offers information regarding how the extent of the microenvironment created by carrier matrix, functionality, activators, and bound proteins preserved a proper orientation of BCL molecules towards the carrier surface with the preservation of the active conformation. Thus, the best microenvironments were ordered as follows: silicas, then acrylic carriers, whereas cellulose-based carriers appeared to lack feasibility for use in BCL immobilization.

2.2. Thermal, operational and storage stability of BCL preparations

Although the absolute activity of immobilized preparations is the most often used parameter for the selection of the best procedures and carriers for a given enzyme coupling, the stability of a bound enzyme plays a dominant role in practical applications. Thus, all enzyme-carrier preparations were tested for their thermal, storage and operational stabilities (Fig. 1). It was expected that the lowest stabilities should be observed in the case of immobilization by adsorption (Nos. 3, 5, 9, 11) due to the high probability of enzyme desorption in aqueous solutions, especially at elevated temperature (70 °C). However, this appears to be true only for cellulose-based carriers, although all preparations were more stable by factors ranging from 2.4 (No. 11) to even 15 (No. 2) with regard to native PCL (6.6%). The highest thermal stability was noted for the carriers 2, 4, 5 of the silica family, whereas the highest activities

Table 1
Immobilization efficiency of lipase bound to silica (ZS), acrylic (A) and cellulose-based (G) carriers. Ads – enzyme-carrier preparations obtained by adsorption.

No [–]	Functionality [–]	Protein		Activity			Affinity ratio [–]	Active in bound
		Bound [mg·mL^{-1}]	Yield [%]	Expected [U·mL^{-1}]	Measured [U·mL^{-1}]	Yield [%]		
1	ZS-glycidyl	1.29	53.7	8648	6650	30.5	0.74	56.2
2	ZS-NH_2	1.71	76.0	8200	2708	12.0	0.48	33.0
3	ZS-NH_2,ads	0.96	64.3	12,492	3139	17.4	1.08	25.1
4	ZS-CH_3, NH_2	0.80	35.6	4235	2965	13.2	0.53	70.0
5	ZS-CH_3, NH_2,ads	0.87	57.9	12,804	1308	7.2	1.22	10.2
6	A-OH	0.92	51.5	20,791	1987	9.42	1.91	9.6
7	A-COOH	1.15	65.2	19,911	1654	8.10	1.52	8.3
8	A-NH_2	1.50	84.7	28,482	849	2.94	1.20	3.0
9	A,ads	0.32	21.1	14,098	2641	14.6	3.65	18.7
10	G-OH	0.83	54.3	34,898	219	0.6	1.86	0.6
11	G-OH,ads	0.14	9.1	17,211	211	1.2	10.2	1.2

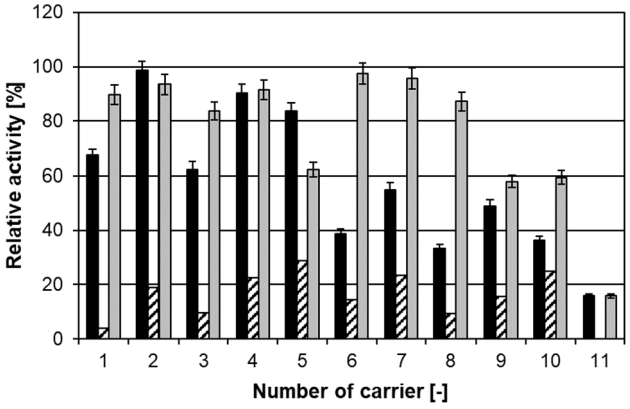


Fig. 1. Relative thermal, operational and storage stabilities of BCL immobilized by covalent attachment or by adsorption on the carriers (numbers as in Table 1). Thermal stability – activity after incubation at 70 °C for 4 h (black); operational stability measured as activity under 20 successive batch hydrolysis experiments of 4-NPP (striped); storage stability determined as activity after 30 days of incubation at 4 °C (gray). Thermal stability of native lipase was 6.6%.

after 30 days of storage were noted for carriers 1, 2, 4 (silica) and 6, 7, 8 (acrylic copolymer). Combining both types of stabilities, the best enzyme-carrier preparations seem to be numbered as 2 and 4, with preparation 2 exhibiting slightly better thermostability.

Operational stability is also considered as an important factor for immobilized catalysts. The idea is to keep the same portion of immobilized enzyme under reaction conditions; in batch reaction runs, the enzymes are exposed to decreasing substrate and growing product concentrations. The interactions of substrate/product combinations with the carrier matrices could also play an important role in processes observed; this was the case in our experiments (Fig. 2). In each case, rapid activity attenuation after the first reaction was noted – most likely caused by the sorption of fatty acid molecules – followed by stepwise decreases. After storage, the preparations were rinsed intensively and the desorbed product was removed (yellow washings), making lipase active centers more accessible. A slow decrease in activity was observed in all the sets of batch processes during the working day, while activity increased following night breaks. This could be because the parameter depicting operational stability has no absolute character and we primarily considered the first processes of the day. Regarding the operational stability, preparations 2, 4, 5 (silicas), 7 (acrylic) and 10 (cellulose) appeared to be best. Surprisingly, the most stable preparation was that resulting from the adsorption of BCL on silica carrier (No. 5).

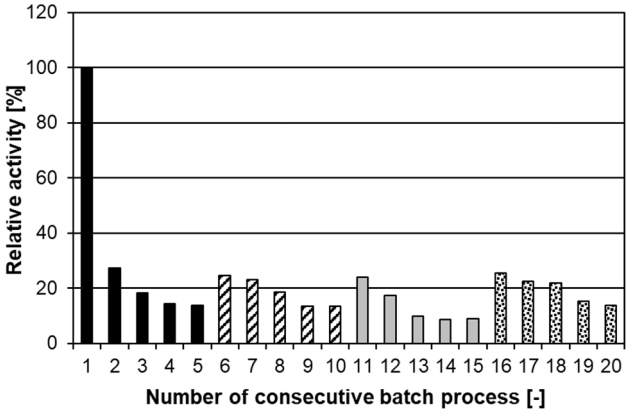


Fig. 2. Representative relative activities of BCL immobilized on Granocel carrier (preparation No. 10) in the series of consecutive batch processes. Bars of the same pattern were tested in the same day. The reaction rate obtained in the first run was set as 100%. Reaction conditions: rotary shaker 60 rpm, pH 8.2, 37 °C, 1.65 mM 4-NPP as the substrate, time of a single process: 1–2 h.

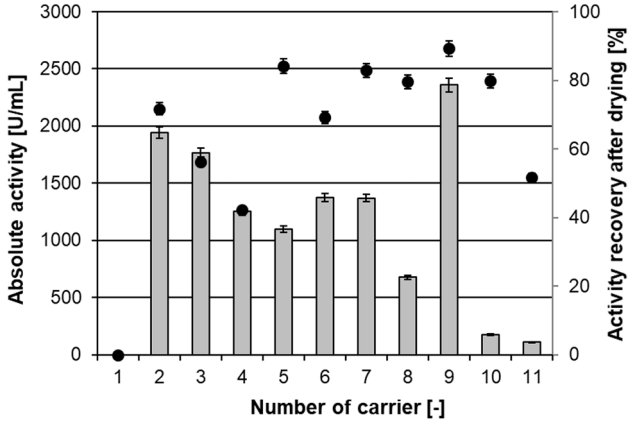


Fig. 3. Absolute hydrolytic activity after drying (gray bars) and relative recovery of activity after drying (black dots) determined for immobilized preparations of BCL after washing with cold acetone and subsequent drying.

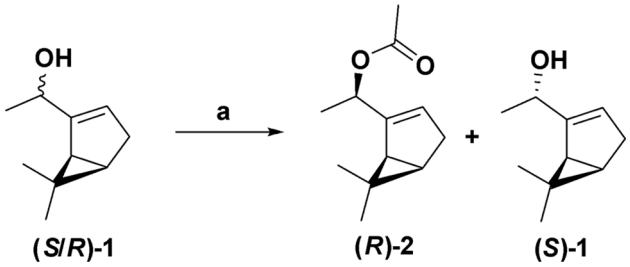
When searching for the best preparations for hydrolytic reaction with immobilized lipase, one should consider the combination of stabilities: this results in the choice of preparations number 2 and 4. However, the highest thermal stability of preparation number 2 (99% retained activity) indicates that it is the preparation of choice.

One topic of interest was the extent to which the enzyme preparation selected for reactions in water-rich media would be suitable for reactions in water-restricted media. In these reactions, the enzyme preparations must be dry and activities should be as high as possible due to the low reaction rates of enzymes in organic solvents; the situation is additionally complicated by the presence of artificial substrates [26,27]. As there are no rules for the selection of the best groups of carriers, all preparations were dried after washing with cold acetone (Fig. 3). In this case, combining the highest absolute hydrolytic activities after drying (preparations No. 9 and 2) with the highest relative recovery activities after drying (No. 9, 5, 7) allowed the determination of the acrylic carrier with BCL immobilized by a simple adsorption (No. 9) as the ideal choice. The type of carrier best for transesterifications in organic solvent remained a mystery; therefore, for the next set of experiments the following carriers: No. 2 (covalent attachment, the best for hydrolysis), No. 5 (the highest operational stability among adsorbed BCL), No. 7 (covalent attachment, high activity recovery after drying), No. 9 (adsorption, the highest activity recovery and the highest absolute activity after drying), and No. 10 (covalent attachment, represents cellulosic carrier) were chosen for further studies.

2.3. Transesterification in a batch reactor

As a model for transesterification reactions, (+)-(*SR*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol was chosen due to its value as a possible component of fragrances. Kuriata and co-workers [28] showed the potential of Amano PS lipase powder from *Burkholderia cepacia* (BCL, *Pseudomonas cepacia*) in obtaining a pure diastereoisomer of (*R*)-acetate from this alcohol and determined its interesting odor properties, with fruity and herbal-balsamic notes. In this study, 5 previously selected immobilized preparations were used for the transesterification of this compound over 24 and 48 h (Scheme 1, Table 2).

Among the studied preparations, BCL adsorbed on acrylic carrier turned out to be the best when using longer reaction times. Surprisingly, excellent conversion was achieved after 24 h of reaction with a preparation based on cellulose; however, it did not increase over the next 24 h. In the case of silica carriers, preparations were less active and conversion increments were lower after prolonged time. Thus, acrylic carriers with BCL bound via adsorption and via covalent binding were selected for operational stability tests.



Scheme 1. Kinetic resolution of (+)-(*S*/*R*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo [3.1.0]hex-2-en-2-yl)]ethanol. **a -** Immobilized *Burkholderia cepacia* lipase.

Table 2
Substrate conversion and diastereomeric excess (d.e.) of model (*SR*)-alcohol after 24 and 48 h transesterification in the batch reactor with the use of BCL immobilized by adsorption (ads) or by covalent binding. Reaction conditions: rotary shaker 100 rpm, 37 °C, 4 mg/mL (*SR*)-alcohol in hexane, 0.1 mL of immobilized BCL.

Carrier No.	Functionality [–]	Substrate conversion [%]		*de.* of *R*-acetate [%]
		24 h	48 h	
2	ZS-APT	3.3	4.3	> 99
5	ZS-APM, ads	3.5	5.2	> 99
7	A-COOH	14.0	24.0	> 99
9	A, ads	17.7	32.0	> 99
10	G-OH	18.5	16.6	> 99

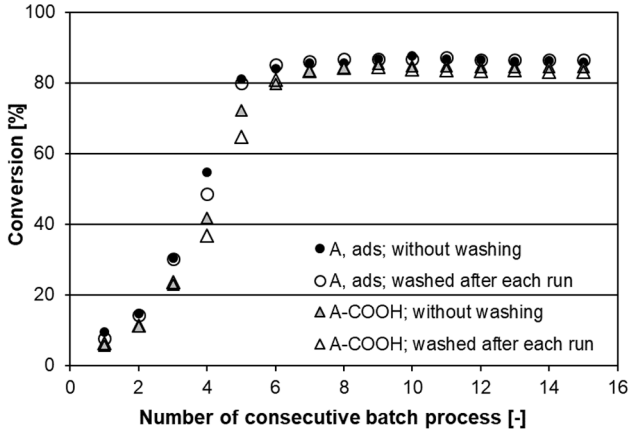


Fig. 4. Substrate conversion upon the transesterification of (+)-(*SR*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol over 15 consecutive reaction runs using lipase immobilized covalently on the acrylic carrier via –COOH groups (grey and white triangles) or by adsorption (black and white circles). Enzyme preparations were washed with hexane after each run (white circles and triangles) or were used directly in the next run (black circles or gray triangles). Reaction conditions: rotary shaker 100 rpm, 37 °C, 4 mg/mL (*SR*)-alcohol in hexane, 0.1 mL of immobilized BCL, 24 h for a single run.

The quality of lipase preparations immobilized by the two methods on acrylic carrier was evaluated on the basis of operational stability over several consecutive batch processes with the same portion of the preparations (Fig. 4). Surprisingly, initially low substrate conversions increased gradually for two ensuing runs, which was followed by a more rapid increase in the fourth and fifth reactions, ultimately reaching over 80% conversion in the next 10 runs. It must be strongly indicated that without operational stability experiments, the industrial potential of lipase immobilized on acrylic carrier could be ignored.

We suspected that the reason for this behavior lies in the substrate/product sorption on the carrier. However, the carrier with lipase bound by covalent attachment adsorbed 2.3% of the substrate, in comparison to 5.7% by its counterpart, suggesting a marginal influence of this

Table 3
Substrate/product sorption on carriers with BCL immobilized by adsorption (ads) or by covalent attachment.

Carrier No.	Functionality [–]	Substrate sorption [mg]	Product sorption [mg]
2	ZS-APT	0.0	4.0
5	ZS-APM, ads	3.8	3.0
7	A-COOH	5.7	7.2
9	A,ads	2.3	2.7
10	G-OH	0.9	18.4

parameter on the phenomenon observed (Table 3). Thus, the saturation profiles in Fig. 4 can be caused by product sorption, and this was indirectly visualized by lower conversion when enzyme preparations were washed with hexane after each run (see runs 4 and 5 in Fig. 4). It was proven by an additional experiment on substrate/product sorption on the tested preparations (Table 3). As it can be seen, in all the cases but one product sorption was stronger than the substrate. The phenomenon of the affinity/sorption of substrates/products to the carriers' surface was underlined in our previous studies on operational stability of laccase immobilized on acrylic-, cellulose-, and silica-based carriers [14,29]. Moreover, it was shown that adsorption capacity of indigo carmine was lower when the carrier with immobilized enzyme was used, reaching value of 78.5 mg/g [14].

When discriminating between two tested enzyme preparations, it is clear that immobilization by adsorption allowed the achievement of almost complete substrate conversion (maximal value is 85% due to stereoisomer ratio) in the fifth run; however, differences were negligible in the next reactions. We suspect that the lower protein load on the carrier in the case of adsorptive immobilization (Table 1) caused the more rapid saturation of the matrix with the product, as in the case of substrate. Thus, the selection of the best preparation should be based on the primary advantage of adsorptive binding: immobilization simplicity.

3. Conclusion

In the study, lipase from *Burkholderia cepacia* was used as the biocatalyst of the hydrolytic reaction and kinetic resolution processes performed for 4-NPP hydrolysis and the acylation of ((+)-(S/R)-1-[(1S,5R)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol, respectively.

Initially, 11 carriers differing in matrix composition (acrylic, silica, cellulose) and the type of reactive functional groups (e.g., –glycidyl, –OH, –COOH, $–NH_2$) were taken under consideration for lipase immobilization by means of adsorption and/or covalent attachment. In searching for the best preparation for hydrolytic reaction, discrimination parameters such as activity and thermal, operational and storage stability were determined; this resulted in the selection of a silica carrier with amine functional groups, activated with glutaraldehyde, for lipase covalent attachment. However, it must be noted that the interaction of substrate/product combinations with the carrier matrices plays an important role in consecutive processes.

In the case of the selection of preparations suitable for executing reactions in organic solvents, the reactivity and resistance of bound lipase to drying were used as discrimination parameters. For that reason, five preparations were chosen for the kinetic resolution processes of (+)-(*S*/*R*)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol carried out for 24 and 48 h, with acrylic carriers with BCL bound via adsorption (A,ads) and via covalent binding (A-COOH) selected for operational stability tests. Surprisingly, saturation-like profiles were observed for both preparations in the first five runs, followed by increases in substrate conversion from 10 to 80%, which approaches the value characteristic of equilibrium (85%). Most likely, this result stemmed from product sorption onto the carrier. Finally, irrespective of the type of immobilization and the presence or absence of washing

procedure between runs, both preparations were equally effective in kinetic resolution with conversions of approximately 85% and *de* > 99%. Thus, the system proposed in our study can be regarded as an exceptionally attractive solution for the perfume and cosmetic industries.

The general conclusion is that the sorption of reagents might influence the obtained results in unpredictable ways, for example, by causing the apparent decay of reactivity in hydrolytic and/or transesterification reactions, as was observed in our case. Thus, the industrial potential of immobilized lipase should be evaluated only on the basis of consecutive reactions and/or the testing of reactant sorption onto the immobilization matrix.

4. Experimental protocols

4.1. General methods and materials

Lipase Amano PS solid (≥30,000 unit/g) from *Burkholderia cepacia* (*Pseudomonas cepacia*; PCL), 4-nitrophenyl palmitate (4-NPP), 2-propanol, Triton X-100, gum arabic from acacia tree, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), vinyl acetate, divinyl sulfone (DVS), trihydroxymethylaminomethane (tris), Lowry reagent and bovine serum albumin were purchased from Sigma-Aldrich (Germany). Glutaraldehyde (GA) was from Merck (Germany). Other chemicals, all of analytical grade, were supplied by Avantor (Poland). (+)-3-carene was from Acros (Belgium) and was used without further purification.

Silica gel with small mesopores (ZS; IE Int. Enzymes Ltd., USA) was functionalized as presented previously [12] and kindly donated by Dr. Katarzyna Szymańska from the Silesian University of Technology (Poland). Cellulose-based carriers (Granocel; G) were prepared according to the procedure presented previously [11]. Acrylic carriers with –COOH, –OH and NH_2 functional groups were kindly donated by the Tarchomin Pharmaceutical Plant (Poland).

Chiral gas chromatography (CGC) was conducted on a Shimadzu GC-2010 Plus gas chromatograph equipped with an FID detector and CycloSil-B capillary column [30% heptakis(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-ß-cyclodextrin in DB-1701 ((14%-cyanopropylphenyl)-methylpolysiloxane) stationary phase; 30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent, Santa Clara]. The injection port was held at 200 °C. The split ratio was 10:1, and 1 µL of the sample was injected. The oven temperature was maintained at 120 °C for 12 min with a constant nitrogen carrier gas flow of 1.5 mL·min^{-1}. FID temperature was maintained at 230 °C. Quantitative analyses were conducted with an external standard method. The standard (+)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol **(*S*/*R*)-1** was synthesized as described below (Section 3.2.) and its purity was > 99%. All analyses were performed in duplicate. The mean analytical error was ± 1.1%.

4.2. Synthesis of the (+)-1-[(1S,5R)-6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)]ethanol

Secondary allylic alcohol (+)-1-[(1*S*,5*R*)-6,6-dimethylbicyclo [3.1.0]hex-2-en-2-yl)]ethanol **(*S*/*R*)-1** was synthesized according to a known procedure [28,30]. Briefly, the monoterpene hydrocarbon (+)-3-carene was transformed to ozonide, which was subsequently reduced with zinc powder to ketoaldehyde. Dicarbonyl intermediate was subjected to the intramolecular aldol condensation. Bicyclic enone was then reduced with lithium aluminum hydride to the **(*S*/*R*)-1** alcohol in the form of a diastereoisomeric mixture in a 15:85 ratio (*S*:*R*), which was determined by chiral gas chromatography (CGC).

4.3. Lipase activity measurements

Hydrolytic activity was measured as described previously [24]. Briefly, the reaction mixture contained 0.05 M Tris-HCl buffer at pH 8.2, 4‰ Triton X-100, 1‰ gum Arabic, and 1.65 mM 4-NPP. Reaction (37 °C) progress was monitored spectrophotometrically (410 nm, Cintra 303 spectrophotometer), and the product concentration was calculated from the linear dependence of absorbance versus time (the initial reaction rate). The enzyme activity unit [U] was defined as the amount of the enzyme that caused the formation of 1 µmol 4-nitrophenol in 1 min under the reaction conditions.

The activity measurement of immobilized BCL was carried out in a similar manner as above, but shake flasks (Laboplay; 60 rpm, 37 °C) containing 10–20 mg of the wet preparation were utilized instead. The samples of reaction mixture were withdrawn from the flask in 1 min intervals, the absorbance values were measured, and the mixtures were returned to the flask.

Protein concentration was determined by Lowry's method using bovine serum albumin as a protein standard. All analyses were performed in triplicate. The mean analytical error was less than ± 2.5% for protein measurements and activity assays, while the error was less than ± 2.0% and ± 7.5% for native and immobilized enzymes, respectively.

4.4. PCL immobilization

The procedure for BCL immobilization was described in detail by Hrydziuszko et al. [24]. In the case of covalent preparation, the following functional groups were activated: –COOH with EDAC (pH 5.2), OH with DVS (pH 8.2) and NH_2 with GA (pH 7.0). Protein coupling onto carriers with glycidyl moieties (pH 8.2) or by adsorption (pH 7.0) was performed without an activating step. After filtration or centrifugation (MCFs; 8000 rpm, 15 min), 5 mL properly prepared carriers were added to 5 mL of enzyme solution with an adequate pH (30 mg·mL^{-1}) and left for 24 h at 4 °C with occasional stirring. The excess of protein was removed by washing the carrier with buffers of varying pH and ionic strength [12]. In all eluates, enzyme activity and protein concentration were measured. Finally, the obtained preparations were stored in buffer (pH 7.0) at 4 °C and were washed several times with the buffer before use. For transesterification reactions, dry immobilized preparations obtained by washing several times with cold acetone and drying under vacuum were used. All dry preparations were stored at 4 °C. Protein yield (activity yield) was calculated as a difference between the total milligram of protein (the total units, U) in the enzyme solution used for immobilization and that in the supernatants, multiplied by 100 and divided by the total milligram of protein (U) in the initial solution.

4.5. Thermal, operational and storage stability of BCL preparations

The thermal stability analysis of free and immobilized BCL was performed by the incubation of preparations in the buffer at 70 °C for 4 h followed by 1 h of cooling; the reduced activity was then measured under standard conditions. Thermal stability was calculated as a residual activity in relation to the activity of control samples incubated at room temperature.

The operational stability of immobilized preparation was determined in consecutive batch operations under standard conditions. In all performed processes, the initial reaction rate was measured and the ongoing process was monitored until substrate depletion (approximately 2 h). After the end of the reaction, the immobilized BCL was washed intensively with the buffer and the next batch process was initialized with the same enzyme sample. The reaction rate in the first run was set as 100%.

Storage stability was tested by keeping preparation in the buffer at 4 °C for 30 days. The activity measured before storage was set as 100%.

4.6. Transesterification in a batch reactor

A general procedure of (*SR*)-alcohol transesterification with native BCL was presented in the work of Kuriata *et al.* [28]. In the case of the

batch process, 24 mg of the substrate, 45 μL of vinyl acetate, 40 mg of powdered molecular sieves (3 Å mesh) and 0.1 mL of dry enzyme were added to 6 mL of hexane in an Erlenmeyer flask. The reaction was run in the shaker (100 rpm) at 37 °C for 24 or 48 h, and then immobilized PCL was filtered and the substrate concentration was measured by CGC.

Selected preparations (0.1 mL) were tested towards operational stability over 15–20 successive runs in the shaker in the same manner as activity tests. Each run lasted 24 h followed by the aspiration of the reaction mixture, and the remaining enzyme preparation was directly used for the next run or was washed several times with hexane before subsequent reaction. Filtered postreaction mixtures were analyzed by CGC.

4.7. Substrate sorption

Substrate sorption was tested after the inactivation of BCL immobilized on tested carriers (No. 2,5,7,9,10) by either adsorption or by the formation of covalent bonds *via* incubation in 50% ethanol at 70 °C for 24 h and redrying. For experiments, 0.1 mL of enzyme preparation was added to 6 mL of hexane containing substrate or product (4 mg/mL), and the slurry was agitated in the shaker (100 rpm) at 37 °C for 2 days, followed by measuring substrate/product concentration in filtrates by CGC.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.041.

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